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The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades

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Björkbacka, Harry, Katherine A. Fitzgerald, François Huet, Xiaoman Li, James A. Gregory, Melinda A. Lee, Christine M. Ordija, Nicole E. Dowley, Douglas T. Golenbock, and Mason W. Freeman. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiol Genomics* 19: 319–330, 2004. First published September 14, 2004; doi:10.1152/physiolgenomics.00128.2004.—Myeloid differentiation protein-88 (MyD88) is a signal adaptor protein required for cytokine production following engagement of Toll-like receptors (TLRs) by their cognate ligands. Activation of both TLR-3 and TLR-4, however, can engage signaling events independent of MyD88 expression. The relative importance of these MyD88-dependent and -independent signaling pathways in the macrophage response to lipopolysaccharide (LPS) is unknown. Here we define these events using microarray expression profiling of LPS-stimulated macrophages taken from MyD88-null and wild-type mice. Of the 1,055 genes found to be LPS responsive, only 21.5% were dependent on MyD88 expression, with MyD88-independent genes constituting 74.7% of the genetic response. This MyD88-independent gene expression was predominantly transcriptionally regulated, as it was unaffected by cycloheximide blockade of new protein synthesis. A previously undescribed group of LPS-regulated genes (3.8%), whose induction or repression was significantly greater in the absence of MyD88, was also identified by these studies. The regulation of these genes suggested that MyD88 could serve as a molecular brake, constraining gene activity in a subset of LPS-responsive genes. The findings generated with LPS stimulation were recapitulated by exposure of macrophages to live *Escherichia coli*. These expression-profiling studies redefine the current dogma of TLR-4 signaling and establish that MyD88, although essential for some of the best-characterized macrophage responses to LPS, is not required for the regulation of the majority of genes engaged by macrophage exposure to endotoxin or live bacteria.

monocytes/macrophages; lipopolysaccharide; gene regulation; signal transduction; cellular activation

THE ACTIVATION OF THE INNATE immune response by infectious microorganisms requires the integration of complex networks of signal transduction pathways, leading to the induction of specific sets of genes (18). Toll-like receptors (TLRs) have emerged as a major recognition and signaling component of the mammalian host defense. TLRs recognize molecular products derived from all the major classes of microbes including bacteria, viruses, yeast, and fungi. Elucidating the cellular responses following the activation of individual TLRs is fundamental to our understanding of innate immunity.

TLR superfamily members share a conserved Toll/interleukin-1 resistance (TIR) domain, which is also present in the interleukin-1 receptor (IL1R) and related proteins, such as SIGIRR and ST2 (27, 48). Engagement of these receptors by their respective ligands allows the recruitment of one or more TIR domain-containing adapter molecules. These adapters, myeloid differentiation protein-88 (MyD88), Mal (also known as TIRAP), TRIF (also known as TICAM-1), or TRAM (also known as TIRP, TICAM2), provide a structural platform for the recruitment of kinases and downstream effector molecules (for a complete review see Refs. 31, 47). The complex signaling network initiated by the TIR domain-mediated interaction of TLRs and adapter proteins ultimately dictates the distinct pattern of gene expression that one observes in response to different TLR agonists.

MyD88, the first TIR-domain adapter molecule to be described, is essential for signaling by all IL1R/TLR family members with the exception of TLR-3 (15, 33). The recruitment of MyD88 to oligomerized TIR domains engages NF-κB and MAPK signaling cascades, resulting in the expression of proinflammatory cytokines, such as TNF (5, 19, 32). Consistent with these observations, the production of TNFα is impaired in MyD88-deficient macrophages following stimulation by various TLR ligands or IL-1β (1, 23). Although MyD88 initially appeared to be required for signal transduction events engaged by TLR activation, certain TLR agonists, such as lipopolysaccharide (LPS), have been shown to generate signals in the absence of MyD88. These responses include the upregulation of costimulatory molecules on dendritic cells (22), caspase-1-dependent release of IL-18 (38), and expression of type I interferons (IFN-β) and interferon-stimulated genes (24). To clarify the role of MyD88, we set out to determine the relative contribution of MyD88-dependent and -independent signaling events in the totality of macrophage responses to endotoxin or live *Escherichia coli*. To accomplish this, we conducted genome-wide expression profiling experiments in macrophages taken from MyD88-null and wild-type (WT) mice. Surprisingly, we found that the preponderance of LPS or *E. coli*-responsive genes was regulated by the MyD88-independent signaling network. Although the MyD88-independent genes include many that are known to be important mediators of innate immunity, a large group of genes in this class are of unknown function. These findings indicate that recently identified adaptor molecules, other than MyD88, that lie downstream of TLR-4 play a more important role in the response to

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bacterial engagement by macrophages than was previously recognized.

EXPERIMENTAL PROCEDURES

Materials. MyD88-deficient mice were a gift from S. Akira (Osaka, Japan) (1) and were backcrossed onto a C57BL/6 background for a minimum of six generations, theoretically yielding mice that are genetically ~98.5% C57BL/6. LPS derived from *E. coli* strain O111:B4 was purchased from Sigma, dissolved in deoxycholate, and re-extracted by phenol:chloroform as described (14). *E. coli* O111:B4 (smooth strain) gram-negative bacteria were purchased from American Type Culture Collection (ATCC, Manassas, VA). Endotoxin-free poly I:C was from Amersham Pharmacia (Piscataway, NJ) and was used in the absence of lipid reagents. Sendai virus was from Charles River Laboratories (Wilmington, MA). Malp-2 was from EMC Microcollections (Tuebingen, Germany). R-848 was from GLSynthesis (Worcester, MA). CpG DNA 1668 ODN was from MWG Biotech, and cycloheximide was from Sigma (St. Louis MI). The plasmids, pCDNA3-MyD88-AU1, pEF-Bos-Trif-Flag, and NF-κB-luciferase, used in cell transfection experiments have been previously described (10). IRF3–5D was from John Hiscott (Montreal, Quebec, Canada). p65-HA was from Tom Maniatis (Harvard, MA). The human Vig1 promoter (450 bp) was cloned from Thp-1 genomic DNA into pGL-3 enhancer (Promega, Madison, WI).

Cell culture and RNA isolation. Mice were anesthetized with isoflurane and then killed by cervical dislocation. Bone marrow was isolated from 6- to 8-wk-old C57BL/6 WT and MyD88-deficient mice. Macrophages from these marrows were cultured in DMEM media supplemented with 10% FBS and 15% L929 conditioned media [a source for colony-stimulating factor (CSF)-1] for 8 days. Embryonic fibroblasts were derived from WT or MyD88-deficient *day 14* embryos by trypsinization and cultured in DMEM, 20% FCS with antibiotics. Thioglycolate-elicited peritoneal-exudate macrophages were cultured as described (35). All experimental procedures involving animals conformed to NIH guidelines for animal research and have been approved by the Massachusetts General Hospital Animal Care Committee. For mRNA expression studies by quantitative reverse transcriptase PCR (QRT-PCR) and microarray analysis in bone marrow-derived macrophages (BMDMs), cells were treated at *day 8* ex vivo for 2 h with 10 ng/ml LPS or live *E. coli* bacteria (log phase; 1 bacteria per 1 macrophage) for 2 h, and their gene expression profiles were compared with that of mock-treated cells incubated for the same time. Cells from four individual mice of each genotype were used, and each mouse served as its own mock control. Total RNA for microarray analysis and quantitative PCR was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy columns (Qiagen, Valencia, CA). RNA quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Microarray analysis. The Operon *Mus musculus* ver. 1.1 probe set (Qiagen) consisting of over 13,000 oligonucleotide probes (70-mers) was printed in the Massachusetts General Hospital (Cambridge, MA) microarray core facility using an Omnigrid 100 (GeneMachines, San Carlos, CA) on CodeLink activated slides (Amersham, Piscataway, NJ). RNA was reverse transcribed and differentially labeled with Cy3 and Cy5 dyes (Amersham) using the Atlas PowerScript fluorescent labeling kit (BD Biosciences, Palo Alto, CA). Labeled samples were hybridized overnight using an automated hybridization station (Genomic Solutions; Perkin-Elmer, Boston, MA). Fluorescent images from the arrays were acquired using a microarray scanner and its accompanying software (GenePix 4000B microarray scanner; Axon Instruments, Union City, CA). Data was stored and further quality controlled using the GeneTraffic software (Iobion Informatics, La Jolla, CA) and the BioArray Software Environment (BASE) (36). Higher-level data and statistical analysis was performed using the GeneSpring (Silicon Genetics, Redwood City, CA) software and Gene Expression Dynamics Inspector v. 2.0.5 (GEDI; **http://web1.tch.harvard.edu/research/ingber/GEDI/gedihome.htm**) (7). For complete microarray data sets visit the ParaBioSys web site (**http://pga.mgh.harvard.edu/**) or the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (**http://www.ncbi.nlm.nih.gov/projects/geo/**; accession numbers GSE1383, GSE1384, GSE1385, and GSE1386).

QRT-PCR. Random-primed (Promega) cDNAs were generated by reverse transcription of total RNA samples with SuperScript II (Invitrogen). QRT-PCR analysis was performed with an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) using 2× QuantiTect SYBR Green PCR Kit (Qiagen). Standard curves used to determine the relative abundance of unknown samples were derived from serially diluted gel-purified PCR products or cloned plasmids. Data are expressed as fold changes of gene expression in LPS-treated samples compared with mock-treated samples. The expression of β-actin was used as a control. Primers were designed using Primer3 (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA) or a public PrimerBank for PCR primers (49) (Table 1).

ELISA. BMDMs at *day 8* ex vivo were plated and cultured in 96-well plates (5×10^4 cells/well) for 24 h prior to treatment with MALP2 (0.01, 0.1, 1, 10 nM), poly I:C (0.1, 1, 10, 100 μg/ml), LPS (0.1, 1, 10, 100 ng/ml), R-848 (0.01, 0.1, 1, 10 μM), CpG DNA

Table 1. *Primers*

Gene	Forward Primer (5′)	Reverse Primer (3′)	cDNA Product Size, bp
Cox2	GCTGTACAAGCAGTGGCAAA	GCTCGGCTTCCAGTATTGAG	318
IL-1β	CAGGCAGGCAGTATCACTCA	AGGCCACAGGTATTTTGTCG	219
IL-6	AACGATGATGCACTTGCAGA	GAGCATTGGAAATTGGGGTA	285
IP-10	AAGTGCTGCCGTCATTTTCT	CATTCTTTTTCATCGTGGCA	200
Irf1	GGAAGGGAAGATAGCCGAAG	ACTCACTCAGGAGGGCAAGA	341
Irg1	CACTCTAAAGGACGGGACCA	GGAGCAGGAAACCAAAACAA	337
M-CSF	ATGGACACCTGAAGGTCCTG	GCTGGAGAGGAGTCTCATGG	303
MIP-1β	TTCTGTGCTCCAGGGTTCTC	GAGGAGGCCTCTCCTGAAGT	282
PAI-1	ACGTTGTGGAACTGCCCTAC	TTTTGCAGTGCCTGTGCTAC	306
RANTES	CCCTCACCATCATCCTCACT	CTTCTTCTCTGGGTTGGCAC	218
TNFα	CAGTTCTATGGCCCAGACCCT	CGGACTCCGCAAAGTCTAAG	450
Ifit1	TGGCCGTTTCCTACAGTTTC	GGAGCATTGGAACACTTGGT	329
Mlp	TCTTGTGCTGTGCCTAGTGG	GGGGTTTGGCCATTAAAAGT	233
Vig1-pending*	AACCCCCGTGAGTGTCAACTA	AACCAGCCTGTTTGAGCAGAA	140
Ifit3*	AGTGAGGTCAACCGGGAATCT	TCTAGGTGCTTTATGTAGGCCA	188
Tyki	GGCAATTATCTCGTGGCTTC	GGGTTCTCCATACGCTGGTA	334
β-Actin	TTGAACATGGCATTGTTACCAA	TGGCATAGAGGTCTTTACGGA	675

*See Ref. 49.

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(0.005, 0.05, 0.5, 5 μM), or infection with Sendai virus (0.1, 1, 10, 100 hemagglutination units/ml). Cell culture supernatants were removed at 0, 1, 2, 4, 6, 8, and 24 h and analyzed for the presence of TNFα, IL-6, IL-12p40, granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), RANTES, and IP-10 by ELISA according to the manufacturer's recommendations (R & D Systems, Minneapolis, MN).

Transcription factor binding site identification. The sequences of genes represented on the microarrays, spanning the region from 1,000 bp upstream of the start site of transcription to 500 bp downstream of that site, were downloaded from **http://siriusb.umdnj.edu:18080/EZRetrieve/index.jsp** (55), and Repeat Masker (**http://www.repeatmasker.org/**) was used to mask out repeats. NF-κB and interferon-stimulated response element (ISRE) sites were defined by TRANSFAC 6.0 weight matrices (**http://www.gene-regulation.com**), and a similarity scoring method was used to find matching sequence sites. For each position along the sequence, a match score to the weight matrix minus the minimal possible score divided by the difference of the maximal score and the minimal weight matrix score was calculated and multiplied by 100. A resulting score larger than 85 was considered a match. One insertion (or deletion) in the $k \pm 1$ bases from the current position was allowed and a match recorded if the calculated result score, excluding the inserted (or deleted) position, was larger than 85.

Transfection assays. TLR-3-expressing HEK293 cells were seeded into 96-well plates at a density of 1.5×10^4 cells/well and transfected 24 h later with 40 ng of the indicated promoter-luciferase constructs and a thymidine kinase *Renilla*-luciferase reporter gene (TK-luc; Promega) (40 ng/well) using GeneJuice (Novagen, Madison, WI). Cells were also cotransfected with expression vectors for MyD88, TRIF, p65, or IRF3–5D as indicated. After 24 h, cells were lysed and reporter gene activity was measured using the Dual Luciferase Assay System (Promega). Data were normalized to TK-luc and are expressed as the mean stimulation ± SD.

RESULTS

Role of MyD88 in TLR signaling. To examine the requirement for MyD88 in TLR signaling, we treated WT C57BL/6 and MyD88-deficient BMDMs with a variety of TLR agonists at varying concentrations. The induction of TNFα (reported to be MyD88 dependent when activated by TLR-4; Ref. 23) and RANTES (MyD88 independent; Ref. 10) were measured. MyD88-dependent induction of TNFα was observed in macrophages upon treatment with LPS (TLR-4), Malp-2 (TLR-2), CpG DNA (TLR-9), and R-848 (TLR-7) but not upon treatment with the TLR-3 ligand poly I:C or following infection with Sendai virus (Fig. 1*A*). The expression of RANTES was impaired following stimulation with Malp-2, CpG DNA, or R-848. In contrast, RANTES was induced normally following poly I:C and LPS stimulation or infection with Sendai virus (Fig. 1*B*). These findings suggest that both TLR-3 and TLR-4 signaling pathways have distinctive properties that distinguish them from the other TLRs. TLR-3 signaling is unique among TLRs in that it does not require MyD88, whereas TLR-4 can employ both MyD88-dependent and -independent signaling pathways.

LPS and E. coli elicit identical responses in murine macrophages. To delineate the role of MyD88 in TLR-4 signaling, we used microarray analysis to examine the gene expression profile of WT C57BL/6 and MyD88-deficient macrophages following LPS and live *E. coli* stimulation. Four conditions were tested: *1*) WT BMDMs stimulated with LPS vs. controls (medium only), *2*) MyD88-null BMDMs stimulated with LPS vs. controls, *3*) WT BMDMs stimulated with living *E. coli* vs. controls, or *4*) MyD88-null BMDMs stimulated with living *E. coli* vs. controls. A concentration of 10 ng LPS/ml was chosen to approximate the endotoxin concentration encountered when cells are treated with live *E. coli* bacteria at a density of ~1 bacterium per macrophage. We found 1,055 genes (7.9% of the 13,369 genes represented on the array) to be significantly induced or repressed (fold change ≠ 1; $P < 0.01$; $n > 4$; Benjamini-Hochberg correction) in at least one of the four conditions tested. The Benjamini-Hochberg correction, when used at a significance level of $P < 0.01$, predicts that only 1% of the genes deemed significant by this test (<11 genes in this experiment) can be expected to be false positives. The LPS- and *E. coli*-induced gene expression profiles observed in WT macrophages closely resemble previously published LPS- and pathogen-induced gene programs in human macrophages and dendritic cells (3, 17, 29, 30, 39). Among the most highly responsive genes in our data set were cytokines, chemokines, and other proinflammatory mediators, including TNFα, IL-1β, IL-12p40, IL-6, Gro1, IP-10, MIP-1α, MIP-1β, RANTES, and COX-2.

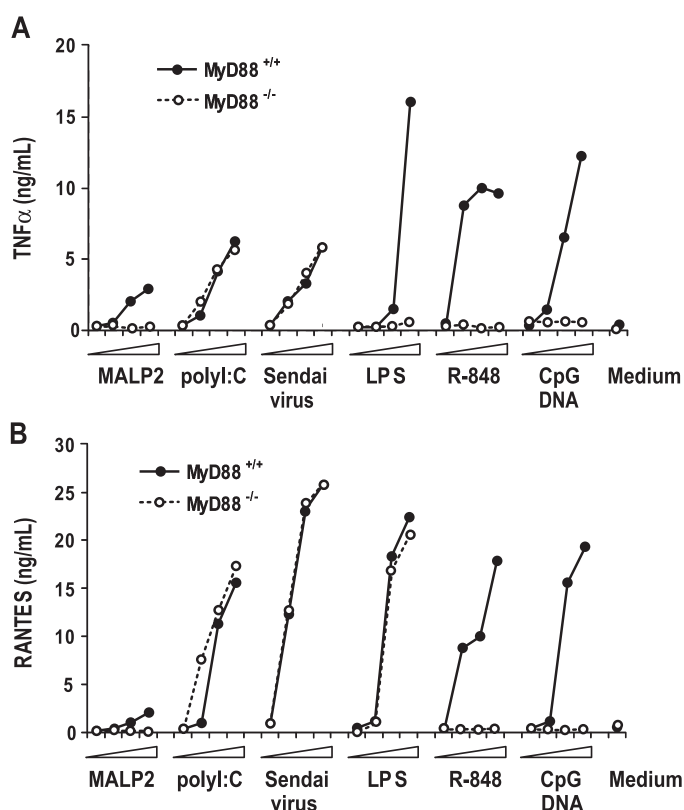

Fig. 1. Myeloid differentiation protein-88 (MyD88) plays a unique role in Toll-like receptor-3 (TLR-3) and TLR-4 signaling. TNFα (*A*) and RANTES (*B*) were measured in supernatants by ELISA after stimulation for 24 h with several different TLR agonists. The agonists used were the TLR-2 agonist MALP2 (0.01, 0.1, 1, 10 nM), the TLR-3 agonists poly I:C (0.1, 1, 10, 100 μg/ml) and Sendai virus (0.1, 1, 10, 100 hemagglutination units/ml), the TLR-4 agonist lipopolysaccharide (LPS; 0.1, 1, 10, 100 ng/ml), the TLR-7 agonist R-848 (0.01, 0.1, 1, 10 μM), and the TLR-9 agonist CpG DNA (0.005, 0.05, 0.5, 5 μM).

Next we compared the *E. coli* vs. the LPS response by one-way ANOVA analysis. Surprisingly, only 13 genes ($P <$ 0.01) responded differentially to the *E. coli* and the LPS treatment among the 1,055 genes determined to be responsive

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to either stimulus. This number nearly matches the number of genes whose expression would be predicted to differ by chance (∼11 genes), and thus it appears that the *E. coli* and LPS stimulations produced virtually identical gene expression profiles. In Fig. 2*D*, the similarity in macrophage responses to LPS and *E. coli* is depicted using a self-organizing map (SOM) clustering algorithm (GEDI; Ref. 7) that graphically portrays the expression of the 1,055 responsive genes.

MyD88-dependent, MyD88-independent, and "MyD88-modulated" pathways mediate the LPS response. We next set out to group genes based on their requirement for MyD88. Genes can be either induced or repressed by LPS treatment, the response can be either MyD88 dependent or independent, and the MyD88 dependence can be directional: that is, the absence of MyD88 can either increase or decrease the LPS response. To populate these theoretical groups (illustrated in Fig. 2*A*), we started by determining which genes were induced or repressed following LPS stimulation in WT macrophages. For those genes whose expression changed, we next determined whether this change required MyD88 to be present. MyD88-dependent genes were initially defined as those whose absolute change in response to LPS was significantly reduced in the absence of MyD88 ($P < 0.01$, one-way ANOVA using Benjamini-Hochberg correction). The expression of a few genes that failed to reach this statistical measure of significance was noted to be consistently different in WT and MyD88-deficient cells. In

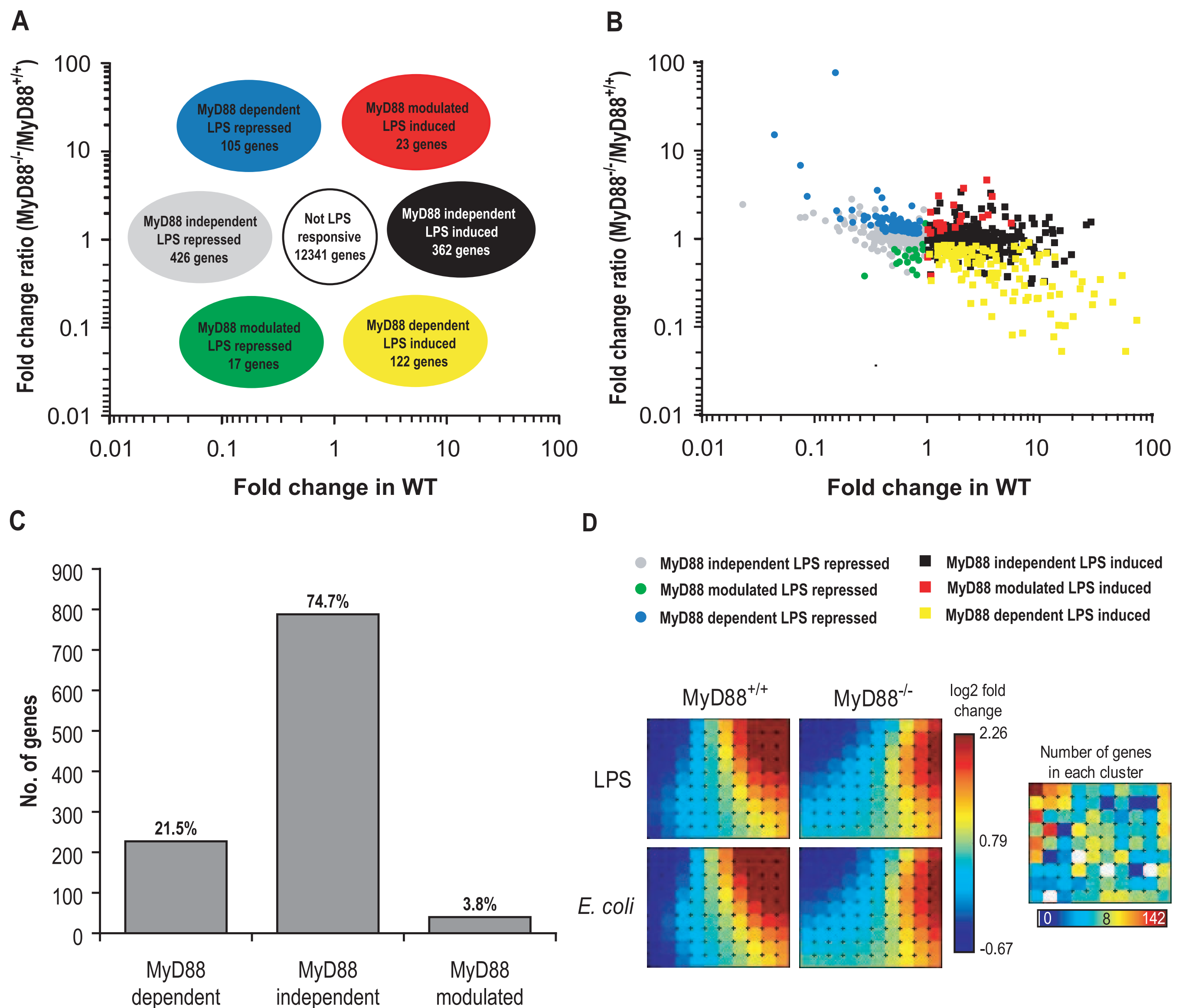

Fig. 2. LPS signaling is mediated by MyD88-dependent, MyD88-independent, and MyD88-modulated pathways. The LPS response in wild-type (WT) and MyD88-deficient macrophages was classified into seven theoretical groups (*A*), defined by the WT response to LPS treatment (*x*-axis) and the MyD88 dependency of the response (*y*-axis). Fold changes in gene expression upon LPS treatment were compared with mock treatment, assessed by microarray analysis (*B*). The LPS response consisting of MyD88-dependent, MyD88-independent, and MyD88-modulated genes is dominated by MyD88-independent pathways. Percentages reflect the number of genes in the category shown divided by the total number of genes that responded to LPS (*C*). A graphical mosaic of LPS-responsive gene expression clusters produced by a self-organizing map algorithm (GEDI) illustrates the similarity of gene responses to LPS and *E. coli* (*D*).

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order not to exclude these genes, we added a second criterion by which a gene could qualify as MyD88 dependent. This criterion required that the difference in expression between MyD88-null and WT cells differed by >4-fold (average calculated for $n > 4$ replicates) upon LPS or *E. coli* treatment. In total, nine genes, including IL-12p40, were added to the MyD88-dependent gene list using this criterion (see *P* values for IL-12p40 in Table 2).

In the statistical analysis, we also noted a small group of genes that were significantly dependent on MyD88 but showed increased induction (or repression) in the absence of MyD88, rather than decreased induction, as might have been expected. Hence, if the absolute response to LPS was greater in the absence of MyD88, then the genes were classified as "MyD88 modulated" (Fig. 2, *A* and *B*). The remaining genes that were LPS responsive, but neither dependent nor modulated by MyD88 as defined above, were considered to be MyD88 independent.

About half of the genes were induced (507 genes of 1,055 or 48.1%) by LPS treatment and half were repressed. This 50% ratio was largely preserved when broken down into the various types of MyD88 responses as just defined (Fig 2*A*). However,

Table 2. *Representative genes of the categorized LPS-induced gene program in macrophages*

Gene	Fold Change			Fold Change	
	WT (n = 8)	MyD88$^{-/-}$ (n = 8)	$P_{\Delta}^{\mathrm{MyD88}}$	WT + CHX (n = 3)	$P_{\Delta}^{\mathrm{CHX}}$
LPS-induced genes (fold upregulated)					
MyD88 dependent					
Interleukin-1β	73 (46–117)	9.3 (3.4–26)	<0.0001	36 (16–77)	0.047
Prostaglandin-endoperoxide synthase 2 (Cox-2)	58 (19–183)	3.1 (1.5–6.2)	0.00032	14 (7.7–25)	0.20
Chemokine (C-X-C motif) ligand 1 (CXCL1; KC; Gro1)	54 (27–107)	23 (11–45)	0.0067	69 (49–97)	0.69
Interleukin-1α	45 (18–109)	8.9 (5.4–15)	<0.0001	29 (21–41)	0.57
Tumor necrosis factor	35 (21–57)	12 (9.5–15)	<0.0001	23 (12–43)	0.53
Serine (or cysteine) proteinase inhibitor, clade E, member 1 (Serpine1; PAI-1)	31 (15–62)	7.4 (5.1–11)	<0.0001	2.8 (1.2–6.5)	0.047
Chemokine (C-X-C motif) ligand 2 (MIP-2)	30 (21–42)	5.4 (3.2–9.1)	<0.0001	33 (20–54)	0.83
Chemokine (C-C motif) ligand 4 (MIP-1β)	24 (13–43)	7.7 (4.6–13)	<0.0001		
Interleukin-10 (IL-10)	23 (15–35)	5.4 (2.4–12)	0.00052	1.0 (0.8–1.2)	0.00023
Interleukin-12β (IL-12p40)	17 (8.5–34)	1.7 (0.2–13)	0.021		
Interleukin-6 (IL-6)	15 (6.7–32)		0.00016		
Colony stimulating factor 2 (granulocyte-macrophage) (Csf2; GM-CSF)	13 (6.3–25)	1.8 (1.3–2.3)	0.00044	2.8 (0.8–10)	0.39
MyD88 independent					
Immunoresponsive gene 1	29 (21–39)	44 (31–62)	0.43	22 (12–41)	0.66
Chemokine (C-X-C motif) ligand 10 (IP-10)	26 (17–40)	37 (18–74)	0.99	30 (20–44)	0.78
Interferon regulatory factor 1	12 (9.3–16)	22 (13–38)	0.13	17 (15–20)	0.17
Mus musculus NIH 3T3 chemokine rantes (Scya5) gene, complete cds (RANTES; CCL5)	15 (11–22)	19 (12–29)	0.48	6.6 (5.6–7.8)	0.028
Icos ligand (Icosl)	13 (8.4–20)	16 (11–25)	0.97	22 (19–26)	0.19
C-type lectin, superfamily member 9 (Clecsf9)	13 (8.8–20)	15 (11–19)	0.84	19 (11–33)	0.48
Tnf receptor-associated factor 1 (Traf1)	9.0 (5.0–16)	13 (6.4–28)	0.92	19 (17–21)	0.17
Interferon-induced protein with tetratricopeptide repeats 1 (Ifit1)	3.7 (2.1–6.6)	13 (6.8–25)	0.040	1.7 (1.4–2.1)	0.16
Colony stimulating factor 1 (macrophage) (Csf1; M-CSF)	11 (8.3–15)	12 (6.9–22)	0.39		
MARCKS-like protein (Mlp)	6.4 (4.6–8.9)	11 (8.5–14)	0.21	7.3 (6.9–7.6)	0.63
MyD88 modulated					
Viral hemorrhagic septicemia virus (VHSV) induced gene 1	3.4 (2.1–5.5)	17 (7.5–39)	0.00088	1.9 (1.1–3.5)	0.38
Toll-like receptor 2 (TLR-2)	5.6 (4.7–6.7)	8.3 (6.8–10)	0.0013	4.0 (3.1–5.2)	0.26
Thymidylate kinase homolog mRNA, complete cds (Tyki)	2.1 (1.4–3.3)	8.2 (6.0–11)	0.00080	1.3 (1.2–1.1)	0.36
Interferon-induced protein with tetratricopeptide repeats 3 (Ifit3)	1.7 (1.3–2.4)	4.4 (2.6–7.3)	0.0020	0.9 (0.8–1.1)	0.054
LPS-repressed genes (fold downregulated)					
MyD88 dependent					
Thioredoxin interacting protein	23 (49–10)	1.5 (1.9–1.2)	<0.0001	2.9 (3.9–2.2)	0.022
Lymphoblastomic leukemia	12 (23–5.9)	3.8 (4.3–3.3)	0.0056	1.7 (3.1–1.0)	0.031
RIKEN cDNA 2700062C07 gene	6.4 (8.1–5.0	3.2 (3.7–2.7)	0.00049	1.7 (2.2–1.2)	0.01
Isovaleryl CoA dehydrogenase putative (Ivd)	5.9 (7.4–4.7)	3.4 (4.0–3.0)	<0.0001	1.2 (1.4–1.0)	0.00070
MyD88 independent					
Cannabinoid receptor 2 (macrophage)	43 (99–19)	14 (44–4.8)	0.18	1.5 (1.8–1.3)	0.0028
cDNA sequence BC003323	13 (28–6.4)	7.3 (11–4.9)	0.23	3.4 (5.8–2.0)	0.11
Hematopoietically expressed homeobox (Hhex)	12 (38–3.9)	6.7 (8.9–5.0)	0.16	1.5 (2.7–0.9)	0.074
MyD88 modulated					
RIKEN cDNA 4933411B03 gene	1.9 (2.5–1.4)	3.5 (4.2–2.9)	0.0026	1.8 (2.8–1.1)	0.93

Bone marrow-derived macrophages (BMDMs) were treated with 10 ng/ml of lipopolysaccharide (LPS) for 2 h, and their gene expression profiles were compared with that of mock-treated cells incubated for the same time using two-color oligonucleotide microarrays. Cells from four individual mice of each genotype were used. Each mouse served as its own mock control, and expression was assayed in duplicate with reversal of the dyes used for labeling control and stimulated samples (n = 8 microarrays per genotype). Cells from three wild-type (WT) mice were treated with 10 ng/ml of LPS and 10 μM cycloheximide for 2 h. The data are reported as fold changes upon LPS treatment, compared with mock treatment. Average fold changes are followed by a 95% confidence interval within parenthesis. Probability values for the effect of MyD88 deletion ($P_{\Delta}^{\mathrm{MyD88}}$) and the effect of cycloheximide inhibition of the LPS response in WT macrophages ($P_{\Delta}^{\mathrm{CHX}}$) were calculated by t-test.

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those genes whose expression was induced had larger percentage changes in expression than the genes that were repressed (note the distribution along the *x*-axis in Fig. 2*B*).

MyD88-independent gene expression dominates the LPS response. Although recent studies (22, 24, 38) had prepared us for the possibility that a few genes would not require MyD88 for their activation by LPS stimulation, we expected the number to be quite small and anticipated that those might be worth analyzing by individual experimental interrogation. In contrast to this prediction, 788 (74.7%) of the 1,055 genes that met our criteria as LPS responsive were MyD88 independent. Only 227 (21.5%) genes were MyD88 dependent (Fig. 2*C*). The number of MyD88-modulated genes was small (40 genes or 3.8%). A striking feature of the data is the predominance of genes induced or repressed to the same extent by LPS in WT and MyD88-deficient cells. As our criteria for classification of genes as MyD88 dependent were quite stringent, we thought it possible that they might have led to the overestimation of the genes classified as MyD88 independent. We therefore examined the levels of differential gene expression in the subset of genes classified as MyD88 independent. This analysis demonstrated that more than 82% of these genes had an average LPS-stimulated expression level in the WT macrophages that was <30% different from the MyD88-deficient macrophages. With current microarray methodology, expression differences of this magnitude typically represent variability in the assay measurement rather than significant differences in gene expression. Thus our finding of the predominance of MyD88-independent gene expression in response to LPS stimulation is unlikely to be a consequence of our choice of criteria in defining MyD88 dependence. The predominance of MyD88-independent gene expression is readily visualized in the GEDI mosaic graph (Fig. 2*D*), where the majority of colorized clusters persists in the MyD88-null cells compared with the WT macrophages. Thus LPS induces a vast gene expression program that is independent of MyD88 activity, although many of the best characterized LPS responses do, in fact, require MyD88.

MyD88-dependent pathways regulate many classic markers of inflammation. The expression of many well-characterized markers of inflammation, including IL-1β, Cox-2, TNFα, IL-10, IL-12, and IL-6, required MyD88 for optimal expression (Table 2). We measured the MyD88-dependent expression of TNFα, MIP-1β, IL-1β, PAI-1, Cox-2, and IL-10 using QRT-PCR (Fig. 3*A*) to verify the microarray results. Simultaneous measurement of the housekeeping gene, β-actin, indicated no change in its expression in the comparison of WT to MyD88-deficient macrophages (Fig. 3*B*). The protein expression of many of the MyD88-dependent inducible genes was also measured by ELISA, to further validate the RNA expression results. As seen in Fig. 3*C*, TNFα, IL-6, IL-12p40, and GM-CSF production was severely attenuated in macrophages taken from MyD88-deficient mice. Thus the microarray data for MyD88-dependent expression of several of the most important

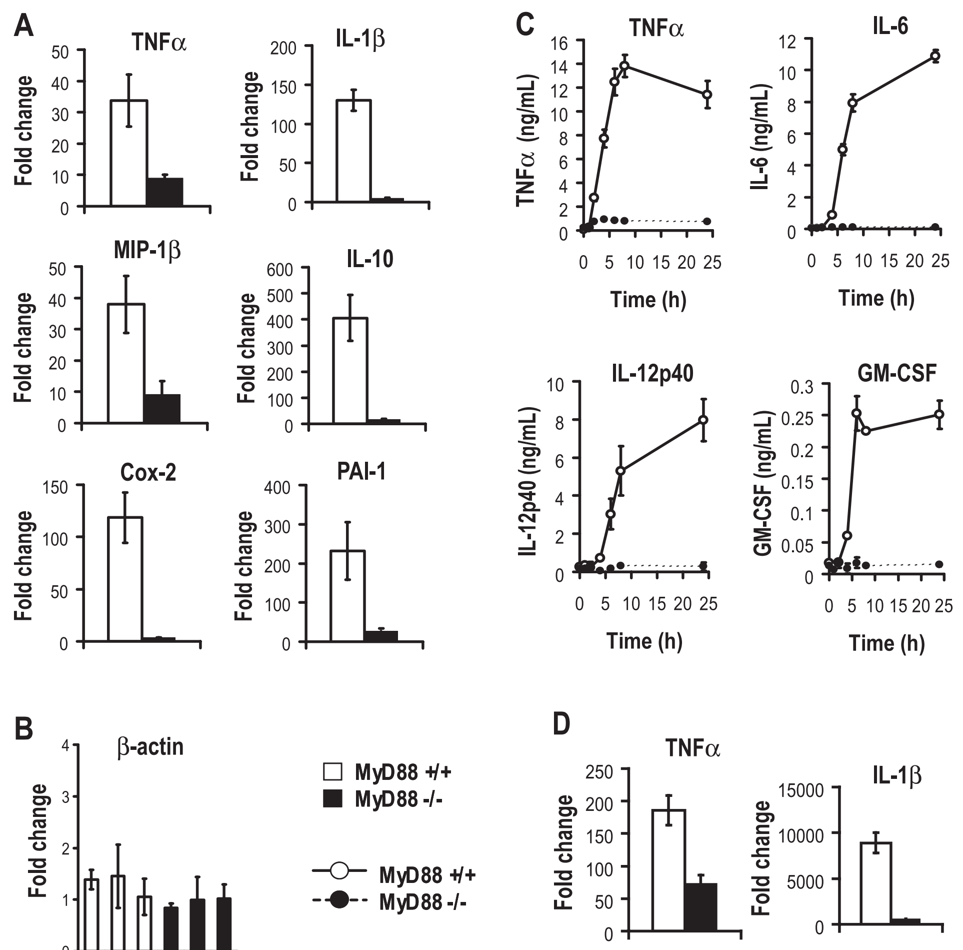

Fig. 3. QRT-PCR and protein expression analysis of MyD88-dependent gene expression. Total RNA was isolated from macrophages 2 h after stimulation with 10 ng/ml LPS or mock treatment. *A*: Total RNA from four mice was pooled, and fold changes in gene expression were determined in triplicate by comparison to the mock-treated sample using QRT-PCR. Since IL-1β expression could not be detected by QRT-PCR in unstimulated cells, this level was assigned the value of the lowest detection limit of the assay to estimate an expression ratio. *B*: QRT-PCR quantification of β-actin in all the reverse-transcribed total RNA samples used for normalization of the data in *A*, and in Figs. 4*A* and 5*A*. *C*: supernatants were collected from macrophages taken from two WT and two MyD88-null mice and stimulated for the time indicated with LPS, and TNFα, IL-6, IL-12p40, and GM-CSF levels were measured in triplicate by ELISA. *D*: total RNA was isolated from thioglycolate-elicited peritoneal-exudate macrophages 2 h after stimulation with 10 ng/ml LPS, and the fold change in gene expression (compared to a mock-treated sample) was quantified by QRT-PCR.

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macrophage inflammatory genes was confirmed using independent measurements of both mRNA and protein production.

MyD88-independent pathways regulate known inflammatory responses as well as many unknown genes. Several of the most highly induced genes were found to be MyD88 independent (Table 2). Some of these genes have previously been shown to participate in the immune response, such as Irf1, IP-10, RANTES, and M-CSF, and their expression was confirmed by QRT-PCR (Fig. 4*A*). However, the functions of many of the MyD88-independent genes are unknown. In the very large group of genes determined to be MyD88 independent, only IP-10, Irg1, IFN-β, and Ifit1 have previously been reported to respond to LPS stimulation via MyD88-independent signaling pathways (24). Using QRT-PCR analysis, we verified the MyD88-independent expression of several genes not previously known to fall into this category, including Irf1, MARCKS-like protein (Mlp), and M-CSF (Fig. 4*A*). ELISA analysis of RANTES and M-CSF established that protein expression also mirrored the mRNA data for MyD88-independent genes (Fig. 4*B*).

BMDMs were cultured in cytokine-rich medium for several days prior to experimentation. The possibility existed that the large number of genes expressed independently of MyD88 activity that we observed reflected the ex vivo maturation conditions used in the culturing of the BMDMs. Thus we measured the LPS-induced activation of two MyD88-dependent genes (TNFα and IL-1β) and two MyD88-independent genes (RANTES and Irf1) in thioglycolate-elicited peritoneal-exudate macrophages by QRT-PCR to assess their dependence on the macrophage preparative conditions (Fig. 3*D* and 4*C*). Although the magnitudes of the MyD88-dependent gene responses were at least 10-fold higher in the peritoneal macrophages than in the BMDMs, the MyD88-dependent and -independent classifications did not differ between these macrophage preparations.

MyD88-modulated genes reveal a novel regulatory function of MyD88. A third group of genes identified in these experiments were those whose expression was induced by LPS to higher levels or repressed to lower levels in macrophages

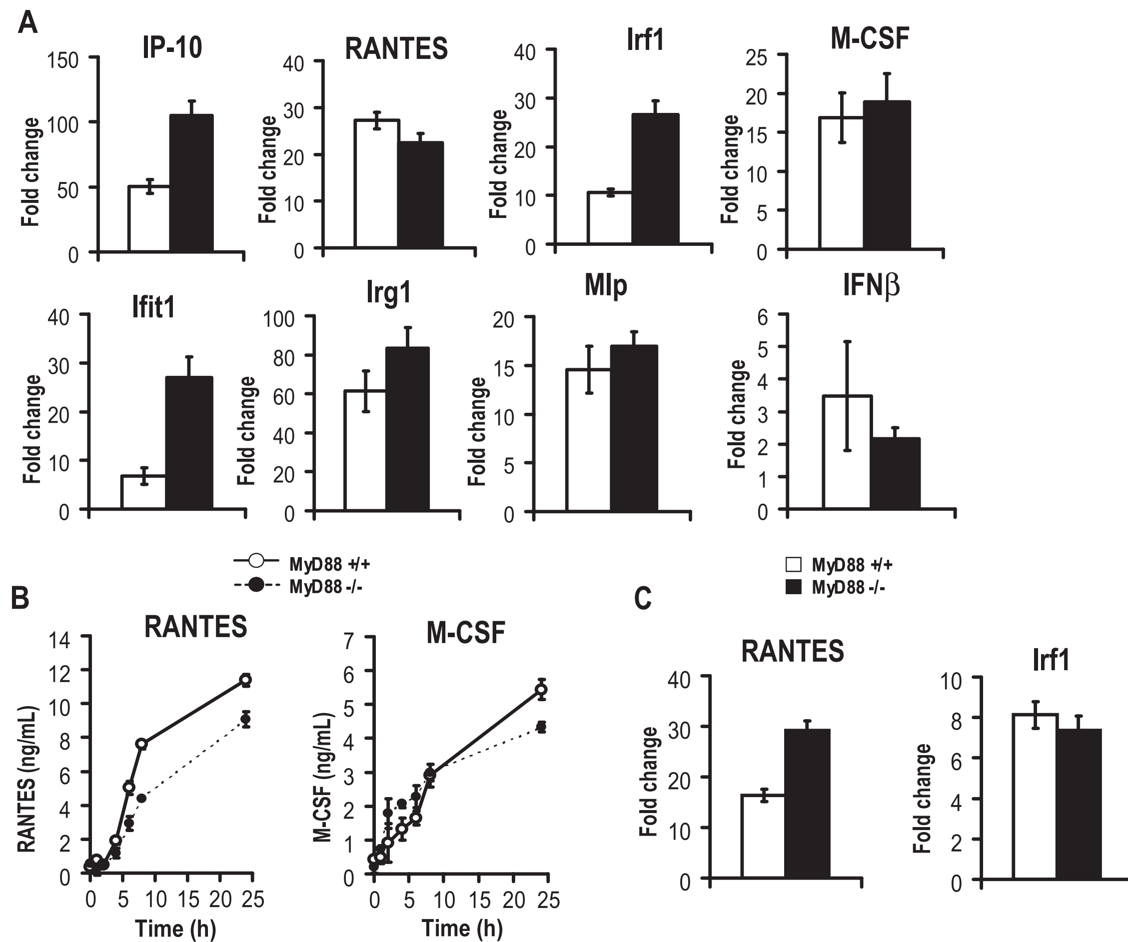

Fig. 4. QRT-PCR and protein expression analysis of MyD88-independent gene expression. Total RNA was isolated from macrophages 2 h after stimulation with 10 ng/ml LPS or mock treatment. *A*: total RNA from four mice was pooled, and fold changes in gene expression were determined in triplicate by comparison to the mock-treated sample using QRT-PCR. *B*: supernatants were collected from macrophages taken from two WT and two MyD88-null mice and stimulated for the time indicated with LPS, and RANTES and M-CSF levels were measured in triplicate by ELISA. *C*: Total RNA was isolated from thioglycolate-elicited peritoneal-exudate macrophages 2 h after stimulation with 10 ng/ml LPS, and the fold change in gene expression (compared to a mock-treated sample) was quantified by QRT-PCR.

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lacking MyD88 activity. These we termed "MyD88 modulated." The modulation of viral hemorrhagic septicemia virus (VHSV) induced gene 1 (Vig1-pending, also known as Viperin and cig5), interferon-induced gene with tetratricopeptide repeats 3 (Ifit3), and thymidylate kinase (Tyki) was confirmed using QRT-PCR (Fig. 5*A*). The QRT-PCR data suggests there may be more MyD88-modulated genes than can be classified using microarray data. Both Ifit1 and Irf1 were classified as MyD88 independent because their expression levels did not differ significantly, using our chosen cutoff value ($P < 0.01$). With QRT-PCR methodology, however, these would have been categorized as MyD88 modulated (Fig. 4*A* and Table 2). The existence of MyD88-modulated genes was further established by measuring LPS induction in WT and MyD88-deficient mouse embryonic fibroblasts (MEFs) by QRT-PCR (Fig. 5*B*). The LPS- and poly I:C-induced response was greater in MyD88-deficient MEFs compared with MyD88-containing MEFs. Both IL-6 and RANTES protein expression measured by ELISA were induced by LPS in WT MEFs (data not shown).

Although the MyD88-modulated gene group comprised only a small percentage (3.8%) of the LPS-responsive genes on the arrays, they provided an insight into a potentially novel MyD88 function. This function can be viewed as constraining the responsiveness of genes (either induction or repression) to endotoxin stimulation. To investigate whether MyD88 modulation also occurs downstream of TLR-3, a Toll receptor whose signaling appears to be entirely dependent on the expression of TRIF, we treated WT and MyD88-deficient MEFs with the double-stranded RNA mimetic poly I:C for 2 h and assayed the induction of MyD88-modulated genes by QRT-PCR (Fig. 5*B*). The expression of Vig1-pending, Ifit3, and Tyki was increased in the absence of MyD88 after poly I:C stimulation, suggesting that MyD88 may also influence the expression of genes induced by the TLR-3 pathway.

MyD88-independent gene expression does not depend on new protein synthesis. Interferon-responsive genes (IRGs) are components of an anti-viral signaling cascade shared by TLR-3 and TLR-4 and are regulated, in part, by the transcription activator, interferon regulatory factor (IRF)-3 (4). The type I interferon IFN-β is an example of one such gene, whose expression can activate a second wave of gene expression via the type I interferon receptor and the JAK/STAT pathways (4, 45). As only one (myxovirus resistance protein 1; $P_{\Delta}^{\mathrm{MyD88}} \sim 0.0013$) of 19 LPS-responsive genes annotated as viral response genes or IRGs was MyD88 dependent, we wondered whether the induction of many of the MyD88-independent genes could be explained by secondary transcriptional events that were dependent on the generation of protein produced in the initial wave of gene induction. To test this possibility, WT macrophages were treated with LPS in the presence of 10 μM of the protein synthesis inhibitor cycloheximide (26). Analysis of this issue was limited to genes whose average expression changed at least fourfold or more in response to LPS, as determined in three or more independent microarray experiments. Genes were deemed cycloheximide sensitive if the induction by LPS alone or by LPS plus cycloheximide was significantly different (*t*-test; $P < 0.05$). Furthermore, gene repression, should it exist for a given gene or set of genes, is often reduced or eliminated by cycloheximide treatment as transcripts become protected from degradation, resulting in enhanced stability of mRNAs (26). We found that the majority of those genes whose expression was repressed by endotoxin were not repressed in the presence of cycloheximide (15 of 18 genes or 83%; Table 2). In contrast, the majority of those genes that were upregulated after LPS stimulation were not sensitive to cycloheximide (65 of 97 genes or 67%; Table 2). MyD88-independent genes still constituted the majority of LPS-responsive genes in the cycloheximide-treated cells (41 of 65 genes or 63%), establishing that MyD88-independent signaling does not simply reflect widespread secondary transcriptional events. These data confirm previous studies by others showing that IP-10 and Irf1 are primary response genes (4, 12) and classifies the expression of several other genes as part of a primary or secondary response pathways (Table 2).

Vig1-pending and other genes not requiring MyD88 for maximal induction are regulated by concordant NF-κB and IRF-3 activation. We were particularly interested in MyD88-independent antiviral response genes such as RANTES, IP-10, and Ifit1, which can be activated without new protein synthesis and are known to be induced by coordinate IRF-3 and NF-κB activation. This suggested that the presence of IRF-3 and NF-κB transcription factor binding sites might represent a signaling module activating at least a subset of the MyD88-

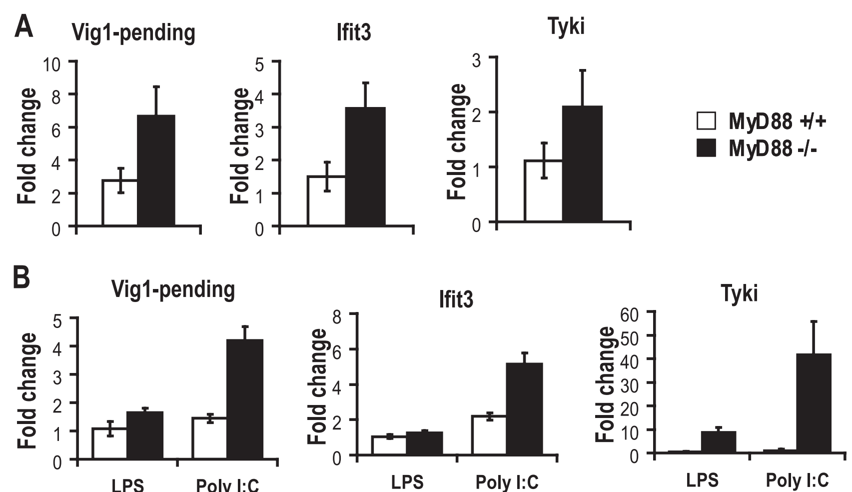

Fig. 5. QRT-PCR expression analysis of MyD88-modulated genes. Total RNA was isolated from macrophages 2 h after stimulation with 10 ng/ml LPS or mock treatment. The fold change in gene expression was determined by comparison to the mock-treated sample using QRT-PCR (*A*). Total RNA was isolated from mouse embryonic fibroblasts (MEFs) 2 h after stimulation with 100 ng/ml LPS (*B*) and 100 μg/ml poly I:C (*C*), and the fold change in gene expression (compared to a mock-treated sample) was quantified by QRT-PCR

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independent genes. Hence, we examined the promoter regions of all of the genes arrayed on our slides for both NF-κB and IRF transcription factor binding sites. The known IRF-3/ NF-κB response genes (RANTES, IFNβ, IP-10, and Ifit1) contained both ISRE and NF-κB sites within 300 bp of the transcription start site and no greater than 100 bp apart from each other. In addition, Irg1, Vig1-pending, and Ifit3, which respond robustly to LPS, share these same tandem site characteristics, suggesting that they are also regulated by concordant IRF-3 and NF-κB activation (Fig. 6A).

To explore the concordant IRF-3 and NF-κB activation further, we examined the inducibility of the Vig1-pending promoter, a gene strongly induced by LPS, in HEK293 cells expressing TLR-3. As seen in Fig. 6B, two strong activators of NF-κB, IL-1β and TNFα, both failed to induce Vig1-pending reporter gene activity. In contrast, stimulation of cells with poly I:C or infection with Sendai virus, which activate both NF-κB and IRF-3, strongly induced reporter gene activity, suggesting that activation of NF-κB alone is insufficient to induce Vig1-pending promoter activation. Recently the TIR domain-containing adapter molecules TRIF and TRAM have been shown to regulate MyD88-independent gene expression via IRF-3. Overexpression of either of these two adapters or MyD88 results in the activation of their downstream signal transduction pathways (8). We examined the ability of either MyD88 or TRIF to activate the Vig1-pending reporter gene (Fig. 6C) in transfected HEK293 cells. Overexpression of MyD88 did not induce Vig1 promoter activity, whereas overexpression of TRIF induced a vigorous Vig1 promoter response. Vig1 promoter activity was also induced by the constitutively active IRF3–5D mutant (Fig. 6D), but not by the p65 subunit of NF-κB, again suggesting that activation of NF-κB alone is insufficient to drive this promoter. This is in agreement with the inability of either IL-1 or TNFα to induce Vig1, as

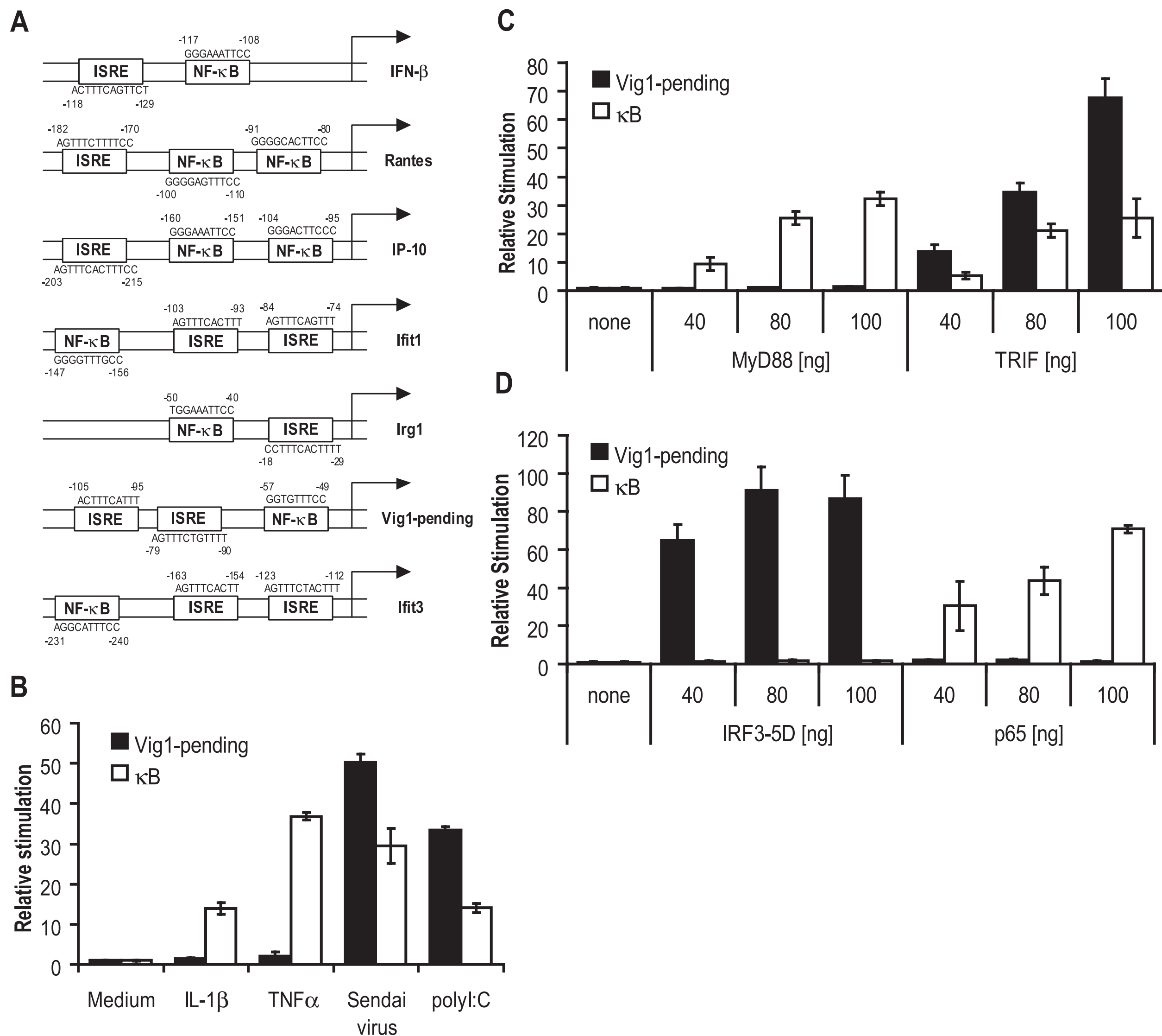

Fig. 6. *A*: Vig1-pending is activated by NF-κB and IRF-3. Schematic representation of NF-κB and ISRE transcription factor binding sites in LPS-induced genes. *B*: TLR-3-expressing HEK293 cells were transfected with luciferase reporter genes under the control of Vig1-pending and NF-κB promoters and activated by IL-1β, TNFα, Sendai virus, and poly I:C. *C*: Vig1-pending and NF-κB promoter activation when cotransfected with MyD88 or TRIF. *D*: Vig1-pending and NF-κB promoter activation when cotransfected with constitutively active IRF-3 or p65.

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these cytokines activate NF-κB but will not activate reporter gene expression from an ISRE. Furthermore, the induction of Vig1 by TRIF was inhibited by coexpression of dominant-negative forms of both NF-κB and IRF signaling (the IκB super-repressor or IRF-3 dominant negative) (data not shown). Taken together, these observations confirm that the MyD88-independent pathway regulator, TRIF, induces Vig1 and that expression of Vig1 requires activation of both NF-κB and IRF-3. Although several MyD88-independent genes contained NF-κB and ISRE binding sites, and are likely critical early mediators of MyD88-independent signaling events, it is clear that additional signaling pathways remain to be identified that would account for the expression activity of the large number of MyD88-independent genes that apparently lack proximal ISRE binding sites.

DISCUSSION

The present study illustrates the use of expression profiling to compare and classify the LPS response in cells that do and do not express critical mediators of that response. This study has some inherent limitations that must be recognized. To conduct these experiments, it was necessary to select a single time point to perform the comparison. As previous work has indicated that LPS responses can be delayed rather than lost in mice lacking key mediators in the TLR pathway (23), our classifications of genes as MyD88 dependent or independent are necessarily limited to the time course we have chosen. To fully understand the complex signaling network induced by LPS over time, additional time course experiments will be required. In addition, our understanding of the transcriptional regulation of this gene network is quite rudimentary, making it unwise to generalize from the limited Vig1-pending transcriptional analysis undertaken here.

In view of the toxicity of gram-negative bacteria, it is not surprising that LPS activates such a rich gene expression program (~1,000 genes or almost 8% of the genes probed in this experiment), all of which are likely induced via TLR-4. The data presented here show that LPS and *E. coli* induce almost identical gene expression profiles, suggesting that the contribution of TLRs other than TLR-4 to the *E. coli* response is either minor or overlapping with the TLR-4 response. The data are the latest confirmation of a long-held (but nevertheless controversial) dogma, i.e., that the LPS portion of the outer leaflet of gram-negative bacteria is the predominant activator of the innate immune system. In this respect, the influence of endotoxin appears to be far greater than other cell wall constituents, including immunologically important molecules such as lipopeptide, peptidoglycan, and flagellin.

MyD88-deficient mice do not need to be housed in a pathogen-free environment to survive and have been shown to mount a host defense response to polymicrobial peritonitis (51) and local *Staphylococcus aureus* infection, despite blunted cytokine responses (41). This suggests that MyD88-independent signaling pathways play important roles in the control of endogenous host microflora as well as in the defense against some virulent pathogens. However, MyD88-deficient mice are more vulnerable to certain infections, such as systemic challenge with *S. aureus* (46), *Listeria monocytogenes* (6), and *Pseudomonas aeruginosa* (41), indicating that MyD88 plays a vital role in normal host defense against important pathogens. The genes downstream of the NF-κB pathway are clearly critical to this host defense response and were clearly dysregulated in the absence of MyD88.

NF-κB, JNK, and p38 MAPK are essential for the induction of TNFα and are activated downstream of MyD88 (40, 42). In MyD88-deficient cells, NF-κB, JNK, and p38 MAPK all exhibited delayed activation. The lack of LPS-inducible TNFα protein production in MyD88-null cells may be due to this delayed activation, although it is difficult to understand how the modestly altered kinetics of these responses might translate into such a profound defect in protein production, especially when so many other LPS responses are entirely intact. Alternatively, MyD88 might regulate an unknown key factor essential for TNFα transcription, mRNA stabilization, translation, or secretion. It should also be noted that not all reports (47) have shown the same failure of MyD88-deficient macrophages to secrete cytokines in response to LPS as presented here and elsewhere (23). Our data also suggest that results obtained in macrophages from different sources by different experimental protocols may not always be directly comparable. The use of thioglycolate to elicit the macrophage exudate in the peritoneum may result in cells that are primed to be more responsive to subsequent inflammatory stimuli. Such sensitization has been observed, for instance, for IL-6 production in macrophages when primed with either low doses of LPS (13) or M-CSF (43).

To avoid detrimental uncontrolled inflammation, the innate immune response must constantly strike a balance between activation and inhibition. Following LPS stimulation, macrophages enter a transient state where a second LPS stimulus is blunted or absent. This so-called "endotoxin tolerance" suggests that inhibitory pathways may become activated that downregulate the LPS response. The "MyD88 modulation" phenomena described herein may reflect a requirement for MyD88 in this attenuation response. MyD88 could directly inhibit LPS activation pathways or alternatively could be required for the induction of an inhibitory molecule. Recently a number of negative regulators of LPS signaling have been described that could modulate expression by either of these mechanisms. These proposed negative regulators, including a short splice variant of MyD88 ($MyD88_s$) (20), Irak-M (25), SOCS-1 (28), PI3K (11), and the TIR domain containing receptors T1-ST2 (44) or SIGGIR (48). These proteins represent attractive candidates responsible for the MyD88 modulation observed in this study.

Our study suggests that the majority (3/4) of the host response to LPS is regulated independently of MyD88 and that this response involves a diversity of host activities in addition to the well-characterized type I interferon response. LPS signaling appears to uniquely utilize all four of the known TIR domain-containing adapter molecules [MyD88, Mal (also known as TIRAP) (9, 16), TRIF (also known as TICAM-1) (33, 54), and TRAM (also known as TIRP/TICAM-2) (2, 10, 34, 53)] to activate its full transcriptional response. Published reports indicate that TRAM may be the "master adapter" for LPS responses (53). TRAM contains an N-myristoylation site that targets this adapter to TLR-4 in resting cells (D. C. Rowe, K. A. Fitzgerald, D. T. Golenbock, unpublished observations). Both targeted deletion and small interfering RNA (siRNA) silencing experiments have demonstrated a nearly absolute requirement for TRAM in the TLR-4 pathway (10, 53).

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In contrast to MyD88 and Mal, which are thought to function as a heterodimer to regulate MyD88-dependent pathways, TRIF seems to bridge both MyD88-independent and MyD88-dependent pathways. TRIF-deficient (52) and TRIF-mutant mice (15) fail to induce IFNβ, IP-10, Irf1, RANTES, TNFα, IL-6, and IL-12p40. TRIF has been shown to interact with TRAF6, a component of the MyD88-dependent pathway that activates NF-κB and controls the expression of genes such as TNFα and IL-6 (21, 37). A recent study from Holzmann and coworkers (50) examining the gene expression profile following LPS stimulation in dendritic cells taken from mice carrying a loss of function mutation in TRIF found that ~50% of the LPS response was dependent on TRIF. Genes appearing to be TRIF dependent are classified as both MyD88-independent and MyD88-dependent genes in our study, consistent with the ability of TRIF to bridge both MyD88-independent and MyD88-dependent pathways.

A genome-wide approach will be critical to defining the individual contribution, and possible redundancy, of the different adapter molecules and pathways to the LPS-induced response in immune cells. Once established, the differences in adapter molecule utilization can be exploited for the rationale design of anti-inflammatory therapies that inhibit certain subsets of deleterious inflammatory responses while leaving host defense mechanisms intact. The data we report here provide an initial roadmap to defining the adaptor specificity of the host innate immune response.

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REFERENCES

1. **Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, and Akira S.** Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9: 143–150, 1998.
2. **Bin LH, Xu LG, and Shu HB.** TIRP, a novel Toll/interleukin-1 receptor (TIR) domain-containing adapter protein involved in TIR signaling. *J Biol Chem* 278: 24526–24532, 2003.
3. **Boldrick JC, Alizadeh AA, Diehn M, Dudoit S, Liu CL, Belcher CE, Botstein D, Staudt LM, Brown PO, and Relman DA.** Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc Natl Acad Sci USA* 99: 972–977, 2002.
4. **Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, Rao G, Sun R, Haberland M, Modlin R, and Cheng G.** IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17: 251–263, 2002.
5. **Dunne A and O'Neill LA.** The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE* 2003 Feb 25 (171): re3.
6. **Edelson BT and Unanue ER.** MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. *J Immunol* 169: 3869–3875, 2002.
7. **Eichler GS, Huang S, and Ingber DE.** Gene Expression Dynamics Inspector (GEDI): for integrative analysis of expression profiles. *Bioinformatics* 19: 2321–2322, 2003.

7a. **Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, and Golenbock DT.** LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *J Exp Med* 198: 1043–1055, 2003.

8. **Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, and Maniatis T.** IKKϵ and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4: 491–496, 2003.
9. **Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA, and O'Neill LA.** Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413: 78–83, 2001.
10. **Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, and Golenbock DT.** LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *J Exp Med* 198: 1043–1055, 2003.
11. **Fukao T and Koyasu S.** PI3K and negative regulation of TLR signaling. *Trends Immunol* 24: 358–363, 2003.
12. **Hattori Y, Akimoto K, Matsumura M, Tseng CC, Kasai K, and Shimoda S.** Effect of cycloheximide on the expression of LPS-inducible iNOS, IFN-beta, and IRF-1 genes in J774 macrophages. *Biochem Mol Biol Int* 40: 889–896, 1996.
13. **Hirohashi N and Morrison DC.** Low-dose lipopolysaccharide (LPS) pretreatment of mouse macrophages modulates LPS-dependent interleukin-6 production in vitro. *Infect Immun* 64: 1011–1015, 1996.
14. **Hirschfeld M, Ma Y, Weis JH, Vogel SN, and Weis JJ.** Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* 165: 618–622, 2000.
15. **Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, and Beutler B.** Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424: 743–748. 2003.
16. **Horng T, Barton GM, and Medzhitov R.** TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol* 2: 835–841, 2001.
17. **Huang Q, Liu D, Majewski P, Schulte LC, Korn JM, Young RA, Lander ES, and Hacohen N.** The plasticity of dendritic cell responses to pathogens and their components. *Science* 294: 870–875, 2001.
18. **Janeway CA Jr and Medzhitov R.** Innate immune recognition. *Annu Rev Immunol* 20: 197–216, 2002.
19. **Janssens S and Beyaert R.** A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem Sci* 27: 474–482, 2002.
20. **Janssens S, Burns K, Tschopp J, and Beyaert R.** Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. *Curr Biol* 12: 467–471, 2002.
21. **Jiang Z, Mak TW, Sen G, and Li X.** Toll-like receptor 3-mediated activation of NF-κB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-β. *Proc Natl Acad Sci USA* 101: 3533–3538, 2004.
22. **Kaisho T, Takeuchi O, Kawai T, Hoshino K, and Akira S.** Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* 166: 5688–5694, 2001.
23. **Kawai T, Adachi O, Ogawa T, Takeda K, and Akira S.** Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122, 1999.
24. **Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, and Akira S.** Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 167: 5887–5894, 2001.
25. **Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, and Flavell RA.** IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110: 191–202, 2002.
26. **McCarthy JE.** Posttranscriptional control of gene expression in yeast. *Microbiol Mol Biol Rev* 62: 1492–1553, 1998.
27. **Mitcham JL, Parnet P, Bonnert TP, Garka KE, Gerhart MJ, Slack JL, Gayle MA, Dower SK, and Sims JE.** T1/ST2 signaling establishes it as a member of an expanding interleukin-1 receptor family. *J Biol Chem* 271: 5777–5783, 1996.
28. **Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, Seki E, Sato S, Takeuchi O, Takeda K, Akira S, Yamanishi K, Kawase I, Nakanishi K, and Kishimoto T.** SOCS-1 participates in negative regulation of LPS responses. *Immunity* 17: 677–687, 2002.
29. **Nau GJ, Richmond JF, Schlesinger A, Jennings EG, Lander ES, and Young RA.** Human macrophage activation programs induced by bacterial pathogens. *Proc Natl Acad Sci USA* 99: 1503–1508, 2002.

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30. **Nau GJ, Schlesinger A, Richmond JF, and Young RA.** Cumulative toll-like receptor activation in human macrophages treated with whole bacteria. *J Immunol* 170: 5203–5209, 2003.
31. **O'Neill LA, Fitzgerald KA, and Bowie AG.** The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol* 24: 286–290, 2003.
32. **O'Neill LA, Fitzgerald KA, and Bowie AG.** The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol* 24: 286–289, 2003.
33. **Oshiumi H, Matsumoto M, Funami K, Akazawa T, and Seya T.** TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 4: 161–167, 2003.
34. **Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, and Seya T.** TIR-containing adapter molecule (TICACM-2): a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-beta. *J Biol Chem* 278: 49751–49762, 2003.
35. **Perera PY, Mayadas TN, Takeuchi O, Akira S, Zaks-Zilberman M, Goyert SM, and Vogel SN.** CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. *J Immunol* 166: 574–581, 2001.
36. **Saal LH, Troein C, Vallon-Christersson J, Gruvberger S, Borg A, and Peterson C.** BioArray Software Environment (BASE): a platform for comprehensive management and analysis of microarray data. *Genome Biol* 3: SOFTWARE0003, 2002.
37. **Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, and Akira S.** Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 171: 4304–4310, 2003.
38. **Seki E, Tsutsui H, Nakano H, Tsuji N, Hoshino K, Adachi O, Adachi K, Futatsugi S, Kuida K, Takeuchi O, Okamura H, Fujimoto J, Akira S, and Nakanishi K.** Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1β. *J Immunol* 166: 2651–2657, 2001.
39. **Shaffer AL, Rosenwald A, Hurt EM, Giltnane JM, Lam LT, Pickeral OK, and Staudt LM.** Signatures of the immune response. *Immunity* 15: 375–385, 2001.
40. **Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, and Jongeneel CV.** Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J Exp Med* 171: 35–47, 1990.
41. **Skerrett SJ, Liggitt HD, Hajjar AM, and Wilson CB.** Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against *Pseudomonas aeruginosa* but not *Staphylococcus aureus*. *J Immunol* 172: 3377–3381, 2004.
42. **Swantek JL, Cobb MH, and Geppert TD.** Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol Cell Biol* 17: 6274–6282, 1997.
43. **Sweet MJ, Campbell CC, Sester DP, Xu D, McDonald RC, Stacey KJ, Hume DA, and Liew FY.** Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. *J Immunol* 168: 392–399, 2002.
44. **Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D, and Liew FY.** A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol* 166: 6633–6639, 2001.
45. **Takaoka A and Taniguchi T.** New aspects of IFN-alpha/beta signalling in immunity, oncogenesis and bone metabolism. *Cancer Sci* 94: 405–411, 2003.
46. **Takeuchi O, Hoshino K, and Akira S.** Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J Immunol* 165: 5392–5396, 2000.
47. **Vogel SN, Fitzgerald KA, and Fenton MJ.** TLRs: differential adapter utilization by Toll-like receptors mediates TLR-specific patterns of gene expression. *Mol Intervent* 3: 466–477, 2003.
48. **Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, Towne J, Sims JE, Stark GR, and Li X.** SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. *Nat Immunol* 4: 920–927, 2003.
49. **Wang X and Seed B.** A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* 31: e154, 2003.
50. **Weighardt H, Jusek G, Mages J, Lang R, Hoebe K, Beutler B, and Holzmann B.** Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. *Eur J Immunol* 34: 558–564, 2004.
51. **Weighardt H, Kaiser-Moore S, Vabulas RM, Kirschning CJ, Wagner H, and Holzmann B.** Cutting edge: myeloid differentiation factor 88 deficiency improves resistance against sepsis caused by polymicrobial infection. *J Immunol* 169: 2823–2827, 2002.
52. **Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, and Akira S.** Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301: 640–643, 2003.
53. **Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, and Akira S.** TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* 4: 1144–1150, 2003.
54. **Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, and Akira S.** Cutting Edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 169: 6668–6672, 2002.
55. **Zhang H, Ramanathan Y, Soteropoulos P, Recce ML, and Tolias PP.** EZ-Retrieve: a web-server for batch retrieval of coordinate-specified human DNA sequences and underscoring putative transcription factor-binding sites. *Nucleic Acids Res* 30: e121, 2002.

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