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Transcriptional regulation of lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) expression in murine macrophages: role of interferon regulatory factors 1 (IRF-1) and 2 (IRF-2)

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Abstract

Activation of TLRs is most closely associated with induction of pro-inflammatory gene expression; however, expression of many other genes, including the TLR genes themselves, has also been shown to be modulated following TLR engagement. A large family of nuclear transcription factors, the interferon regulatory factors (IRFs), have been implicated in TLR signaling leading to pro-inflammatory gene expression. Given that IRF-1 and IRF-2 counter-regulate the transcriptional activity of many genes, we hypothesized that IRF-1 and IRF-2 might also regulate TLR gene expression following LPS stimulation of murine macrophages. mRNA derived from medium- or LPS-treated primary peritoneal macrophages was analyzed for TLR gene expression using quantitative real-time PCR. In wild-type macrophages, LPS up-regulated expression of TLRs 1–3 and 6–9 steady-state mRNA, while TLR4 mRNA was modestly down-regulated. IRF-$2^{-/-}$ macrophages responded to LPS with dysregulated expression of TLR3, TLR4, and TLR5 mRNA, whereas IRF-1 deficiency dampened LPS-induced mRNA expression for TLR3, TLR6, and TLR9. Functional studies revealed aberrant TLR3 signaling in IRF-$2^{-/-}$ macrophages. Collectively, these findings reveal an additional level of complexity associated with TLR transcriptional regulation and suggest that the *trans*-acting factors, IRF-1 and IRF-2, contribute to the innate immune response to infections by regulating TLR gene expression.

Keywords

Gene expression; interferon regulatory factors; macrophage; Toll-like receptor; transcriptional regulation

Introduction

Toll-like receptors (TLRs) are type I transmembrane glycoproteins that act to sense invading microbial structures.[1] To date, 13 murine TLRs have been identified, with 10 of these being implicated in responsiveness to conserved microbial moieties, such as LPS, double-stranded RNA, flagellin, and unmethylated CpG DNA.[1,2] TLRs can function as homodimers or heterodimers.[3–5] Thus, the array of detectable ligands may be theoretically larger than that

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already described. Structurally, TLRs bear distinct characteristics. In the ectodomain, TLRs contain 19–25 tandem leucine-rich repeat (LRR) motifs; intracellularly, they contain a conserved Toll/IL-1 resistance (TIR) homology domain. LRRs facilitate co-receptor and/or ligand recognition, while the TIR domain mediates protein–protein interactions with downstream adapter molecules and kinases necessary for intracellular signaling.

The signaling cascades that result upon TLR activation are complex. For example, the prototype TLR4 agonist, LPS, activates the receptor and signals through two major intracellular pathways, namely the 'MyD88-dependent' and 'MyD88-independent' pathways.[1] Activation of the MyD88-dependent pathway results in the rapid translocation of NF-κB into the nucleus, which leads to brisk production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α. In contrast, the MyD88-independent pathway primarily activates IRF-3, which dimerizes and translocates into the nucleus to induce transcription of responsive genes, including IFN-β. Once induced, IFN-β can act in an autocrine fashion on macrophages via the IFN-α/β receptor to induce activation of the JAK–STAT pathway that, in turn, generates a secondary wave of transcription factors that enable transcription of STAT-1-dependent genes.[6] In response to specific TLR agonists, TLRs use distinct combinations of adapter molecules to respond with activation of a unique repertoire of transacting factors, and ultimately, gene expression.[2,7]

IRF-3 belongs to a large family of transcription factors called interferon regulatory factors (IRFs), many of which have been implicated in TLR signaling.[8] The IRFs constitute a family of 9 closely related transcription factors that exhibit a broad range of activities within the cell (reviewed by Taniguchi *et al.*[9]). All IRF members contain a conserved N-terminal domain that recognizes interferon stimulated response elements (ISREs)/IRF elements (IRF-Es) within the promoters of IRF-regulated genes. Originally, IRF-1 and IRF-2 were identified for their ability to regulate IFN-β gene expression antagonistically: IRF-2 was found to compete with the transcriptional activator, IRF-1, for binding sites within the IFN-β promoter, due to its lack of a transactivation domain.[10,11] However, it was nearly a decade later that IRF-3, rather than IRF-1, was found to be the major transactivator for the induction of IFN-β gene expression.[12,13] To date, IRF-3, -4, -5, -7, and -8 have been implicated in TLR signaling.[14–19] Additionally, some members of the IRF family, such as IRF-1 and IRF-2, have been shown to contribute to the transcriptional regulation of genes induced by TLR4-mediated signaling, and both IRF-$1^{-/-}$ and IRF-$2^{-/-}$ mice have been found to be resistant to endotoxicity.[20–22]

In addition to up-regulation of pro-inflammatory gene expression, TLR stimulation also results in modulation of TLR gene expression itself.[23–29] For example, when mouse macrophages are stimulated with LPS, there is a very modest and transient decrease in TLR4 gene expression,[23,25,29] whereas TLR2 and TLR3 mRNA are strongly up-regulated (reviewed by Rehli[30]). However, the molecular mechanisms by which TLRs are regulated transcriptionally still have not been well characterized, although several studies have implicated the roles of various transcription factors in TLR transcriptional regulation.[29,31–36] In one study, it was reported, based on promoter analysis, that IRF-1 and IRF-2 may contribute to TLR3 gene expression.[35] Given that several members of the IRF family are essential for the induction of TLR-responsive genes and that IRF-1 and IRF-2 display

stimulatory and inhibitory transcriptional activities, respectively, we hypothesized that IRF-1 and IRF-2 might also participate more broadly in the transcriptional regulation of TLRs. Using LPS as the stimulus, a potent TLR4 agonist that has been implicated as a key inflammatory mediator of Gram-negative bacterial infections, we systematically characterized the gene expression profiles of TLRs 1–9 in macrophages from mice with targeted mutations in either IRF-1 or IRF-2 using quantitative real-time PCR to follow the kinetics of TLR gene expression. It was found that IRF-1 and IRF-2 contributed to the transcriptional regulation of some, but not all, TLRs, basally or in response to LPS. In particular, TLR3 gene expression and TLR3-mediated signaling were found to be significantly aberrant in macrophages derived from IRF-2-deficient mice. Collectively, the data suggest a novel level of regulation in which the *trans*-acting factors, IRF-1 and IRF-2, contribute to the innate immune response to infections by regulating the expression of TLR genes.

Materials and Methods

Reagents

Protein-free, phenol/water extracted LPS from *Escherichia coli* K235 was purified as described.[37] Other TLR agonists that were used in the study included poly I:C (Amersham Biosciences, Piscataway, NJ, USA), Pam2Cys (EMC Microcollections GmbH, Germany), and CpG ODN 1826 (InvivoGen, San Diego, CA, USA).

Mice

Mice homozygous for targeted mutations in either IRF-1 or IRF-2 were generated as previously described.[38] IRF-$1^{-/-}$ mice, back-crossed to C57BL/6J mice for 11 generations, and wild-type C57BL/6J mice, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Two in-bred colonies of IRF-$2^{-/-}$ mice, maintained in our laboratory, were used in this study. The first colony was originally obtained from Dr Tak Mak (Amgen Institute, Toronto, Canada). These mice were back-crossed to C57BL/6J mice for 3–5 generations at the time of receipt, as described elsewhere.[22] These mice have been maintained as an in-bred colony in our laboratory since 1996. IRF-$2^{+/+}$ mice derived from this colony were used as controls for IRF-$2^{-/-}$ mice. As described elsewhere,[38] the IRF-$2^{-/-}$ mice do not breed well homozygously. Therefore, IRF-$2^{-/-}$ males were mated to IRF-$2^{+/-}$ females to obtain IRF-$2^{-/-}$ and IRF-$2^{+/-}$ mice, and IRF-$2^{+/-}$ or IRF-$2^{+/+}$ breeding pairs were used obtain background-matched IRF-$2^{+/+}$ control mice. The second IRF-$2^{-/-}$ colony was obtained from Dr Gary Splitter (University of Wisconsin) who had back-crossed these mice to C57BL/6J mice for > 20 generations. C57BL/6J mice were used as wild-type controls for the second colony. No differences were observed in the responses of the two colonies (data not shown). The genotypes of all mice used in the study were confirmed. Mice were typically used at 6–8 weeks of age. Animals were housed in cages with filter tops in a laminar flow rack and received food and acid water *ad libitum*. For *in vivo* studies, mice were injected intraperitoneally (i.p.) with either a sub-lethal (25 μg per mouse, ~1.2–1.4 mg/kg body weight) or a 90% lethal dose (35 mg/kg body weight) of LPS. All experiments were conducted with institutional approval. For *in vivo* experiments, mice were injected i.p. with

saline or the indicated dose of LPS and livers extracted at the time points indicated in the figure caption. Livers were subjected to total RNA extraction as described below.

Tissue culture

Primary peritoneal macrophages were collected for *in vitro* studies. Mice were injected i.p. with 3 ml of sterile 3% thioglycollate broth and were sacrificed 4 days later. Cells were extracted from the peritoneal cavity with ice-cold Hanks' Balanced Salt Solution. Extracted cells were resuspended in RPMI 1640 (CellGro, Herndon, VA, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 0.3% sodium bicarbonate, and 2% fetal calf serum (FCS). Cells were plated in 6-well tissue culture plates at 4×10^6 cells/well and incubated overnight at 37°C in 5% CO_2 atmosphere. Cultures were washed twice with PBS to remove non-adherent cells and were subsequently treated with the indicated concentrations of TLR agonists over the indicated time intervals. In all *in vitro* experiments, TLR agonists were used at concentrations determined in other studies (data not shown) to elicit optimal gene expression: LPS (10 ng/ml), poly I:C (100 μg/ml), P2C (30 ng/ml), and CpG (2.5 μM).

Preparation of total RNA and cDNA

Total RNA from cultured macrophages or from mouse livers was extracted using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX, USA), followed by isopropanol precipitation at −20°C. After washing twice with 80% ethanol, the RNA pellet was resuspended in nuclease-free distilled water. A 1-μg aliquot of RNA was used for oligo(dT)-primed cDNA synthesis (Promega Reverse Transcription System, Madison, WI, USA), according to the manufacturer's instructions. The reaction was carried out on an ABI GeneAmp PCR System 9700 with the following thermal profile: 42°C for 60 min and 95°C for 5 min. The completed reaction was diluted 1:5 with water, and 2 μl of the diluted cDNA was used for real-time PCR analysis.

Real-time PCR for steady-state mRNA quantification

Quantitative real-time PCR primer sets for each of 9 mouse TLR genes were designed using ABI Prism® PrimerExpress™ v.2.0 software and were synthesized by the Biopolymer and Genomics Core Facility (UMB). In most cases, primers were designed to overlap adjacent exon boundaries to exclude detection of genomic DNA. Primer sequences for the housekeeping gene HPRT were described previously.[22] Real-time PCR was performed on ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Amplification occurred in a 25 μl reaction volume that contained 20 ng cDNA from the reverse transcribed reaction, 0.3 μM each of sense and antisense primers, and 12.5 μl of 2× SYBR Green Master Mix (Applied Biosystems) under the following thermal conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by a dissociation stage. The mRNA levels of the genes of interest were normalized to HPRT, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[39] The quantitative real-time PCR primers that were used are listed in Table 1.

Western analysis for detection of phosphorylated ERK-1/2 and STAT-1

Primary peritoneal macrophages, plated overnight at 4×10^6 cells per well in 6-well plates, were stimulated with TLR agonists for 30 min or 2 h. Following treatment, cells were washed with ice-cold PBS containing 1 mM sodium orthovanadate and then lysed in freshly prepared lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM NaF, 1% Triton X-100, 2 mM EDTA, 100 mM NaCl) supplemented with 1 mM sodium orthovanadate and 1× complete protease inhibitor cocktail (Roche). Whole cell lysates were centrifuged at 14,000 *g* for 15 min at 4°C. Supernatants were collected for protein quantification by the Bradford assay. Lysates were boiled in Laemmli buffer for 5 min, resolved in pre-cast 10% Tris-HCl SDS-PAGE gel (BioRad), and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk/TBS-Tween-20, 0.1% (TBS-T) at room temperature for 1 h and then washed 3 times in TBS-T. Membranes were probed overnight with primary antibodies under gentle agitation at 4°C. Primary antibodies directed against phosphorylated STAT-1 (Y701) (Zymed Laboratories, Inc., South San Francisco, CA, USA; 1:500 dilution), phosphorylated ERK-1/2 (Cell Signaling Technology, Beverly, MA, USA; 1:1000 dilution), and β-actin (Santa Cruz Technologies, Santa Cruz, CA, USA; 1:1000 dilution) were used. Membranes were subsequently washed 3 times with TBS-T and incubated for 1 h with secondary HRP-conjugated, goat anti-rabbit IgG (Cell Signaling Technology; 1:2000 dilution) or donkey anti-mouse IgG (Amersham Biosciences, Piscataway, NJ, USA; 1:10,000 dilution). Finally, after washing the membranes 5 times in TBS-T, bands were detected using ECL Plus reagents (Amersham Biosciences), according to the manufacturer's directions.

Results

When activated by their cognate ligands, TLRs elicit strong pro-inflammatory responses.[1] In addition to the up-regulation of genes that encode many pro-inflammatory mediators, TLR-mediated signaling can also lead to modulation of gene expression patterns of the TLR genes themselves. In this study, we examined the roles of the two counter-regulatory nuclear transcription factors, IRF-1 and IRF-2, in the transcriptional regulation of nine functional murine TLRs that are conserved between mouse and human.[2]

Selective transcriptional regulation of macrophage TLRs by IRF-2

This study was initiated with an analysis of IRF-$2^{-/-}$ mice since the breeding colony was already established in our laboratory. Primary peritoneal macrophages from background matched IRF-$2^{+/+}$ and IRF-$2^{-/-}$ mice were stimulated with LPS (10 ng/ml) over time. Quantitative real-time PCR analysis revealed that, in response to LPS, IRF-$2^{+/+}$ macrophages displayed an up-regulation in steady-state mRNA for TLRs 1, 2, 3 and 6–9 (Fig. 1; filled bars). As had been previously described,[23,25,29] when murine macrophages were stimulated with LPS, TLR4 mRNA was transiently and modestly down-regulated in wild-type macrophages. TLR5 mRNA was not significantly modulated by LPS stimulation *in vitro*. The absence of IRF-2 resulted in gene expression profiles that were altered for some TLRs. While there was virtually no difference between IRF-$2^{-/-}$ and IRF-$2^{+/+}$ macrophages in the gene expression profiles of TLRs 1, 2, and 6–9, IRF-2 deficiency dysregulated gene expression profiles for TLRs 3–5 (Fig. 1). The most striking difference was in the expression

of TLR3 mRNA. At basal levels, IRF-2$^{-/-}$ macrophages express approximately one-third of the level of TLR3 mRNA present in the wild-type macrophages. Additionally, whereas there was a marked increase in TLR3 mRNA in IRF-2$^{+/+}$ macrophages in response to LPS, IRF-2 deficiency resulted in greatly diminished TLR3 gene expression at all time points examined (Fig. 1). TLR5 mRNA expression was increased about 6-fold in unstimulated IRF-2$^{-/-}$ macrophages, but was not modulated by LPS treatment. On the other hand, while statistically significant, the effects of IRF-2 deficiency were not as dramatic for TLR4 mRNA expression. In the IRF-2$^{-/-}$ macrophages, TLR4 exhibited kinetics similar to that seen in the wild-type cells up to 8 h post-LPS stimulation; however, baseline mRNA levels were higher in the IRF-2$^{-/-}$ macrophages. Overall, IRF-2 appears to contribute largely to the transcriptional regulation of TLR3, and to a lesser extent, TLR4 and TLR5, in murine primary peritoneal macrophages.

Furthermore, the diminished basal and LPS-induced gene expression patterns observed for TLR3 in IRF-2-deficient macrophages were reproducible *in vivo*. Initially, we challenged control and IRF-2$^{-/-}$ mice i.p. with either saline or a sub-lethal dose of LPS (25 μg per mouse) and examined their livers for TLR3 mRNA expression at 3-h post-exposure. Consistent with the *in vitro* data, basal expression in IRF-2$^{-/-}$ mice was significantly lower than controls, and TLR3 gene expression was up-regulated to a much lesser extent than exhibited by LPS-challenged wild-type mice (Fig. 2A). However, there was no significant difference in the basal or LPS-induced expression of TLR4 and TLR5 mRNA between IRF-2 knockout and wild-type mice (data not shown). In a more comprehensive *in vivo* time-course experiment, we quantified TLR3 mRNA expression in liver samples from IRF-2$^{-/-}$ and wild-type mice following i.p. administration of LPS at 35 mg/kg body weight (approximately an LD_{90} in C57BL/6J mice). Again, we observed a much lower level of TLR3 gene expression in the livers of IRF-2$^{-/-}$ mice at all time points examined (Fig. 2B). Thus, IRF-2 appears to serve as a major transcriptional regulator of constitutive and LPS-induced TLR3 both *in vitro* and *in vivo*.

Diminished basal TLR3 gene expression in IRF-2$^{-/-}$ macrophages results in hyporesponsiveness to poly I:C

TLR3 and TLR4 signaling increases expression of IFN-β which then acts in an autocrine manner to stimulate STAT-1 phosphorylation through type I IFN-receptor.[6,40] Based on our findings (Figs 1 and 2) and, in particular, the reduced basal TLR3 mRNA in IRF-2$^{-/-}$ cells, we speculated that IRF-2$^{-/-}$ macrophages would fail to respond normally to TLR3 agonists. Using the TLR3 agonist, poly I:C, we compared the downstream responses in wild-type and IRF-2$^{-/-}$ macrophages by Western analysis. As a positive control, macrophages were also stimulated with the TLR4 agonist, LPS. In both mouse strains, LPS induced strong levels of phosphorylated STAT-1 (pSTAT-1 Y701), although the levels were slightly higher in the IRF-2$^{-/-}$ macrophages (Fig. 3A). This is consistent with the increased production of IFN-β previously described for the IRF-2$^{-/-}$ mice.[41,42] However, stimulation of IRF-2$^{-/-}$ macrophages with poly I:C failed to induce detectable pSTAT-1 in contrast to control macrophages (Fig. 3A). Furthermore, whereas LPS induced comparable levels of phosphorylated ERK-1/2 (pERK-1/2) in both wild-type and knockout macrophages, poly I:C stimulation exhibited greatly diminished ERK-1/2 activation in IRF-2$^{-/-}$ macrophages

compared to pERK-1/2 levels in the IRF-$2^{+/+}$ macrophages (Fig. 3A). The hypo-responsiveness of IRF-$2^{-/-}$ macrophages to poly I:C stimulation, as assessed functionally by measuring phosphorylation status of STAT-1 and ERK-1/2, were observed up to 4 h post-stimulation (data not shown). These functional results are congruent with the depressed basal TLR3 gene expression patterns observed in IRF-2-null macrophages and mice (Figs 1 and 2).

The findings in Figure 3A were extended to an analysis of poly I:C-induced IFN-β mRNA expression. Because basal TLR3 mRNA expression is lower in IRF-$2^{-/-}$ macrophages, poly I:C stimulation of IRF-$2^{-/-}$ macrophages was predicted to elicit poor IFN-β production, resulting in decreased STAT-1 phosphorylation (Fig. 3A). As expected, we found that while poly I:C stimulation resulted in a robust production of IFN-β in the IRF-$2^{+/+}$ macrophages, IRF-$2^{-/-}$ cells showed relatively weak IFN-β mRNA induction (Fig. 3B). Additionally, whereas poly I:C stimulation strongly up-regulated TLR3 mRNA levels in the IRF-$2^{+/+}$ macrophages, it only weakly induced TLR3 gene expression in the IRF-$2^{-/-}$ cells (Fig. 3C). Thus, the absence of detectable poly I:C-induced pSTAT-1 in IRF-$2^{-/-}$ macrophages is consistent with the observation that TLR3 and IFN-β mRNA expression are diminished in the absence of IRF-2. Taken together, the data suggest that while IRF-2 deficiency is normally associated with a de-repression of IFN-β expression,[41,42] the resultant reduction in expression of TLR3 supersedes this, as basal TLR3 mRNA levels are significantly lower in IRF-$2^{-/-}$ macrophages.

Selective transcriptional regulation of macrophage TLRs by IRF-1

Similar to IRF-2, deficiency in IRF-1 resulted in altered LPS-induced gene expression patterns for some, but not all, TLRs. Relative to control C57BL/6J macrophages, IRF-1-deficient primary peritoneal macrophages showed dampened gene expression patterns for TLRs 3, 6, and 9 in response to LPS (Fig. 4). Notably, the dampened gene expression profile for TLR3 due to IRF-1 deficiency was not as dramatic as that observed in IRF-2-deficient macrophages (Figs 1 and 4). Collectively, it appears that both IRF-1 and IRF-2 may contribute positively to the transcriptional regulation of TLR3, with IRF-2 being the major regulator. Interestingly, whereas there was a partial depression in mRNA for TLR3 and TLR6, IRF-1 deficiency resulted in the complete loss of LPS inducibility of TLR9 mRNA, while the basal levels remained similar between IRF-1 wild-type and knockout macrophages (Fig. 4). However, when we analyzed the livers of IRF-1 knockout and wild-type mice for TLR gene expression following a sub-lethal LPS challenge, we did not observe any difference between the two mouse strains in the mRNA expression of TLRs 3, 6, and 9 (Fig. 5), nor for the remaining TLRs (data not shown). We also compared TLR-mediated signaling in IRF-$1^{+/+}$ versus IRF-$1^{-/-}$ peritoneal macrophages when stimulated with poly I:C (TLR3), Pam2Cys (TLR2/6), or CpG DNA (TLR9). TLRs 3 and 6 signaled normally in both IRF-$1^{-/-}$ and C57BL/6J macrophages, as assessed by the ability of poly I:C and Pam2Cys, respectively, to stimulate ERK-1/2 phosphorylation (Fig. 6A). While CpG DNA was still able to signal through TLR9 in IRF-$1^{-/-}$ macrophages, the intensity of ERK-1/2 phosphorylation at 30 min and 2 h was less pronounced than observed for the wild-type C57BL/6J macrophages (Fig. 6B). Collectively, these data show that despite the absence of

IRF-1, the ability of TLRs 3, 6, and 9 to signal in response to their corresponding agonists was still largely intact.

Discussion

TLRs play important roles in the innate immune response against bacteria, viruses, fungi, and parasites in the initial, early stages of infection. TLRs are poised to respond rapidly and potently to invading microbes to alert the host defense system and initiate and orchestrate appropriate immune reactivity. Weiss *et al.*[28] recently proposed a model where expression of TLRs is modulated temporally in order to generate effective immune responses to different classes of pathogens. Consistent with this model, we showed in the present study that LPS stimulation, representative of Gram-negative bacterial infections, resulted in the modulation of TLR gene expression over time in murine primary macrophages. Our results suggest that exposure to one TLR agonist may alter subsequent responsiveness to other TLR agonists. Oftentimes, bacterial infections are followed by viral infections and present clinically as complicating superinfections.[43–45] More importantly, virus infections have been shown to sensitize the host for bacterial products such as LPS.[46–48] Although there are many possible mechanisms by which this might happen, alterations in TLR expression induced by the primary infection may account for some of these observations.[26,28,49,50]

We showed that IRF-1 and IRF-2 contribute to the transcriptional regulation of TLRs 3, 4, 5, 6, and 9. IRF-1 deficiency dampened or abolished LPS-induced mRNA expression of TLRs 6 and 9, respectively, in the macrophages (Fig. 4). However, IRF-2 deficiency had no effects on the LPS inducibility of TLRs 6 and 9 (Fig. 1). Consistent with our data, a recent study demonstrated that IRF-1 is recruited directly to the distal regulatory region in the murine TLR9 promoter, which is necessary for optimal IFN-β-induced TLR9 gene expression,[51] With regard to TLR6, it is unclear at the moment whether the effects of IRF-1 deficiency on the reduction in LPS inducibility of TLR6 mRNA expression are direct or indirect. At this point, we cannot disregard the possibility that IRF-1 may be necessary for the expression of additional protein(s) that are required for maximal LPS-induced gene expression of TLR6.

The transient and modest down-regulation of TLR4 mRNA upon LPS stimulation was not affected by deficiencies in either IRF-1 or IRF-2 (Figs 1 and 4). This finding implies that IRF-1 and IRF-2 do not play a role in LPS-induced modulation of TLR4 transcription. Consistent with this hypothesis, Roger *et al.*[29] recently demonstrated that LPS stimulation did not inhibit mouse TLR4 promoter activity, but instead lowered the TLR4 mRNA half-life by almost 50%.

At the basal level, IRF-2-deficient macrophages exhibited higher TLR4 mRNA expression, as compared to wild-type control macrophages (Fig. 1). IRF-1 deficiency did not alter basal TLR4 mRNA expression (Fig. 4). These findings suggest that IRF-2 may directly function as a transcriptional repressor of TLR4 gene expression or may function co-operatively with other transcription factors, such as other members of the IRF family, to regulate basal TLR4 gene expression negatively. Rehli *et al.*[31] previously identified an IRF/PU.1 binding site in the proximal promoter of human TLR4. In that study, it was reported that PU.1 and ICSBP/IRF-8 were required for full basal activity of the human TLR4 promoter. The mouse TLR4

promoter also contains an IRF/Ets element.[29] From the work of Bovolenta *et al.*,[52] it was demonstrated that interferon consensus sequence binding protein (ICSBP; also called IRF-8) bound tightly to IRF-2 and conferred co-operative DNA binding activity upon both proteins; the association of ICSBP/IRF-8 with IRF-1 was relatively less stable and did not result in cooperative binding. Both ICSBP/IRF-8 and IRF-2 have been described to function as transcriptional suppressors and attenuators.[11,41,53] However, ICSBP by itself does not bind DNA; it is only in the presence of IRF-2, which is constitutively expressed, that ICSBP/IRF-8 can form complexes with and then can bind to DNA. Thus, one could envision a scenario where ICSBP/IRF-8, in complex with IRF-2, dampens the transcription of TLR4 to submaximal levels. In the absence of IRF-2, however, ICSBP/IRF-8 fails to bind to the IRF/Ets element, and thereby cannot exert its suppressive effects, and results in the observed elevated basal level of TLR4 mRNA expression (Fig. 1). Congruent with this hypothesis, Roger *et al.*[29] recently reported that while the IRF/Ets element within mouse TLR4 promoter was not required for TLR4 gene activation, removal of the proximal region within the TLR4 promoter, which contains the IRF/Ets element, resulted in enhanced TLR4 promoter activity. Further studies will be necessary to clarify the potential roles of IRFs in the transcriptional regulation of mouse TLR4. Likewise, the gene expression of TLR5 may be subjected to similar transcriptional regulation by the IRFs.

With regard to TLR3 mRNA expression, IRF-2 deficiency resulted in a much more drastic depression in TLR3 mRNA compared to IRF-1 deficiency, and this was reflected functionally (compare Figs 3 and 6). The incomplete abrogation of TLR3 gene expression in either IRF-$1^{-/-}$ or IRF-$2^{-/-}$ macrophages, however, suggests the potential involvement of other transcriptional regulators. Since the IRF system is known to have a high degree of redundancy, other IRFs may partially replace IRF-1 or IRF-2 (reviewed by Taniguchi *et al.*[9]). The results observed for TLR3 gene expression in IRF-$1^{-/-}$ macrophages are consistent with the established role of IRF-1 as a transcriptional activator. However, the diminished patterns of TLR3 gene expression in IRF-2-deficient cells imply that IRF-2 may function, in this case, as a transcriptional activator, rather than its normal role as a transcriptional repressor or attenuator. Although rare, IRF-2 has been described as a transcriptional activator for several genes, such as VCAM-1,[54] histone H4,[55] $gp91^{phox}$,[56] and, more recently, non-muscle myosin heavy chain II-A.[57] Additionally, Heinz *et al.*[35] previously suggested that IRF-2 may function as a transcriptional activator of TLR3, based on their results from promoter mutational analysis. Collectively, our results suggest that both IRF-1 and IRF-2 may function as positive transcriptional regulators of TLR3.

In line with this observation, we identified by computational transcriptional factor binding analysis several ISREs/IRF-Es that are present in the promoter region and scattered throughout TLR3 genomic DNA (data not shown). Interestingly, we also identified by the same method a large region in the second intron of murine TLR3 that putatively contains multiple ISREs/IRF-Es in tandem repeat. While it remains to be confirmed experimentally whether IRF-1 and IRF-2 actually bind to this region of DNA in the second intron, we speculate that this DNA region may contribute to the enhancer complex involved in the transcription of TLR3. Recently, Chung and Kawamoto[57] reported that IRF-2 functions as a transcriptional activator via an ISRE in the enhancer region that is located in the first intron of the non-muscle myosin heavy chain II-A (NMHC-A) gene. Additionally, Brooks *et al.*[58]

previously reported that the enhancer necessary for optimal transcription of the human apoB gene was located in the second intron. Future studies focusing on the contribution of the putative second-intron ISREs to TLR3 transcriptional regulation should reveal its significance.

In summary, we have systematically characterized basal and LPS-induced gene expression profiles of TLRs 1–9 in primary murine peritoneal macrophages from wild-type mice, as well as from mice with targeted mutations in IRF-1 or IRF-2. Macrophages from both knockout strains exhibited altered LPS-induced gene expression patterns for some, but not all, TLRs, in comparison to wild-type controls. The transcription factors, IRF-1 and IRF-2, appear to play a role in the gene expression of TLRs 3, 4, 5, 6, and 9. In particular, LPS-induced gene expression for TLR3 was diminished in both IRF-$1^{-/-}$ and IRF-$2^{-/-}$ macrophages, and basal TLR3 mRNA was strongly depressed in the IRF-$2^{-/-}$ cells. Additionally, we showed that TLR3 signaling was significantly dysregulated in IRF-$2^{-/-}$ macrophages. The results from the present study suggest that exposure to one TLR ligand intricately tailors the subsequent immune response to other TLR agonists. The data also implicate a novel level of regulation in which the *trans*-acting factors, IRF-1 and IRF-2, contribute to the innate immune response to infection by regulating expression of TLR genes.

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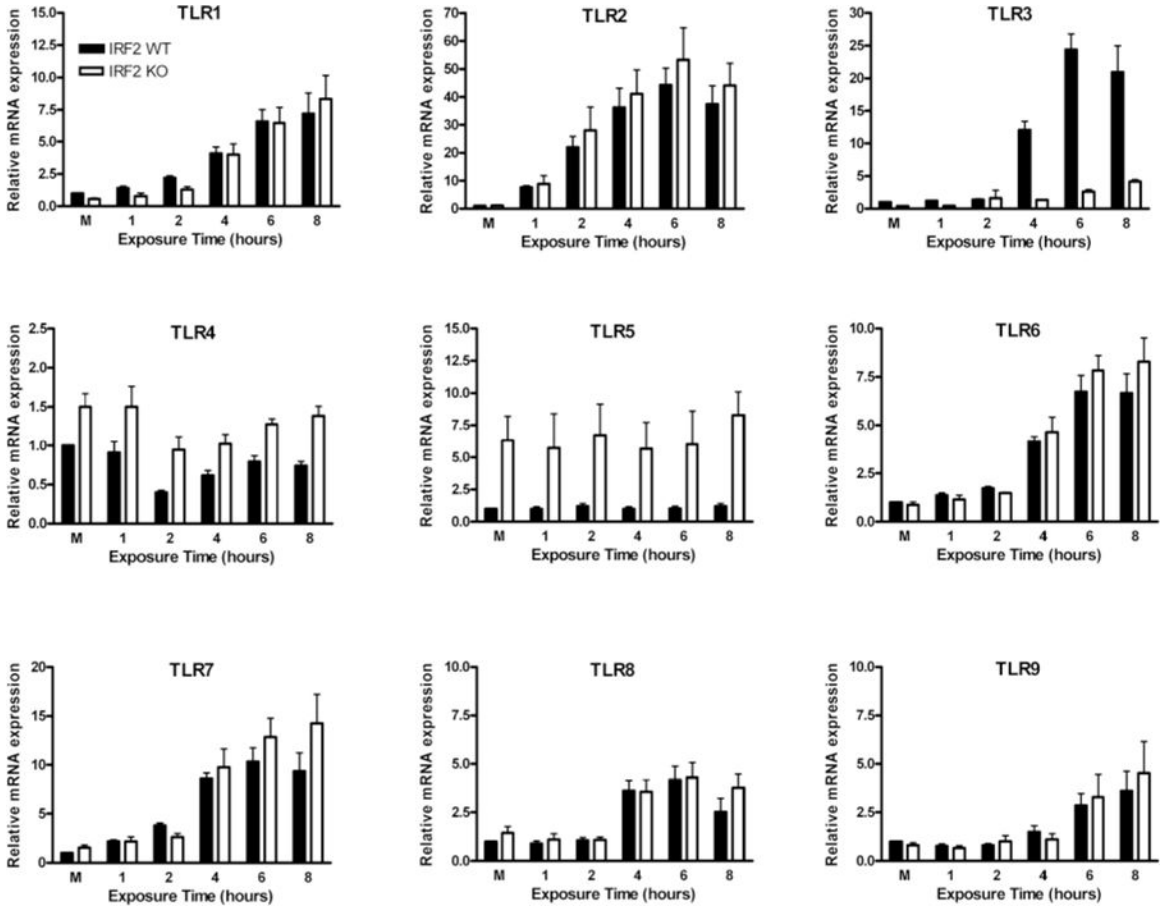

Fig. 1.

Basal and LPS-induced mRNA expression profiles for TLRs 1–9 in primary peritoneal macrophages from IRF-$2^{+/+}$ (filled bars) and IRF-$2^{-/-}$ mice (open bars). Cells were stimulated with LPS (10 ng/ml) over the indicated time intervals. Results are the mean ± SEM of 4–6 separate experiments. M, medium only.

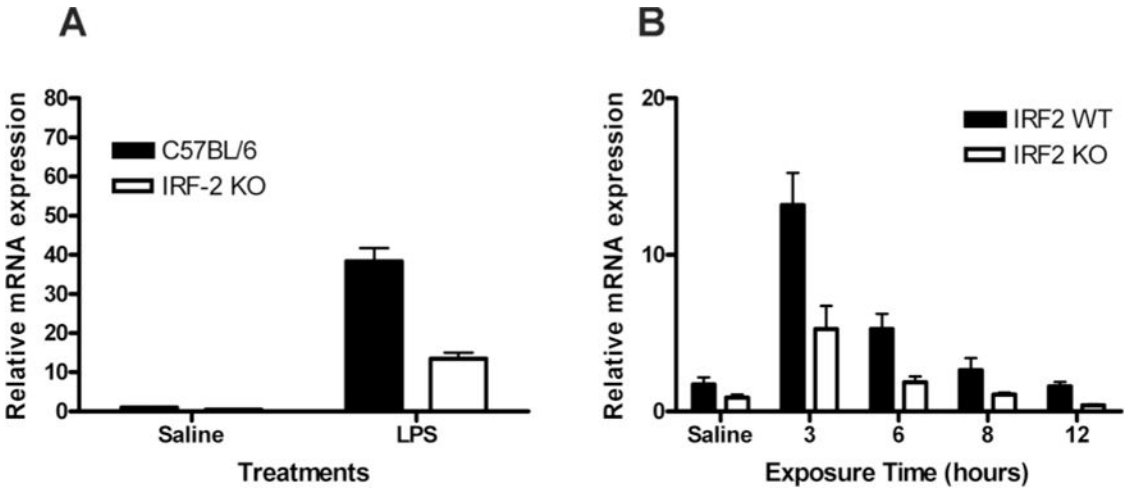

Fig. 2.

In vivo TLR3 mRNA expression in IRF-2 mice in response to LPS. (A) TLR3 mRNA expression in liver samples collected from IRF-$2^{+/+}$ C57BL/6 (filled bars) and IRF-$2^{-/-}$ (open bars) mice 3 h after challenge with either saline or a sub-lethal dose of LPS (25 μg per mouse). Results were derived from 3 mice per strain per treatment. (B) TLR3 mRNA expression in liver samples collected from IRF-$2^{+/+}$ (filled bars) and IRF-$2^{-/-}$ (open bars) mice challenged with a high dose of LPS (35 mg/kg body weight) over the indicated time intervals. Four mice from each strain were assigned to each treatment group, and the experiment was carried out twice. Results represent mean ± SEM for both experiments.

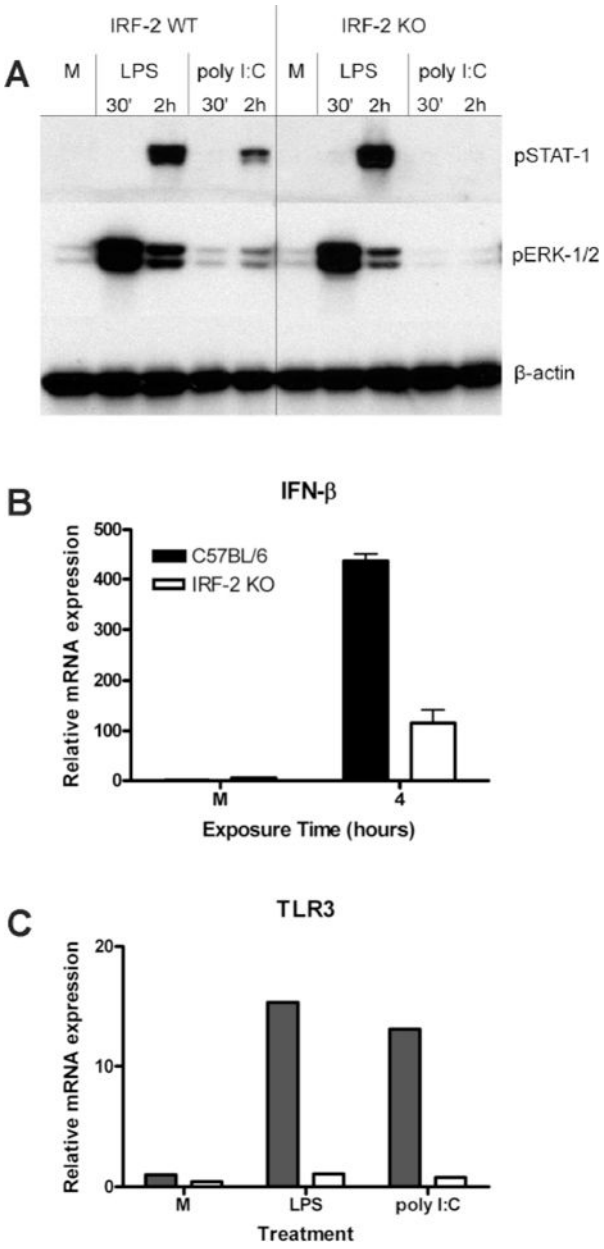

Fig. 3.

IRF-2 regulates responsiveness to TLR3 and TLR4 agonists. (A) Phosphorylation patterns of STAT-1 (Y701) and ERK-1/2 in IRF-$2^{+/+}$ and IRF-$2^{-/-}$ primary peritoneal macrophages. Cells were treated with LPS (10 ng/ml) or poly I:C (100 μg/ml) for 30 min or 2 h. β-Actin was used as the loading control. Data are representative of 3 separate experiments. M, medium only. (B) IFN-β mRNA expression profile in response to poly I:C (100 μg/ml) stimulation. Results are the mean ± SEM of two separate experiments. M, medium only. (C) TLR3 mRNA expression in IRF-$2^{+/+}$ (filled bars) and IRF-$2^{-/-}$ (open bars) macrophages in response to a 4-h stimulation with LPS (10 ng/ml) or poly I:C (100 μg/ml). Results are representative of two separate experiments that showed similar trends. M, medium only.

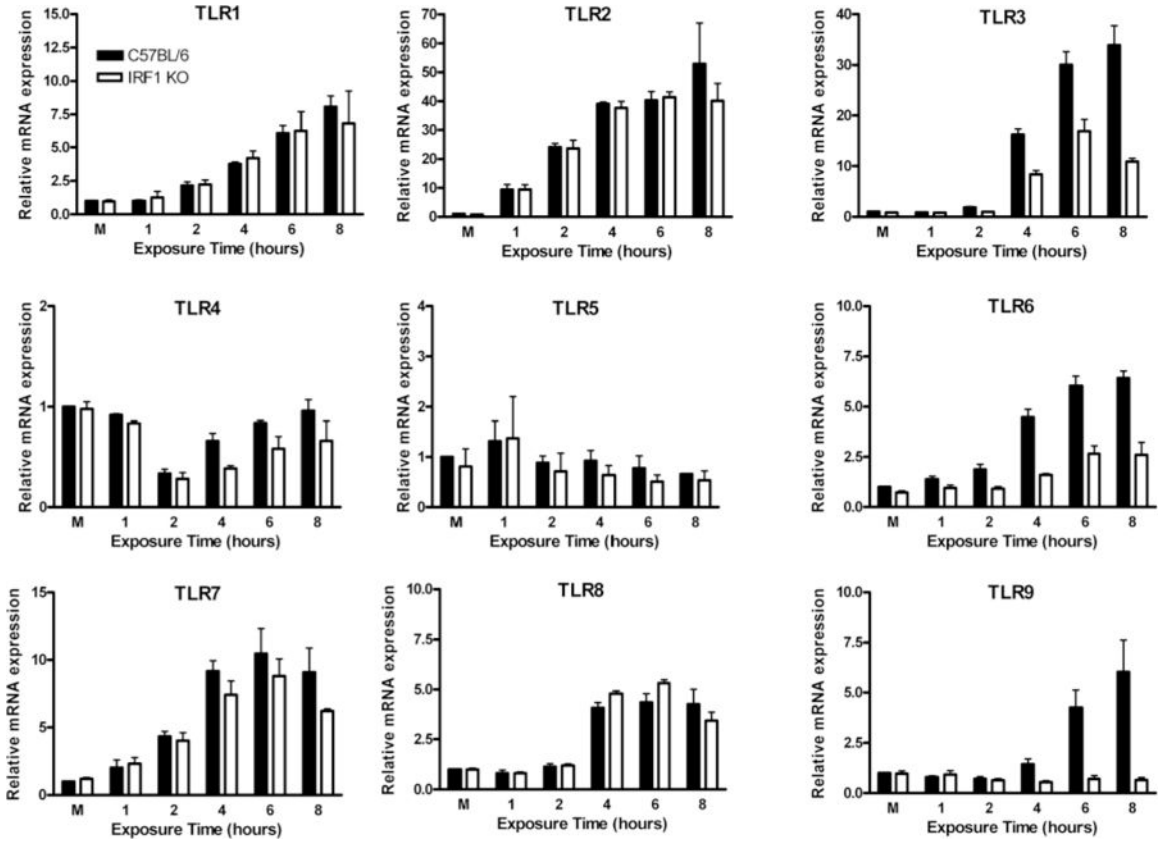

Fig. 4.

Basal and LPS-induced mRNA expression profiles for TLRs 1–9 in primary peritoneal macrophages from C57BL/6J (filled bars) and IRF-$1^{-/-}$ mice (open bars). Cells were stimulated with LPS (10 ng/ml) over the indicated time intervals. Results are the mean ± SEM of four separate experiments. M, medium only.

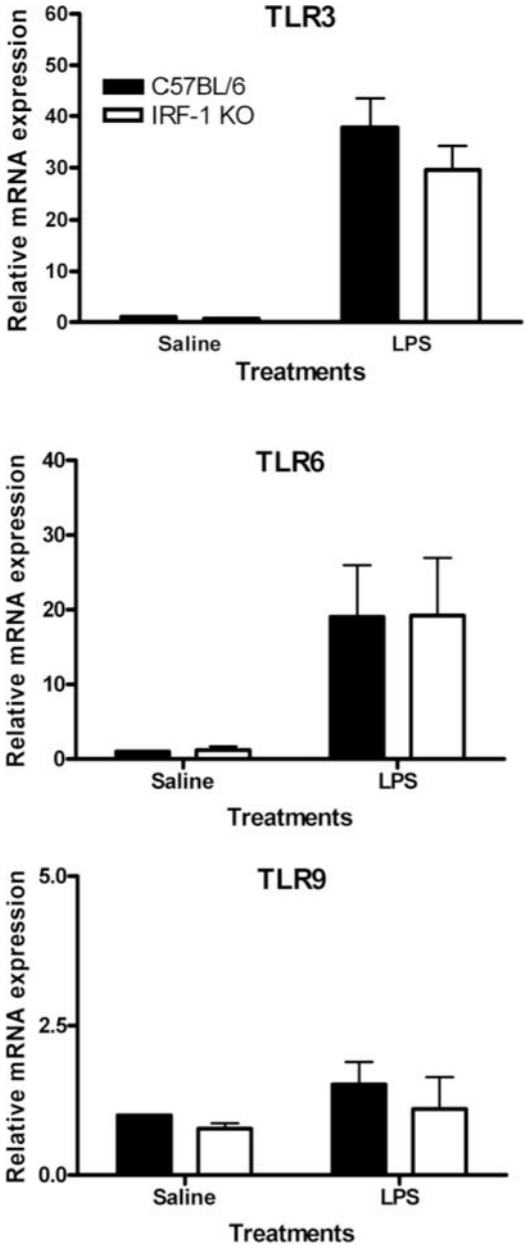

Fig. 5.

mRNA expression profiles for TLRs 3, 6, and 9 in liver samples collected from C57BL/6J (filled bars) and IRF-$1^{-/-}$ (open bars) mice 3 h after challenge with either saline or a sublethal dose of LPS (25 μg per mouse); 3 mice per strain per treatment.

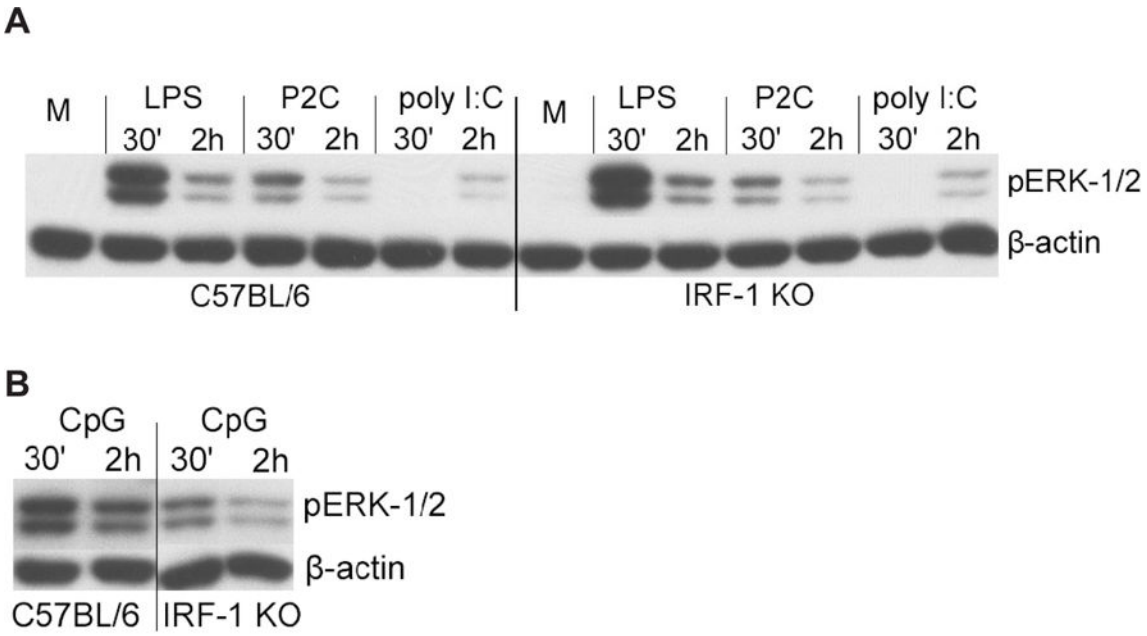

Fig. 6.

ERK-1/2 phosphorylation in C57BL/6J and IRF-$1^{-/-}$ primary peritoneal macrophages. Cells were treated with: (A) LPS (10 ng/ml), Pam2Cys (30 ng/ml), poly I:C (100 μg/ml); or (B) CpG (2.5 μM) for 30 min or 2 h. β-Actin was used as the loading control. Data are representative of two separate experiments. M, medium only.

Table 1

Primer sequences for quantitative real-time PCR

Target genes	Primer sequences
HPRT	5′-GCT GAC CTG CTG GAT TAC ATT AA-3′ (S)
	5′-TGA TCA TTA CAG TAG CTC TTC AGT CTG A-3′ (AS)
TLR1	5′-TGG ACA CCC CTA CAG AAA CGT-3′ (S)
	5′-AAT TTG GTT TAG TCA TTG TAT GGC C-3′ (AS)
TLR2	5′-CTG GAG CAT CCG AAT TGC A-3′ (S)
	5′-CAT CCT CTG AGA TTT GAC GCT TT-3′ (AS)
TLR3	5′-CCA GAA GAA TCT AAT CAA ATT AGA TTT GTC-3′ (S)
	5′-TTT TGC TAA GAG CAG TTC TTG GAG-3′ (AS)
TLR4	5′-GGC AAC TTG GAC CTG AGG AG-3′ (S)
	5′-CAT GGG CTC TCG GTC CAT AG-3′ (AS)
TLR5	5′-CAC TCC CTC GGA GAA CCC A-3′ (S)
	5′-GGC CTT GAA AAA CAT CCC AAC-3′ (AS)
TLR6	5′-AAA GTC CCT CTG GGA TAG CCT CT-3′ (S)
	5′-TGC TTC CGA CTA TTA AGG CCA-3′ (AS)
TLR7	5′-ACA GAA ATC CCT GAG GGC ATT-3′ (S)
	5′-CAG ATG GTT CAG CCT ACG GAA G-3′ (AS)
TLR8	5′-TGT CTA TAG AAC ATG GAA AAC ATG CC-3′ (S)
	5′-CAG AGG ACA GCA GAC AAA AGC AC-3′ (AS)
TLR9	5′-GGG CCC ATT GTG ATG AAC C-3′ (S)
	5′-CTT GGT CTG CAC CTC CAA CA-3′ (AS)
IFN-β	5′-CTC GGA CCA CCA TCC AGG-3′ (S)
	5′-CAC TTG AAG AGC TAT TAC TGG AGG G-3′ (AS)