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Genetic regulation of endotoxin-induced airway disease

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Abstract

To identify novel genes regulating the biologic response to lipopolysaccharide (LPS), we used a combination of quantitative trait locus (QTL) analysis and microarray-based gene expression studies of C57BL/6J × DBA/2J(BXD) F2 and recombinant inbred (RI) mice. A QTL affecting pulmonary TNF- α production was identified on chromosome 2, and a region affecting both polymorphonuclear leukocyte recruitment and TNF- α levels was identified on chromosome 11. Microarray analyses of unchallenged and LPS-challenged BXD RI strains identified approximately 500 genes whose expression was significantly changed by inhalation of LPS. Of these genes, 28 reside within the chromosomal regions identified by the QTL analyses, implicating these genes as high priority candidates for functional studies. Additional high priority candidate genes were identified based on their differential expression in mice having high and low responses to LPS. Functional studies of these genes are expected to reveal important molecular mechanisms regulating the magnitude of biologic responses to LPS.

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Lipopolysaccharide (LPS), a major component of the cell membrane of gram-negative bacteria, elicits a vigorous innate immune response in many animals, including humans. The magnitude of this inflammatory response affects the progression and outcome of several diseases, including sepsis [16], septic shock [3,5], and progressive airway disease [21,22]. For example, relatively weak responses to LPS can result in life-threatening bacteremia [reviewed in 16], whereas overly vigorous responses can lead to an equally dangerous septic shock [3,5]. LPS is also a component of air pollution and contributes to airway reactivity in agricultural workers [22] and asthmatics [reviewed in 17]. The development of novel therapies for these individuals would be facilitated by an improved understanding of the genes that function in the biologic response to LPS. Some of these genes are likely to also participate in the innate responses to other stimuli, including bacteria, viruses, and

fungi. Thus, identifying novel genes that affect the response to LPS might have considerable impact on the future care of individuals in a wide variety of inflammatory settings.

The biologic response to inhaled LPS is initiated by a receptor complex that includes the toll-like receptor (TLR) 4, CD14, and MD-2. This complex, together with either of the adaptor molecules MyD88 or TIRAP (Mal), initiates a signaling cascade leading to the activation of the transcription factor NF κ B and ultimately to the recruitment of leukocytes and production of proinflammatory cytokines such as tumor necrosis factor (TNF)- α [recently reviewed in 2]. However, much remains to be learned about the molecular mechanisms that regulate this response. Studies of both mice and humans suggest that the magnitude of this response is regulated by multiple, uncharacterized gene interactions [1,15]. For example, the inbred mouse strains C57BL/6J and DBA/2J share a common sequence encoding the Tlr4, a membrane-spanning molecule essential for LPS-induced signaling, yet C57BL/6J mice respond less vigorously to LPS than do DBA/2J mice [15]. This finding suggests that analysis of C57BL/6J × DBA/2J(BXD) recombinant inbred (RI) or F2 mice might be used to identify

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genetic polymorphisms underlying this differential responsiveness. Accordingly, we have studied BXD recombinant mice using two independent gene identification strategies: quantitative trait locus (QTL) mapping and microarray-based gene expression analysis. Individually, each of these approaches usually yields more candidate genes than can be feasibly pursued by functional analysis. However, by combining these two approaches, we have identified a relatively small number of high priority candidate genes that either reside within identified QTL or are differentially expressed between high- and low-responder RI mice.

Results

BXD RI mice display a wide range of phenotypic responses to LPS

To identify genes contributing to the differential responses to inhaled LPS of C57BL/6J and DBA/2J mice, we initially exposed 32 different strains of BXD RI mice to aerosolized LPS prepared from *Escherichia coli*. Biologic responses to this challenge were assessed by the number of polymorphonuclear leukocytes (PMN) in the lung airspace and by levels of TNF- α . The phenotypes of the BXD RI strains ranged

from less responsive than C57BL/6J mice to more responsive than DBA mice (Fig. 1). The gradual increase in response from hyporesponsive to hyperresponsive strains suggests the existence of multiple QTL. Moreover, there was little correlation between TNF- α levels and PMN levels, suggesting the existence of separate QTL affecting these two phenotypic traits. Some strains, however, had low levels of both TNF- α and PMN. Of particular note, the strain BXD29 was essentially unresponsive to LPS in both phenotypic assays. The extreme nature of this phenotype suggested that it might result not from one or more QTL, but to a spontaneously occurring mutation in a gene encoding a protein required for the LPS response. Subsequent genetic and flow cytometric analyses demonstrated that this BXD29 strain has a mutation at the *tlr4* locus (D. Cook, manuscript in preparation).

Identification of a QTL for PMN recruitment

To identify QTLs affecting the differential responses to LPS in the BXD RI mice, marker regression analyses were performed using publicly available software. Several suggestive QTLs were identified, but none reached statistical significance (data not shown). To increase the statistical power of this analysis, we generated 250 individual BXD F2 mice, exposed them to aerosolized LPS, and measured their

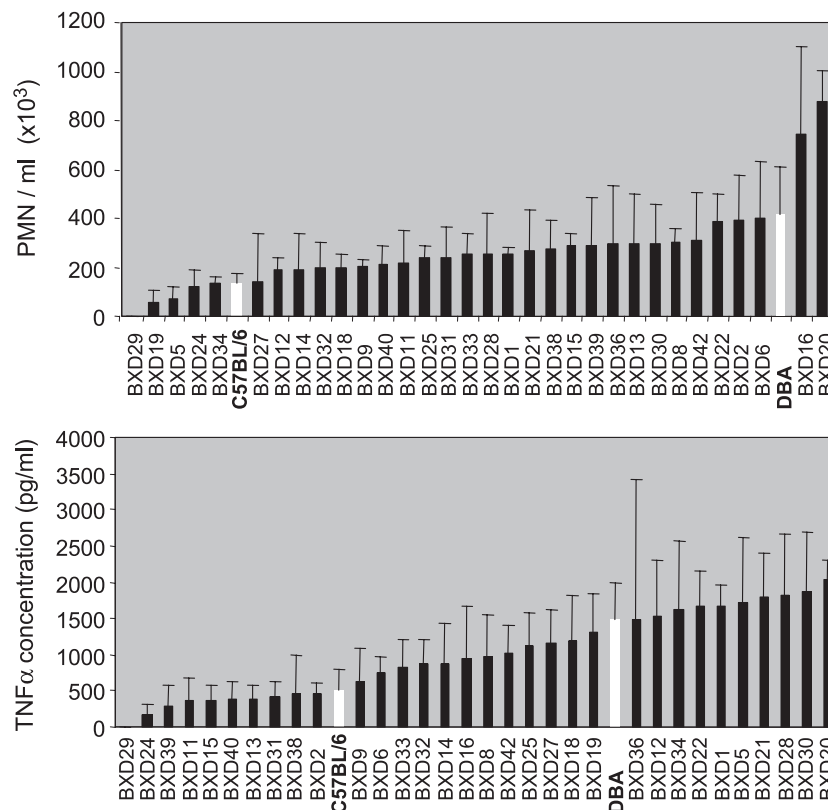


Fig. 1. Phenotypic analysis of BXD RI mice. Mice were harvested immediately after a 4-h exposure to aerosolized LPS. Whole-lung lavage was performed and the fluid assayed for the number of PMN/ml and for TNF- α . The parental strains are indicated by white rectangles and error bars represent standard deviation ($n \geq 5$ mice per strain). Strain BXD29 was essentially unresponsive, probably due to a spontaneously occurring mutation at the *tlr4* locus (D. Cook, manuscript in preparation), and was excluded from the microarray analyses presented here.

levels of PMN and TNF- α in the lung. These F2 mice displayed a wide range of responses similar to that seen in the BXD RI mice (data not shown). Preliminary QTL experiments with DNA prepared from these F2 mice were conducted using a quantitative PCR-based, genome-wide scan comparing parental allele frequencies in separate DNA

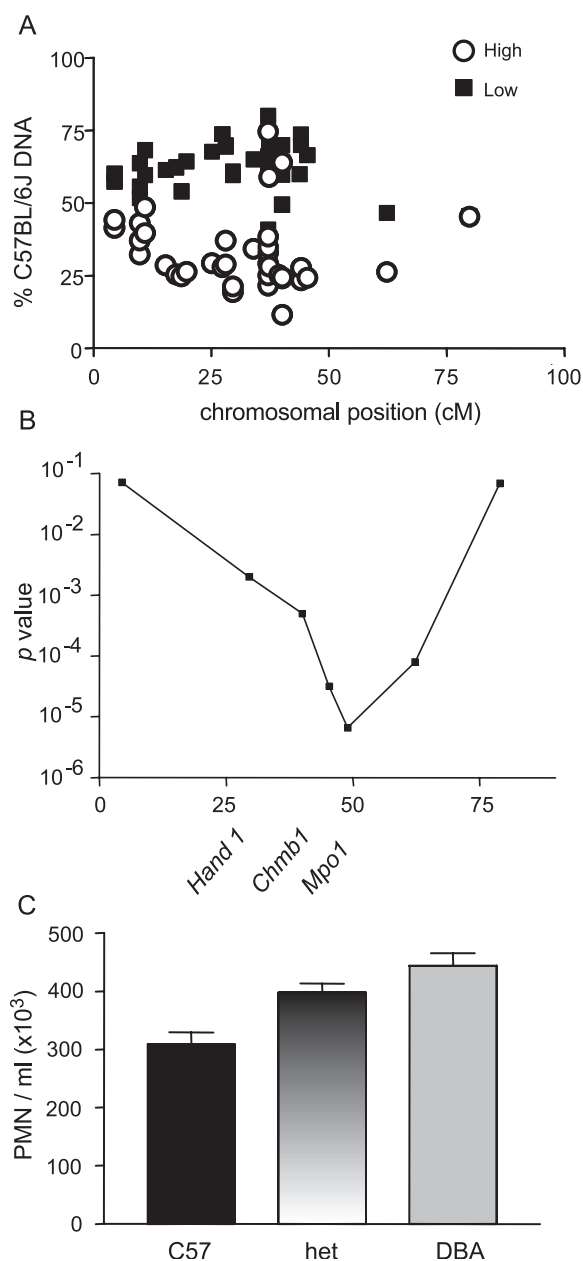


Fig. 2. Identification of a QTL on chromosome 11 for PMN recruitment (*lpl1*). (A) Identification. Parental allele frequencies are shown for DNA pools prepared from BXD F2 mice with high (open circles) or low (solid squares) PMN/ml of lavage fluid. Chromosomal positions for each locus assayed are indicated. (B) Correlation of phenotype and genotype. The p values (t test) for the differences between mean PMN values of mice homozygous for C57BL/6J or DBA/2J DNA are shown for selected loci near the candidate QTL. (C) Phenotypic contribution of *lpl1*. The mean PMN shill/ml value for C57BL/6J and DBA/2J DNA-homozygous and -heterozygous mice are shown for the locus having the lowest p value.

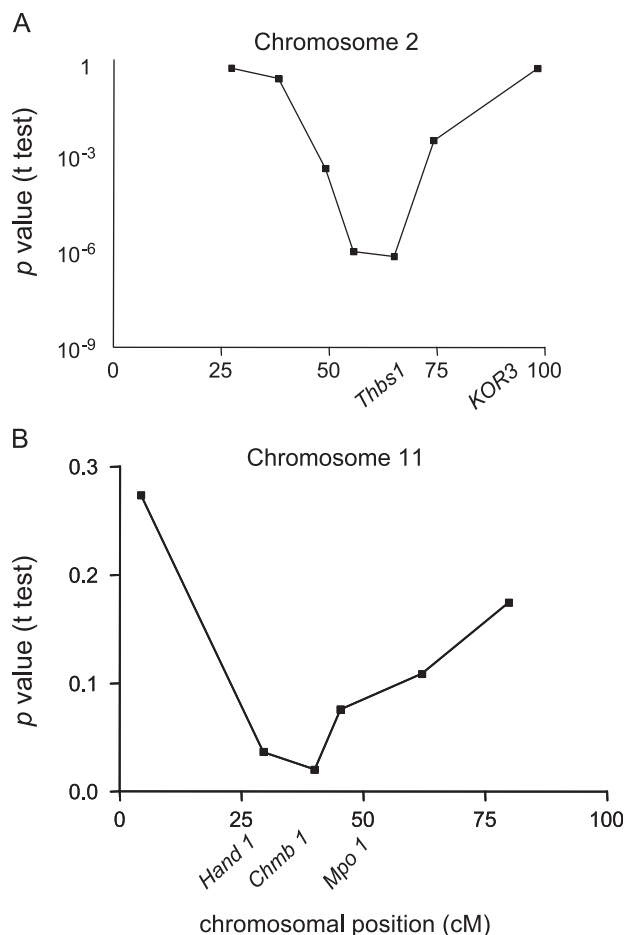


Fig. 3. Localization on chromosomes 2 and 11 of QTL associated with LPS-induced pulmonary TNF- α levels (*lpt11* and *lpt12*). The p values (two-tailed t test) for the differences between mean PMN values of individual mice homozygous for C57BL/6J or DBA/2J DNA are shown for selected loci near candidate QTL on chromosomes (A) 2 and (B) 11.

pools prepared from 25 mice having high and low PMNs, respectively [8]. This genome scan revealed that a region on chromosome 11 was represented predominantly by C57BL/

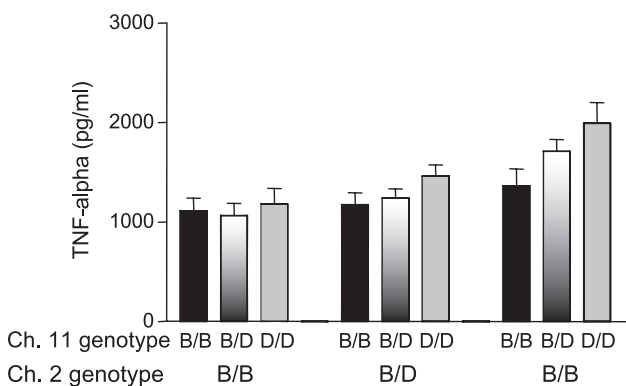


Fig. 4. Interrelationship of *lpt11* and *lpt12*. Pulmonary TNF- α levels in mice having various combinations of parental alleles C57BL/6J (B) and DBA/2J (D) at *lpt11* and *lpt12* are shown. Note that for mice homozygous for C57BL/6J DNA at *lpt12*, TNF- α production is not affected by the genotype at *lpt11*.

6J DNA in the low-responder pool and by DBA/2J DNA in the high responder pool (Fig. 2A). This locus was designated LPS-induced pulmonary PMN locus 1 (*lpp1l*).

To confirm the existence of *lpp1l* and to map more precisely its position, we determined the parental strain identities of all 250 BXD F2 mice at several loci on chromo-

some 11 spaced by intervals of approximately 5 cM. Comparison of the mean PMN values for mice homozygous for C57BL/6J or DBA/2J at these loci revealed that *lpp1l* resides at or near 50 cM (Fig. 2B) and is highly significant ($p < 10^{-5}$; two-tailed *t* test). F2 mice having DBA/2J DNA at this region had a mean PMN concentration approximately 30% higher

Table 1
Gene induction in the lung following inhalation of LPS

GenBank Accession No.	Annotation	Function	Mean LPS induction ratio	C57BL/6J (low PMN; low TNF)	BXD19 (low PMN; high TNF)	BXD5 (low PMN; high TNF)	BXD39 (med PMN; low TNF)	BXD42 (med PMN; high TNF)	BXD16 (high PMN; med TNF)	DBA/2J (high PMN; high TNF)
BE306167	Serum amyloid A3	Acute-phase response	19.0	11.9	25.9	24.0	19.4	11.8	28.4	11.3
BG070106	24p3 lipocalin	Apoptosis	13.7	12.6	16.7	11.8	20.8	9.0	15.9	9.0
BG063925	Metallothionein II	Stress response	9.5	6.4	8.9	10.5	24.9	4.2	6.2	5.6
BG063775			8.8	1.2	0.7	0.8	55.5	1.4	1.2	0.8
AW741846	PPAR γ -binding protein	Transcription	7.2	5.6	13.3	11.1	1.0	2.2	11.9	5.0
BG080268	KC precursor	Chemokine	6.8	4.2	4.8	10.7	8.9	1.5	8.3	9.4
BG072801	S100A9	Calcium binding	5.2	4.0	3.6	5.3	15.3	1.2	3.6	3.4
C85808			5.1	3.7	5.8	7.9	8.7	2.6	2.8	4.4
BG065761*	B94	Signal transduction	5.1	5.1	7.5	6.8	5.9	2.2	4.6	3.7
BG088898			4.7	3.9	5.3	3.5	8.2	2.5	6.9	3.0
BG071318			4.5	5.0	6.4	2.5	7.1	3.5	2.7	4.1
BG071239			4.3	4.8	7.6	3.3	4.8	2.8	2.5	4.3
BG065735	C-type lectin	Cell surface receptor	4.3	5.4	2.6	8.8	6.0	1.4	3.2	2.7
AI181127	gp49A	Signal transduction	4.1	4.4	4.6	4.3	4.9	1.7	5.4	3.6
AW538759*			4.1	5.6	4.4	4.6	4.0	3.3	3.4	3.7
BG077818	Metallothionein I	Stress response	4.0	2.5	5.1	1.8	8.1	3.6	2.8	4.1
BG078274	I κ B α chain	Transcription	3.8	4.1	5.0	3.7	5.4	2.0	2.6	3.8
BG067341			3.7	3.8	5.4	3.0	5.8	2.0	3.2	2.8
BG068331			3.6	3.3	3.8	3.4	6.2	2.7	3.4	2.6
BG067620*			3.6	3.1	4.8	4.7	5.1	1.9	3.0	2.5
BG067349	Mac. C-type lectin Mincle	Cell surface receptor	3.6	3.2	2.0	6.5	2.2	1.3	6.2	3.8
BG066678			3.6	4.1	3.9	3.4	5.4	2.0	3.4	2.9
C86354*	Protease-nexin 1	Thrombin inhibitor	3.5	3.8	4.7	4.4	4.1	2.2	2.9	2.5
BG078398	MARCKS-like protein	PKC substrate	3.5	3.5	2.7	3.7	5.3	1.8	4.7	2.7
BG067670			3.5	3.8	5.5	2.7	5.0	2.1	3.1	2.1
BG066267			3.5	2.8	3.4	4.4	5.7	2.0	3.1	2.8
BG064781	CTP synthase	Nucleotide metabolism	3.4	3.9	1.9	3.5	3.7	2.4	4.6	3.8
BG076991	SOCS-3	Signal transduction	3.3	4.0	3.5	3.3	4.0	2.1	3.7	2.7
BG069325	Nef-associated factor 1	CD4 regulation	3.3	2.6	5.0	4.1	4.2	1.9	3.1	2.4
BG076041*			3.3	4.9	5.4	3.5	2.3	1.8	3.1	2.3
BG067419*	OIG37	Cell growth inhibitor	3.3	3.2	3.0	3.0	8.7	1.3	2.2	1.5
BG080688	CSF-1	Cytokine	3.3	3.4	3.2	4.0	3.0	2.1	4.6	2.7
BG084405			3.3	3.0	3.4	4.4	4.1	1.8	2.9	3.2
BG071169			3.3	3.6	2.8	2.9	4.7	3.1	1.9	3.7
BG068491*			3.2	3.1	3.5	3.3	5.1	2.3	2.9	2.4
BG073108*	G7e		3.1	2.8	4.6	3.9	5.1	1.9	2.0	1.6
BG063041			3.1	1.4	2.5	6.1	2.5	2.0	2.4	4.6
BG068648*			3.0	3.9	5.2	3.7	1.0	2.3	3.1	2.0
BG066914			2.9	0.8	0.4	8.6	6.3	1.5	1.0	1.5
BE373183	Stearoyl-CoA desaturase	Lipid metabolism	2.8	3.0	3.5	1.0	3.2	2.8	2.8	3.6
BG074378			2.8	3.1	4.8	2.5	2.8	1.7	2.6	2.1

Ratios of gene expression for LPS-challenged/unchallenged mice are shown for each strain studied and for mean expression across all strains. The 50 most highly induced genes are presented; for additional genes, see Web site. Phenotypic responses for each strain are indicated, based on data shown in Fig. 1. Genes whose induction ratios differ significantly between strains having a high and low PMN response are denoted with an asterisk.

than mice having C57BL/6J DNA at this position (Fig. 2C). Although this difference might not be physiologically meaningful, the statistical significance of this difference demonstrates that a gene impacting innate immune responses resides within the QTL and that a polymorphism in this gene accounts for a large proportion of the difference in PMN recruitment between the two parental strains. Approximately 300 genes reside within the 35 cM encompassing *lpp11*, including several genes encoding proteins having a plausible role in the pathway leading to PMN recruitment: interferon regulatory factor-1, TNF- α -induced protein-1, and several CC chemokines.

Identification of QTLs associated with TNF- α levels

To identify QTLs associated with pulmonary TNF- α levels, we determined parental allele frequencies in separate DNA pools created from high- and low-TNF- α -producing BXD F2 mice. Loci associated with this phenotype were identified on chromosome 11 and on chromosome 2 and designated LPS-induced pulmonary TNF- α locus 1 (*lptl1*) and *lptl2*, respectively. Genotyping analysis was performed on all F2 mice at several loci on these two chromosomes to confirm the QTLs and to map more precisely their positions

(Fig. 3). *lptl1* is located near cM 45 on chromosome 11, at or near *lpp11*, the locus affecting PMN recruitment. Of these two loci, *lptl2* has a larger impact on TNF- α levels. Mice having DBA/2J DNA at *lptl2* have a 59% increase in TNF- α levels compared to mice having C57BL/6J DNA at *lptl2* (1745 \pm 96 and 1100 \pm 83 pg/ml, respectively). The current boundaries of *lptl2* encompass approximately 160 known genes, including several genes whose known or suspected actions are consistent with a possible role in the biologic response to LPS. These genes include two zinc-finger proteins, interleukins 1 α and 1 β , CD59a antigen, and CD44 antigen. The chromosomal locations of the two identified QTLs collectively exclude most genes having known roles in the signaling response to LPS, including those for Tlr4, lipid binding protein, MyD88, MD2, interleukin-1 α receptor-associated kinases, TNF- α -associated factors, I κ B, NF κ B, and TNF- α itself.

Interrelationships between the QTL associated with levels of TNF- α

To investigate the relationship between the two identified QTLs, we compared responses of mice having different combinations of parental strain alleles at these loci. Mice

Table 2
Additional genes differentially expressed in high and low LPS responder strains

GenBank Accession No.	Annotation	Function	BXD strains						
			Low PMN responders			p	High PMN responders		
			BXD19	C57	BXD5		BXD42	DBA	BXD16
BG072805			0.3	0.7	0.3	0.01	1.1	1.2	1.1
BG085649	Similar to KIAA0963		2.8	3.1	2.9	0.02	1.4	1.7	2.4
BG068040			1.0	1.2	1.1	0.04	1.0	0.8	0.8
BG088587	Beclin 1	Tumor suppressor/ autophagy	1.3	1.1	1.1	0.04	1.0	0.9	1.0
AU017639			0.3	0.7	0.7	0.04	1.5	1.0	1.1
BG079067	β 2-ad. receptor	Cell signaling	1.5	1.6	1.6	0.05	1.0	1.3	0.5
BG067350			2.1	1.9	1.7	0.05	1.5	1.5	1.7
BG068359	Ceruloplasmin	Fe ⁺ metabolism	2.7	2.4	2.5	0.05	1.6	2.1	2.2
BG070406			0.7	0.6	0.4	0.05	1.7	0.8	1.2
BG072324			0.7	0.7	0.3	0.05	1.0	1.4	0.9
			Low TNF α responders		p	High TNF α responders			
			C57	BXD39		BXD19	BXD5	DBA	
BG077031			1.4	1.4	0.01	0.7	0.8	0.9	
BG065111	Histone H2A	Chromatin	1.5	1.6	0.01	2.5	2.5	2.1	
AW543947			1.7	1.9	0.02	2.3	2.5	2.3	
AW229040	Lysyl oxidase	ECM	1.5	1.0	0.02	2.4	2.9	2.3	
BG074387			2.7	3.0	0.02	2.2	2.2	1.9	
BG078967	DAG acyltransferase	Metabolism	0.8	0.5	0.03	1.2	1.0	1.1	
BG066984	N2	Amino acid transport	1.0	1.1	0.03	0.4	0.6	0.7	
BG068664			0.9	0.9	0.04	0.5	0.7	0.7	
BG071417			0.7	0.2	0.04	1.1	1.1	1.0	
AA408602	Chaperonin 10-like		1.2	1.5	0.04	0.5	0.3	0.9	
C77656	PP peptidase	Protein metabolism	2.4	2.3	0.05	1.5	1.9	1.9	
AI314900	GAS 6	Growth factor	0.3	0.7	0.05	1.0	1.0	0.9	

p values were calculated with a t test using mean gene induction ratios corresponding to the low- and high-responder strains as measured by levels of PMN and TNF α in whole-lung lavage fluid.

having C57BL/6J DNA at *lptl2* had relatively low TNF- α levels, irrespective of the parental strain identity at *lptl1* (Fig. 4). In contrast, a progressive increase in TNF- α levels was seen in mice having C57BL/6J DNA, heterozygous DNA, or DBA2/J DNA at *lptl2*. Mice homozygous for DBA2/J DNA at both QTLs had mean TNF- α production values at least as high as that of the high responder parental strain, DBA/2J. This finding suggests that these two QTL are the major loci contributing to the differential responsiveness of C57BL/6J and DBA/2J mice and that C57BL/6J DNA at *lptl2* has a dominant effect on pulmonary TNF- α levels.

Identification of LPS-induced genes

To identify genes whose steady-state mRNA levels are changed by inhaled LPS, microarray-based gene expression studies were conducted on both unchallenged and LPS-challenged mice of the C57BL/6J and DBA/2J parental strains, as well as on four BXD RI strains (BXD5, BXD16, BXD19, and BXD42) that displayed either high or low phenotypic responses. This analysis identified approximately 500 known genes or expressed sequence tags (ESTs) whose expression significantly changed following LPS exposure in at least one BXD RI strain. A subset of these genes having a mean expression change of at least 2.8-fold across the six strains is shown in Table 1. The most highly induced gene was that for lipocalin 24p3, which induces apoptosis in cultured hematopoietic cells [6]. The induction of lipocalin in the lung following inhalation of LPS suggests that this gene might have a causal role in apoptosis seen in pulmonary neutrophils and endothelium after LPS exposure [4,9]. Other LPS-induced genes included those for metallothioneins I and II, the chemokine KC, the apoptotic factor B94, and the inhibitor of NF κ B function I κ B. Many genes not previously associated with the LPS response were also identified, including several genes having functions consistent with a plausible role in the response to LPS. These include the inhibitor of apoptosis protein 1 (IAP-1), which is up regulated by NF κ B in vascular smooth muscle [7], and GP49, an immunoglobulin-like molecule associated with production of eicosanoids and cytokines [14]. The gene whose expression was most consistently decreased in LPS-exposed-lungs encodes the Ets-related transcription factor, suggesting that this protein might negatively regulate the LPS response.

Association of gene expression level with biologic response to LPS

Microarray analyses typically yield many more genes than can be feasibly pursued by functional studies. To stratify candidate genes in an objective manner and thereby obtain a relatively small number of high priority candidate genes, we used two different approaches. First, we compared gene expression changes in mice having high or low biologic responses to LPS. This analysis identified 30 genes whose mean change in expression after LPS was signifi-

cantly different between low- and high-responder strains, as determined by relative levels of pulmonary PMN or TNF- α (Tables 1 and 2). Among these genes are several uncharacterized ESTs as well as some known genes, including B94, originally described as a novel TNF- α -inducible primary response gene in endothelial cells [25].

LPS genes residing in identified QTL

As a second approach to stratifying genes identified by microarray analysis, we combined the use of positional

Table 3
LPS-induced genes within QTL

GenBank Acc. No.	Annotation	Induction ratio C57BL/6J	Induction ratio DBA/2J	High/low ratio (TNF)	High/low ratio (PMN)
<i>Chromosome 11</i>					
BG063430	Eukaryotic initiation factor 4A	2.2	1.8	0.8	0.7
BG077569	Zinc-finger protein 91	1.3	0.9	2.2	0.4
BG064979		0.9	0.9	0.7	1.5
BG078838	EGFR-binding protein 7	0.5	1.1	0.7	1.6
BG065736		1.1	0.8	0.3	0.9
BG079623		0.6	1.2	0.9	2.2
BG066671		1.0	0.8	1.6	1.0
BG066947		1.7	0.9	0.9	1.1
C81585		0.8	0.8	1.1	1.4
BG067642		2.0	2.0	1.0	0.8
BG081711		0.9	0.7	0.8	1.3
BG069859		1.1	0.7	3.0	0.4
BG070652		0.8	0.9	3.0	0.4
BG084700		1.4	1.4	3.4	0.3
BG072235		1.3	1.0	0.8	1.4
BG072851		0.9	1.0	2.2	0.5
BG086752		0.8	1.0	0.9	1.0
BG076245	IGTP	1.6	1.6	0.9	0.8
<i>Chromosome 2</i>					
BG063885		2.2	1.8	1.3	0.7
BG078835		0.5	1.1	2.6	0.9
BG079704	Cleavage stimulation factor	2.4	0.8	1.8	0.3
BG067121		1.2	1.2	1.2	1.0
BG067899		1.1	0.6	0.6	0.9
BG068522		0.9	0.9	0.5	1.0
BG069000		1.4	0.9	0.9	0.4
BG069473	Ets homologous factor	2.0	2.4	0.9	0.9
BG069668		0.5	1.0	1.4	1.5
BG073335		0.7	1.0	1.9	0.7

Genes residing within the identified QTL (chromosome 2, 50–75 cM; chromosome 11, 25–60 cM) are listed, together with their fold change following exposure to LPS in the parental strains, C57BL/6J and DBA/2J. Also shown is the ratio of gene expression between high and low responders as measured by levels of TNF- α and PMN. Note that most induced elements within these regions do not correspond to known genes.

cloning together with microarray analysis to identify 28 genes whose expression was significantly changed by LPS and that also reside within the boundaries of the identified QTL (Table 3). These genes have an increased likelihood of having a *cis*-acting polymorphism that is causally linked to the LPS response and therefore represent high priority candidate genes.

Discussion

The studies described here were carried out to identify genes, other than *tlr4*, that have a causal role in the innate immune response to inhaled LPS. Two independent strategies were used: microarray-based gene expression analysis and QTL identification. The microarray analysis identified approximately 500 genes whose expression was significantly changed in at least one of the mouse strains studied. These genes can be grouped into three categories: genes whose induction by LPS has been previously described in other systems, genes not previously associated with the LPS response, and uncharacterized ESTs.

Many of the genes identified by our microarray studies have been previously identified as being induced by LPS in cultured cells such as macrophages [23]. Our results demonstrate that these genes are also induced *in vivo* and suggest they might be functionally relevant to the biologic response to LPS in the lung. Several genes not previously associated with the response to LPS have properties consistent with a plausible role in that response. For example, IAP-1 has not been described as an LPS-inducible gene, although it is up regulated by NF κ B in vascular smooth muscle [7]. IAP-1 might contribute to the expansion of vascular or airway smooth muscle following exposure to LPS, providing a molecular link between TLR4 function and smooth muscle proliferation [20]. Another up regulated gene of this class, GP49A, encodes an immunoglobulin-like receptor that causes calcium mobilization, eicosanoid production, and cytokine gene transcription through its incorporation into cell membranes [14]. Although most genes whose expression changed following inhalation of LPS had increased expression, some, including the Ets-related transcription factor, were consistently down regulated in LPS-exposed lungs.

Many genes identified by the microarray analyses are likely to have causal roles in the biologic response to LPS. Demonstrating causality requires proof that a change in the level or activity of the encoded gene product measurably affects that biologic response. Due to time and resource requirements, these experiments cannot easily be performed on a large number of genes. Therefore, it is important to prioritize candidate genes identified by microarray studies. Genes have previously been prioritized based on their absolute expression levels or relative change in expression following a stimulus. However, this approach suffers from several inherent drawbacks that lead to both false positives

and false negatives. First, it is likely that many highly expressed genes function relatively late in the response cascade and may therefore not have a causal role. Second, following LPS inhalation, leukocytes are recruited to the lung and remain in the interstitium or airspace even after lavage and vascular perfusion. Therefore, mRNAs corresponding to genes constitutively expressed in these recruited leukocytes are more abundant in lungs of challenged mice than in unchallenged mice even though rates of transcription may not change. Finally, important genes that are highly induced in a minor cell population such as dendritic cells will effectively be “diluted” in RNA prepared from whole lung.

An alternative strategy, which we have used here, is to prioritize LPS-induced genes that reside within a QTL associated with a biologic response. These QTL were identified based on differences in innate immune phenotypes due to polymorphisms between C57BL/6J and DBA/2J mice. Depending on the nature of the polymorphism, it might have either a large or a small effect on innate immune responses. Therefore, the magnitude of the difference between low and high responders does not necessarily reflect the relative importance of the causal gene in innate immunity. Regardless of the relative impact of the polymorphism, the high statistical significance associated with the difference between genotypes indicates with a high probability that a gene associated with innate immunity exists within the mapped region.

The strain-specific phenotypic differences we see might be due to a polymorphism affecting expression of that gene. It is this type of polymorphism that is most likely to be identified by combining QTL and gene expression analyses. However, a coding region polymorphism might also be identified by this procedure if the expression level of that gene is significantly changed by LPS. Genes whose expression is not changed by LPS will not be identified by this strategy, although polymorphisms distinguishing the two parental inbred strains can theoretically be identified by a strain-specific comparison of the coding regions of genes within the QTL. Together, these types of analyses should reveal all categories of genes underlying the identified QTLs.

Many LPS-induced genes not located within the boundaries of the identified QTL may nonetheless have important roles in the biologic response. Genes whose steady-state levels of mRNA influence a biologic response can be identified by comparing transcript levels in responsive and unresponsive recombinant inbred lines. The statistical power of the present analysis is limited by the relatively small number of BXD RI strains analyzed by microarray. Future studies will increase the power of analysis by determining expression levels of select genes in all 32 RI strains using quantitative PCR.

Ultimately, functional studies will be required to identify LPS-induced genes having a causal role in the biologic response. Preliminary *in vitro* tests for causality might

include the use of small interfering RNA molecules to determine the consequences of gene-specific interference on TNF- α production in LPS-challenged cells in culture. Together with subsequent gene-targeting studies, this approach should lead to an improved understanding of the pathogenesis of LPS-induced airway disease, sepsis, and septic shock. Moreover, the general strategy described here should be applicable to any mouse model of human disease and should therefore facilitate identification of many genes causally related to human disease.

Methods

Mice

All mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in the vivarium at Duke University. All procedures were carried out according to the Duke University guidelines for animal use and care.

Quantitative PCR-based genotyping assays

Real-time PCR assays were performed using a 7700 thermocycler (Applied Biosystems, Foster City, CA, USA) using standard conditions recommended by the manufacturer. Sequences corresponding to parental strain allele-specific primers were obtained from the Roche mouse SNP Web site. Percentages of parental strain DNAs were calculated using the formula $\% = 100 \times 1/(1 + 2^{\Delta C_t})$, where C_t is the PCR cycle number (C) at which the intensity of fluorescence crosses a set threshold (t). For genome-wide analysis of F2 populations, parental allele frequencies at 305 different loci were determined. High- and low-responder DNA pools were created by combining equal quantities of DNA from the 25 mice having the highest and lowest biologic responses, respectively [8].

Endotoxin exposures and phenotypic analyses

Mice were exposed to a monitored, 4-h exposure to an aerosol of LPS. The target concentration of LPS in this aerosol was $6 \mu\text{g}/\text{m}^3$, as previous studies have shown this dose to be comparable to that received by agricultural workers in an 8-h work day [11,18]. The actual concentration of LPS in the chamber was determined by placing filters in the exhaust tubing that were periodically collected, and the levels of LPS on them were determined using a *Limulus* amoebocyte lysate assay (QCL-1000; Whittaker Bioproducts, Walkersville, MD, USA). Measured concentrations of LPS ranged from 4.7 to $6.16 \mu\text{g}/\text{m}^3$. Following the exposure, mice were sacrificed by CO_2 , their lungs lavaged, and differential cell counts done as previously described [15]. TNF- α levels were assayed using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

RNA preparation

Total RNA was prepared using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to procedures provided by the manufacturer.

Microarrays

Mice of the parental strains C57BL/6J and DBA/2J, as well as the RI strains BXD5, BXD19, BXD39, and BXD16, were sacrificed following their exposure to aerosols of LPS, and their airways and pulmonary vasculature were washed with PBS to remove leukocytes that might otherwise contribute to the RNA preparation. RNAs prepared from lungs of these mice, and from their unchallenged counterparts, were labeled and used to interrogate cDNA microarrays prepared using the National Institute on Aging 15k Clone Set [12,24], with each clone spotted twice per array. Probes were made from total RNA and hybridized to the arrays as described by Hegde et al. [10], with minor modifications. The hybridization scheme was a combination of the loop and reference designs described by Kerr and Churchill [13], with the reference being pooled total RNA from both exposed and unexposed DBA/2J and C57BL/6J mice. Each pair of samples to be compared was hybridized twice, with the samples labeled with the opposite dye for the second hybridization. The mean Cy3 and Cy5 values from each spot were extracted from the images of the hybridized slides using TIGR Spotfinder [19]. For each strain, the hybridization data from the relevant models (e.g., BXD5 exposed to LPS, BXD5 unexposed, and the reference) was normalized using loess regression implemented in MIDAS [19] with smoothing parameter set to 0.3. Normalized data from both direct and inferred comparisons through a common reference were used to calculate relative expression ratio comparisons between exposed and unexposed animals within each strain. Significantly differentially expressed genes were identified using an intensity-dependent Z score [26] with a threshold of $Z > 1.96$ (95% confidence). The union of all significantly regulated genes was then collected and a one-parameter t test was used to identify genes that significantly differentiated ($p < 0.005$ by permutation testing) high and low responders.

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