INFECTION AND IMMUNITY, Jan. 2005, p. 532–545 Vol. 73, No. 1
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.1.532–545.2005

Central Role of Toll-Like Receptor 4 Signaling and Host Defense in Experimental Pneumonia Caused by Gram-Negative Bacteria

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Received 29 July 2004/Returned for modification 7 September 2004/Accepted 2 October 2004

Toll-like receptor 4 (TLR4) has been identified as a receptor for lipopolysaccharide. However, the precise role of TLR4 in regulating gene expression in response to an infection caused by gram-negative bacteria has not been fully elucidated. The role of TLR4 signaling in coordinating gene expression was assessed by gene expression profiling in lung tissue in a mouse model of experimental pneumonia with a low-dose infection of *Klebsiella pneumoniae*. We analyzed four mouse strains: C57BL/6 mice, which are resistant to bacterial dissemination; 129/SvJ mice, which are susceptible; C3H/HeJ mice, which are susceptible and have defective TLR4 signaling; and their respective control strain, C3H/HeN (intermediate resistance). At 4 h after infection, C57BL/6 and C3H/HeN mice demonstrated the greatest number of genes, with 67 shared induced genes which were TLR4 dependent and highly associated with the resistance phenotype. These genes included cytokine and chemokine genes required for neutrophil activation or recruitment, growth factor receptors, MyD88 (a critical adaptor protein for TLR signaling), and adhesion molecules. TLR4 signaling accounted for over 74% of the gene expression in the C3H background. These data suggest that early TLR4 signaling controls the vast majority of gene expression in the lung in response to an infection caused by gram-negative bacteria and that this subsequent gene expression determines survival of the host.

It is now known that recognition of lipopolysaccharide (LPS) by the host is mediated by Toll-like receptor 4 (TLR4) and is responsible for the early innate immune response to this agent (3, 23). However, the precise role that TLR4 plays in coordinating gene expression in response to an intact gram-negative infection where LPS is only one of many virulence factors remains unclear. It has been previously reported in experimental animal models of TLR deficiency that TLR4-deficient mice are more susceptible to *Salmonella* sp. infection (10) and that TLR2-deficient mice are more susceptible to *Staphylococcus aureus* infection (29). In both models, reduced survival was associated with uncontrolled bacterial growth.

The genomic response of purified cell populations such as dendritic cells (15) and macrophages (21) in response to purified bacterial ligands like LPS or whole organisms have revealed distinct genetic programs. Huang and colleagues showed that dendritic cells respond to *Escherichia coli*, *Candida albicans*, and influenza virus infection with both distinct and shared gene expression profiles. *E. coli* infection resulted in the greatest number of gene expression changes, with 118 of 685 genes being unique to *E. coli* and 166 being shared between influenza, *C. albicans*, and *E. coli*. Nau and colleagues showed similar findings of shared transcriptional programs in human macrophages but also demonstrated that LPS resulted in a gene expression profile similar to that of live *E. coli*, suggesting that LPS controls the dominant response in macrophages to this organism (21, 22). Furthermore, that group showed that infection of human peripheral blood mononuclear cells with *Mycobacterium tuberculosis* poorly induced interleukin-12 (IL-12) p40 and IL-15 compared to *E. coli* or *S. aureus*, which may in part explain immune evasion by this organism (21).

Despite these data, it remains unclear on a genomic scale what the contribution of TLR4 signaling is in the lung in response to infection by gram-negative bacteria. To address this question, we performed gene expression profiling using whole lungs of TLR4-deficient C3H/HeJ mice and C3H/HeN mice with intact TLR4 as well as resistant (C57BL/6) and susceptible (129/SvJ) strains of mice in an experimental model of *Klebsiella pneumoniae* infection. We chose this organism because it is capable of eliciting pneumonia with very small inocula (35, 36), and the growth curves of this bacteria are similar in these mouse strains from time 0 to 16 h, such that changes in gene expression would not be due to changes in organism burden over this time course (36). We chose C3H mice since the C3H/HeJ mutation was initially characterized in these mice by using C3H/HeN as a control which is nearly isogenic, with the exception of the TLR4 mutation (28). $TLR4^{-/-}$ mice have been made on the 129/SvJ background with subsequent backcrossing to C57BL/6; however, significant 129/SvJ alleles still exist in this strain (14). Moreover, since 129/SvJ mice show susceptibility to infections caused by gram-negative bacteria independent of TLR4, as outlined below, data from $TLR4^{-/-}$ mice may be confounded by these 129 alleles.

By using susceptible TLR4 mutant C3H/HeJ mice (23), it was shown that only 42 genes out of 14,700 genes were significantly induced by twofold or more at 4 h compared to a total of 184 genes in resistant C57BL/6 mice or 130 genes in resistant C3H/HeN mice which were induced. These data demon-

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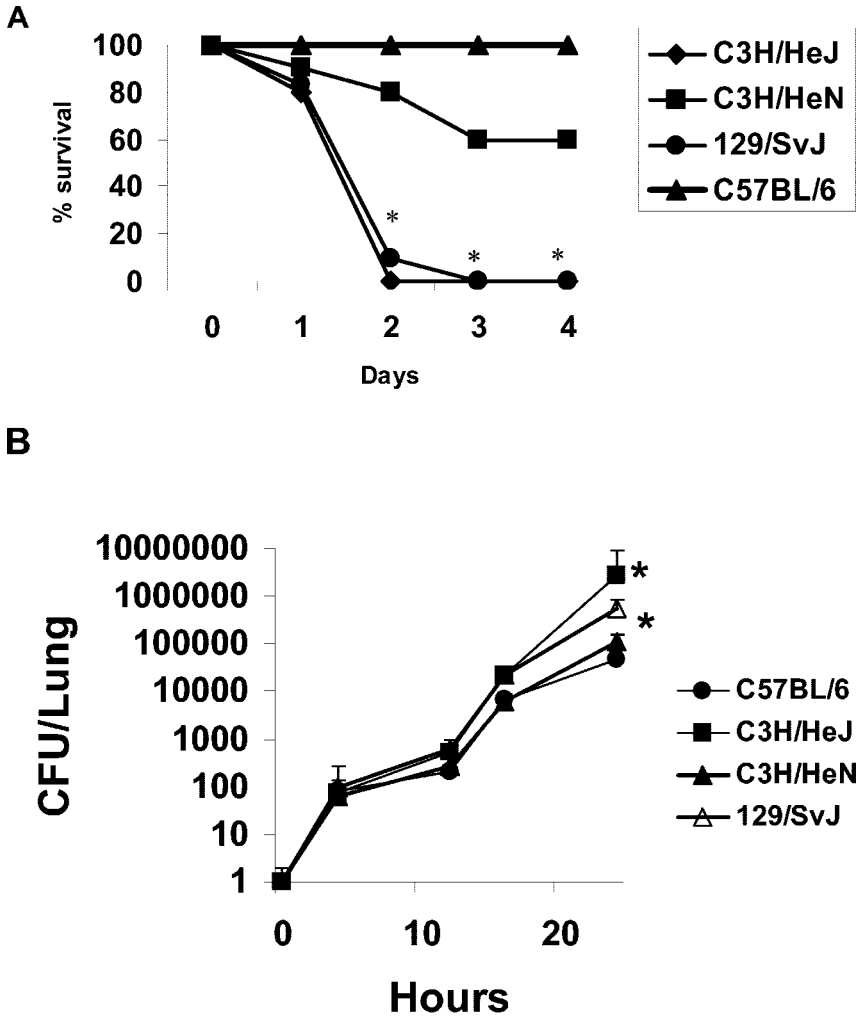

FIG. 1. Panel A: reduced survival of mice deficient in TLR4 signaling. 129/SvJ, C3H/HeJ, C3H/HeN, and C57BL/6N ($n = 10$ mice per group) were challenged with intratracheal inoculation of 10^4 CFU of *K. pneumoniae*, and the survival rate was recorded every 24 h. An * denotes significant difference ($P < 0.05$; log-rank test) compared to C57BL/6 mice. Panel B: lung bacterial burden in each mouse strain over time ($n = 4$ to 6 mice per time point). An * indicates a P value of <0.05 compared to C57BL/6 mice.

strate that TLR4 is critical for gene induction and accounts for over 81% of acute gene expression changes in C57BL/6 mice and over 74% of acute gene expression changes in C3H/HeN mice. Moreover, we identified 67 genes that were shared between resistant C57BL/6 and C3H/HeN mice which were clearly TLR dependent. Although TLR4 signaling was critical in early gene expression, hierarchal clustering showed that TLR4 mutant mice "catch up" by 16 h, as evidenced by the fact that gene expression profiles in C3H/HeJ mice at 16 h cluster with C57BL/6 and C3H/HeN mice with intact TLR at 4 h. This result may be due to other TLR pathways such as TLR2 or TLR9 which recognize lipopeptides (25) and CpG DNA (12), respectively.

MATERIALS AND METHODS

Mice. C3H/HeJ and 129/SvJ male mice (6 to 8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, Maine), while C3H/HeN and C57BL/6 male mice (6 to 8 weeks old) were obtained from the National Cancer Institute (Frederick, Md.). All mice were maintained according to protocol approved by the Institutional Animal Care and Use Committee. Mice were provided with sterile food, water, and filtered air and were housed with 12-h light-dark cycles in the Louisiana State University Health Sciences Center Animal Care Facility.

Bacteria. *K. pneumoniae* strain ATCC 43816 serotype 2 (American Type Culture Collection, Rockville, Md.) bacteria were grown in 100 ml of tryptic soy broth (Difco, Sparks, Md.) for 18 h at 37°C. After 18 h, 1 ml of the culture was added to a fresh 100 ml of tryptic soy broth and grown for 2 h at 37°C. The culture was centrifuged at 2,700 × *g* for 15 min, and the supernatant was discarded. The bacterial pellet was washed twice with phosphate-buffered saline (PBS) and serially diluted to the desired concentration. The concentration of bacteria was measured by calculating the number of CFU on tryptic soy agar plates (Remel, Lenexa, Kans.).

Experimental animal procedures. All mice were anesthetized with 50 μl of PBS-diluted ketamine-xylazine (50 to 150 mg/kg). The mice were intratracheally inoculated with 10^4 CFU of *K. pneumoniae*/ml in a 50-μl volume. At 0, 4, and 16 h postinoculation, the mice were euthanized, the heart and both lungs were excised, and the right ventricle was flushed with PBS. The right lung was isolated, homogenized in 1 ml of Trizol (Invitrogen, Carlsbad, Calif.), and placed at −80°C.

Preparation of labeled cRNA. RNA was extracted from tissues in Trizol (Invitrogen) according to manufacturer's protocol. The SuperScript Choice system (GIBCO/BRL) in combination with a T7-$(T)_{24}$ DNA primer (5′-GGCCAGTG AATTGTAATACGACTCACTATAGGGAGGCGG-d$(T)_{24}$-3′; Integrated DNA Technologies) was used to synthesize cDNA from total RNA. The first-strand DNA

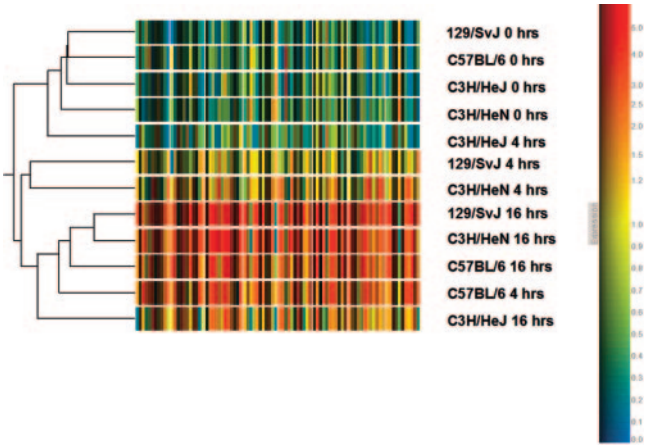

FIG. 2. Cluster analysis showing relative RNA message levels of genes found differentially expressed in C57BL/6N mice in response to *K. pneumoniae* intratracheal challenge at 0, 4, and 16 h postchallenge. Gene lists were generated by using algorithms from Affymetrix MAS version 5.0 and mined by using Affymetrix DMT version 3.0, and lists were imported into Genespring version 6.0 in order to generate a cluster. Only those genes that were significantly upregulated and that had a greater than threefold change in gene expression in all strains were used to generate a hierarchical cluster using a standard correlation. A total of 90 genes met these requirements (see Table S1 in the supplemental material [http://www.medschool.lsuhsc.edu/genetics/genechip_record.asp]). All strains at the zero-hour time point clustered together. Overall gene expression is similar for 129/SvJ and C3H/HeJ mice at 4 h in that they share a common node. Interestingly, the 16-h time point for C3H/HeJ shows a gene expression pattern similar to that of C57BL/6N at just 4 h, indicating that key TLR4-dependent immunomodulators produced within 4 h of *K. pneumoniae* challenge contribute to the overall survival phenotype.

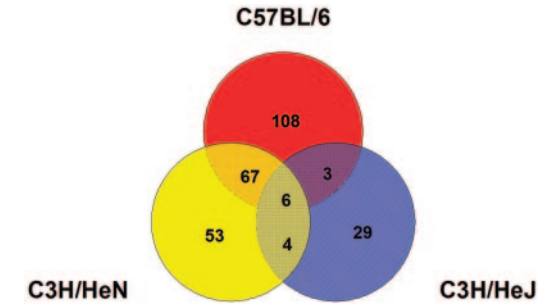

FIG. 3. Venn diagram displaying genes upregulated at 4 h postchallenge that are both common and unique to three strains of mice: C57BL/6N, C3H/HeN, and C3H/HeJ (LPS hyporesponsive). Data were analyzed by using Affymetrix software MAS version 5.0 and DMT version 3.0. Genes that were included had to be significantly upregulated at 4 h versus the zero-hour time point and had to have a fold change between these two conditions of two or higher. Lists were imported into Genespring software version 6.0 (Silicon Genetics) for graphic illustration. The list of 29 genes unique to C3H/HeJ mice are TLR4 independent in nature, whereas the list of 67 genes shared between C57BL/6N and C3H/HeN mice are dependent and should correlate with overall resistance patterns in mice.

synthesis reaction mixture contained 5 μg of total RNA, 100 pmol of T7-$(T)_{24}$ primer, 500 μM each deoxynucleoside triphosphate, and 200 U of reverse transcriptase (Superscript II Reverse; Gibco/BRL). The reaction mixture was incubated for 1 h at 42°C. Second-strand cDNA synthesis was carried out at 16°C for 2 h in a total volume of 170 μl using 10 U of *E. coli* DNA ligase, 40 U of *E. coli* DNA polymerase I, and 2 U of *E. coli* RNase H in the presence of 200 μM each deoxynucleoside triphosphate. Following the second-strand cDNA synthesis, 10 U of T4 DNA polymerase was added, and the samples were incubated for 5 min at 16°C. The reaction was stopped by the addition of 0.5 M EDTA, and samples were phenol-chloroform extracted by using Phase-Lock gels (Eppendorf 5 Prime, Boulder, Colo.). Samples were then precipitated overnight at −20°C with 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol. With this double-stranded DNA serving as a template, a biotinylated antisense cRNA was synthesized by using the Enzo Bioarray High-Yield RNA transcript labeling kit (Affymetrix). Reactions were run according to the manufacturer's instructions. The reaction mixture was incubated at 37°C for approximately 5 h. Samples were then precipitated overnight at −20°C and subsequently resuspended in 20 μl of diethyl pyrocarbonate-treated water. Forty micrograms of biotinylated antisense cRNA was fragmented by heating the sample to 94°C for 35 min in a volume of 40 μl of fragmentation buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate.

Microarray analysis of lung RNA. To determine which gene expression profiles correlated with host resistance to *K. pneumoniae* and which of those genes were regulated by TLR4 in the lung, microarray analysis of lung RNA at time points early in the infection (4 and 16 h) was performed. Mice were euthanized at 0, 4, and 16 h ($n = 4$ to 9 mice per time point) postinoculation, the right lung was harvested and homogenized, and the total RNA was isolated. Replicate samples were individually prepared and hybridized onto separate microarrays as outlined below. Subsequent enzymatic reactions were carried out with 5 μg of total RNA to generate labeled and fragmented cRNA which were then hybridized to Affymetrix MGU74AV2 microarrays.

Microarray processing. U74Av2 chips (Affymetrix) were prehybridized with 200 μl of 1× hybridization buffer (100 mM MES, 1 M Na^+, 20 mM EDTA, 0.01% Tween 20) for 10 min at 45°C in an Affymetrix Genechip Hybridization Oven 640 at 60 rpm. Hybridization was done in a final volume of 300 μl containing 15 μg of fragmented biotinylated cRNA, 50 pmol of control oligonucleotide B2 (Affymetrix), eukaryotic hybridization controls (Affymetrix), 0.1 mg of herring sperm DNA/ml, and 0.5 mg of acetylated bovine serum albumin/ml in 1× hybridization buffer. The samples were heated to 95°C for 5 min and 45°C for an additional 5 min and then briefly spun down. Two hundred microliters of the hybridization cocktail was added to the standard arrays, and hybridizations were carried out for 16 h at 45°C with mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed, and the arrays were washed by using a fluidics station (Affymetrix). Hybridized arrays were stained for 10 min at 25°C with streptavidin-R phycoerythrin (10 μg/ml; Molecular Probes), followed by staining with biotinylated goat anti-streptavidin antibody (3 μg/ml; Sigma Chemical) for 10 min at 25°C. Genechips were then stained once again with streptavidin-R phycoerythrin for 10 min at 25°C. Probe arrays were scanned with a confocal laser scanner (Agilent) at a wavelength of 570 nm. Pixel intensities were then measured, and expression signals were analyzed by using a commercial software package (Microarray Suite [MAS], version 5.0; Affymetrix). LIMS version 3.0 (Affymetrix), Data Mining Tools (DMT) version 3.0 (Affymetrix), and Genespring version 6.0 (Silicon Genetics) were used to perform data analysis.

Microarray data analysis. Microarray data were generated by using Affymetrix (http://www.affymetrix.com) protocols. Absolute expression transcript levels were normalized for each chip by globally scaling all probe sets to a target signal intensity of 500. Three statistical algorithms (detection, change call, and signal log ratio) were then used to identify differential gene expression in experimental and control samples. The detection metric (presence, absence, or marginal) for a particular gene was determined by using default parameters of the MAS software. Transcripts that were absent under both control and experimental conditions were eliminated from further consideration. Statistical significance of signals between the control and experimental conditions ($P \leq 0.05$) for individual transcripts was determined by using the *t* test and Mann-Whitney test. Batch analyses in which pairwise comparisons between individual experimental and control chips were made in order to generate a change call and a signal log ratio value for each transcript were performed with MAS. We defined a positive change call as one in which greater than 50% of the change calls for any one transcript were increased or marginally increased for upregulated genes and decreased or marginally decreased for downregulated genes. Finally, the median value of the signal log ratios from each comparison file was calculated. Signal log ratio values were converted from $\log_2$ and expressed as fold changes. In addition, only those genes that met the above-mentioned criteria and that had a median signal log ratio of greater than or equal to 1 for upregulated transcripts and less than or equal to 1 for downregulated transcripts were kept in the final list of genes.

For hierarchical clustering, genes were included in the final lists if they passed the following filter requirements when the 4- or 16-h time point was compared to the 0-h controls within the same strain: elimination of Absent to Absent genes

TABLE 1. Mean TLR4-dependent gene fold changes categorized by function

Affymetrix probe ID	Gene symbol	Genbank accession no.	Change (fold) at 4 vs 0 h			Results in C3H/HeJ mice at 4 vs 0 h[a]	Description
			C57BL/6	129/SvJ	C3H/HeN		
Cytokine/chemokine							
101160_at	*Cxcl2*	X53798	51.27	41.9	13.18	A	Chemokine (C-X-C motif) ligand 2/MIP-2
102218_at	*IL-6*	X54542	26.91	4.89	25.11	A	IL-6
102424_at	*Ccl3*	J04491	8.51	3.05	3.05	A	Chemokine (C-C motif) ligand 3/MIP-1α
102629_at	*Tnf*	D84196	9.45	5.54	3.34	A	TNF
102736_at	*Ccl2*	M19681	32.90	6.73	6.23	A	Chemokine (C-C motif) ligand 2/MCP-1
103486_at	*IL1b*	M15131	21.56	6.68	4.82	A	Interleukin 1β
104388_at	*Ccl9*	U49513	4.99	1.42	2.25	A	Chemokine (C-C motif) ligand 9
94755_at	*IL1a*	M14639	9.45	5.10	4.82	A	IL-1α
95349_g_at	*Cxc11*	J04596	61.39	25.99	25.63	A	Chemokine (C-X-C motif)/KC-GRO
98772_at	*Cxc15*	U27267	28.05	3.46	12.64	A	Chemokine (C-X-C motif) ligand 5
94142_at	*G-CSF*	M13926	2.83	2.13	3.97	A	Granulocyte colony-stimulating factor
102914_s_at	*A1b*	U23778	3.76	1.93	2.60	A	Hematopoietic-specific early response A1-b
Receptors							
102658_at	IL-12	X59769	12.38	3.39	8.63	A	IL-1 receptor, type II
931198_at	*Csf3r*	M58288	4.41	1.58	3.20	A	Colony-stimulating factor 3 receptor (granulocyte)
93430_at	*Cmkor1*	AF000236	4.06	1.67	3.78	A	Chemokine orphan receptor 1
93871_at	*IL-1m*	L32838	8.06	3.18	2.89	A	IL-1 receptor antagonist
102255_at	*Osmr*	AB015978	3.03	1.33	2.08	Chg	Oncostatin receptor
102663_at	*Plaur*	X62700	4.03	2.19	2.77	NS	Urokinase plasminogen activator receptor
101410_at	*mCPE-R*	AB000713	4.06	1.88	5.35	A	CPE receptor
98088_at	*CD14*	X13333	7.84	2.82	4.17	Fld<2	LPS receptor
99413_at	*CCR1*	U29678	6.96	3.05	2.63	*A	Chemokine (C-C) receptor 1
Apoptosis							
101979_at	*Gadd45g*	AF055638	3.34	2.17	2.20	A	Growth arrest and DNA damage-inducible 45 gamma
99392_at	*Tnfaip3*	U19463	17.75	9.45	3.76	A	TNF-α-induced protein 3
Signal/transduction							
102430_at	*Myd88*	X51397	2.10	1.16	2.11	A	Myeloid differentiation primary response gene 88
96747_at	*Arhu*	AW121294	2.48	1.56	3.12	A	*ras* homolog gene family, member U
98423_at	*Gjb2*	M81445	2.14	2.41	4.89	A	Gap junction membrane channel protein beta 2
92232_at	*SOCS-3*	U88328	8.28	3.94	4.50	A	Suppressor of cytokine signaling 3
92534_at	*GEM*	U10551	2.23	1.56	4.41	A	GTP binding protein
Adhesion							
102805_at	*Ceacam1*	M77196	2.89	1.11	3.53	A	CEA-related cell adhesion molecule 1
103005_s_at	*CD44*	X66084	3.07	1.48	2.08	A	CD44 antigen
96752_at	*Icam1*	M90551	2.28	1.47	2.51	Fld<2	Intercellular adhesion molecule
Enzymes							
102905_at	*Casp4*	Y13089	2.57	1.05	2.25	A	Caspase 4, apoptosis-related cysteine protease
103341_at	*Ctps*	U49350	2.89	1.40	2.01	A	Cytidine 5′-triphosphate synthase
104509_at	*Ch25h*	AF059213	9.25	2.19	2.75	A	Cholesterol 25-hydroxylase
104671_at	*Ampd3*	D88994	3.05	1.67	2.22	A	AMP deaminase 3
99985_at	*TxNR*	AB027565	3.70	1.92	3.56	Chg	Thioredoxin reductase 1
98473_at	*Arg2*	AF032466	4.20	2.39	3.89	A	Arginase type II
94297_at	FK506 binding protein 5	U16959	6.02	2.57	2.81	A	FK506 binding protein 5
103024_at	*Adam8*	X13335	3.18	4.41	8.75	A	A disintegrin and metalloprotease domain 8
Transcriptional factors							
102955_at	*NFIL3/E4BP4*	U83148	3.03	1.89	2.55	A	Nuclear factor, IL-3 regulated
104712_at	*C-MYC*	L00039	6.32	2.58	5.58	A	Myelocytomatosis oncogene
Other							
102780_at	*Tx01*	Z31362	3.81	3.03	3.11	A	
103887_at	*MRP14*	M83219	5.86	6.36	5.03	NS	Migration inhibitory factor-related protein 14
92315_at	*Slfn4*	AF099977	4.20	1.13	2.17	A	Schlafen4
93861_f_at	Endogenous murine leukemia virus modified polytropic provirus	M17327	3.05	1.16	2.33	A	Endogenous murine leukemia virus modified polytropic provirus

Continued on following page

TABLE 1—*Continued*

Affymetrix probe ID	Gene symbol	Genbank accession no.	Change (fold) at 4 vs 0 h			Results in C3H/HeJ mice at 4 vs 0 h[a]	Description
			C57BL/6	129/SvJ	C3H/HeN		
92471_i_at	Slfn2	AF099973	2.36	1.95	2.30	A	Schlafen2
103448_at	MRP8	M83218	5.86	6.50	4.56	NS	Intracellular Ca^{2+} binding protein
93573_at	MT1	V00835	7.89	5.70	6.23	Fld<2	Metallothionein I
99915_at	SDGF	L41352	2.46	2.33	2.53	A	Amphiregulin
103328_at	I-TRAF	U59864	2.39	1.62	2.33	A	TRAF-interacting activator
93104_at	BTG1	Z16410	2.07	1.48	2.38	Fld<2	B-cell-transclocation gene 1 protein
98067_at	P21	U09507	2.19	2.10	2.66	A	Cyclin-dependent kinase inhibitor 1A
97689_at	Coagulation factor III	M26071	3.36	3.39	2.35	Chg	Coagulation factor III

[a] Fold changes were not shown for those genes that had detection calls of absent (A) in the 4-h treated group; that had an inappropriate change call (Chg), such that there was not a call of increased or marginally increased in greater than half of the comparisons made; that had a fold change less than 1 (Fld<2); or that were not determined to be significant (NS) by parametric and nonparametric tests with a P cutoff value of ≤0.05.

(genes denoted to be absent in control and experimental conditions); statistical significance by Student's *t* test and Mann-Whitney test; change calls of "increased" for upregulated genes and "decreased" for downregulated genes; and a fold change of >3 or <−3. Parametric (*t* test) and nonparametric (Mann-Whitney test) statistical tests were performed on these data to obtain those genes that were potentially normally distributed across groups and those that were not. Self-organizing maps were generated by using DMT with a subset of genes that were found to be up- and downregulated by the above-described methods in C57BL/6 mice infected with *K. pneumoniae* at 4 h compared to the 0-h time point. The same subset of genes was used for hierarchical clustering using Genespring version 6.0 on all strains of mice at 0, 4, and 16 h following inoculation.

Alveolar macrophage isolation. Male C57BL/6, C3H/HeN, C3H/HeJ, or 129/SvJ mice were anesthetized with intraperitoneal pentobarbital and sacrificed by exsanguination. Thereafter, the lungs were lavaged through an intratracheal catheter with prewarmed (37°C) calcium and magnesium-free PBS supplemented with 0.6 mM EDTA. A total of 10 ml was used in each mouse in 0.5-ml increments with a 30-s dwell time. The lavage fluids were pooled and centrifuged at 300 × *g* for 10 min, and the cells were collected. To ensure that each cell preparation was enriched for macrophages, 10,000 cells were cytospun onto slides and stained with hematoxylin and eosin. Cell preparations were generally >98% enriched for alveolar macrophages.

Cytokine assays. Alveolar macrophages were isolated from C57BL/6, C3H/HeN, C3H/HeJ, and 129/SvJ mice as described above. Macrophages were adjusted to 5×10^4 in RPMI 1640 medium with 10% fetal bovine serum and 1% PenStrep (Gibco) and added to a volume of 100 μl in individual wells of a 96-well plate (in duplicate). The macrophages were allowed to attach for 1 h at 37°C, and nonadherent cells were washed away. Thereafter, heat-killed *K. pneumoniae* (70°C for 30 min) was added to experimental wells at 10^4 CFU/well in a volume of 100 μl of RPMI 1640 medium with 10% fetal bovine serum and 1% PenStrep. Controls included macrophages cultured in the presence of LPS (10 μg/ml; List Biological Laboratories, Campbell, Calif.) or medium alone (control for spontaneous production). The macrophage cultures were allowed to incubate for 6 h at 37°C, 5% CO_2, and thereafter, supernatants were harvested for quantification of macrophage inflammatory protein 2 (MIP-2), IL-6, tumor necrosis factor alpha (TNF-α), KC-GRO, and granulocyte colony-stimulating factor (G-CSF) by using the Bio-Plex protein array system (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. The concentrations of each cytokine and chemokine were determined by using Bio-Plex Manager version 3.0 software (Bio-Rad). Data are expressed as picograms per milliliter.

Statistical analysis of lung CFU and cytokine protein and fold change data. Data were analyzed by using StatView statistical software (Brainpower Inc., Calabasas, Calif.). Comparisons between groups were analyzed by analysis of variance with a Scheffe follow-up test. Survival was analyzed by log-rank testing. Significance was accepted at a *P* value of <0.05.

RESULTS

Reduced survival of mice deficient in TLR4 signaling. Four strains of mice were chosen to investigate the role of TLR4 signaling in host lung defense in a *K. pneumoniae* model: C57BL/6, 129/SvJ, C3H/HeN, and C3H/HeJ. The C3H/HeJ strain has a mutant TLR4 allele which results in defective TLR4 signaling, rendering it hyporesponsive to LPS (23), while the C3H/HeN mice have a normal TLR4 allele. Lung inoculums were based on previous pilot studies, whereby the 50% lethal dose at 12 days was determined to be 3×10^4 CFU in C57BL/6 mice (data not shown). Four strains of mice were inoculated intratracheally with 10^4 CFU of *K. pneumoniae* (ATCC 43816, serotype 2), and their survival was recorded in 24-h intervals for 96 h. Wild-type control mice, C57BL/6 and C3H/HeN mice, had a 100 and 60% survival rate, respectively, after 96 h, while the remaining susceptible strains had a 100% mortality rate within the same time period. Of these strains, the C3H/HeJ and 129/SvJ mice shared a phenotype of drastically reduced survival, with death resulting 48 to 72 h following inoculation (Fig. 1A). Bacterial lung burdens of *K. pneumoniae* were determined postinoculation in a separate group of animals at 4, 12, 16, and 24 h ($n = 4$ to 6 mice per time point) by quantitative culture of lung homogenates (Fig. 1B). As previously described for C57BL/6 and IL-17 receptor knockout mice (36), all mice had similar lung bacterial burdens within the first 16 h, with C3H/HeJ and 129/SvJ mice developing statistically significantly higher lung bacterial burdens at 24 h (Fig. 1B). Moreover, bacterial dissemination was assessed by quantitative cultures of spleen homogenates at the same time points. However, by 24 h, five out of six 129/SvJ, four out of six C3H/HeJ, and two out of six C3H/HeN mice had spleen cultures that were positive for *K. pneumoniae*, whereas zero out of six C57BL/6 mice were bacteremic at this time point. By 48 h, three out of six C3H/HeN and only one out of six C57BL/6 mice had positive spleen cultures. 129/SvJ and C3H/HeJ mice were moribund at this time point and 100% bacteremic. Thus, as previously observed in this model, mortality was associated with bacterial dissemination from the lungs (36).

Microarray analysis of lung homogenates. Hierarchal cluster analysis revealed distinct patterns among the four murine strains at 4 h that correlate closely with the survival outcome as seen in Fig. 1. Specifically, the mouse strains with the greatest susceptibility to bacterial dissemination, 129/SvJ and C3H/HeJ, clustered near each other at 4 h. Moreover, as visually seen in the cluster (Fig. 2) the C3H/HeJ mice failed to upregulate a number of genes by 4 h that were observed in C3H/HeN or

TABLE 2. Mean TLR4-independent gene fold changes categorized by function[a]

Affymetrix probe ID	Gene symbol	Genbank accession no.	Change (fold) at 4 vs 0 h			Results in C3H/HeJ mice at 4 vs 0 h	Description
			C57BL/6	129/SvJ	C3H/HeN		
Transcriptional Factors							
161348_r_at	*Pdlim1*	AV149007	−0.06	−0.09	0.55	2.18	PDZ and Lim domain 1 (elfin)
104155_f_at	*Atf3*	U19118	0.8	1.74	−0.07	3.54	Activating transcription factor 3
96418_r_at	*Hox-2.4*	M18399	−0.02	0.22	0.73	1.87	Homeobox (Hox2.4) region
Enzymes							
161357_r_at	*Gstm2*	AV207739	0.05	−0.37	0.62	3	Glutathione *S*-transferase, mu2
101179_at	*DEAD/H*	D50494	−0.53	−0.22	−0.16	2.98	Aspartate-glutamate-alanine-aspartate box polypeptide 6
Receptor							
101771_r_at	*CNR5*	AB008180	−0.13	0.17	0.5	3.92	Cadherin-related neural receptor 5
Heat shock							
100946_at	*Hspab*	AF109906	0.43	0.09	−1.98	5.15	Heat shock protein 1B
93875_at	*Hspa1a*	M12571	−0.23	0.37	−1.79	4.98	Heat shock protein 1A
96254_at	*Dnajb1*	AB028272	−0.14	0.18	−0.86	2.06	DNA J homolog, subfamily B, member 1
Other							
92958_at	NA	AI849135	0.16	−0.21	0.18	1.04	NA
98590_at	*SYND4*	D89571	0.16	−0.35	0.48	1.13	Ryudocan core protein
96680_at	NA	AI835630	0.6	−0.19	0.51	2.2	NA
162420_r_at	NA	AV290470	−0.16	0.08	0.21	2.28	NA
99849_at	NA	C85523	0.13	0.72	0.77	1.51	NA
96526_at	NA	AW228840	0.14	0.02	0.44	1.55	NA
160169_at	NA	AI851407	0.69	−0.01	0.14	1.96	NA
104046_at	NA	AI854141	0.06	−0.09	0.49	1.42	NA
161348_r_at	NA	AV149007	−0.06	−0.09	0.55	2.18	NA
160949_at	NA	AI848924	0.41	−0.15	0.24	2.12	NA
99162_at	NA	AW122398	0.67	0.3	0.88	2.04	NA
160989_r_at	NA	AA717225	−0.16	−0.81	1.07	2.34	NA
161561_r_at	NA	AV264321	−0.63	0.04	0.31	3.9	NA
160979_at	NA	AW120820	−0.36	−0.11	−0.31	1.5	NA
104343_f_at	NA	AI845798	0.38	0.58	0.38	1.44	NA
102381_at	NA	AA619207	0.56	0.14	0.65	1.49	NA
162496_r_at	NA	AV153195	0.04	0.42	0.55	2.82	NA
161869_s_at	NA	AV265258	−0.09	−0.85	−0.32	1.82	NA
101869_s_at	β-*globin*	J00413	−0.41	−0.61	−0.87	1.68	β-Globin major gene
AFFX-b-ActinMur/M12481_M_st	β-*actin*	M12481	−0.14	−0.36	0.06	1.53	Cytoplasmic β-actin
103534_at	β1-*globin*	V00722	−0.38	−0.63	−0.82	2.23	β-1-globin
102727_at	*BDNF*	X55573	0.18	−0.07	−0.06	2.22	Brain-derived neurotrophic factor
99500_at	*SLC12A2*	U13174	0.37	0.09	0.43	1.11	Solute carrier family 12, member 2

[a] NA, not applicable.

C57BL/6 mice. The defect in gene expression in C3H/HeJ mice resulted in this strain at 4 h actually clustering with time zero control mice (Fig. 2). The hyporesponsiveness in gene expression observed in C3H/HeJ mice suggests a critical role for TLR4 signaling and subsequent TLR4 gene expression in mediating host resistance to *K. pneumoniae* infection. The fact that the 129/SvJ strain clustered near C3H/HeJ mice at 4 h also suggests that there may be defects in downstream genes regulated by TLR4 in this mouse strain. This distinction for the 129/SvJ and C3H/HeJ mice was lost at 16 h, as the 129/SvJ mice clustered together with both C3H/HeN and C57BL/6 mice at 16 h. This is despite the fact that there are significant differences in rates of bacteremia at 24 h across these strains. These data suggest that the gene expression profile at 4 h is critical to controlling bacteremia and survival. Lastly, of note is that C3H/HeJ mice at 16 h cluster with C3H/HeN and C57BL/6 mice 4 h into the infection, further suggesting that a defect in LPS recognition delays the gene expression of analogous genes expressed in mice capable of recognizing LPS in response to infection. In 129/SvJ mice, these genes are eventually expressed by 16 h but at a time when it may be too late to affect their survival. Taken together, our results indicate that the expression of critical genes downstream of the TLR4 pathway at 4 h is important in clearing gram-negative infections.

Role of TLR4 signaling and pulmonary gene expression. Next, these lists of differentially expressed genes were incorporated into a Venn diagram by using Genespring version 6.0 (Fig. 3) except that genes were now included if they had a fold change of ≥ 2 or ≤ -2 in order to increase sensitivity. Specifically, genes that were found to be upregulated at 4 h following

TABLE 3. Mean fold changes of upregulated genes common to all strains

Affymetrix probe ID	Gene symbol	Genbank accession no.	Change (fold) at 4 vs 0 h			Results in C3H/HeJ mice at 4 vs 0 h	Description
			C57BL/6	129/SvJ	C3H/HeN		
Enzymes							
102049_at	*pdk*	AJ001418	1.80	1.5	1.64	4.21	Pyruvate dehydrogenase kinase-like protein
104647_at	*griPGHS*	M88242	1.69	0.89	1.17	3.39	Glucocortoid-regulated inflammatory prostaglandin G/H synthase
99649_at	*GCLC*	U85414	2.26	1.32	2.08	3.17	Glutamate cysteine ligase (gammylcysteine synthetase), catalytic
Other							
101561_at	*MT2*	K02236	3.27	2.65	2.82	2.36	Metallothionein 2
100325_at	*gp49*	M65027	3.24	1.42	2.49	1.6	Glycoprotein 49

K. pneumoniae infection for strains C57BL/6, C3H/HeN, and C3H/HeJ were compared to each other to observe both shared and unique genes between lists. Key gene lists can be found in Tables 1 to 3, along with information regarding fold changes for each strain at 4 h versus its own 0-h time point. The fold changes for specific genes from certain murine strains were not included in the final lists of differentially expressed transcripts if these genes did not meet the above-mentioned filtering requirements, and the exact reasons for their exclusion are denoted in the tables. The mouse strain most resistant to bacteremia, C57BL/6, showed the greatest number of induced genes (184) meeting at least a twofold change, followed by C3H/HeN and129/SvJ, with 130 and 78 genes, respectively. C3H/HeJ showed the least amount of alteration in gene expression, with only 42 genes changing from 0 to 4 h. Moreover, the two strains showing the greatest level of host resistance shared the greatest number of genes, 67 (Fig. 3). In addition to these 67 common genes, C57BL/6 mice have an additional set of 108 unique genes (Table 4) and C3H/HeN mice had 53 unique genes (Table 5) that were significantly upregulated by 4 h. Because this set of 108 and 53 unique genes may represent TLR4-dependent genes but may be strain specific to C3H or C57, we focused on the 67 shared genes that were not expressed in C3H/HeJ mice as genes that were clearly TLR4 dependent in nature. This set of genes constituted approximately 29% of the genes uniquely upregulated by both control strains, C57BL/6 and C3H/HeN (Table 1). The products of these genes can be categorized by functional ontology, with cytokines/chemokines and receptors constituting the majority of genes found in this group. As expected, several known TLR4-related genes reside in this group of shared genes, including TNF-α, MIP-2 (Cxcl2), IL-1β, IL-6, CD14, and MyD88, further suggesting that the expression of these genes is critical for early infection clearance in the lungs and the ultimate survival of the host. Moreover, this analysis revealed genes that may be critical for host defense but that were heretofore not known to be directly regulated by TLR4, such as G-CSF, a molecule critical for regulating neutrophil responses to bacterial infection (8), and I-TRAF (24), a molecule involved in TNFR II signaling.

This Venn diagram analysis also revealed a list of 29 TLR4-independent genes that were found to be uniquely upregulated in C3H/HeJ mice at 4 h, including a number of transcriptional factors, enzymes, receptors, and heat shock proteins (HSPs) (Table 2). The 29 genes in Table 2 fall in to two broad categories: ones which are significantly more upregulated in C3H/HeJ mice than controls and genes that are upregulated in C3H/HeJ mice but downregulated at 4 h in the control strains. These latter genes, such as HSP 1A and 1B, appear to be negatively regulated by TLR signaling in control mice (Table 2). Of the 270 genes found in all three lists, only 6 are found to be upregulated by all strains (Table 3) which include a number of transcripts known or believed to be involved in the regulation of glucose (PDK), inflammation (GriPGHS), and glutathione production (GCLC). In addition to these genes, there were genes that were selectively upregulated in C3H/HeN mice compared to C3H/HeJ mice (Table 5). Among these were Rabb33b, which is involved in cellular vesicular transport; Rgs16, a regulator of G protein-coupled receptor signaling which is inducible by LPS and found to be expressed in dendritic cells (26); NF-κB; and IL-2-inducible T-cell kinase (Itk), involved in activation of T cells (34). Taken together, these data suggest that TLR4-mediated recognition of LPS in the lung is critical for the early and coordinated expression of a variety of genes controlling inflammation, granulopoeisis adhesion of neutrophils, and control of genes such as Itk which may be critical for adaptive immunity. We focused our subsequent analysis on a few key transcripts which have been shown to be critical in this model in either knockout or antibody neutralization studies (7, 8, 18, 20).

Detection of key TLR4-dependent transcripts. The signal values of several important TLR4-dependent genes were plotted for each strain at the 4-h time point, including TNF-α, IL-1β, IL-6, MIP-2 and KC-GRO (Cxcl1) (Fig. 4A). These values are suggestive of the relative transcript abundance in a sample and were generated with algorithms found in MAS version 5.0 (Affymetrix). The numbers for individual replicates in a group were averaged together and plotted by using Excel software. Both C57BL/6 and C3H/HeN mice showed significantly higher levels of these transcripts at the 4-h time point, while the C3H/HeJ mice showed a minimal signal response. Moreover, the 129/SvJ mice showed lower levels of these transcripts than control mice.

Detection of key TLR4-dependent proteins from isolated macrophages. TLR4 is most abundantly expressed on alveolar macrophages and dendritic cells (1, 16, 32). As these are some of the first cells to encounter an invading pathogen in the lung and to verify that changes in signal levels were reflected at the protein level, we examined protein production for TLR4-de-

TABLE 4. Genes (108) that are upregulated 4 h following *K. pneumoniae* infection and are unique to C57BL/6 mice

Affymetrix probe ID	Gene symbol	GenBank accession no.	Mean change (fold) in C57BL/6 mice at 4 vs 0 h	Results in C3H/HeN mice at 4 vs 0 h[a]	Description
102712_at	*Saa3*	X03505	47.8	A	Serum amyloid A3
94769_at	*Mmp8*	U96696	29.4	A	Matrix metalloproteinase 8
92948_at	*Csf2*	X03020	19.3	A	Colony-stimulating factor 2 (granulocyte macrophage)
93858_at	*Cxcl10*	M33266	17.1	A	Chemokine (C-X-C motif) ligand 10
160564_at	*Lcn2*	X81627	11.8	NS	Lipocalin 2
95951_at	*Clecsf8*	AF061272	11.2	A	C-type lectin
96515_at	*Il4i*	U70430	10.9	A	IL-4 induced 1
95303_at	*EST*	AA144469	10.9	Chg	IMAGE-597616
103465_f_at	*Saa2*	U60438	9.3	A	Serum amyloid A2
98774_at	*Irg1*	L38281	9.25	A	Immunoresponsive gene 1
96153_at	*Ngp*	L37297	8.8	A	Neutrophilic granule protein
94688_at	*Mad*	X83106	8.2	A	Max dimerization protein
97106_at	*Map3k8*	D13759	8.2	A	Mitogen-activated protein kinase kinase kinase 8
97783_at	*Ccl17*	AJ242587	8.1	A	Chemokine (C-C motif) ligand 17
161689_f_at	*Il1r2*	AV223216	7.6	NS	IL-1 receptor, type II
162206_f_at	*Socs3*	AV374868	7.3	NS	Suppressor of cytokine signaling 3
104692_at	*Selp*	M72332	7.2	A	Selectin, platelet
92217_s_at	*gp49b*	V05265	7.2	NS	Glycoprotein 49B
99333_at	*Sele*	M80778	5.9	A	Selectin, endothelial cell
102816_at	*Serpina 3m*	X69832	5.9	A	Serine protease inhibitor, clade A, member 3M
94761_at	*Ccl7*	X70058	5.0	A	Chemokine (C-C motif) ligand 7
95808_g_at	*GCSFR*	V05894	4.9	A	Colony-stimulating factor, granulocyte receptor pseudogene
101912_at	*EST*	AI01967	4.6	Chg	IMAGE-1364952
101800_at	*Fprrs2*	AF071180	4.6	NS	*N*-formylpeptide receptor-like 2 gene
160489_at	*Tnfaip2*	L24118	4.4	NS	TNF-α-induced protein 2
103839_at	*SPHK1a*	AF068748	4.1	A	Spingosine kinase
160608_at	*Rab20*	AI854462	3.8	A	RAB20, member RAS oncogene family
92276_at	*Map3k6*	AB021861	3.5	A	Mitogen-activated protein kinase kinase kinase 6
102114_f_at	*Angpt14*	AI326963	3.5	A	Angiopoietin-like 4
97197_r_at	*EST*	C78850	3.5	NS	Unknown
96704_at	*Sfn*	AF058798	3.4	A	Stratifin
101820_at	*Ly-6G1*	X70920	3.4	A	Neurotoxin homologue, exons 1–3
102798_at	*Adm*	U77630	3.4	Chg	Adrenomedullin
102239_at	*Bcl3*	M90397	3.3	A	B-cell leukemia/lymphoma 3
102921_s_at	*Tnfrsf6*	M83649	3.3	Chg	TNF receptor superfamily, member 6
94147_at	*Serpine1*	M33960	3.3	Chg	Serine (or cysteine) proteinase inhibitor, clade E, member 1
93869_s_at	*Ald*	U23781	3.3	Fld<2	Hematopoietic-specific early response A1-d gene
160894_at	*Cebpd*	X61800	3.2	NS	CCAAT/enhancer binding protein (C/EBP), delta
102707_f_at	*Serpina 3C*	X61597	3.2	A	Serine (or cysteine) proteinase inhibitor, clade A, member 3C
104333_at	*G7e*	U69488	3.2	NS	Viral envelope-like
98299_s_at	*Slfn3*	AF09997	3.2	A	Schlafen 3
104374_at	*Spi2/eb4*	M64086	3.1	Chg	Spi2 proteinase inhibitor
102337_s_at	*FCRII*	M31312	3.1	A	Mouse beta Fc receptor type II
94137_at	*I18rb*	L13239	3.0	A	IL-8 receptor, beta
102021_at	*Il4ra*	M27960	3.0	Fld<2	IL-4 receptor, alpha
92472_f_at	*Slfn2*	AF099973	2.9	NS	Schlafen 2
102860_at	*Spi2/eb1*	M64085	2.9	A	Spi2 prot
93037_i_at	*Anxa1*	M69260	2.9	Chg	Annexin A1
94747_at	*Csf2rb1*	M34397	2.9	A	IL-3 receptor, beta chain 1
93411_at	*EST*	AI52789	2.8	Chg	IMAGE-1478197
95784_at	*Pira*	U96682	2.8	A	Paired immunoglobulin like receptor A1
97949_at	*Fgl2*	M16238	2.7	Fld<2	Fibrinogen-like protein 2
97203_at	*Mlp*	X61399	2.7	NS	MARCKS-like protein
96119_s_at	*Angpt14*	AA797604	2.7	NS	Angiopoietin-like 4
102313_at	*Gch*	L09737	2.6	NS	GTP cyclohydrolase 1
92925_at	*Cebpb*	M61007	2.6	NS	CCAAT/enhancer binding protein (C/EBP), beta
93058_at	*Elf-1A*	AF026481	2.6	NS	Eukaryotic translation initiation factor 1A
93860_i_at		M17327	2.6	NS	Mouse endogenous murine leukemia virus modified polytropic provirus
160353_i_at	*Mapkapk2*	X76850	2.5	NS	Map kinase-activated protein kinase 2
96042_at	*Sod2*	L35528	2.5	NS	Superoxide dismutase 2, mitochondrial
99957_at	*Mmp9*	X72795	2.5	A	Matrix metalloproteinase 9
160645_at	*Birc2*	U88908	2.5	NS	Baculoviral IAP repeat-containing 2
AFFX-MurFAS_at	*Tnfrsf6*	M83649	2.5	NS	TNF receptor superfamily, member 6
102362_i_at	*Junb*	U20735	2.5	NS	Jun-B oncogene
104406_at	*Ptges*	AI060798	2.4	A	Prostaglandin E synthase

Continued on following page

TABLE 4—*Continued*

Affymetrix probe ID	Gene symbol	GenBank accession no.	Mean change (fold) in C57BL/6 mice at 4 vs 0 h	Results in C3H/HeN mice at 4 vs 0 h[a]	Description
160646_at	*Gsr*	AI851983	2.4	NS	Glutathione reductase I
94351_r_at	*Nqo1*	U12961	2.4	A	NAD(P)H dehydrogenase, quinine 1
97525_at	*Gyk*	U48403	2.4	NS	Glycerol kinase
97950_at	*Xdh*	X75129	2.4	NS	Xanthine dehydrogenase
102264_at	*Slfn1*	AF099972	2.4	A	Schlafen 1
94556_at	*Snx10*	A1746846	2.4	NS	Sorting nexin 10
95785_s_at	*Rab7*	Y13361	2.4	NS	RAB7, member RAS oncogene family
102957_at	*Lcp2*	U20159	2.4	NS	Lymphocyte cytosolic protein 2
95706_at	*Lgals3*	X16834	2.4	NS	Lectin galactose binding soluble 3
101464_at	*Timp1*	V00755	2.4	NS	Immunoresponsive gene 1
93328_at	*Hdc*	X57437	2.4	NS	Histidine decarboxylase
160583_at	*Xlkd1*	AA880988	2.3	NS	Extracellular link domain-containing 1
99384_at	*Pim1*	M13945	2.3	A	Proviral integration site 1
102806_g_at	*Ceacam1*	M77196	2.3	NS	CEA-related cell adhesion molecule 1
160975_at	*EST*	AI504338	2.3	NS	IMAGE-963414
100328_s_at	*Akr1b8*	U96684	2.3	A	Ado-keto reductase family 1, member B8
92251_f_at	*EST*	AA960657	2.3	A	IMAGE-2076183
103091_at	*Relb*	M83380	2.2	A	Avian reticuloendotheliosis viral (v-rel) oncogene related B
93202_at	*Nt5e*	L12059	2.2	NS	5′ nucleotidase, ecto
101554_at	*Nfkbia*	U57524	2.2	Fld<2	Nuclear factor of kappa light chain gene enhancer in B cells inhibitor, alpha
96562_at	*Slc11a1*	L13732	2.2	A	Solute carrier family 11 member 1
99461_at	*Hcls1*	X84797	2.2	A	Lyn substrate 1
100884_at		U04204	2.2	Chg	Fibroblast growth factor regulated protein
101908_s_at	*Bgp2*	AF101164	2.2	A	Biliary glycoprotein 2 long isoform
162198_f_at	*EST*	AV373027	2.2	NS	Unknown
94085_at		M34603	2.2	NS	Proteoglycan secretory granule
160359_at	*EST*	AI746846	2.2	NS	Unknown
96295_at	*Psat1*	AW122030	2.1	NS	Phosphoserine aminotransferase 1
102310_at	*Cc122*	AF052505	2.1	A	Chemokine (C-C motif) ligand 22
99387_at	*Fpr1*	L22181	2.1	NS	Formyl peptide receptor 1
161132_at	*Sce1*	AA727482	2.1	NS	Screllin
99160_s_at	*EST*	AW227647	2.1	NS	IMAGE-2654099
93624_at	*EST*	AI225296	2.1	NS	Unknown
100397_at	*Tryrobp*	AF024637	2.1	NS	TYRO protein tyrosine kinase binding protein
160108_at	*Nupr1*	AI852641	2.1	NS	Nuclear protein 1
160387_at	*EST*	AI853900	2.1	NS	Unknown
94821_at	*Xbp1*	AW123880	2.1	Fld<2	X-box binding protein 1
101923_at	*Pla2g7*	U34277	2	A	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
101996_at	*Ptpn2*	M80739	2.0	Chg	Protein tyrosine phosphatase, nonreceptor type 2
94224_s_at	*Ifi205*	M74123	2.0	Chg	Interferon-activated gene 205
92877_at	*Tgfbi*	L19932	2.0	NS	Transforming growth factor, beta induced
97825_at	*Perp*-pending	AI854029	2.0	A	p53 apoptosis effector related to Pmp22

[a] Fold changes were not shown for those genes that had detection calls of absent (A) in the 4-h treated group; that had an inappropriate change call (Chg), such that there was not a call of increased or marginally increased in greater than half of the comparisons made; that had a fold change less than 1 (Fld<2); or that were not determined to be significant (NS) by parametric and nonparametric tests, with a P cutoff value of ≤0.05.

pendent transcripts in ex vivo-treated alveolar macrophages. In these studies, 50,000 alveolar macrophages from each murine strain were harvested, plated, and incubated with heat-killed *K. pneumoniae* for 6 h, and cytokines and chemokines were measured by using a Bio-Plex 18 assay as described in Materials and Methods. The trends identified by surveying the signal values of specific genes were replicated in these protein assays. Specifically, C57BL/6 and C3H/HeN strains showed higher levels of MIP-2, IL-6, KC-GRO, G-CSF (Fig. 4B), and TNF-α (Fig. 4C) in culture supernatants, as opposed to that of the TLR4 mutant C3H/HeJ. The 129/SvJ mice had lower levels of these proteins at the same time point, consistent with the signal value trends seen in Fig. 4A. *E. coli* LPS-treated cells were also included as positive controls in this study and showed levels of cytokine/chemokine production across mouse strains similar to those found with heat-killed *K. pneumoniae* (data not shown). Control wells lacking any stimulus revealed cytokine/chemokine levels of <50 pg/ml.

Comparison of fold change values over time for key transcripts. Although TLR4 is critical for early gene expression, TLR4-deficient mice clustered with mice with intact TLR4 at 16 h (Fig. 2), suggesting that they have a delayed response. To confirm this hypothesis, we analyzed eight known genes related to or regulated by TLR4 (TNF-α, IL-1β, IL-6, MIP-2, KC-

TABLE 5. Genes (53) that are upregulated 4 h following *K. pneumoniae* infection and are unique to C3H/HeN mice

Affymetrix probe ID	Gene symbol	GenBank accession no.	Mean change (fold) in C3H/HeN mice at 4 vs 0 h	Results in C57BL/6 mice at 4 vs 0 h[a]	Description
99367_at	*EST*	AA434661	9.1	NS	IMAGE-818356
94375_at	*Hk2*	Y11666	4.2	Fld<2	Hexokinase 2
103891_i_at	*Eli2*	AI197161	4.1	Fld<2	Elongation factor RNA polymerase II
92294_at	*EST*	AW060793	3.6	NS	Unknown
100302_at	*Maff*	AB009694	3.4	Chg	v-Maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)
104701_at	*Bhlhb2*	Y07836	3.2	Fld<2	Basic helix-loop-helix domain containing, class B2
103438_at	*Dio2*	AF096875	2.8	NS	Deiodinase, iodothyronine, type II
97142_at	*EST*	C80153	2.7	Chg	Unknown
99864_at	*Adora2b*	AA608277	2.7	Chg	Adenosine A2b receptor
103892_r_at	*EST*	AI197161	2.6	Fld<2	IMAGE-1494691
99529_f_at	*Rnf138*	AB025011	2.6	NS	Ring finger protein 138
104612_g_at	*Wdr26*	AI854008	2.6	Fld<2	WD repeat domain 26
92738_at	*Gdnf*	D49921	2.6	NS	Glial cell line-derived neurotrophic factor
102984_g_at	*Madh1*	U58992	2.1	Fld<2	MAD homolog 1
160095_at	*Lox*	D10837	2.5	NS	Lysyl oxidase
95681_f_at	*Ppplr2*	AW049584	2.5	Chg	Protein phosphatase I, regulatory subunit 2
93981_at	*Plat*	J03520	2.4	Chg	Plasminogen activator
99032_at	*Rasd1*	AF009246	2.4	NS	RAS, dexamethasone induced 1
97740_at	*Dusp16*	A1642662	2.4	Fld<2	Dual specificity phosphatase 16
95978_at	*EST*	AA414964	2.4	Chg	Unknown
93882_f_at	*Tgoln2*	D50032	2.4	Chg	*trans*-Golgi network protein 2
97890_at	*Sgk*	AW046181	2.4	Fld<2	Serum/glucocorticoid-regulated kinase
103362_at	*Ptger4*	D13458	2.4	Fld<2	Prostaglandin E receptor 4 (subtype EP4)
94489_at	*Prp4aI*	U84411	2.3	Fld<2	Protein tyrosine phosphatase 4a 1
102292_at	*Gadd45a*	U00937	2.3	Fld<2	Growth arrest and DNA-damage-inducible 45 alpha
104534_at	*Pgm1*	AA623974	2.3	Fld<2	Phosphoglucomutase 1
98892_at	*Lpin1*	AI846934	2.3	NS	Lpin1
104494_at	*EST*	AI642098	2.3	NS	Unknown
103062_at	*Rab33b*	AB004664	2.3	NS	RAB33B, member of RAS oncogene family
96777_at	*Sf3b1*	AW049372	2.2	NS	Splicing factor 3b, subunit 1
100065_r_at	*Gja1*	M63801	2.2	Fld<2	Gap junction membrane channel protein alpha 1
94378_at	*Rgs16*	U94828	2.2	Fld<2	Retinally abundant regulator of G-protein signaling mRGS-r
104257_g_at	*Pscdbp*	AI120844	2.2	Fld<2	Pleckstrin homology, Sec7, and coiled-coil domains, binding protein
94088_at	*Ptbp2*	AW228429	2.2	Chg	Polypyrimidine tract binding protein 2
98540_g_at	*Cops2*	AF071312	2.2	Chg	COP9 (constitutive photomorphogenic homolog), subunit 2
94662_at	*EST*	AA409766	2.2	NS	Unknown
100010_at	*Klf3*	U36340	2.2	NS	Kruppel-like factor 3
98608_at	*Etf1*	AI845886	2.1	Fld<2	Eukaryotic translation termination factor 1
93471_at	*Slc4a7*	AI594427	2.1	Chg	Solute carrier family 4, sodium bicarbonate cotransporter, member 7
100064_f_at	*Gja1*	M63801	2.1	Fld<2	Gap junction membrane channel protein alpha 1
100509_at	*Rnf19*	AW121012	2.1	Fld<2	Ring finger protein 19
92638_at	*Ppp2ca*	Z67745	2.1	NS	Protein phosphatase 2a, catalytic subunit alpha isoform
103708_at	*Eifla*	AI132207	2.1	Fld<2	Eukaryotic translation initiation factor 1A
104340_at	*Mbd1*	AF072240	2.1	Fld<2	Methyl-CpG binding domain protein 1
101437_at	*Stk2*	AF039574	2.1	NS	Serine/threonine kinase 2
103451_at	*Ptk2b*	AI835159	2.0	Chg	PTK2 protein tyrosine kinase 2 beta
100297_at	*Wdr26*	AA693125	2.0	NS	WD repeat domain 26
98946_at	*Wsb-1*	AF033186	2.0	Fld<2	WD-40-repeat-containing protein with a SOCS box 1
99087_at	*D10Ertd749e*	AW060179	2.0	Chg	DNA segment chromosome 10
103028_at	*Itk*	D14042	2.0	NS	IL-2-inducible T-cell kinase
103614_at	*Nfkb2*	AW047899	2.0	Fld<2	Nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100
94415_at	*EST*	AA710439	2.0	Fld<2	IMAGE-1165752
103288_at	*Nrip1*	AF053062	2.0	NS	Nuclear receptor-interacting protein 1

[a] Fold changes were not shown for those genes that had detection calls of absent (A) in the 4-h treated group; that had an inappropriate change call (Chg), such that there was not a call of increased or marginally increased in greater than half of the comparisons made; that had a fold change less than 1 (Fld<2); or that were not determined to be significant by parametric and nonparametric tests with a P cutoff value of ≤ 0.05.

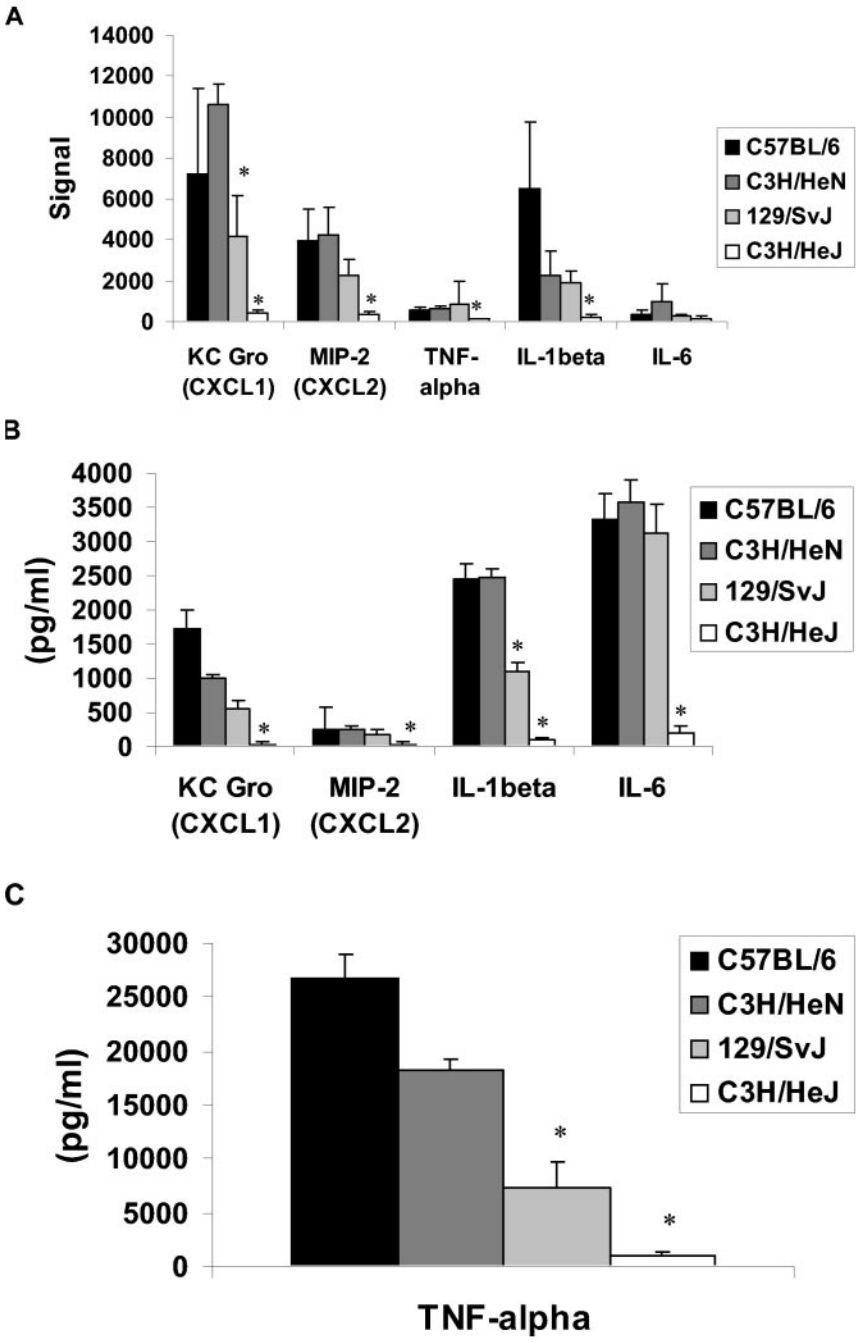

FIG. 4. A: presence of key TLR4-dependent transcripts in lung samples generated in four different strains of mice 4 h after intratracheal challenge with *K. pneumoniae*. Signal values were generated by using algorithms found in MAS software version 5.0 (Affymetrix), and biological replicates for each strain were averaged together and plotted by using Excel. Control strains, C57BL/6 and C3H/HeN, showed higher signal values, while the 129/SvJ strain showed lower levels of signal for almost all genes displayed. The C3H/HeJ strain showed a minimal signal response for these genes, as expected. Data are expressed as means ± standard errors of the means (SEM) ($n = 4$ to 9 mice per group; an * denotes a P value of <0.05 compared to C57BL/6 mice). B: protein concentrations in macrophage supernatants by Bio-Plex assay of four TLR4-dependent immune factors in four strains of mice after 6 h of ex vivo incubation with heat-killed *K. pneumoniae.* These levels are consistent with the RNA expression patterns established in the signal plot for the same factors, thus confirming that RNA levels may be predictive of a survival phenotype. Data are expressed as means ± SEM ($n = 4$ to 6 mice per group; an * denotes a P value of <0.05 compared to C57BL/6 mice). C: presence of TNF-α protein as measured by enzyme-linked immunosorbent assay in supernatants of macrophages isolated from four strains of mice and incubated with *K. pneumoniae* for 6 h. Data are expressed as means ± SEM ($n = 4$ to 6 mice per group; an * denotes a P value of <0.05 compared to C57BL/6 mice).

GRO, CD14, and Myd88) and graphed their fold change from time zero by strain and experimental time point (Fig. 5). As illustrated, C3H/HeJ mice had significantly lower fold change values for all genes at 4 h than mice with intact TLR4 ($P <$ 0.001; analysis of variance) compared to those at 16 h (Fig. 5), where they had a fold induction similar to that of a strain with intact TLR4 at 4h. The results for C3H/HeN and 129/SvJ at 4 h gave similar expression patterns which were also depicted in

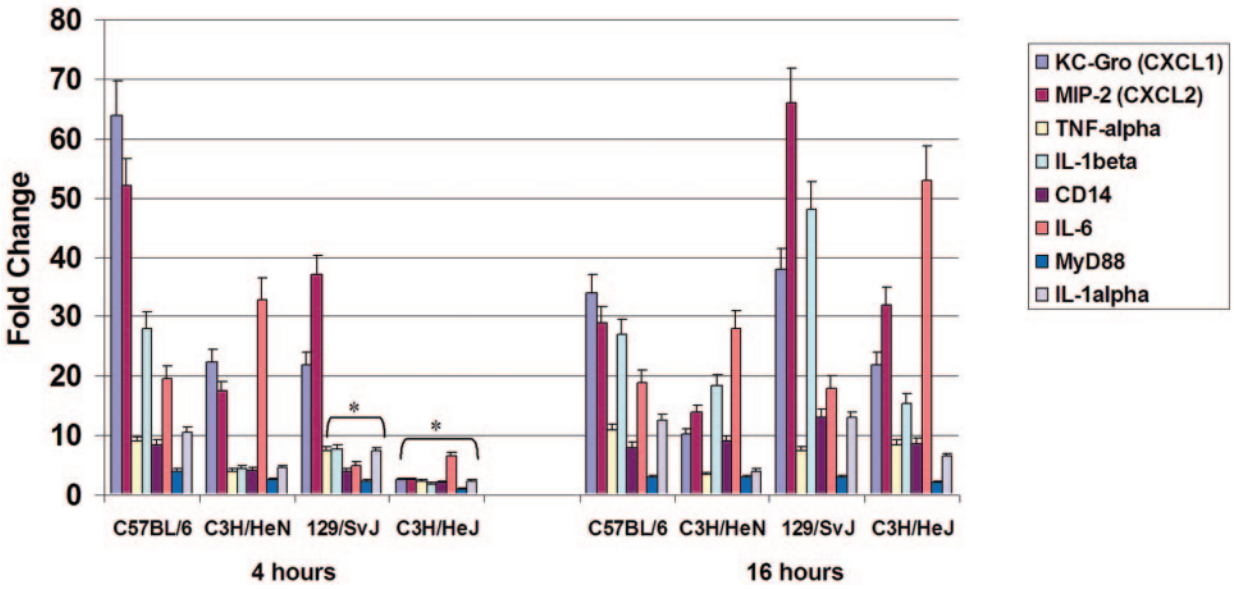

FIG. 5. Analysis of eight known related genes regulated by TLR4 by strain. In order to verify our hierarchical clustering, we plotted the average fold change values of eight highly expressed TLR4-related genes (KC-GRO, MIP-2, TNF-α, IL-1β, CD14, IL-6, MyD88, and IL-1α) by strain and time. Data are expressed as means ± SEM (an * denotes a P value of <0.05 compared to C57BL/6 mice).

the hierarchical cluster, whereby these two strains at this time point clustered together. This graph also demonstrated the same trends seen in the hierarchical cluster, where C3H/HeN, 129/SvJ, and C3H/HeJ showed delayed expression in these genes at 4 h but at 16 h appeared to catch up to the expression values seen at the 4-h time point in C57BL/6 mice. Thus, this analysis independently confirms data from hierarchical clustering (Fig. 2).

DISCUSSION

The development of pneumonia is a complex interaction between virulence factors of the invading pathogen, the inoculum size, and host factors. The results outlined in these studies suggest that TLR4 recognition of invading pathogens is a critical early response pathway that regulates genes ultimately responsible for successful host defense against gram-negative bacteria.

TLR4 signaling was responsible for a diverse set of genes involved in innate immunity, such as IL-1β and TNF-α; chemokines involved in host defense and granulopoeisis, including MIP-2, KC-GRO, MIP-1α, and G-CSF (6, 19); and receptors expressed in lung cells and recruited immune cells. Further support of our data is provided by the fact that antibody neutralization or genetic disruption of MIP-2 (8), TNF (17, 20), MIP-1α (18), or the urokinase receptor (9) results in impaired host defense against gram-negative bacteria. Some of the genes in Table 1, such as CD14, MyD88, and CD44, may be upregulated due to cell recruitment into the lung rather than a true increase in endogenous gene expression. We chose 4 h for our first time point since this time point represents the beginning of significant neutrophil recruitment into the lung, and we postulated that this time point would largely represent endogenous genes, but we cannot exclude an effect of marginated cells in the lung that may account for this set of genes appearing in our list of TLR-dependent genes. Furthermore, our data show that TLR was required for nearly all of the cytokine/chemokine gene expression at 4 h in our model, as these growth factors are notably absent in our TLR4-independent or shared genes (Tables 2 and 3). Moreover, this microarray analysis revealed that TLR4 appears to be critical for downregulation of HSPs (Table 2). Recent evidence supports HSP 70 as an endogenous stimulus for TLR signaling (2, 5, 31), and downregulation of HSPs in the control strains may occur in an effort to downregulate the inflammatory response.

Studies in this model have suggested that macrophages which express TLR4 are critical to early host defense (4). To confirm some of these critical genes at the protein level, we performed ex vivo stimulations with macrophages from the mouse strains. These ex vivo experiments confirmed the role of TLR4 in the protein production of cytokines and chemokines. However, our data do not allow us to determine the role of resident alveolar macrophages in the overall production of the TLR4 pathway in the lung. Using microarray analysis, Weighardt and colleagues have recently shown that over 40% of the gene expression in purified dendritic cells is Trif dependent rather than MyD88 dependent (33). Moreover, 129/SvJ mice have also been reported to have a defect in the upregulation of costimulatory molecules on dendritic cells in response to double-stranded RNA, and this appears to be due to a single gene defect (13). As this strain clearly also has attenuated host defense against *K. pneumoniae* and has altered expression of genes downstream of TLR4, this phenotype may be explained by a defective adaptor protein that plays a role in host defense against gram-negative bacteria and recognition of double-stranded RNA. Moreover, due to these defects in the 129/SvJ mice, phenotypes obtained in knockout mice backcrossed to C57BL/6 mice must be interpreted with caution until these 129/SvJ alleles are elucidated (13).

Of note, TLR4-deficient mice clustered with mice with intact TLR4 at 16 h, suggesting that they have a delayed response. As

TLR4 is the critical LPS receptor, we speculate that this change in gene expression at 16 h may involve other MyD88-dependent pathways such as TLR2 (25, 30) or TLR9 (12) which could come into play later in this model of infection. In support of this speculation, it has been recently been reported that MyD88 knockout mice fail to upregulate TNF-α even 24 h after a pulmonary challenge with *Pseudomonas aeruginosa*, suggesting that mice lacking MyD88 signaling do not show this catch-up phenomenon (27). Additionally, the whole organ gene expression profiling approach clearly misses some genes with are indirectly TLR dependent. For example, IL-17A and IL-17F, which regulate lung neutrophil recruitment into the lung and host defense in this model, were absent on the chip algorithm but have been demonstrated to be TLR dependent by a Taqman approach (11). Thus, our approach is biased towards the most abundantly expressed transcripts and does not exclude more subtly expressed genes that may be equally critical for host defense. Moreover, our data are limited to one strain of *K. pneumoniae*, and some of the TLR4-dependent genes may be under indirect control of TLR4 signaling via TLR4-dependent activation of a signaling molecule or transcription factor. Nevertheless, these data do show that TLR4 signaling is a critical early response pathway and accounts for 120 of the 162 genes that change in 4 h in C3H mice or 74% of gene expression. This coordinated gene expression plays a key role in determining lung host defense to live gram-negative bacteria. We postulate that the ability to rapidly express TLR4-related genes in response to a bacterial challenge permits containment of infection and survival, while delayed expression of these genes results in bacterial dissemination and mortality.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AA010384, HL061271 (J.K.K.), and AI051677 (J.E.S.) and the Louisiana Gene Therapy Research Consortium.

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Editor: F. C. Fang