Genome-Wide Profiling of *In Vivo* LPS-Responsive Genes in Splenic Myeloid Cells

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Lipopolysaccharide (LPS), the major causative agent of bacterial sepsis, has been used by many laboratories in genome-wide expression profiling of the LPS response. However, these studies have predominantly used *in vitro* cultured macrophages (Macs), which may not accurately reflect the LPS response of these innate immune cells *in vivo*. To overcome this limitation and to identify inflammatory genes *in vivo*, we have profiled genome-wide expression patterns in non-lymphoid, splenic myeloid cells extracted directly from LPS-treated mice. Genes encoding factors known to be involved in mediating or regulating inflammatory processes, such as cytokines and chemokines, as well as many genes whose immunological functions are not well known, were strongly induced by LPS after 3 h or 8 h of treatment. Most of the highly LPS-responsive genes that we randomly selected from the microarray data were independently confirmed by quantitative RT-PCR, implying that our microarray data are quite reliable. When our *in vivo* data were compared to previously reported microarray data for *in vitro* LPS-treated Macs, a significant proportion (~20%) of the *in vivo* LPS-responsive genes defined in this study were specific to cells exposed to LPS *in vivo*, but a larger proportion of them (~60%) were influenced by LPS in both *in vitro* and *in vivo* settings. This result indicates that our *in vivo* LPS-responsive gene set includes not only previously identified *in vitro* LPS-responsive genes but also novel LPS-responsive genes. Both types of genes would be a valuable resource in the future for understanding inflammatory responses *in vivo*.

INTRODUCTION

When microbes enter our bodies, the innate immune system initially detects these microbes by recognizing highly conserved microbial structures called pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). In an infected host, PAMPs are sensed by a diverse set of germline-encoded receptors called pattern recognition receptors (PRRs)

that are present in the extracellular space, the cell membrane and the intracellular space (Lee and Kim, 2007a). Membrane-associated PRRs are highly expressed in innate immune antigen-presenting cells such as macrophages (Macs) and dendritic cells (DCs), which are located at the major sites of microbial entry as well as other immune tissues (Takeuchi and Akira, 2010). Upon sensing the invading microbes, PRRs on innate immune cells initiate various intracellular signaling cascades leading to inflammatory responses that are essential for early pathogen clearance and for inducing later adaptive immune responses mediated by T and B cells (Iwasaki and Medzhitov, 2010; Lee and Kim, 2007b).

Toll-like receptors (TLRs), a type of membrane-associated receptors, are the best studied signal-inducing PRRs, and they commonly activate the transcription factors (TFs) nuclear factor (NF)- κ B and activator protein-1 (AP-1), leading to the production of inflammatory cytokines and chemokines. Among TLRs, TLR4 initiates many diverse signaling pathways and activates also the TF interferon regulatory factor 3 (IRF3), leading to the production of type I interferons (IFNs), which are critical for antiviral defense (Takeuchi and Akira, 2010). Lipopolysaccharide (LPS), the major cell wall component of Gram-negative bacteria, is the best known TLR4 agonist and induces a very strong inflammatory response. LPS is also known to be the major causative agent of bacterial sepsis which is a common cause of death in intensive care units in hospitals (Riedemann et al., 2003).

Because of its ability to induce strong inflammation and cause sepsis, LPS has been widely used to identify genes important for inflammatory responses on a genomic scale. However, most of these studies used *in vitro* cultured cells such as bone-marrow-derived Macs (BMDMs) (Koziczak-Holbro et al., 2008), peritoneal Macs (PMs) (Matsushita et al., 2009) or Raw264.7 cells (a representative Mac cell line) (Bilban et al., 2006; Murray et al., 2005) to measure the LPS effect. However, the LPS response of *in vitro* cultured Mac cells may not well reflect the *in vivo* LPS response of Macs. There have also been examples of genome-wide studies of *in vivo* LPS-responsive genes using tissues such as liver and spleen (Hammer et al., 2006; Yoo and Desiderio, 2003). However, these studies used

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whole tissues that contain various other cells in addition to the inflammation-initiating innate immune cells, which may result in blunting or negating the LPS-induced gene expression changes in the innate immune cells.

To overcome these problems and to screen for inflammatory genes relevant to *in vivo* biology, we profiled gene expression patterns of non-lymphoid, splenic myeloid cells directly extracted from LPS-treated mice using a mouse whole-genome microarray. The results re-identified many previously known LPS-responsive genes and also identified a significant number of novel genes that cannot be easily identified from the previous microarray analyses on *in vitro* cultured Mac cells. As LPS-responsive genes selected from our microarray data were validated independently by quantitative RT-PCR (qRT-PCR) in this study, we believe that our list of LPS-responsive genes is reliable and valuable in the future understanding of inflammatory responses *in vivo*.

MATERIALS AND METHODS

In vivo LPS treatment and tissue collection

All mice were housed in the specific pathogen-free facility of the Laboratory Animal Research Center of Yonsei University. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the Yonsei University, Korea. Three groups of 6- to 8-week-old C57BL/6 male mice ($n = 3$ per group) were intraperitoneally injected with either phosphate-buffered saline (PBS; pH 7.4, Invitrogen) or 12.5 mg/kg LPS (from *E. coli* O55:B5 strain, Sigma) dissolved in PBS. At 3 h or 8 h after LPS injection and 8 h after PBS injection, the mice were sacrificed, and their spleens were collected in ice-cold PBS separately for each group of samples. The collected, pooled spleens (three spleens per each sample type) were ground against a 70 μ m Falcon cell strainer (Becton Dickinson Labware). The disaggregated cells were then filtered through the strainer. After red blood cells (RBCs) were lysed using RBC lysis solution (Qiagen), the splenocytes were washed once with PBS and were resuspended in PBSF buffer [PBS plus 2% fetal bovine serum (FBS; HyClone)].

Preparation of non-lymphoid splenocytes by depleting T and B cells using MACS (magnetic-activated cell sorting)

The following steps were performed at 4°C if not specified. A small portion of the resuspended cells was stained in PBSF buffer for FACS (fluorescence-activated cell sorting) analysis of the splenocyte populations before purification using the following combinations of antibodies (Abs) according to the manufacturer (BD Pharmingen)'s recommendations: CD11b-FITC (clone M1/70), CD19-PE (clone 1D3), CD3-PerCP (clone 145-2C11) and CD45-APC (clone 30-F11). The remaining splenocytes were resuspended in MACS bead buffer containing PBS (pH 7.2), 0.5% bovine serum albumin (BSA) and 2 mM EDTA (pH 8.0) and stained with CD90 (Thy1.2)-microbeads (magnetic microbeads conjugated to monoclonal rat anti-mouse CD90 Ab, Miltenyi Biotec) and CD19-microbeads (Miltenyi Biotec) for 20 min. Cells depleted of CD90-positive (T cells) and CD19-positive (B cells) cells were then prepared by running the stained cells on a MidiMACS system using an LD MACS column (Miltenyi Biotec) and obtaining the flow through following the manufacturer's recommendations. A small portion of the MACS-purified cells were also stained with the fluorescently labeled Abs listed above, and the depletion percentage was determined by FACS analysis (Fig. 1). Only the samples with good purity (i.e., approximately 90% or more of the purified cell fractions are T- and

B-cell negative) were used in further analysis.

RNA preparation, microarray data collection and analysis of the splenic myeloid cells

The majority of the non-lymphoid splenocytes prepared by MACS sorting as above were pelleted at 1,500 rpm in a micro-centrifuge for 5 min at 4°C and lysed immediately in Buffer RLT (Qiagen RNeasy Mini kit). Total cellular RNA was purified following the manufacturer's recommendations including the on-column DNase I treatment step and analyzed using the RNA 6000 NanoKit on an Agilent BioAnalyzer 2100 system (Agilent Technologies). Discrete 28S and 18S rRNA peaks with good purity (OD_{260}/OD_{280} ratio of 1.9-2.1) were observed for all samples. Approximately 500 ng of purified RNA from each sample type was used for microarray analysis. All of the RNA amplification, cRNA labeling, microarray hybridization (CodeLink Mouse Whole Genome Bioarray, GE Healthcare), data collection and normalization steps were performed by Digital Genomics (Korea) following the manufacturer's recommendations. Hierarchical clustering analysis was performed using Pearson's correlation coefficient (r) as a measure of similarity and the average linkage method as the clustering algorithm with Gene Cluster 3.0 (originally developed by Michael Eisen) (Eisen et al., 1998).

Confirmation of microarray data by quantitative RT-PCR (qRT-PCR)

RNA prepared from each sample was first reverse-transcribed to cDNA using SuperScript® II Reverse Transcriptase (Invitrogen). The expression level of each individual gene was then measured by quantitative PCR (qPCR). qPCR was performed in a 20 μ l reaction with SYBR Green I dye as a detection reagent. The reaction contained 1 μ l cDNA, 8 pmol of each primer, 1 $\times$ EF-Taq reaction buffer (Solgent, Korea), 0.5 unit EF-Taq DNA Polymerase, 0.75 mM $MgCl_2$, 0.2 mM dNTPs, 5% DMSO and 1 $\times$ SYBR Green I dye [using an 1:10,000 dilution of the stock reagent (S7563, Invitrogen)]. The PCR protocol was as follows: initial denaturation at 95°C for 1 min, then 45 cycles of 95°C for 15 s, 60°C for 25 s and 72°C for 30 s. The PCR was analyzed in real time on a LightCycler 480 (Roche, Germany). Quantification was then performed using the comparative C_T method ($\Delta\Delta C_T$). The results were expressed as a ratio of each gene signal over the control *Gapdh* signal. The primers used for PCR are displayed in Supplementary Table 1.

In vitro LPS treatment of PMs and Raw264.7 cells

To collect resident PMs, the intraperitoneal spaces of 6- to 8-week-old C57BL/6 male mice were washed with cold PBS (pH 7.4). The extracted cells were then plated on 60 mm tissue culture plates (Nalgene) at a density of 2×10^6 in DMEM medium (Invitrogen) with 10% FBS, penicillin (100 units/ml; Invitrogen) and streptomycin sulfate (100 μ g/ml; Invitrogen). After a 4 h incubation to allow attachment, non-adherent cells were extensively washed with PBS. The adherent cells were then treated with LPS (100 ng/ml) for 3 h or 8 h or with PBS for 8 h. Raw264.7 cells obtained from the American Type Culture Collection were treated in the same way. Total RNA was isolated from these cells using TRIzol® Reagent (Invitrogen) and analyzed as described above for qRT-PCR.

Comparison of microarray data between *in vitro* and *in vivo* LPS-treated samples

The gene expression microarray data for *in vitro* LPS-treated Mac cells were downloaded from the public database Gene Expression Omnibus (GEO) at the National Center for Biotech-

Fig. 1. Representative FACS analysis of splenocytes from LPS-treated mice before and after T and B cell depletion. Male mice 6–8 weeks old ($n = 3$ per group) were intraperitoneally injected with either PBS or 12.5 mg/kg LPS dissolved in PBS. Spleens were collected at 8 h (A) after PBS injection (PBS), or at 3 h (LPS3) (B) or at 8 h (LPS8) (C) after LPS injection. The single-cell suspensions derived from these spleens were then stained and analyzed by FACS. Left panels are the analyses of cells before MACS bead depletion using CD3-PerCP and CD19-PE antibodies (Abs); middle and right panels are the analyses of cells after the bead depletion using CD3-PerCP/CD19-PE Abs and CD45-APC/CD11b-FITC Abs, respectively. Data are representative of at least three independent experiments.

nology Information: bone-marrow-derived Macs (BMDMs) (GSE9037) (Koziczak-Holbro et al., 2008), peritoneal Macs (PMs) (GSE14891) and Raw264.7 cells (GSE1906). For comparison, microarray data of untreated samples and samples treated for 2 h or 4 h with LPS were used. The data used for BMDM were from untreated samples and samples treated for 4 h with LPS; data used for PM were from untreated samples and samples treated for 2 h or 4 h with LPS and the data used for Raw264.7 were from untreated samples and samples treated for 2 h or 4 h with LPS. For each experiment with each different type of cells, we first selected genes as LPS-responsive genes if their signals from at least one type of LPS-treated samples resulted in both more than 3x the average signal and more than 3-fold higher signal compared to that from the untreated sample, and we gave the strongest fold induction values as representatives of the selected genes. Then to compare LPS-responsive genes between *in vivo* and *in vitro*-treated samples, for a given gene, we first chose the largest fold induction value among the fold induction values of all of the *in vitro* samples and considered it as a representative of the *in vitro* sample for the gene. We then compared the representative fold induction value of the *in vitro* sample with that of our *in vivo* sample. Hierarchical and K means clustering analyses were performed to measure fold induction similarity among samples using the Gene Cluster 3.0.

RESULTS AND DISCUSSION

Gene expression microarray analysis of non-lymphoid splenocytes from LPS-treated mice

To determine *in vivo* LPS-responsive gene expression profiles of innate immune cells, we chose to analyze the spleen, a secondary lymphoid organ that monitors the circulating blood and is easily accessible for sampling. At 3 h and 8 h after LPS treatment (samples referred as “LPS3” and “LPS8”, respectively), when the proportion of target cells [CD3 (T cell marker)-negative and CD19 (B cell marker)-negative cells] does not change significantly (Fig. 1), as well as at 8 h after vehicle PBS treat-

ment (sample referred as “PBS”), spleens were sampled. Because spleens are composed of mainly T and B cells which are not the major responding cells following acute LPS stimulation such as in our experimental setting (Fig. 1 and see below), we first enriched for innate immune cells by depleting T and B cells from the single-cell suspensions of splenocytes. Approximately 90% or more of the purified cells were negative for both CD3 and CD19, and the majority of such cells were CD11b-positive myeloid cells: the purified samples contained on average 76% of CD11b⁺ cells, ranging from 73% to 79%, implying that potential cell type difference among the samples is minor (Fig. 1 and data not shown). RNAs were prepared from the three types of samples (PBS, LPS3 and LPS8), for which three mice were used per sample. Genome-wide microarray analysis was then performed using the CodeLink Mouse Whole Genome Bioarray, which has approximately 33,000 transcripts, including over 30,000 well-annotated mouse genes. The entire collected, normalized data set is listed in Supplementary Table 2.

To determine if the enrichment of innate immune cells by depleting T and B cells is reflected in the gene expression profile, expression levels for major hematopoietic lineage-specific marker genes were analyzed (Table 1). Marker genes for B cells (Cd19 and Pax5) and T cells [T cell receptor complex CD3 components and T cell receptor coreceptors (Cd4, Cd8a and Cd8b1)] were undetectable or barely detectable in all the analyzed samples (Lee et al., 2004; Nutt et al., 1999), supporting the conclusion that the T and B cell depletion was indeed well achieved as planned. As expected, the expression of the common hematopoietic cell marker gene *Ptprc* (CD45) was reasonably strongly detected for all of the analyzed samples. A major marker gene for myeloid cells such as monocytes, Macs and granulocytes, *Itgam* (encoding CD11b) (Rose et al., 2012), was moderately expressed in most samples (Fig. 1 and Table 1). Another gene that is mainly expressed in monocytes and Macs, *Cd14* (the LPS coreceptor gene), was moderately expressed in the PBS sample but was strongly induced by LPS stimulation. A marker gene for DCs, *Itgax* (encoding CD11c)

Table 1. Expression values for hematopoietic lineage-specific marker genes

Probe_name	Gene_symbol and description	PBS	LPS3	LPS8
GE35179	Cd19, B cell precursor and mature B cells	1.1*	2.0	1.5
GE118205	Pax5 (Paired box gene 5), a B-cell specific transcription factor	0.8	0.9	0.9
GE119777	Cd3z (CD3 antigen, zeta), T-cell receptor complex	2.0	3.4	4.1
GE35185	Cd3g (CD3 antigen, gamma), T-cell receptor complex	7.2	4.8	2.8
GE34828	Cd3e (CD3 antigen, epsilon), T-cell receptor complex	0.3	0.2	0.0
GE36571	Cd4 (CD4 antigen), T-cell receptor coreceptor	10.9	5.8	5.4
GE32954	Cd8a (CD8 antigen, alpha), T-cell receptor coreceptor	3.9	6.4	1.4
GE35189	Cd8b1 (CD8 antigen, beta1), T-cell receptor coreceptor	2.9	6.3	2.7
GE35970	Ptpcr, leukocyte common antigen CD45	80.9	58.5	22.8
GE34658	Itgam (integrin alpha M), CD11b, monocyte/macrophage/granulocyte cell marker	23.2	8.1	19.7
GE35176	Cd14, monocytes/macrophage cells, LPS coreceptor	17.7	412.1	279.9
GE37899	Itgax (integrin alpha X), CD11c (Dendritic cell marker)	35.3	9.6	4.6
GE116598	Ncam1, CD56 (Natural killer cell marker)	0.6	0.9	1.1
GE1406127	Gypa (glycophorin A), precursor and mature red blood cell marker	1.0	2.7	13.5

*Signal value for each gene; average signal value for the whole gene set is 9.0

Fig. 2. A total of 498 transcripts were induced by LPS *in vivo* more than 3-fold with signal values 3-fold above the average. (A) Venn diagram showing the numbers of transcripts induced by LPS at only the 3 h time point (208), at only the 8 h time point (106) or at both time points (184) among the 498 transcripts. (B) Heat map of the results of hierarchical clustering analysis on the 498 transcripts. Each row represents a transcript, and the first, second and third columns represent signals for PBS, LPS3 and LPS8, respectively. LPS3-specific transcripts are in cluster C1, transcripts up-regulated at both LPS3 and LPS8 are in cluster C2, and LPS8-specific transcripts are in cluster C3. Red and green represent up-regulation and down-regulation, respectively, and intensity reflects the degree of differential expression (darker colors indicate larger differences).

(Rose et al., 2012), was marginally detected in LPS-treated samples, whereas moderate expression was observed in the PBS sample. Natural killer cell marker genes, such as Ncam1 (encoding CD56), were not detected in these samples, whereas the RBC marker gene Gypa (glycophorin A) (Lee et al., 2004) was mostly undetectable except for the LPS8 sample, which showed marginal expression. These results, together with the FACS data in Fig. 1, suggest that the highly enriched innate immune cells in the samples are mostly composed of myeloid cells such as Macs and granulocytes.

Biological processes enriched in LPS-induced genes

The average signal for the whole transcript data set was 9.0. To enrich for biologically significant LPS-induced genes, we chose 3x the average signal as a signal intensity cut-off and a 3-fold signal difference as a differential expression cut-off. The number of transcripts whose signals from at least one type of LPS-treated sample gave both more than 3x the average signals (27.0) and a more than 3-fold higher signal compared to that from the PBS-treated sample was 498 (see the list and descrip-

tion of the transcripts in Supplementary Table 3). Among these, 392 and 290 transcripts were up-regulated in the LPS3 and LPS8 samples, respectively, and 184 transcripts were up-regulated at both time points (Fig. 2A). Hierarchical clustering analysis performed on the 498 LPS-induced transcripts showed similar time-specific and overlapping LPS response gene expression patterns (Fig. 2B).

To determine which biological processes or pathways these LPS response genes belonged to, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and three Gene Ontology (GO) biological categories were searched with the 498 transcripts, using a web-accessible bioinformatics resource, the Database for Annotation, Visualization and Integrated Discovery (DAVID) resource (Huang da et al., 2009) with the default settings. The biological processes that were significantly enriched in the LPS-responsive gene group at each time point are listed in Table 2. The most significantly represented KEGG pathway for both groups of LPS-responsive genes was the "Toll-like receptor signaling pathway" (p -value $< 1.30 \times 10^{-7}$ for LPS3 and p -value $< 1.60 \times 10^{-4}$ for LPS8) (Table 2). KEGG

Table 2. Biological processes significantly enriched in LPS-responsive 498 transcripts

Sample types	Enriched biological processes/pathways	Transcript number	p-value*
LPS3	KEGG Pathways		
392 transcripts	Toll-like receptor signaling pathway	14	1.30E-07
(316 DAVID IDs)**	Cytokine-cytokine receptor interaction	18	7.90E-06
	MAPK signaling pathway	15	6.70E-04
	Type I diabetes mellitus	7	1.50E-03
	p53 signaling pathway	6	1.10E-02
	Apoptosis	6	2.70E-02
	Jak-STAT signaling pathway	8	4.00E-02
	GO terms (Biological process)		
	Immune response	44	3.90E-20
	Immune system process	51	4.70E-18
	Inflammatory response	21	8.90E-11
	Response to wounding	22	5.80E-09
	Defense response	33	1.30E-08
	Response to external stimulus	25	1.80E-07
	Response to biotic stimulus	17	1.20E-06
	Response to other organism	14	1.70E-06
	Response to virus	9	1.80E-06
	Response to stress	31	3.20E-06
	Apoptosis	27	5.60E-06
	Chemotaxis	10	3.50E-05
	Response to stimulus	75	6.70E-05
	Multi-organism process	14	8.20E-05
	Regulation of apoptosis	19	1.10E-04
	Response to chemical stimulus	19	3.70E-04
	Cell differentiation	45	4.10E-04
	Acute-phase response	5	5.50E-04
	Induction of apoptosis	10	5.60E-04
	Positive regulation of programmed cell death	11	8.90E-04
	GO terms (Molecular function)		
	Chemokine activity	9	1.00E-07
	Chemokine receptor binding	9	1.30E-07
	Cytokine activity	16	2.60E-06
	G-protein-coupled receptor binding	9	5.40E-06
	Nucleotide receptor activity	7	3.80E-04
	Protein binding	115	4.20E-04
	GO terms (Cellular component)		
	External side of plasma membrane	10	3.70E-04
LPS8	KEGG Pathways		
290 transcripts	Toll-like receptor signaling pathway	10	1.60E-04
(261 DAVID IDs)	Cell cycle	8	5.50E-03
	p53 signaling pathway	6	8.90E-03
	Jak-STAT signaling pathway	9	9.90E-03
	Ubiquitin mediated proteolysis	8	1.40E-02
	Cytokine-cytokine receptor interaction	11	2.00E-02
	GO terms (Biological process)		
	Immune system process	42	1.80E-14
	Immune response	30	2.40E-11

(Continued)

Table 2.

Sample types	Enriched biological processes/pathways	Transcript number	<i>p</i> -value*
	Apoptosis	23	2.90E-05
	Negative regulation of cellular process	28	3.60E-05
	Negative regulation of biological process	29	4.30E-05
	Response to other organism	11	6.10E-05
	Cell death	23	6.50E-05
	Response to stress	25	7.50E-05
	Inflammatory response	12	1.00E-04
	Regulation of apoptosis	17	1.50E-04
	Positive regulation of cellular process	25	2.00E-04
	Multi-organism process	12	2.90E-04
	Cell cycle process	19	3.30E-04
	Response to biotic stimulus	12	3.50E-04
	Positive regulation of biological process	26	6.00E-04
	Regulation of cell cycle	14	6.30E-04
	GO terms (Molecular function)		
	Protein binding	101	2.40E-05
	Interleukin binding	6	1.60E-04
	Cytokine binding	7	5.60E-04
	GO terms (Cellular component)		
	External side of plasma membrane	10	1.10E-04
	Cell surface	12	2.20E-04

*For KEGG pathways, ones showing *p*-value < 0.05 with minimum five-associated transcripts, and for GO terms, ones showing *p*-value < 0.001 with minimum five-associated transcripts, were listed as biologically significant.

**Among 392 transcripts, 316 were identifiable and analyzed in the DAVID resource.

pathways involved in cytokine and chemokine activity and signaling, such as “cytokine-cytokine receptor interaction” and “Jak-STAT signaling pathway”, were also significantly represented in both the LPS3 and LPS8 groups (Table 2). Consistent with these results, among GO biological categories, processes involved in immunological responses, such as “immune response”, “immune system process”, and “inflammatory response”, were significantly represented in both LPS-responsive gene groups (Table 2). In agreement with these results, the top three GO molecular functions enriched in the LPS3 group were “chemokine activity”, “chemokine receptor binding” and “cytokine activity”, and two of the three functions most enriched in the LPS8 group were “interleukin binding” and “cytokine binding” (Table 2). In terms of GO cellular components, regardless of the time point, the “external side of plasma membrane” GO term was the most highly enriched in the LPS-responsive gene set. These results are in good agreement with the expectation that the genes induced by LPS, one of the strongest inflammation inducers, should be those encoding factors mediating immune responses and host defense. Biological processes related to apoptosis and cell death were also significantly represented in the LPS3 and LPS8 groups (two KEGG pathways, “p53 signaling pathway” and “apoptosis”, and four apoptosis-related GO terms were highly represented for the LPS3 group; one KEGG pathway, “p53 signaling pathway”, and three apoptosis-related GO terms were represented for the LPS8 group) (Table 2), implying that our LPS stimulus (12.5 mg/kg) may be strong enough to induce apoptosis in certain myeloid cells.

More detailed analysis of highly LPS-induced genes

To analyze more strongly LPS-induced genes, transcripts showing both more than 5-fold (45.0) the average signal and more than 5-fold more signal in at least one type of LPS sample compared to the PBS sample were selected. This process resulted into 184 highly LPS-responsive transcripts. Because the GO biological terms used in Table 2, such as “immune response” and “immune system process”, were rather broad, by inspecting known and putative functions of the selected genes that are reported in the literature, the genes were grouped into more specific functional or molecular categories for further discussion (see the list of the categorized 184 transcripts in Table 3).

Chemokines and cytokines

Genes for chemokines that recruit diverse leukocytes to inflammatory sites were strongly up-regulated at an early time point by LPS (Table 3). The gene for the CC chemokine Ccl3 (Mip1a), which has the capability to recruit diverse immune cell types such as monocytes, T cells, NK cells, basophils, eosinophils, DCs and hematopoietic progenitors, was very highly induced by LPS at the 3 h time point and less so at the 8 h time point (49-fold for LPS3 and 13-fold for LPS8). The gene for Ccl4 (Mip1b), another CC chemokine that can also recruit similarly diverse leukocytes such as monocytes, T cells, DCs and NK cells, was strongly induced only at LPS3 (7-fold for LPS3 and 1.3-fold for LPS8) (Le et al., 2004). The gene encoding the C chemokine Xcl1 (lymphotactin), which is capable of recruiting diverse immune cells such as B cells, T cells, NK cells and neutrophils, was also strongly induced only at LPS3 (16-fold for

Table 3. LPS-responsive transcripts showing both more than 45.0 signal value and more than 5-fold induction

Functional category	LPS3*	LPS8**
Chemokine/receptors	Ccl3, Ccl4, Xcl1, Cxcl2, Cxcl9, Cxcl10	Ccl3, Cxcl2, Cxcl9, Cxcl10
Cytokine/receptors	Il1b, Il1f9, Il1rn, Il1r2, IFN γ , Ebi3	Il1rn, Il1r2, IFN γ
Pathogen sensors (PRRs)	TLR2, CD14, Clecsf8, Ddx58, Ifih1, Oas2, Oas3, Oasl1, Oasl2, Zbp1	CD14, Ddx58, Ifih1, Oas2, Oas3, Oasl1, Zbp1
IFN-inducible genes	Trim56, Mx1, Isg20, Ifi35, Tgtp, Ifi203, Ifi204, Ifi205, A1595338 (Gbp6), Ifi1, Ifit1, Igtp, Olfr56	Isg20, Tgtp, Ifit1, Igtp, Olfr56
Acute-phase response	Saa3, Orm1	Saa3, Orm1
Stress response/homeostasis	Hspa1a, Sod2, Mt1, Slc7a11	Mt1
Apoptosis/cell death/Cell cycle	Traf1, Bcl2a1b, Parp14, Serpina3g, Tnfsf6, Gzmb, Pml, Gadd45b, Casp4, Ier3, Tes, Cdkn1a, Ccnd2, Vdac1	Serpina3g, Lgals9, Pml, Casp4, Ier3, Nusap1, Vdac1
Negative regulation of inflammatory signaling	Socs1, Socs3, Trim30, Fln29, Usp18, Nfkbia	Socs1, Usp18
Gene transcription	IRF7, Jun, Batf, Lhx4, Nr4a3, Vezf1, Zfhx1a, SpiC, Bcor11, Etv3, Trim14, Phf11	IRF7, Idb3, Etv3, Phf11
Immune cell differentiation/function	Cd86, Tnfrsf5, Pdcd1lg1, Hdc, Dusp2, Trem1, Ikbke, Fcgr1, Mmp8, Il4i1, Sfn, Ly6a, Cd69, Btla, Slfn4, Ibrdc3, Gypc, Hbb-bh1, Hba-a1	Cd86, Pdcd1lg1, Hdc, Ikbke, Fcgr1, Fcrl3, Mmp8, Il4i1, Ly6a, CD69, Ibrdc3, Gypc, Eraf, Hbb-bh1, Hba-a1
G-protein coupled receptors/signaling	Fpr1, Fpr-rs2, Fprl1, P2ry10, P2ry14, Gpr171, Gpr109b, Ccr12, Mrgpra2, Rgs1, Gng11	Fpr1, Fpr-rs2, Fprl1, P2ry10, Ccr12, V1ri9
Miscellaneous	Sema4c, Ehd1, Car13, Alas1, Eif1ay, Fscn1, Pla1a, Pde4c, Ctrb1, Xpo4, Rhof, Actr1a, Gch1, Parp9, Nt5c3, Pyp, Basp1, Plec1, Bace2, Gp49a, Marcks, Fmnl2, D11Erd759e, AW049306	Car13, Fscn1, Mtpn, Aldh1a1, Basp1, Plec1, Gp49a, Lrg1, Fmnl2, Tm4sf8
Unknown/EST	A1481105, 4933415I03Rik, 5830458K16Rik, Irg1, 2010002M12Rik, MGC58382, 5730508B09Rik, Fchsd2, Haghl, Ms4a6d, Ogfr11	4933415I03Rik, Irg1, MGC58382

Transcripts showing more than 5-fold (45.0) average signal and also more than 5-fold more signal in LPS3 (*) or LPS8 (**) sample compared to signal of PBS sample.

LPS3 and 1.3-fold for LPS8). However, genes encoding several CXC chemokines such as Cxcl2 (GRO α), Cxcl9 (Mig) and Cxcl10 (IP-10), which recruit more specialized cells such as neutrophils and T cells, were strongly up-regulated at both LPS3 and LPS8 (400-fold and 260-fold for Cxcl2, 40-fold and 30-fold for Cxcl9 and 150-fold and 65-fold for Cxcl10, respectively) (Le et al., 2004). These chemokine expression patterns suggest that at very early time points such as LPS3, innate immune myeloid cells such as Macs produce diverse types of chemokines that are capable of recruiting diverse immune cell types and that at later time points such as LPS8, such cells produce less diverse chemokines that recruit more specialized cell types such as T cells and neutrophils.

In contrast to the diverse and strong chemokine gene induction following LPS stimulation, only a few types of cytokines were strongly induced by LPS (Table 3). Several genes for IL-1 family members, such as IL1 β /IL1 α (a major pro-inflammatory hetero-dimeric cytokine), IL1f9 (pro-inflammatory) and IL1rn (also called IL1ra; IL1 receptor antagonist), and the gene for the IL1 decoy receptor IL1r2, were strongly induced in the LPS3 samples (5.4-fold for IL1 β , 37.3-fold for IL1 α , 5.9-fold for IL1f9, 35.0-fold for IL1rn and 18.2-fold for IL1r2) (Towne et al., 2004). Among these cytokine genes, the induction of the pro-inflammatory factor IL1 β /IL1 α was reduced at the later time point

LPS8 (2.9-fold for IL1 β and 9.2-fold for IL1 α at LPS8) whereas the factors IL1rn and IL1r2 inhibiting the function of IL1 were still strongly induced at this time point (18.5-fold for IL1rn and 18.6-fold for IL1r2 at LPS8). These results suggest that one mechanism preventing the initial strong inflammatory response mediated by IL1 β /IL1 α from being overactivated *in vivo* involves the use of its competitive antagonist IL1rn and decoy receptor IL1r2.

Antigen-presenting cells such as Macs and DCs can polarize naïve CD4⁺ T cells into several different effector T cell subsets, such as T helper type 1 (Th1) cells, which are critical for intracellular pathogen clearance, and T helper type 2 (Th2) cells, which are critical for extracellular pathogen clearance, by providing different types of cytokines to T cells (Pulendran and Ahmed, 2006). IFN γ , which is critical for polarizing naïve CD4⁺ T cells into Th1 cells and also acts as a Th1 cell effector cytokine, was strongly induced in LPS3 and LPS8 (22.1-fold and 28.2-fold, respectively). Another key Th1-polarizing cytokine, IL12 (a hetero-dimer of IL12 α and IL12 β) (Pulendran and Ahmed, 2006), was also induced at both LPS3 and LPS8 (7.7-fold and 6.8-fold for IL12 α ; 5.2-fold and 7.7-fold for IL12 β , respectively), although their signal intensity values (approximately 10) at LPS3 and LPS8 were not strong enough to be listed in Table 3. Furthermore, the cytokine IL27 (a hetero-dimer of

Ebi3/ IL27p28), which has the capacity to promote Th1 cell differentiation (Villarino et al., 2004), was also strongly induced at LPS3 and less so at LPS8 (6.7-fold and 3.2-fold for Ebi3; 9.9-fold and 2.3-fold for IL27p28, respectively). However, the expression of IL4, the major cytokine promoting Th2 cell differentiation, was negligible and unchanged (signals were approximately 1.5), and the expression of genes encoding major factors important for Th17 cell polarization, such as IL23 and TGF β (Ghoreschi et al., 2010), were not induced by the LPS treatment (Supplementary Table 2). These results suggest that LPS-responding myeloid cells produce a cytokine environment that allows naïve CD4⁺ T cells to differentiate into Th1 cells. This hypothesis is consistent with the known observation that TLR4 signaling promotes Th1 cell polarization (Pulendran and Ahmed, 2006).

Pathogen sensors and IFN-inducible genes

One intriguing observation is that diverse pathogen sensor genes were strongly induced by LPS (Table 3). TLR2, a sensor for the Gram-positive bacterial cell wall component peptidoglycan, and CD14, the coreceptor for LPS, were strongly induced (Akira et al., 2006). Given that several C-type lectin receptors, such as dectin-1 and dectin-2, are known to sense certain fungi and that the closely related C-type lectin receptor Clecsf9 (Mincle) was also shown to recognize the fungal pathogen *Malassezia* (Yamasaki et al., 2009), the LPS-induced gene Clecsf8 may also sense fungi, although its ligand is not yet defined (Akira et al., 2006; Graham et al., 2012; Willment and Brown, 2008). A considerable number of genes that were induced by LPS seem to encode known or potential sensors of viral infection. Two well-known dsRNA sensors, Ddx58 (Rig-I) and Ifih1 (Mda5), which are responsible for detecting diverse RNA viruses (Takeuchi and Akira, 2010), are included in this LPS-induced group. Major members of another dsRNA sensor family, the 2'-5' oligoadenylate synthetase (Oas) family (Kristiansen et al., 2011), including Oas2, Oas3, Oasl1, Oasl2 and Oasl3b, were all induced more than 5-fold by LPS, although Oasl3b was not listed in Table 3 because of its relatively low signal (approximately 15). The cytosolic DNA sensor gene Zbp1 (Z-DNA-binding protein 1; DAI) and its potential downstream regulator Trim56 (tripartite motif protein 56, IFN-inducible) (Tsushima et al., 2010) were also strongly induced by LPS treatment. As might be inferred from the observation that the known and putative viral sensor genes listed above are all IFN-inducible, other types of IFN-inducible genes were also induced by LPS. In addition to several IFN-inducible proteins such as Mx1, Isg20, Ifi35 (IFP35) and Tgtp (T-cell-specific GTPase; Irgb6) (Carlow et al., 1998; Sadler and Williams, 2008; Tan et al., 2008; Zhou et al., 2011) that are known or inferred to mediate antiviral defense, many other genes not directly related to antiviral defense were induced by LPS. These genes include Ifi204 and Ifi205, which have been implicated in regulating cell differentiation and proliferation (Asefa et al., 2004); A1595338 [Gbp6 (guanylate-binding protein 6), an IFN- γ -inducible GTPase], which is involved in bacterial defense (Kim et al., 2011); immunity-related GTPases (IRGs) such as Ifi1 (also called Lrg47), which are required for resistance to a broad range of intracellular bacteria and protozoa; and additional IRG proteins such as Igtg (also called Irgm3) and Olfr56 (Irg-47; also called Irgd), which are involved in host defense against a smaller group of protozoa or bacteria (Taylor, 2007). These results suggest that LPS alerts the host to produce diverse types of sensors against not only Gram-negative bacteria, which should be present inside the host as they produce LPS during natural infection, but also

other pathogens, such as Gram-positive bacteria, fungi, protozoa and viruses, that could access the host internal environment through the already-broken physical or biological barrier that may be evident from the presence of LPS inside the host. The results also suggest that the induction of large numbers of factors involved in defending against pathogens depends on IFNs induced by TLR4 signaling.

Stress response, homeostasis and cell death

As expected from the fact that LPS is the causative agent of bacterial sepsis, several known acute-phase response genes such as IL1 β , Saa3 (serum amyloid A3) and Orm1 (orosomucoid 1) (Cray et al., 2009; Ligresti et al., 2012) were strongly induced by LPS (543-fold and 265-fold for Saa3; 25-fold and 49-fold for Orm1 at LPS3 and LPS8, respectively). In addition, to overcome LPS-induced stress and to maintain internal homeostasis, diverse stress response or detoxifying protein genes were also induced. For example, the gene for heat-shock protein Hspa1a (heat shock protein 1A) (Hageman et al., 2011), which likely helps in resolving unfolded proteins; the gene for Sod2 (superoxide dismutase 2, mitochondrial) (Ferber et al., 2012), which is involved in detoxifying oxidative molecules; the gene for Mt1 (metallothionein 1) (Masters et al., 1994), which is involved in homeostasis and the detoxification of heavy metals such as Zn and Cd; and the gene for Slc7a11 (Xct; cysteine/glutamate transporter) (Sato et al., 2005), which is involved in maintaining the plasma redox balance, were induced. When the stress challenge is strong or persistent, cells must decide whether to undergo apoptosis or to inhibit the apoptotic process and resolve the challenge. In this regard, it is noticeable that a fair number of genes involved in the cell death process were strongly induced (Table 3). Several anti-apoptotic factors induced by LPS include Traf1, a negative regulator of TNF- α -induced cytotoxicity and also a suppressor of antigen-induced apoptosis of T cells (Zapata and Reed, 2002); Bcl2a1b, which has been implicated in suppressing neutrophil apoptosis and pre-T cell apoptosis (Vogler, 2012); Parp14, which is capable of promoting IL-4-treated B cell survival (Cho et al., 2011); and Serpina3g (Spi2A; extracellular serine protease inhibitor), which is capable of promoting the survival of cytotoxic T lymphocytes (Liu et al., 2004). Several pro-apoptotic factors induced by LPS include Tnfrsf6 (FasL, Fas ligand), Lgals9 (galectin-9) and Gzmb (granzyme B), which are known to promote target cell (T cell or infected cells) killing (Elmore, 2007; Wada et al., 1997). The inflammatory caspase Casp4 (also called Casp11), which initiates both inflammatory responses and apoptosis, was also induced (Kang et al., 2000). Although most apoptosis-related genes described above have been clearly shown to be involved in lymphocyte cell death, it remains to be seen whether these factors also regulate apoptosis in myeloid cells. Critical cell cycle regulators such as Cdkn1a (p21), which inhibits cell cycle progression, and Ccnd2, which is essential for control of the cell cycle at the G1/S transition, were also induced by LPS (Malumbres and Barbacid, 2009). Furthermore, Vdac1 (voltage-dependent anion channel 1) (Shoshan-Barmatz et al., 2010), a critical component involved in regulating mitochondrial apoptosis, was also included in this group.

Negative regulation of inflammatory signaling

As excessive inflammation can damage host tissues and organs and lead to septic shock and lethality, negative feedback regulation mechanisms for inflammation must be enacted. Diverse molecules were induced by LPS that may induce this negative regulation (Table 3). Several negative regulators on

key components that mediate TLR signaling were induced by LPS. One such gene is *Socs1*, which inhibits Mal (TIRAP) (Mansell et al., 2006), the key adaptor protein for MyD88-dependent signaling from TLR2 and TLR4, by ubiquitinating activated Mal and thus inducing its proteasomal degradation. Another such gene is *Socs3*, which can inhibit the function of *Traf6* (Frobose et al., 2006), the key signaling molecule for all TLR and IL1 signaling pathways by inhibiting *Traf6* ubiquitination, which is a key required event for mediating downstream signaling. A third such gene is *Trim30* (tripartite motif protein 30), which can induce the degradation of the *Tak1/Tab2/Tab3* complex, the key signaling complex for NF- κ B activation, and *Fln29*, which can also inhibit *Traf6* function by binding to *Traf6* (Richards and Macdonald, 2011). *Usp18* (ubiquitin-specific protease 18; also called *Ubp43*) (Randall et al., 2006), which can suppress IFN receptor signaling was also induced by LPS in this experiment. In addition, the well-known NF- κ B-induced NF- κ B inhibitor *NFkBia* (*Ikb α*) was induced by LPS. As several other reported LPS-inducible negative regulators such as *Tnfrsf5* (*A20*), *Irf4* and *Atf3* (Activating transcription factor 3) (Lee and Kim, 2007b) were not significantly induced in our experimental setting (Table 3), different sets of negative regulators may be more active in different cell types or conditions.

Gene transcription

Genes encoding various factors that are directly involved in regulating gene expression were induced by LPS (Table 3). Several major TFs mediating target gene induction and a TF inhibitor were included in this group. *Irf7*, the key TF necessary for inducing positive feedback amplification of type I IFN production (Honda et al., 2006); *Jun*, the major component of activator protein-1 (AP-1), which is commonly activated in response to TLR stimulation similar to NF- κ B; and *Batf* (Basic leucine zipper transcription factor, ATF-like), a Jun inhibitor functioning by binding to *Jun*, were induced by LPS (Williams et al., 2001). TFs involved in cell differentiation or organ development were also included in this group. These genes include *Lhx4* (LIM homeobox protein 4), which is involved in pituitary development (Mullen et al., 2007); *Nr4a3* (nuclear receptor subfamily 4, group A, member 3; also called *Nor-1*), an orphan steroid receptor important for early embryo development (De-Young et al., 2003); *Vezf1* (Vascular endothelial zinc finger 1), a zinc finger TF that is critical for vascular system development (Kuhnert et al., 2005); *Zfhx1a* (zinc finger homeobox 1a), which is important for T cell development and skeletal development (Higashi et al., 1997; Jin et al., 2008); *Irb3* (Inhibitor of DNA binding 3; also called *Id3*), which is important for T cell subset development (Jones-Mason et al., 2012); and *Spic* (PU.1-related TF), which has been shown to selectively control the development of red pulp Macs in the spleen in addition to its role in B cell development and function (Kohyama et al., 2009). Several transcriptional repressors whose physiological roles are not well known were also induced by LPS. These genes include *Bcor1* (BCL6 co-repressor-like 1), a novel transcriptional co-repressor that functions by interacting with Class II HDACs and recruiting corepressor CtBPs (C-terminal-binding proteins) (Pagan et al., 2007); *Etv3* (Ets variant gene 3; also called *Mets*), an Ets transcriptional suppressor that is known to repress the transcription of cell cycle control genes such as *c-Myc* and *Cdc2* (Hester et al., 2007); and *Trim14* (Tripartite motif-containing 14; also called *Pub*), a PU.1-binding protein that is capable of inhibiting the transcriptional activity of PU.1 that is essential for both myeloid and B cell development (Hirose et al., 2003). Because the roles of a large proportion of the genes

listed above are apparently not directly related to immunological processes, particularly to Mac biology, and because *Spic* (which has long been thought to have major roles in B cell development and function) was recently shown to be involved in the development of a specific subset of Macs, it would be very interesting to test whether the mentioned transcriptional regulators have significant roles in Mac development or function.

Immune cell differentiation and function

As might be expected, a large number of genes induced by LPS belong to the immune cell differentiation and/or function category (Table 3). Several genes encoding factors that are critical for linking innate immune signals to adaptive immune activation were included in this group. *CD86* (B7-2) and *CD80* (B7-1, which displayed weak induction), the key co-stimulatory molecules for T cell activation; *Tnfrsf5* (tumor necrosis factor receptor superfamily, member 5; also called *CD40*), an enhancer of Th1-polarizing cytokine IL12 production; and *Pdcd1lg1* (PD-L1; also called B7-H1, another B7 family member), the ligand for PD-1 that is an important negative regulator for T cell function, were included in this group. Several genes important for inflammation induction and amplification were induced by LPS. These genes include *Hdc* (histidine decarboxylase), the key enzyme required for the production of histamine, which is known to trigger the inflammatory response by increasing the permeability of blood vessels to leukocytes and helping leukocyte recruitment to the infected sites (O'Mahony et al., 2011); *Dusp2* (dual-specificity phosphatase 2, a phosphatase for MAP kinases), which is known to significantly contribute to the induction of pro-inflammatory cytokines under certain inflammatory conditions (Lang et al., 2006); and *Trem1* (triggering receptor expressed on myeloid cells 1), a major amplifier of inflammatory responses that are triggered by bacterial and fungal infections and a crucial regulator of septic shock (Ford and McVicar, 2009). Furthermore, *Ikbke* (inhibitor of κ B kinase epsilon, also called *IKK-i*), one of the major kinases activating *IRF3*, which is the key TF for the expression of type I IFNs such as IFN β (Hemmi et al., 2004), was also included in this group. *Fcgr1* (Fc receptor, IgG, high affinity I), a high-affinity receptor for immunoglobulin IgG2a (van der Poel et al., 2011), and *Fcrl3* (Fc fragment of IgG, low-affinity IIIa, receptor; also called *Fcgr4*), a receptor for both IgG2a/2b and IgE (Hirano et al., 2007), were represented in this group. In addition, genes showing other diverse immunological roles were also induced by LPS. These genes include *MMP8* (matrix metalloproteinase 8), which is critical for cutaneous wound repair and neutrophil migration during inflammation (Tester et al., 2007); *Il4i1* (interleukin 4-induced 1), a secreted L-phenylalanine oxidase that is reported to be able to inhibit T lymphocyte proliferation and function (Lasoudris et al., 2011); *Sfn* (stratifin), which is known to regulate B-cell homeostasis (Su et al., 2011); *Ly6a* (Lymphocyte antigen 6 complex, locus A, also called *Sca-1*), a well-known stem/progenitor cell marker with roles in down-modulating T cell proliferation (Stanford et al., 1997); *CD69*, an early leukocyte activation marker that is capable of regulating the formation of T helper memory cells (Shinoda et al., 2012); *Btla* (B and T lymphocyte-associated), a known inhibitory receptor on T lymphocytes (Watanabe et al., 2003); *Slfn4* (Schlafen-4), a potential modulator of myelopoiesis (van Zuylen et al., 2011); and *Ibrdc3* (IBR domain-containing protein 3; also called *Rnf19b*), a regulator of NK cell-mediated cytotoxicity (Hoover et al., 2009). Thus, upon LPS treatment, complex immune responses by myeloid cells occur simultaneously to induce an adaptive immune response and to initiate, amplify and modulate the inflammatory

Fig. 3. qRT-PCR confirmation of selected LPS-induced genes and expression comparison among splenic myeloid cells and *in vitro* cultured Macs. (A) Signal values of 15 highly LPS-inducible genes derived from our microarray data on splenic myeloid cells were plotted (average signal for the whole data set was 9.0). (B-D) qRT-PCR results of the same set of genes for independently prepared, *in vivo* LPS-treated non-lymphoid splenic myeloid cells (B), for *in vitro* LPS-treated resident PMs (C) and for *in vitro* LPS-treated Raw264.7 cells (D) were plotted. The qRT-PCR signal value for each gene on the y-axis was represented as a ratio of each gene signal over the *Gapdh* signal. Each value is the average of three independent determinations, and the standard deviation was represented as an error bar (B-D). PBS-treated samples are represented as “No”; samples treated with LPS for 3 h are represented as “LPS 3h”; and samples treated with LPS for 8 h are represented as “LPS 8h”. Data are representative of two independent experiments. (E) Signal values of the 15 genes derived from the public microarray data on *in vivo* LPS-treated whole spleen are plotted; LPS 6h represents the sample collected at 6 h after LPS treatment. The average signal for the dataset on the whole spleen was approximately 290, and data for P2ry10, Gpr171 and Fmn2 were not available (NA) in the microarray.

response.

G-protein-coupled receptors (GPCRs) and miscellaneous genes

Diverse types of GPCRs were induced by LPS. Several members of the formyl peptide receptor (FPR) family, which are involved in host defense against pathogens and in sensing endogenous molecules, were induced by LPS. These are Fpr1 (formyl peptide receptor 1; the ortholog of human FPR), which can sense N-formyl-methionyl-leucyl-phenylalanine (fMLP; the classical bacteria-derived formylated peptide), HIV (human immunodeficiency virus)-derived peptides and endogenous molecules such as Annexin 1, and is also important for the defense against at least certain types of Gram-negative bacteria, such as *Listeria monocytogenes* (Migeotte et al., 2006); Fpr-rs2 (formyl peptide receptor, related sequence 2; also called Fpr2), which can sense fMLP (although with a lower affinity than Fpr1), HIV-derived peptides and various endogenous molecules such as Saa, A β ₄₂ (a major form of amyloid deposits in Alzheimer's disease brains) and Annexin 1; and Fpr1 (formyl peptide receptor, related sequence 1; also called Fpr-rs1), which mainly senses the endogenous molecule lipoxin A4 (Migeotte et al., 2006). Several purinergic receptor genes were also induced by LPS. These genes include P2ry10 (purinergic receptor P2Y, G-protein-coupled 10), which is a dual lysophospholipid receptor that is capable of sensing the lipid molecules sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) (Murakami et al., 2008), which regulate diverse biological processes; P2ry14 (purinergic receptor P2Y, G-protein coupled 14; also called Gpr105), which is a UDP-glucose receptor that is involved in modulating gastric function (Bassil et al., 2009); and Gpr171, a putative purinergic receptor whose physiological role is un-

known but that is implicated in regulating myeloid cell differentiation (Rossi et al., 2013). Other diverse GPCRs were also induced by LPS. One such gene is Gpr109b [also called Gpr109a, niacin receptor 1 and Puma-g (protein upregulated in Macs by interferon-gamma)], a nicotinic acid receptor that can mediate the anti-lipolytic effect of nicotinic acid on adipocytes (Tunaru et al., 2003). Another LPS-induced gene is Ccr12 [chemokine (C-C motif) receptor-like 2], a non-signaling receptor for the nonchemokine chemoattractant chemerin, which mediates the presentation of chemerin to its signaling receptor, chemokine-like receptor-1 (Cmklr1) (Zabel et al., 2008), which is involved in amplifying inflammation. In this group of LPS-induced genes, there are some putative sensory receptors such as Mrgpra2 (MAS-related GPR, member A2; also called Mrga2), which belongs to the pain-sensing receptor family (Guan et al., 2010), and V1ri9 (vomeronasal 1 receptor, 19), which belongs to the odorant receptor family (Rodríguez et al., 2002), although the immunological functions of these receptors are unknown. Two GPCR signaling components were also induced by LPS. These components are Rgs1 (Regulator of G-protein signaling 1), which acts as a GTPase-activating protein for G protein alpha subunits (G α s) (Bansal et al., 2007), and Gng11 [guanine nucleotide-binding protein (G protein), gamma 11], a G protein γ subunit (G proteins consist of α , β and γ subunits) that is implicated in the regulation of cellular senescence (Hossain et al., 2006). These signaling components can modulate the transmission of signals from extracellular stimuli detected by GPCRs to intracellular effectors. In the case of Rgs1, it is known that this regulatory protein can desensitize a variety of chemotactic receptors, including receptors for fMLP, leukotriene B4 and C5a (Denecke et al., 1999). As the diverse LPS-responsive GPCRs discussed above can sense both endogenous and exogenous molecules

Table 4. *In vivo*-specific LPS response genes (118 genes) isolated in this study

Novel (83 genes)*				Genes also in whole spleen microarray (14 genes)**	Genes reported as LPS-responsive in literature (21 genes)	
Hba-a1	D17H6S56E-5	Ctrb1	Ras2	Trem1	Gng12	(Larson et al., 2010)
Haghl	Aldh1a1	Ctse	Rps27l	Fscn1	Enpp2	(Freitas et al., 2010)
Gsta4	Adcy6	Cenpf	Ngp	Gadd45g	Fcgr1	(Rodriguez et al., 2007)
Gpr171	Alas1	Cct3	Mtpn	Ebi3	Vdac1	(Qu et al., 2007)
Gng11	Arhgap22	Cd81	Rfc4	Hp	Tnip2	(Lang et al., 2004)
Eif2s2	Actr1a	Bcor1	Riok3	Jun	Spic	(Zhu et al., 2008)
Ddx39	Xpo7	Bst1	Prc1	Aldh1a2	Ly6d	(Matossian-Rogers et al., 1982)
Hnrpd1	Abcb1a	Txn1	Rnf7	Ifng	Cyp26b1	(Zolfaghari et al., 2007)
Etv3	BC019943	Birc5	Mrps28	Il1r2	Ly96	(Shimazu et al., 1999)
Htr6	2610019E17Rik	Ccna2	Myl9	Apex1	Acadl	(Haga et al., 1989)
Klk11	2010002M12Rik	Ttyh3	Rgs1	Cxcl9	Apobec3	(Mehta et al., 2012)
Klf22	2310030N02Rik	Ly6f	Npm1	Cacnb3	Crem	(Harzenetter et al., 2007)
Lhx4	2610524H06Rik	Tipin	Sema4c	Socs2	Ctbp2	(Zhang et al., 2012)
Itgb2l	A230046K03Rik	Lyar	Serpib6b	Orm1	Btla	(Kobayashi et al., 2013)
Tacc3	2700089E24Rik	Ngfrap1	P2ry13		Pxn	(Williams and Ridley, 2000)
Trim56	5330406M23Rik	Npepps	Psmc3		Mt1	(Pagenstecher et al., 1997)
Thoc4	Atpif1	Ruvbl2	Pmrvk		Sfn	(Butt et al., 2012)
Tagap1	Basp1	Ms4a4b	Mrpl12		Ncl	(Miranda et al., 1995)
Tbc1d2	Chst1	Ms4a6d	Samsn1		Sfn9	(Geserick et al., 2004)
Insl6	Ckap4	Nusap1	Mrgpra1		Prdx6	(Erttmann et al., 2011)
Ddt	Cdkn2b	Nme1			Mmp8	(Tester et al., 2007)

*Genes not inducible (< 3-fold) by LPS in whole spleen and not reported as LPS responsive in literature.

**Genes showing more than 3-fold induction by *in vivo* LPS treatment in whole spleen.

and as GPCRs have been reported to be able to cross-talk with TLR pathways (Lattin et al., 2007), it is possible that the receptors identified in this study may be very important modulators of the LPS response *in vivo*, implicating their potential use for treating septic shock induced by bacterial infection.

Genes whose immunological functions are not clear or that are not easily categorized into the list discussed above but whose molecular or cellular roles are at least partially characterized were listed in the functional category “miscellaneous” in Table 3, whereas genes whose roles are not well characterized even at the molecular or cellular level were listed in the “unknown/EST” category. In future, it would be interesting to characterize the immunological roles of these genes, especially from the perspective of inflammatory processes. Because the number of genes in these categories is not small, such a study could reveal many new insights to allow understanding of LPS-induced inflammatory processes *in vivo*.

Validation of microarray data and determining the gene set unique to *in vivo* LPS-induced splenic myeloid cells

To validate the observed gene expression profiles of the non-lymphoid splenocytes, the expression patterns of 15 randomly selected genes from the highly LPS-induced genes discussed above (one or a few genes from most groups were selected, more focusing on relatively unknown genes) were measured by quantitative RT-PCR (qRT-PCR) using independently prepared non-lymphoid splenocyte samples. Most of the 15 tested genes showed LPS-induction patterns and signals quite similar to

those in the non-lymphoid splenocyte microarray experiment (Compare Figs. 3A and 3B), although for several genes, the signal values (e.g., IFN γ and Fscn1) were different or the induction timing (e.g., IL4i1 and P2ry10) was different between the microarray data and qRT-PCR results. These results suggest that our microarray data are reliable.

To determine whether the *in vivo* expression patterns of these highly LPS-induced genes from splenic myeloid cells can be reproduced in similar types of *in vitro* cultured cells and to roughly estimate the extent of unique genes induced by LPS in our splenic myeloid cells, qRT-PCR analysis was performed for the same set of 15 genes using *in vitro* LPS-treated resident PMs and Raw264.7 cells. When the qRT-PCR data between the *in vivo* splenic myeloid cells and the PM cells were compared, the relative signal values (each gene value over the control *Gapdh* value) of most genes were slightly higher in splenic myeloid cells (Compare Figs. 3B and 3C). In addition, the relative signal values of all the genes were much lower in Raw264.7 cells (~1/10) compared to other cells (Figs. 3B-3D). On the basis of these results, we chose a relative signal cut-off of 0.02 for the splenic cells and PM cells and 0.002 for the Raw264.7 cells to determine whether genes in these cells were expressed. A large proportion (60%; 9 genes such as Cxcl10, Oasl1, Zbp1, Trim30, Oasl2, Isg20, Dusp2, IL4i1 and Fmn12) of the 15 tested genes in the PM samples showed similar LPS induction patterns compared to those from splenic myeloid cells with significant signal values, and fewer, but still a significant proportion of genes (53%; 8 genes such as Cxcl10, Oasl1,

Fig. 4. Comparison of microarray data between *in vitro* and *in vivo* LPS-treated samples. Heat maps for the result of hierarchical clustering analysis of the 843 genes (A) that are induced by LPS more than 3-fold (with more than 3-fold the average signal) in at least one cell type and for the result of K Means (number of nodes, 4) clustering analysis on 554 representative LPS-responsive genes (B). Each row represents each gene. The first, second, third and fourth columns in (A) represent LPS fold induction signals from our splenic myeloid cells (SPL), Raw264.7 cells (RAW), BMDM and PM, respectively, and the first and second columns in (B) represent LPS fold induction signals from splenic myeloid cells (*In vivo* SPL) and representative *in vitro* Macs (*In vitro* MAC), respectively. *In vitro*-specific genes are in cluster C1 (98 genes); genes commonly up-regulated in both *in vivo* and *in vitro* samples are in clusters C2 (202 genes) and C4 (136 genes); and *in vivo*-specific genes are in cluster C3 (118 genes). Red, green and black in (A) and (B) and gray in (A) represent up-regulated, down-regulated, unchanged and missing data, respectively; intensity reflects the degree of differential expression (darker colors equate to larger differences).

Zbp1, Trim30, Oasl2, Isg20, Dusp2, and IL4i1) tested in the Raw264.7 samples showed LPS induction patterns similar to those of the splenic myeloid cells. However, some genes (33% of the tested genes), such as SpiC, Serpina3g, P2ry10, Gpr171 and Fscn1, that were shown to be highly LPS-induced in splenic myeloid cells showed a negligible response to LPS in both types of *in vitro* cultured cells (Figs. 3B-3D). These results indicate that a significant proportion (~60%) of LPS-responsive genes selected from our microarray data of splenic myeloid cells also respond to LPS in *in vitro*-cultured primary Macs or Mac cell lines but also that some of the LPS-responsive genes defined in this study are specific to the *in vivo* setting and cannot be easily obtained in an *in vitro* LPS response study.

To determine whether the 15 highly LPS-induced genes tested above could also be selected from the previously reported *in vivo* microarray study that measured gene expression patterns of whole spleens without cell purification after *in vivo* LPS treatment, a public microarray data set for *in vivo* LPS-treated whole spleen was analyzed [Fig. 3E; data analyzed from the Gene Expression Omnibus (GEO) series GSE3565 datasets at NCBI]. Although a large proportion (58.3%, 7 out of 12 genes) of genes with available data were strongly expressed and strongly LPS-inducible [showing more than 3-fold average signal (average is ~290) with more than a 3-fold induction by LPS at the 6 h time point], specific genes such as IFN γ showed weak signals (less than average), and others, such as Trim30, SpiC, Isg20 and Dusp2, were induced very weakly (less than 3-fold; for the case of SpiC, negligible 1.4-fold induction). Because Trim30, Isg20 and Dusp2 were strongly inducible by LPS in resident PMs and even in Raw264.7 cells (Figs. 3C-3D) but showed low degrees of induction (approximately 2.5) in the whole spleen, it seems that contaminating adaptive immune cells indeed blunted the signals from the LPS-responding innate immune cells in the whole spleen data. Thus, whole spleen analysis would exclude the identification of such highly LPS-responding genes as LPS-responsive genes in that study, although these genes are relatively easily identifiable both from previous *in vitro* and our *in vivo* microarray analyses. These

data suggest that a significant proportion of the highly LPS-responsive genes obtained in this study from the microarray analysis on splenic myeloid cells are novel genes whose identification cannot be recapitulated by either *in vitro* cultured cell or whole spleen tissue analysis. Furthermore, because LPS-responsive genes identifiable in the previous *in vitro* microarray analysis are not predictable to also be induced by LPS *in vivo*, the genes that are LPS responsive in both our study and in the previous *in vitro* studies are likely very important for *in vivo* inflammatory processes. This result emphasizes the value of our direct *ex vivo* gene expression profiling of splenic myeloid cells.

To more definitely determine the LPS-responsive genes that are identifiable in both the previously reported *in vitro* Mac microarray and our *in vivo* splenic myeloid cell microarray, as well as genes that are uniquely identifiable in our microarray, we downloaded public data sets and compared these with our data. We used the *in vitro* LPS response microarray data from representative Mac cells such as bone-marrow-derived Macs (BMDMs) (GSE9037) (Koziczak-Holbro et al., 2008), peritoneal Macs (PMs) (GSE14891) (Matsushita et al., 2009) and Raw264.7 (a representative Mac cell line) cells (GSE1906) (Bilban et al., 2006; Murray et al., 2005). Because these microarrays used different microarray platforms and contained different signal values, to compare the datasets, we chose 3x average signal as a signal intensity cut-off and a 3-fold signal difference as a differential expression cut-off for each microarray dataset to select LPS-responsive genes from each different cell type microarray, considering only commonly represented transcripts in all the microarray that have unique gene symbols. This process resulted in 843 genes that were derived from our *in vivo* splenic myeloid cells (360 genes), BMDMs (107 genes), PMs (544 genes) and Raw264.7 (89 genes) (See the list and description for the selected LPS-responsive genes in Supplementary Table 4). When we compared the fold LPS induction for these selected genes, variable patterns of gene induction among different samples were noticeable (Fig. 4A).

To more directly define the LPS-responsive gene sets that are common to both *in vivo* and *in vitro* samples and unique to

our *in vivo* sample, we chose the *in vitro* sample showing the highest fold LPS-induction for a given gene as a representative *in vitro* sample and compared its fold induction with that of our *in vivo* sample, considering only 554 genes whose data were available for both types of samples (Fig. 4B). As expected from the qRT-PCR results above, a large proportion (61.0%; 338/554) of the genes were LPS-inducible in both settings (*in vivo* and *in vitro*), whereas a substantial proportion of genes were specific to the *in vivo* condition (21.3%; 118/554) or the *in vitro* condition (17.7%; 98/554) (Supplementary Table 5). Similar to our *in vivo* LPS response gene analysis above, the common LPS response genes belonged most significantly to GO biological processes related to immunological responses, such as "immune response", "defense response" and "inflammatory response" (Supplementary Table 6). This observation was also quite true for *in vitro*-specific LPS response genes, which were significantly associated with these three groups of processes in addition to the process "regulation of interleukin-6 production". However, *in vivo*-specific LPS response genes belonged significantly to the GO processes related to cell proliferation regulation such as "regulation of cell cycle" and "organelle localization" (necessary for cell division) and metabolic processes such as "retinoic acid metabolic process" and "secondary metabolic process" in addition to the GO processes related to the immune response such as "response to wounding" and "defense response". This analysis of *in vivo*-specific LPS response genes implies that in the *in vivo* situation, splenic myeloid cells undergo significant changes in the cell cycle and cell metabolism in addition to inducing inflammatory responses. To define novel LPS response genes more clearly, the fold inductions of apparent *in vivo*-specific genes (Table 4) were compared to those of whole spleen (Supplementary Table 4). This process resulted in 104 novel LPS-responsive genes *in vivo*. We next surveyed the selected genes in the literature to further narrow down the LPS response genes. This process resulted in 83 truly novel LPS-responsive genes *in vivo* (Table 4): most of these genes (59/83) did not belong significantly to any GO biological processes.

In this study, to overcome the limitations of *in vitro* cultured Mac systems that ignore the influence of other cells, tissues and serum mediators that may exist naturally and respond to LPS administered *in vivo*, we profiled the *in vivo* LPS-responsive gene expression patterns in non-lymphoid, splenic myeloid cells. To our knowledge, this is the first report describing genome-wide expression profiles of these innate immune cells responding *in vivo* to LPS. Our LPS-responsive gene expression profiles of T and B cell depleted, splenic innate immune myeloid cells without culture after *in vivo* LPS treatment confirmed the majority of previously identified *in vitro* LPS-responsive genes (~60% of the LPS-induced genes defined in this study overlap with previous *in vitro* microarray data) and also revealed novel LPS response genes (83 genes) that cannot be identified by previous *in vitro* Mac microarray analysis.

Although our splenic myeloid cell preparations contain minor contamination (~10%) with cells such as T, B, DC and RBC, we believe that most, if not all, of the unique genes defined as highly LPS-inducible in this study derive from real LPS responses of splenic myeloid cells and not from cell contamination differences among PBS and LPS samples because (i) minor T and B cell contamination seems to be uniform among the samples, as expression of various T cell marker genes was not different among the samples (Table 1); (ii) DCs seem to contaminate the PBS sample and not the LPS samples (see the values for CD11c in Table 1), not affecting the LPS-induced gene set; and

(iii) RBCs seem to contaminate the LPS8 sample (see the values for Gypa in Table 1), but the number of LPS-inducible genes uniquely derived from the LPS8 sample was very minor (Table 3). Because we did not purify specific innate immune cells such as Macs for the analysis, a certain portion of our LPS responsive genes could come from the LPS response of non-macrophage cells such as neutrophils. Nonetheless, because these other innate immune cells are also important for initiating or mediating the inflammation process in response to bacterial infection, the LPS-responsive gene set identified in this study should be useful in the future to study how LPS induces and modulates the inflammatory response *in vivo*.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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