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Genes other than TLR4 are involved in the response to inhaled LPS

EVA LORENZ,[1,2] MICHAEL JONES,[3] CHRISTINE WOHLFORD-LENANE,[4] NICOLE MEYER,[4] KATHY L. FREES,[4] NANCY C. ARBOUR,[4] AND DAVID A. SCHWARTZ[1,2,5]
Departments of [4]Medicine and [3]Biostatistics and [2]Department of Veterans Affairs Medical Center, University of Iowa, Iowa City, Iowa 52242; and [1]Department of Medicine and [5]Department of Veterans Affairs Medical Center, Duke University Medical Center, Durham, North Carolina 27710

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Lorenz, Eva, Michael Jones, Christine Wohlford-Lenane, Nicole Meyer, Kathy L. Frees, Nancy C. Arbour, and David A. Schwartz. Genes other than TLR4 are involved in the response to inhaled LPS. *Am J Physiol Lung Cell Mol Physiol* 281: L1106–L1114, 2001.—For several decades, the mouse strains C3H/HeJ and C57BL/10ScNCr have been known to be hyporesponsive to endotoxin or lipopolysaccharide (LPS). Recently, mutations in Toll-like receptor (TLR) 4 have been shown to underlie this aberrant response to LPS. To further determine the relationship between TLR4 and responsiveness to LPS, we genotyped 18 strains of mice for TLR4 and evaluated the physiological and biological responses of these strains to inhaled LPS. Of the 18 strains tested, 6 were wild type for TLR4 and 12 had mutations in TLR4. Of those strains with TLR4 mutations, nine had mutations in highly conserved residues. Among the strains wild type for TLR4, the inflammatory response in the airway induced by inhalation of LPS showed a phenotype ranging from very sensitive (DBA/2) to hyporesponsive (C57BL/6). A broad spectrum of airway hyperreactivity after inhalation of LPS was also observed among strains wild type for TLR4. Although the TLR4 mutant strains C3H/HeJ and C57BL/10ScNCr were phenotypically distinct from the other strains with mutations in the TLR4 gene, the other strains with mutations for TLR4 demonstrated a broad distribution in their physiological and biological responses to inhaled LPS. The results of our study indicate that although certain TLR4 mutations can be linked to a change in the LPS response phenotype, additional genes are clearly involved in determining the physiological and biological responses to inhaled LPS in mammals.

Toll-like receptor 4; Toll genes; lipopolysaccharide; asthma

ENDOTOXIN OR LIPOPOLYSACCHARIDE (LPS), the main component of the cell wall of gram-negative bacteria, has been shown to elicit an inflammatory and physiological response that is mediated through the innate immune system (3). As the first step in host defense against infection, the innate immune response is characterized by the binding of bacterial pathogens by pathogen-recognition receptors such as CD14 and Toll proteins (10). Subsequent to the initial recognition, binding, and signaling, nuclear factor (NF)-κB and activator protein-1 promote transcription of proinflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8] that serve to enhance the inflammatory response by recruiting and activating leukocytes (4, 5). In septic shock, this inflammatory response is amplified to a potentially dangerous level that can in and of itself have detrimental effects. However, failure to activate innate immunity can have deleterious outcomes as well, such as overwhelming bacterial infection. Thus the innate immune response serves as a sentinel balance to alert the host to bacterial invasion and modulate the inflammatory response. As such, innate immunity represents a proximal target for potential intervention in bacterial infections.

Recent evidence has identified Toll-like receptor (TLR) 4 as a major LPS receptor in mammals. This evidence is mainly based on work done in mice. In 1965, the first LPS-hyporesponsive strain, C3H/HeJ, was identified (18) and was subsequently shown to have a mutation in TLR4 (14). This mutation changes a conserved proline to a histidine at position 712 of the open reading frame. In addition, strain C57BL/10ScNCr is also hyporesponsive to LPS. This strain has a deletion on chromosome 4, which encompasses the murine TLR4 locus. Two knockout mouse lines also support a role for TLR4 in the LPS response (6, 8). The knockout lines for both TLR4 and MyD88, a downstream mediator of TLR4 signaling, are unresponsive to LPS (6, 8). The combined genetic evidence from these mouse strains is the strongest evidence yet for a role of TLR4 as a regulator of the response to LPS.

In humans, TLR4 has been associated with LPS signaling. Recently, TLR4 mutations in humans have been associated with a reduced response to inhaled LPS (1). Specifically a mutation at residue 299, which changes a conserved Asp to a Gly, is associated with a reduced response to LPS in vitro (1). However, not everyone with the TLR4 mutations was hyporesponsive to inhaled LPS, and not everyone who was hyporesponsive to inhaled LPS had the TLR4 mutations.

Address for reprint requests and other correspondence: D. A. Schwartz, Pulmonary and Critical Care Medicine, Duke Univ. Medical Center, Research Drive, Room 275 MSRB, DUMC Box 2629, Durham, NC 27710 (E-mail: david.schwartz@duke.edu).

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These findings suggest that there are genes in addition to TLR4 that determine the response to LPS in humans.

To further evaluate the relationship between TLR4 and LPS responsiveness, we genotyped 18 genetically diverse strains of mice for TLR4 and measured the physiological and biological responses of these strains to inhaled LPS. Mouse strains were chosen from a variety of backgrounds to ensure genetic diversity (2). Twelve of the eighteen strains of mice were found to have mutations in TLR4. Although two mutant strains (C3H/HeJ and C57BL/10ScNCr) were clearly hyporesponsive to inhaled LPS, there was a broad physiological and biological response to inhaled LPS among the remaining mutant and wild-type strains. These findings provide evidence that other genes apart from the TLR4 receptor gene are important determinants of the physiological and biological responses to inhaled LPS.

MATERIALS AND METHODS

In this paper, we present the physiological, biological, and genotypic results used to determine the role of TLR4 in the response to inhaled endotoxin in mice. We used a total of 18 mouse strains in our analysis. These mouse strains were chosen to represent various evolutionary backgrounds to ensure genetic diversity (2) with respect to potential polymorphisms in the genes, such as TNF-α (7), that affect airway responsiveness and innate immunity. With regard to the known hyporesponsive strains C3H/HeJ and C57BL/10ScNCr, the respective parental strains were included as well (C3H/HeN and C57BL/10ScSn) to ensure that any sign of hyporesponsiveness to inhaled LPS was not a result of the strain background. In addition, all strains were genotyped for TLR4 (open reading frame and splice sites), the major LPS response receptor in mammals, to avoid any observed LPS hyporesponsiveness that may be due to a stop codon or deletion in the TLR4 gene. Phenotyping was performed by measuring airway reactivity to methacholine pre- and postinhalation of LPS and by evaluating the inflammatory response with ELISA assays performed on lavage fluid. A minimum of four mice were used for the phenotypic analysis, with the exception of strains SEA/GNJ, A/J, SM/J, and KK/HiJ in which five mice were used and strain SOD/Ei in which six mice were used.

TLR4 genotyping. Genomic DNA for each mouse strain was either purchased from Jackson Laboratory or extracted from the kidney according to the standard protocol with the PUREGENE method (Gentra Systems, Minneapolis, MN). Overlapping primers (17) covering the entire TLR4 open reading frame as well as the splice sites were used to amplify the TLR4 genomic DNA. PCRs were done with the PE *Taq* polymerase kit, including nucleotides and buffer. All PCR products were sequenced in both directions, and the sequences were compared with the mouse TLR4 cDNA in the GenBank database (accession no. AF177767).

Aerosol exposure protocol and monitoring. LPS for aerosolization was purchased as lyophilized purified *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO). LPS was solubilized in Hanks' balanced salt solution (HBSS) to a concentration of 1 mg/ml, stored at 4°C, and diluted further in HBSS to the appropriate concentration on the day of the experiment. LPS aerosol was generated and directed into a 20-liter glass exposure chamber with a Collison nebulizer (BGI, Waltham, MA). High-efficiency particle apparatus-filtered air was supplied to the nebulizer at a constant pressure of 20 psi. The chamber atmosphere was exchanged at a rate of 0.25–1.0 changes/min, and LPS concentrations were determined by sampling the total chamber outflow. During the exposure, filter samples were taken from the outflow aerosol at regular intervals and assayed to ensure even endotoxin concentrations. Assays for endotoxin content are described in *Endotoxin exposures*.

Endotoxin exposures. Aerosol exposures were performed in a 20-liter exposure chamber with a Collison nebulizer delivering the endotoxin. The endotoxin concentrations generated by the aerosols during the exposure period were assayed with the chromogenic *Limulus* amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD) with sterile pyrogen-free labware and a temperature-controlled microplate block and microplate reader (405 nm), as previously described (9). Briefly, four separate samples were taken during each 4-h exposure period by drawing air from the exposure chamber through 47-mm binder-free glass microfiber filters (EPM-2000, Whatman, Maidstone, UK) held within a 47-mm stainless in-line air-sampling filter holder (Gelman Sciences, Ann Arbor, MI). Endotoxin was extracted from the filters with pyrogen-free water at room temperature with gentle shaking. The extracts were then serially diluted and assayed for endotoxin with a LAL assay. For this series of experiments, the intended endotoxin concentration used in the exposures was 6 $\mu g/m^3$. LAL assays on the filters used showed that the endotoxin concentration was in the range of 6–6.9 $\mu g/m^3$.

Assessment of pulmonary function. A methacholine challenge test was performed with a whole body plethysmograph 24 h before and immediately after the inhalation of LPS. The exposure protocol used in these experiments allowed us to determine how pulmonary function changes in response to LPS exposure. Mice were placed in an 80-ml whole body plethysmograph (Buxco Electronics, Troy, NY) ventilated by bias airflow at 0.2 l/min. This unit was interfaced with differential pressure transducers, analog-to-digital converters, and computers. The breathing patterns and pulmonary function of each individual mouse were monitored over time, and direct measurement was made of respiratory rate, pressure changes within the plethysmograph and "box flow" (the difference between the nasal airflow of the animal and the flow induced by thoracic movement, which varies in the presence of airflow obstruction because of pulmonary compression). The Buxco system measured both the magnitude of the box pressure variations and the slope of the box pressure. Estimates of airway resistance are expressed as enhanced pause (P_{enh}). P_{enh} = (expiratory time/40% of relaxation time $-$ 1) $\times$ peak expiratory flow/peak inspiratory flow $\times$ 0.67. The validity of P_{enh} as a measure of airway hyperreactivity was examined in a recent publication (15). Lung function was measured at baseline and after stimulation with inhaled methacholine (5, 10, and 20 $\mu g/ml$) according to a standard protocol (15).

Whole lung lavage. At specific time points after the completion of the inhalation exposure and second pulmonary function test, mice were killed, the chest was opened, and the lungs were lavaged in situ via PE-90 tubing inserted into the exposed trachea. A pressure of 25 cmH_2O was used to wash the lungs with 6.0 ml of sterile pyrogen-free saline, 1 ml at a time. The volume was noted, and the cells were pelleted by centrifugation for 5 min at 200 *g*. The supernatant fluid was frozen at -70°C for subsequent use. The residual pellet of cells was resuspended and washed twice in HBSS (without Ca or Mg). After the second wash, a small aliquot of the sample was taken for cell count. The cells were washed once more and resuspended in RPMI medium to a final concentration of 1×10^6 cells/ml. The cells that were present in

10–12 μl of the 1×10^6 ml cell suspension were spun for 5 min onto a glass slide with a cytocentrifuge (Cytospin-2; Shandon Southern, Sewickley, PA). Standard staining was carried out with a Diff-Quik stain set (HARLECO, Gibbstown, NY).

Lung lavage fluid. Murine intercellular adhesion molecule (ICAM)-1 was measured in lung lavage fluid with an ELISA prepared with a hamster monoclonal antibody specific for murine ICAM-1 (Endogen, Woburn, MA). The detection limit of this assay was determined to be 5 ng/ml, and it is specific for mouse ICAM-1. Commercial murine ELISA kits were used for the measurement of TNF-α and macrophage inflammatory protein (MIP)-2 in the lavage fluid (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The detection limits for the ELISA assays were 5 ng/ml for ICAM-1, 23.4 ng/ml for TNF-α, and 7.8 pg/ml for MIP-2.

Statistical analysis. Statistical analyses were performed on the inflammatory variables and pulmonary function variables. The inflammatory variables are based on cell counts and ELISA measurements of TNF-α, MIP-2, and ICAM-1 in the lavage fluid. The pulmonary function variables measured included P_{enh} at the methacholine concentrations pre- and post-LPS exposure indicated in *Assessment of pulmonary function*. Previously, (12) the P_{enh} reading at 20 mg/ml of methacholine has given the best measure for the effect of LPS exposure on pulmonary function. All statistical analysis in this paper is therefore based on readings at the concentration of 20 mg/ml of methacholine and compared with the baseline (0 mg/ml of methacholine).

Graphic displays were made for each strain to describe the data. Differences in the inflammatory and pulmonary function variables among all 18 strains of mice were evaluated with analysis of variance. A few pulmonary function values for individual mice were fairly extreme outliers as judged by the SE value, as seen in Fig. 1*A* by the high SE for strain LP/J, in Fig. 1*B* for strain P/J, in Fig. 2*A* for strain C57BL/10ScSn, and in Fig. 2*B* for strain SM/J. Reanalysis of the data without these outliers was performed. Also, the pulmonary function measures were compared with the outliers in the data but on a log scale to assess whether results were still significant while pulling in the larger values via a log transformation. Comparisons were also made among the six wild-type strains with analysis of variance and among the mutant types. Because results from strains C3H/HeJ and C57BL/10ScNCr were vastly different from the rest with regard to the inflammatory variables, the latter analysis was redone without them. Finally, to compare variables between the mutant types and the wild types, two-sample *t*-tests were used based on the average value per strain. *F*-tests were used to assess the equality of variance between these two groups.

RESULTS

Inbred mice differ with respect to their TLR4 genotype. The genotype analysis showed that a total of six mouse strains had a common allele for TLR4, whereas the remaining 12 strains had a variety of point mutations throughout the open reading frame of the TLR4

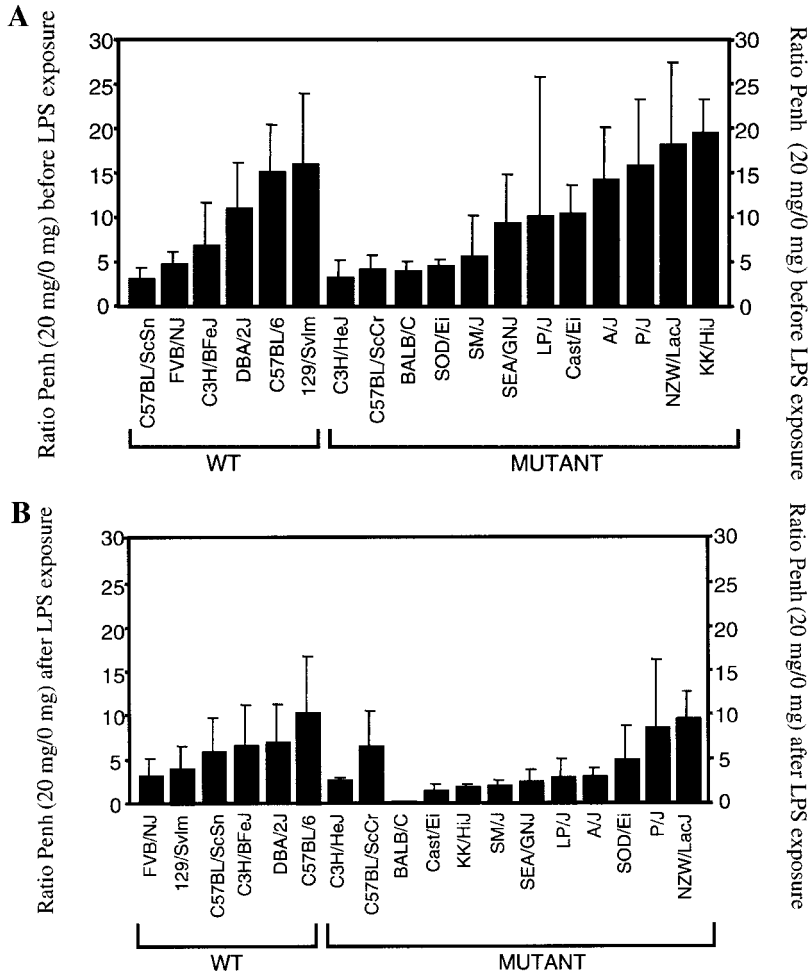

Fig. 1. Effect of lipopolysaccharide (LPS) exposure on the airway response of various mouse strains. *A*: enhanced pause (P_{enh}) before LPS exposure. *B*: P_{enh} after inhalation of 20 mg/ml of methacholine for 5 min after exposure to inhaled LPS for 4 h. WT, wild-type. Values are means ± SE. Relative P_{enh} is given as a ratio of P_{enh} at 20 mg/ml of methacholine to P_{enh} at baseline (saline exposure, 0 mg/ml of methacholine) on either *day 1* (before LPS exposure) or *day 2* (post-LPS exposure).

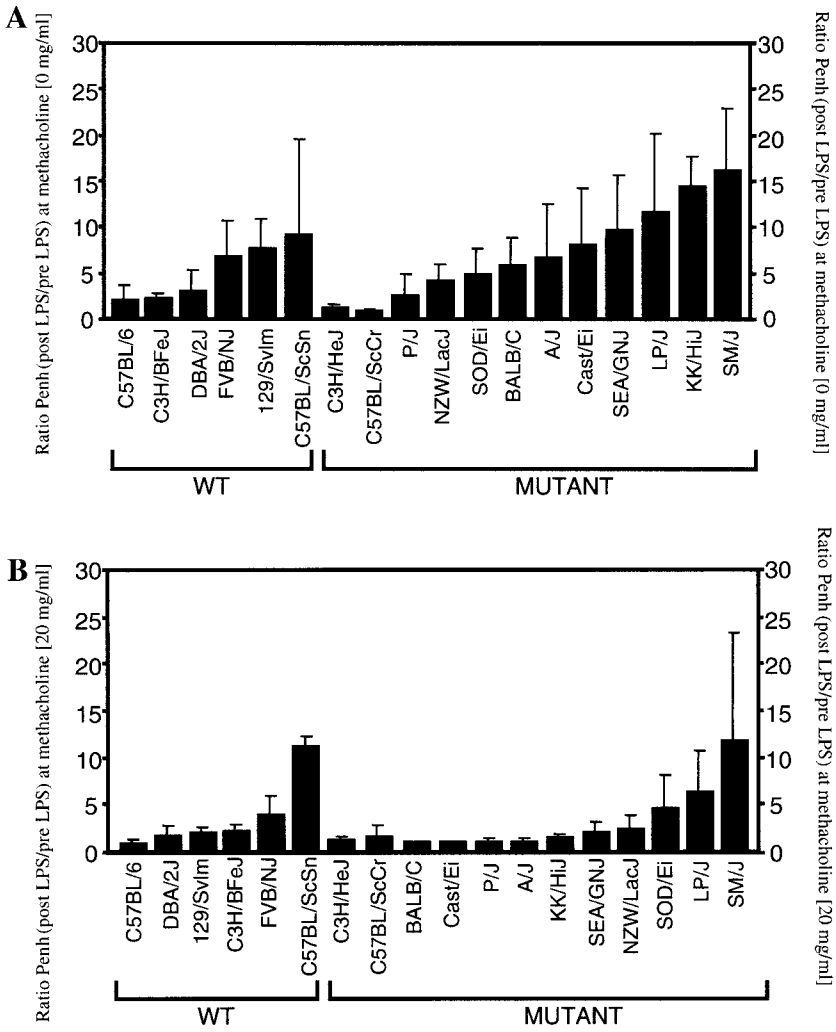

Fig. 2. Relative P_{enh} as a ratio of P_{enh} post-LPS exposure to P_{enh} before LPS exposure. *A*: relative P_{enh} at saline baseline (0 mg/ml of methacholine). *B*: relative P_{enh} after inhalation of 20 mg/ml of methacholine for 5 min. Values are means ± SE.

gene (Table 1). Of these 12 strains with TLR4 mutations, 9 had mutations in highly conserved residues. The results in Table 1 show that Cast/Ei, which has a different evolutionary origin than the other inbred strains used in this study, has a large number of unique mutations in the TLR4 coding region, particularly in the NH_2 terminus of the protein. For the remaining 11 mutant strains, most of the mutations occur in exon 3, the largest exon of mouse TLR4, and are clustered at the COOH terminus of the protein. In addition to the mutations in the open reading frame of the TLR4 gene, we found several mutations in noncoding regions of the genes such as the 5′-untranslated region and introns. These mutations are summarized in Table 2. At this point, it is not known whether any of the strains except C3H/HeJ and C57BL/10ScNCr express TLR4 protein with altered activity, with either reduced or increased signaling activity in response to LPS.

Inbred strains show very different physiological responses to inhaled LPS. We determined the effect of methacholine and LPS inhalation on lung function in 18 different inbred strains of mice. The two known LPS-hyporesponsive strains, C3H/HeJ and C57BL/10ScNCr, when compared with the other strains, were not markedly different in their estimates of airway resistance either before exposure to LPS (Fig. 1*A*) or after inhaling LPS for 4 h (Fig. 1*B*). In fact, the pre-LPS estimates of airway resistance for C3H/HeJ and C57BL/10ScNCr were similar to those for other mouse strains such as C57BL/10ScSn and FVB/NJ, which express the common (or wild-type) TLR4 allele. Even after LPS inhalation, the estimates of airway resistance did not reveal any differences between the hyporesponsive strains C3H/HeJ and C57BL/10ScNCr, and several of the remaining 16 strains used in the study (Fig. 1*B*), such as BALB/c, LP/J, SM/J, A/J, and KK/HiJ, also had similar baseline estimates of airway resistance. Interestingly, most of the strains expressing wild-type TLR4 protein, with the exception of FVB/NJ, had higher airway resistance levels than the hyporesponsive C3H/HeJ strain.

Methacholine-induced airway hyperreactivity revealed interesting strain-dependent variation. Strain C57BL/10ScSn, the wild-type progenitor of the TLR4 deletion mutant C57BL/10ScNCr, was very sensitive to the effect of LPS exposure. Both at baseline and at 20 mg/ml of methacholine, strain C57BL/10ScSn was

Table 1. *Open reading frame mutations in TLR4 by mouse strain*

C3H	BALB/c	KK	Cast	SEA	P/J	A/J	SM/J	SOD	NZW	LP/J	C57BL	Nucleotide	Exon	AA	Domain
			X								*	26384 T-	1	AA 17	Ecto
			X								*	26386 T-	1	AA 17	Ecto
			X								*	26388 C-	1	AA 17	Ecto
			X		X						*	26400 A-G	1	21 Q	Ecto
			X								*	32512 A-G	2	69 K/R	Ecto
			X								*	32546 G-A	2	80 Q	Ecto
		X									*	37754 G-A	3	94 D/N	Ecto
			X								*	37985 T-C	3	171 S/P	Ecto
	X		X	X	X	X		X	X		*	38101 G-A	3	209 M/I	Ecto
				X							*	38130 A-G	3	219 D/G	Ecto
	X			X		X					*	38234 G-A	3	254 V/I	Ecto
			X		X						*	38584 A-G	3	370 L	Ecto
				X							*	38742 A-T	3	423 Q/L	Ecto
					X						*	38794 G-A	3	440 A	Ecto
					X						*	38903 G-T	3	477 A/S	Ecto
										X	*	39020 A-G	3	516 T/A	Ecto
	X		X	X	X	X	X	X	X		*	39199 C-T	3	575 N	Ecto
	X		X	X	X	X		X	X		*	39253 A-C	3	593 E/D	Ecto
		X									*	39273 A-T	3	600 N/I	Ecto
			X								*	39286 A-G	3	604 M/I	Ecto
					X						*	39383 G-A	3	637 V/I	TM
X											*	39608 C-A	3	712 P/H	Cyto
	X			X		X		X	X		*	39631 C-T	3	719 N	Cyto
	X			X		X		X	X		*	39756 G-A	3	761 R/H	Cyto
										X	*	39826 T-C	3	784 Y	Cyto
			X								*	39895 A-G	3	807 R	Cyto

TLR, Toll-like receptor; C3H, C3H/HeJ; KK, KK/HiJ; Cast, Cast/Ei; SEA, SEA/GNJ; SOD, SOD/Ei; NZW, NZW/LacJ; C57BL, C57BL/10ScNCr; AA, amino acid; Q, glutamine; K, lysine; R, arginine; D, aspartic acid; N, asparagine; S, serine; P, proline; M, methionine; I, isoleucine; G, glycine; V, valine; L, leucine; A, alanine; T, threonine; E, glutamic acid; H, histidine; Y, tyrosine; Ecto, ectoplasm; TM, transmembrane; Cyto, cytoplasm. Nucleotide no. and wild-type (*left* letter) and mutated (*right* letter) nucleotides are shown. AA location and wild-type (*left* letter) and mutated (*right*) letter AA are shown. If only 1 AA is shown, no change occurred. Strain C57BL/10ScNCr has a deletion of TLR4 gene. AA 17 was deleted.

almost 10 times more sensitive as measured by pulmonary function assay compared with preexposure levels of airway responsiveness. On the other hand, the TLR4 mutant derivative of C57BL/10ScNCr had almost no change in airway responsiveness as a result of either methacholine or LPS inhalation. Strain C3H/BFeJ, in contrast, showed only slightly more sensitivity to both LPS and methacholine compared with the TLR4 mutant C3H/HeJ strain. This potentially implicates strain background and additional polymorphisms in other genes besides TLR4 as the basis for the lack of hyporesponsiveness.

In general, the variation in airway responsiveness with respect to inhaled LPS seems to be slightly masked by the addition of methacholine. Ratios for saline baseline exposures were significantly higher than the ratios at 20 mg/ml of methacholine. In addition, the airway hyperreactivity at 20 mg/ml of methacholine showed much less variation both in the TLR4 wild-type strains and in the strains expressing mutated forms of the TLR4 protein (Fig. 2, *A* and *B*).

The concentration of LPS-induced polymorphonuclear leukocytes in the airspace is influenced by TLR4 as well as by other genes. The concentration of neutrophils in the lavage fluid after inhalation of LPS has previously been used as an indicator of the inflammatory response to LPS (4). As expected, of all 18 strains tested, the hyporesponsive strains C3H/HeJ and C57BL/10ScNCr had by far the lowest concentration of polymorphonuclear leukocytes (PMNs) in the lavage fluid (Fig. 3). All other strains tested had at least two orders of magnitude more neutrophils in the lavage fluid. The remaining 10 strains with mutations in TLR4 demonstrated a wide range of LPS-induced airway inflammation with PMNs,

Table 2. *Noncoding region mutations in TLR4 by mouse strain*

C3H	BALB/c	KK	Cast	SEA	P/J	A/J	SM/J	SOD	NZW	LP/J	C57BL	Nucleotide	Exon
			X								*	26108 G-C	5′-UTR
			X								*	26173 A-C	5′-UTR
			X								*	26324 A-G	5′-UTR
			X								*	26442 A-G	Intron 1
			X								*	32589 C-T	Intron 2
			X								*	37685 G	Intron 2
	X			X		X	X				*	37685 T10	Intron 2
									X		*	37685 T12	Intron 2

UTR, untranslated region. Strain C57BL/10ScNCr has a deletion of TLR4 gene.

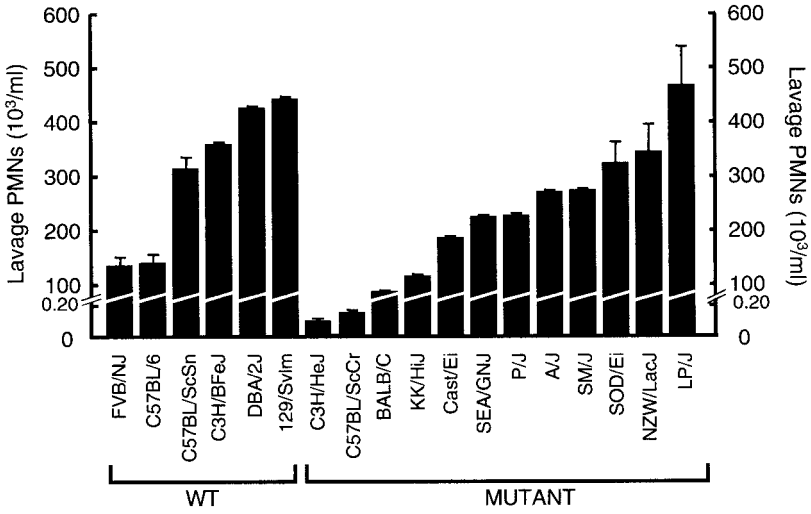

Fig. 3. Concentration of lavage polymorphonuclear leukocytes (PMNs) after a single inhalation challenge with LPS. Values are means ± SE in cells ($\times 10^3$)/ml lavage fluid.

ranging from ~100,000 (BALB/c) to >400,000 (LP/J) cells/ml lavage fluid. There did not appear to be a clear correlation between the TLR4 genotype of these strains and the concentration of neutrophils in the airspace because strains with a shared TLR4 genotype (such as BALB/c and A/J) showed a significant difference in PMNs per milliliter of lavage fluid (Fig. 3). On the other hand, the marked difference between the results for C3H/HeJ and C57BL/10ScNCr mice suggests that the mutations in TLR4 encountered in strains C3H/HeJ and C57BL/10ScNCr, compared with those mutations found in the other TLR4 mutant strains, had a much more profound effect on TLR4 function.

The six strains wild type for TLR4 also demonstrated a wide range of neutrophils in the airspace after inhalation of LPS. Strains such as FVB/NJ and C57BL/6 had values of ~150,000 PMNs/ml, whereas strains such as DBA/2J and 129/Svlm had values of >400,000 PMNs/ml (Fig. 3).

The number of neutrophils in the airspace after inhalation of LPS was specific for each strain ($P < 0.0001$) and clearly showed the exceptionally weak response of the hyporesponsive strains C3H/HeJ and C57BL/10ScNCr compared with all other strains tested.

Inbred strains of mice demonstrate clear differences in the release of specific proteins after inhalation of LPS. A possible cause for the difference in neutrophil migration could be the secretion of cellular proteins such as cytokines, chemokines, and adhesion molecules. All three types of proteins have been implicated in mediating neutrophil migration in response to inhaled LPS. We therefore measured the concentration of TNF-α, MIP-2, and ICAM-1 in the lavage fluid after inhalation of LPS. The hyporesponsive strains C3H/HeJ and C57BL/10ScNCr had the lowest concentrations of TNF-α and MIP-2 of all the strains tested in this study ($P < 0.0001$ for both MIP-2 and TNF-α; Fig. 4, *A* and *B*), suggesting that the lack of TNF-α and MIP-2 secretion may be responsible for the absence of LPS-induced neutrophil infiltration into the airspace. However, the concentration of ICAM-1 in the lavage fluid for both of these strains (C3H/HeJ and C57BL/10ScNCr) was not significantly different from that of the other 16 strains tested ($P = 0.7$), suggesting that the hyporesponsive phenotype of C3H/HeJ and C57BL/10ScNCr cannot be explained by ICAM-1 (Fig. 4*C*).

The remaining 10 TLR4 mutant strains showed a significant variation for both MIP-2 ($P = 0.005$) and TNF-α ($P < 0.0001$) secretion (Fig. 4, *A* and *B*). Although none of the strains had levels as low as C3H/HeJ and C57BL/10ScNCr, strains such as SEA/GNJ (TNF-α) and Cast/Ei (MIP-2) had concentrations of these proteins that were lower than those in the remaining strains. The lack of correlation between MIP-2 and TNF-α secretion among the TLR4 mutant strains seems to indicate that different factors determine the expression levels of both of these proteins. As was observed for C3H/HeJ and C57BL/10ScNCr mice, the lavage fluid concentrations of ICAM-1 were similar throughout the TLR4 mutant strains tested, reaffirming a lack of ICAM-1 involvement in the differential neutrophilic response to inhaled LPS (Fig. 4*C*).

Even though the six wild-type strains shared an identical TLR4 genotype, after the inhalation of LPS, the secreted concentrations of TNF-α and MIP-2 in the lavage fluid were highly variable. Although none of the wild-type strains showed levels as low as C3H/HeJ and C57BL/10ScNCr mice, TNF-α levels showed an almost fourfold variation (Fig. 4*A*) and MIP-2 showed a more than eightfold variation (Fig. 4*B*) among wild-type strains for TLR4. These findings suggest that factors other than the genotype of TLR4 affect the secretion of chemokines and cytokines in response to inhalation of LPS. Identical to the findings in the 12 TLR4 mutant strains, lavage fluid concentrations of ICAM-1 for the six TLR4 wild-type strains were remarkably similar in all strains tested ($P = 0.32$; Fig. 4*C*).

DISCUSSION

Our results suggest that TLR4 is important in the response to inhaled endotoxin in mice but that additional genes are likely involved in modulation of the complex physiological and biological phenotype. For the 18 mouse

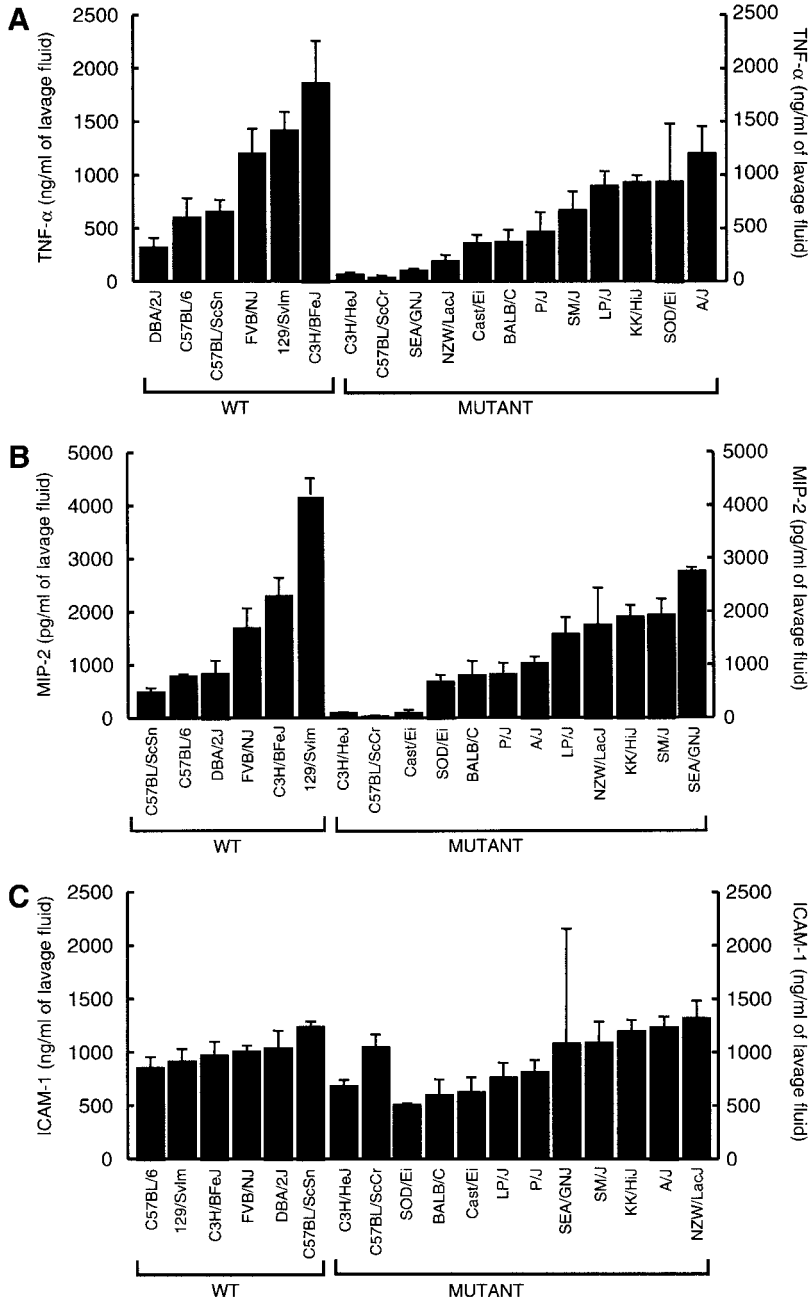

Fig. 4. Concentration of tumor necrosis factor (TNF)-α (*A*), macrophage inflammatory protein (MIP)-2 (*B*), and intercellular adhesion molecule (ICAM)-1 (*C*) in lavage fluid of mice after a single inhalation challenge with LPS. Values are means ± SE.

strains tested, 6 had a common TLR4 allele and 12 had mutations throughout the open reading frame of TLR4. The LPS-hyporesponsive strains (C3H/HeJ and C57BL/10ScNCr) demonstrated a blunted response to inhaled LPS; however, this response was most clearly observed in the concentrations of neutrophils, TNF-α, and MIP-2 in the lavage fluid. Surprisingly, mice with the common allele for TLR4 had substantial variability in their physiological and biological responses to inhaled endotoxin.

As a major LPS receptor in mammals, TLR4 influences a variety of physiological responses that are an important component of the host defense against bacterial pathogens. Although our data indicate that TLR4 influences the physiological response to inhaled LPS, our findings in mice also suggest that other genes may be important in regulating this response. Mutant strains of mice are less responsive physiologically than strains with the common TLR4 allele. Importantly, these findings parallel results in humans in which our laboratory (1) found that mutations in the TLR4 gene were associated with a blunted response to inhaled LPS in some test subjects. Thus both the results presented in this paper and the findings of our laboratory in humans indicate that TLR4 is important in determining the physiological response to inhaled LPS but that other genes are undoubtedly involved.

The ICAM-1 results in this paper suggest that TLR4 is not involved in cell adhesion and that changes in cell adhesion may not be part of the cellular response to inhaled endotoxin. Previous evidence had suggested a

potential involvement of adhesion molecules in mediating the LPS response in mice. ICAM-1-deficient mice have shown an increased resistance to inhaled LPS and lack the airway hyperreactivity of wild-type mice exposed to high doses of LPS (21). In contrast, TNF-α and, to a lesser degree, MIP-2 show a correlation of increased expression with increased responsiveness to inhaled endotoxin and suggest a role for both proteins in mediating the LPS response in mice. This correlates with previous evidence (4) that has shown both cytokines to be regulated by the transcription factor NF-κB, a protein downstream of TLR4 in the LPS response pathway. TNF-α levels have frequently been used as a measure of LPS reactivity because both C3H/HeJ and C57BL/10ScNCr mice lack the increase in TNF-α levels that follows LPS exposure. Our results indicate that TNF-α expression is a reliable indicator for measuring LPS responsiveness in mice.

Besides TLR4, potential genes that would affect cytokine expression and NF-κB translocation include other Toll receptors such as TLR2 that also signal through NF-κB, as well as downstream molecules in the LPS-induced signaling cascade such as MyD88 and MD-2, a cofactor required for TLR4-dependent LPS response (16, 19). The action of other genes that modulate the LPS response in mammals is less well characterized. Ran, a G protein (22), and class II myosin heavy chain molecules (13) have also been indicated as mediators of the mammalian LPS response.

The distinct phenotype of strains C3H/HeJ and C57BL/10ScNCr, both of which have mutations or deletions in the TLR4 gene, suggests that these particular mutations or deletions in TLR4 may specifically affect neutrophil migration. This would explain the near normal levels of neutrophils in the lavage fluid of the Cast/Ei strain, which has numerous mutations spread throughout the TLR4 open reading frame. Further experiments are necessary to determine whether the hyporesponsive phenotype in C3H/HeJ and C57BL/10ScNCr mice is a result of the specific mutation in TLR4 or additional mutations in other genes. There is evidence that mutations other than those in TLR4 contribute to the lack of LPS response in strain C57BL/10ScNCr (11).

The phenotypic variability for endpoints such as TNF-α secretion or neutrophil infiltration underscores the involvement of such additional genes in mediating the immune response to LPS. This scenario would put all such genetic effects downstream of TLR4 and secondary to the initial recognition of LPS by the TLR4 receptor. On the other hand, inherent genetic variability in the TNF-α gene could be the underlying cause for the variability in TNF-α secretion. Naturally occurring mutations in genes involved in neutrophil migration, such as CD11 and CD18, could cause a seemingly hypo- or hyperresponsive phenotype unrelated to the actual TLR4-mediated LPS response. Clearly, more work is necessary to determine the genetic variations in different inbred mouse strains. The ongoing mouse genomic sequencing project will aid in identifying some of the genetic variations in the most commonly used inbred strains.

However, assuming that TLR4 is a key gene in the response to inhaled LPS, the specific mutation in TLR4 in C3H/HeJ mice should be considered further. It is worth noting that the TLR4 protein from mouse strain C3H/HeJ has been found to exert a dominant loss-of-function effect in vitro (20), suggesting an absolute requirement for the presence of the proline residue at position 712 of the open reading frame for protein function. In the same context, mutations in genes other than TLR4 in strains such as DBA/2J and 129/Svlm could result in hyperresponsiveness to inhaled LPS, whereas any of the mutations in TLR4 in hyperresponsive strains such as P/J and NZW/LacJ could constitute gain-of-function mutations. It nevertheless is surprising to see the large number of polymorphisms within the TLR4 gene, given that it is central to the host response to a variety of pathogenic organisms. Smirnova et al. (17) have reported similar findings with respect to the highly polymorphic nature of TLR4 in additional mouse strains as well as in other species. The C3H/HeJ mutation seems to be crucial in reducing the activity of TLR4 because the proximal intracellular domain of TLR4 is highly conserved, whereas the extracellular domain as well as the COOH terminus of the protein varies significantly between mouse strains and species. The recent resolution of the Toll/IL-1 cytoplasmic domain crystal structure (23) seems to support the requirement for a highly organized protein and would explain the varying degrees of polymorphic sites in the different protein domains. It is important to note as well that known polymorphisms in genes such as TNF-α (7) have not been fully characterized for their functional effects, nor have such genes been sequenced in all of the 18 strains tested in this study. Only a full understanding of the functional effects of such polymorphisms in relation to inhaled LPS and to existing polymorphisms in other genes such as TLR4 will yield a complete genetic explanation for the varied response to inhaled LPS. Clearly, more research is necessary to understand the specific role of TLR4 in response to inhaled LPS in mice and potential involvement of these additional genes in this complex phenotype. The results of this study present a first attempt to identify potentially useful strains for further study of this phenotype.

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Present address of E. Lorenz: Wake Forest Univ. School of Medicine, Sect. of Infectious Diseases, Medical Ctr. Blvd., Winston-Salem, NC 27157-1042.

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