Differential Role of Toll-Interleukin 1 Receptor Domain-Containing Adaptor Protein in Toll-Like Receptor 2-Mediated Regulation of Gene Expression of Hepatic Cytokines and Drug-Metabolizing Enzymes^[S]

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ABSTRACT:

Pharmacological activities of drugs are impaired during inflammation because of reduced expression of hepatic drug-metabolizing enzyme genes (DMEs) and their regulatory nuclear receptors (NRs): pregnane X receptor (PXR), constitutive androstane receptor (CAR), and retinoid X receptor (RXR α). We have shown that a component of Gram-positive bacteria, lipoteichoic acid (LTA) induces proinflammatory cytokines and reduces gene expression of hepatic DMEs and NRs. LTA is a Toll-like receptor 2 (TLR2) ligand, which initiates signaling by recruitment of Toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP) to the cytoplasmic TIR domain of TLR2. To determine the role of TIRAP in TLR2-mediated regulation of DME genes, TLR2(+/+), TLR2(–/–), TIRAP(+/+), and TIRAP(–/–) mice were given LTA injections. RNA levels of the DMEs (Cyp3a11, Cyp2b10, and sulfoamino transferase), xenobiotic NRs (PXR and CAR), and nuclear protein levels

of the central NR RXR α were reduced ~50 to 60% in LTA-treated TLR2(+/+) but not in TLR2(–/–) mice. Induction of hepatic cytokines (interleukin-1 β , tumor necrosis factor- α , and interleukin-6), c-Jun NH₂-terminal kinase, and nuclear factor- κ B was blocked in TLR2(–/–) mice. As expected, expression of hepatic DMEs and NRs was reduced by LTA in TIRAP(+/+) but not in TIRAP(–/–) mice. Of interest, cytokine RNA levels were induced in the livers of both the TIRAP(+/+) and TIRAP(–/–) mice, whereas LTA-mediated induction of serum cytokines was attenuated in TIRAP(–/–) mice. LTA-mediated down-regulation of DME genes was attenuated in hepatocytes from TLR2(–/–) or TIRAP(–/–) mice and in small interfering RNA-treated hepatocytes. Thus, the effect of TLR2 on DME genes in hepatocytes was mediated by TIRAP, whereas TIRAP was not involved in mediating the effects of TLR2 on cytokine expression in the liver.

Introduction

The hepatic response to inflammation is characterized by reduced capacity to metabolize drugs because of down-regulation of gene expression of key phase I and phase II drug-metabolizing enzymes (DMEs) (Morgan et al., 2008; Morgan, 2009). Gene expression of DMEs is regulated by basal transcription factors as well as by the nuclear receptors (NRs) pregnane X receptor (PXR), constitutive androstane receptor (CAR), and retinoid X receptor (RXR α) (Xie, 2009). We have shown that treatment of mice with the Gram-positive

bacterial component lipoteichoic acid (LTA) down-regulates the gene expression of hepatic DMEs and NRs (Ghose et al., 2009). LTA can induce release of cytokines and other inflammatory mediators from immune cells including macrophages (Hattori et al., 1997). Infections by Gram-positive organisms accounted for ~50% cases of sepsis from 1979 to 2000 (Martin et al., 2003). Thus, infections by Gram-positive bacteria can induce inflammatory responses, leading to potential alterations in drug metabolism and clearance.

Some DME genes and their regulatory NRs are targeted by a variety of cell-signaling components including c-Jun N-terminal kinase (JNK) and nuclear factor (NF)- κ B, which are activated by LTA (Li et al., 2002; Pascucci et al., 2003; Rochette-Egly, 2003; Oesch-Bartlomowicz and Oesch, 2005). It has been shown that down-regulation of cytochrome P450 (P450) enzymes by lipopolysaccharide (LPS) can be blocked by curcumin, a known inhibitor of JNK, although the mechanism is not known (Cheng et al., 2003). Furthermore, we have shown that inflammation-mediated activation of JNK

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ABBREVIATIONS: DME, drug-metabolizing enzyme; NR, nuclear receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; LTA, lipoteichoic acid; JNK, c-Jun N-terminal kinase; NF, nuclear factor; P450, cytochrome P450; LPS, lipopolysaccharide; TLR, Toll-like receptor; TIRAP, TIR-domain containing adaptor protein; MyD88, myeloid differentiation primary-response protein 88; IL, interleukin; Sultn, sulfoamino transferase; I κ B, inhibitor of nuclear factor- κ B; PCR, polymerase chain reaction; TNF α , tumor necrosis factor- α ; siRNA, small interfering RNA.

results in modification and nuclear export of RXR α , leading to suppression of RXR α -dependent hepatic genes (Li et al., 2002; Ghose et al., 2004; Zimmerman et al., 2006). JNK inhibits glucocorticoid receptor activity, resulting in suppression of CAR genes (Pascucci et al., 2003). Reduction in CAR gene expression in inflammation has also been attributed to the disruption of glucocorticoid receptor-mediated transactivation of the CAR gene by NF- κ B (Assenat et al., 2004). NF- κ B can also interact with the PXR-RXR complex to suppress rifampicin-induced CYP3A4 gene expression by LPS (Gu et al., 2006).

Inflammatory responses in the liver are primarily mediated by the Toll-like receptors (TLRs), which involves the TIR domain-containing adaptor protein (TIRAP)/myeloid differentiation primary-response protein 88 (MyD88)-dependent signaling pathway (Akira and Takeda, 2004; Seki and Brenner, 2008; Takeuchi and Akira, 2010) (Fig. 1). TIRAP interacts with the TIR domains of TLR4 and TLR2 and also the TIR domains of MyD88 (Dunne et al., 2003; Sheedy and O'Neill, 2007), and it functions as a bridging adaptor to recruit

MyD88 to the plasma membrane to initiate TLR signaling (Kagan and Medzhitov, 2006).

Activation of TLRs on Kupffer cells releases cytokines, which alter gene expression in hepatocytes (Su, 2002; Seki and Brenner, 2008) (Fig. 1). Although TIRAP is not known to be involved in signaling by cytokines, MyD88 is involved in IL-1 β signaling (Hornig et al., 2002; Dunne and O'Neill, 2003). Despite the direct link between cytokines and P450 expression, the TLR4 ligand LPS can act on the hepatocytes directly to regulate P450 genes. LPS down-regulates P450 genes in mice with targeted disruptions in the cytokine receptors (Warren et al., 1999, 2001; Ashino et al., 2004), and inactivation of Kupffer cells does not completely block LPS-mediated down-regulation of hepatic genes (Xu et al., 2004). Immunostaining of isolated liver sections has detected the expression of TLR2 and TLR4 in hepatocytes (Galloway et al., 2008). LPS reduces P450 genes in primary hepatocytes (Pagani et al., 1987; Sewer and Morgan, 1997), and LPS can be taken up by hepatocytes by a TLR4-dependent mechanism (Scott and Billiar, 2008). Hepatocytes express transcripts for TLRs 1 to 9 (Liu et al., 2002), and stimulation of hepatocytes with a TLR2 ligand, bacterial lipoprotein, activated NF- κ B and induced serum amyloid A expression (Matsumura et al., 2003). However, the role of hepatocyte-specific TLR2 signaling is not well established.

In this study, we sought to determine the role of the TLR2 signaling pathway in regulating the gene expression of key DMEs [Cyp3a11, Cyp2b10, and sulfoamintotransferase (Sultn)]. We have previously shown that these enzymes were maximally affected by LTA after 8 h of treatment (Ghose et al., 2009). LTA-mediated down-regulation of gene expression of DMEs and the regulatory NRs were completely abrogated in TLR2(-/-) or TIRAP(-/-) mice. Surprisingly, induction of RNA levels of hepatic cytokines by LTA was comparable in TIRAP(+/+) and TIRAP(-/-) mice. However, serum cytokine levels were significantly reduced in LTA-treated TIRAP(-/-) mice compared with wild-type mice. LTA-mediated down-regulation of DME gene expression in primary hepatocytes is dependent on TLR2 and TIRAP. This finding suggests that TLR2 on hepatocytes is directly involved in regulation of DME genes. Furthermore, the hepatic response to inflammation is characterized by multiple intracellular regulatory signaling pathways.

Materials and Methods

Materials. Highly purified LTA (*Staphylococcus aureus*) was purchased from InvivoGen (San Diego, CA) and freshly diluted to the desired concentration in pyrogen-free 0.9% saline before injection. Anti-JNK, phospho-JNK, anti-I κ B, β -actin (Cell Signaling Technology, Danvers, MA), and anti-RXR α (D-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used according to the manufacturer's instructions. Oligonucleotides were obtained from Sigma Genosys (Houston, TX). The sequences of the primers and probes were obtained from the literature as reported previously (Ghose et al., 2004, 2009). All other reagents for real-time PCR were purchased from Applied Biosystems (Foster City, CA).

Animals. Adult male TLR2(+/+) and TLR2(-/-) mice (C57BL/6) were obtained from The Jackson Laboratory (Bar Harbor, ME). TIRAP(+/+) and TIRAP(-/-) mice (C57BL/6 $\times$ SV129; F3) were obtained from Dr. Ruslan Medzhitov (Yale University School of Medicine, New Haven, CT). The animals were maintained in a temperature- and humidity-controlled environment and were provided with water and rodent chow ad libitum. Adult male (8–10 weeks) mice (20–25 g) were given an intraperitoneal injection with 6 mg/kg b.wt. LTA in saline or saline alone. Livers were harvested at 1, 4, and 8 h after treatment as indicated in the figure legends. All animal protocols were approved by the institutional animal care and use committee. Experiments were performed in triplicate and repeated three to four times.

Western Blot Analysis. Nuclear and whole-cell extracts were prepared as described previously (Ghose et al., 2004). The protein concentration was determined by BCA assay according to the manufacturer's protocol (Thermo

FIG. 1. Inflammation-associated cell-signaling pathways in the liver. Activation of TLR2 on Kupffer cells (KCs) recruits the adaptor proteins and kinases to induce proinflammatory cytokines (IL-1 β , TNF α , and IL-6). These cytokines bind to specific receptors (IL-1R, IL-6R, and TNF-R) on hepatocytes to alter gene expression in the hepatocytes.

Fisher Scientific, Waltham, MA). The cell extracts were analyzed by immunoblotting. Equal amounts of protein were resolved on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membrane. The membranes were probed with anti-phospho-JNK, anti-JNK, anti-I κ B α , anti- β -actin, anti-RAR α , and anti-RXR α antibodies. Signals were developed by a standard enhanced chemiluminescence method following the manufacturer's protocol (PerkinElmer Life and Analytical Sciences, Waltham, MA) and quantified by densitometer using ImageQuant software.

Real-Time Quantitative PCR Analysis. Total RNA was isolated from mouse liver tissues using TRIzol reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. cDNA synthesis was performed using a High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems). In brief, each amplification reaction (25 μ l) contained 50 to 100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer, 200 nM fluorogenic probe, and 15 μ l of TaqMan Universal PCR Master Mix. Quantitative expression values were extrapolated from standard curves and were normalized to cyclophilin. The sequences of the primers and probes were obtained from the literature or purchased from Applied Biosystems, as reported previously (Ghose et al., 2004, 2009).

Serum Cytokine Analysis. Serum IL-1 β , IL-6, and TNF α levels were determined by an enzyme-linked immunosorbent assay (BD OptEIA IL-1 β , IL-6, and TNF α Mouse ELISA Kits; BD Biosciences, San Diego, CA) according to the manufacturer's directions.

Primary Mouse Hepatocyte Culture and Treatment. Primary hepatocytes were isolated from adult male mice according to the two-step perfusion procedure using 0.025% collagenase, as described previously (Berry and Friend, 1969; Li et al., 2002). Cells were plated at a density of 250,000 cells/ml in six-well Primaria plates (BD, Franklin Lakes, NJ). Cells were allowed to attach for 3 to 5 h in Williams' E medium (Invitrogen) containing 10% fetal bovine serum, penicillin (10,000 U/ml), streptomycin (10,000 μ g/ml), gentamicin (50 μ g/ml), glutamine (2 mM), insulin (2.5 μ g/ml), transferrin (2.5 μ g/ml), sodium selenite (2.5 ng/ml), and glucagon (4 ng/ml). To ensure that the structural and functional integrity of hepatocytes are comparable between preparations, hepatocytes were screened for viability with the trypan blue dye exclusion method soon after isolation. Hepatocyte preparations with more than 95% viability were used for these experiments. The purity of the hepatocyte cultures was determined to be more than 99% by flow cytometric analysis. These criteria are routinely used to assess the viability and purity of hepatocyte

preparations (Scott and Billiar, 2008; Scott et al., 2009). The absence of Kupffer cell contamination in the hepatocyte culture was further determined by the lack of cytokine RNA induction by LTA treatment of hepatocytes (Supplemental Fig. 1).

The cells were maintained for 48 h with a daily change of medium, after which transient transfection was done with SMARTpool siRNA targeting TLR2, TIRAP, or a control nontargeting siRNA (Dharmacon RNA Technologies, Lafayette, CO) at a final concentration of 30 nM siRNA/well. Transfection was performed using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). After 24 h, cells were washed with phosphate-buffered saline and medium was replaced with serum-free medium before saline or LTA (1 μ g/ml) treatment. After 8 h of treatment, RNA was isolated for real-time PCR analysis as described above. Protein levels of TLR2 and TIRAP was determined by immunoblotting.

Statistical Analysis. Statistical analysis of the results was performed using SigmaStat (Systat Software, Inc., San Jose, CA). Data are reported as means $\pm$ S.D. Differences between experimental groups were assessed for statistical significance by the Mann-Whitney test. $p < 0.05$ was considered significant.

Results

TIRAP Is Involved in TLR2-Mediated Regulation of DME Gene Expression. Gene expression of key phase I and phase II enzymes is down-regulated in inflammation; however, the mechanism by which this suppression occurs is not fully understood (Morgan et al., 2008; Morgan, 2009). We have previously shown that LTA, a known ligand for TLR2, can modulate the expression of hepatic DMEs, NRs, and cytokine gene expression (Ghose et al., 2009). Because LTA had maximal effects on gene expression of Cyp3a11, Cyp2b10, and Sultn (Ghose et al., 2009), RNA levels of these DMEs were determined in saline- or LTA-injected TLR2(+/+) and TLR2(-/-) mice (Fig. 2A). There was no significant decrease in RNA levels of the DMEs between the saline-injected TLR2(+/+) and TLR2(-/-) mice (data not shown). However, 8 h after LTA injection, RNA levels of these DMEs were suppressed 70 to 80% compared with the saline control in the TLR2(+/+), but not TLR2(-/-) mice.

The adaptor protein TIRAP is involved in mediating the intracellular signaling events initiated by the activation of TLR2 and TLR4 on

FIG. 2. Regulation of DME gene expression by TLR2 and TIRAP in vivo. TLR2(+/+) and TLR2(-/-) mice (A) and TIRAP(+/+) and TIRAP(-/-) mice (B) were given intraperitoneal injections of 6 mg/kg LTA or saline (SAL), and livers were harvested after 8 h. $n = 6$ /group. RNA was isolated from the livers and analyzed by TaqMan real-time PCR, with primers and probes specific for Cyp3a11, Cyp2b10, and Sultn. All data were presented as $\pm$ S.D. and standardized for cyclophilin RNA levels. Expression in saline-injected mice was set to 1; fold change after LTA treatment was compared with the saline controls. *, significant difference ($p < 0.05$).

the cell surface (Hornig et al., 2002; Akira and Takeda, 2004). Because TIRAP is the first adaptor in the TLR2 signaling pathway, we hypothesized that the TLR2-mediated effect on DME genes would be significantly attenuated in the absence of TIRAP. Similar to our observations in TLR2 mice, we saw a significant reduction in Cyp3a11, Cyp2a4, and Sultn RNA levels in TIRAP(+/+) mice upon LTA injection (Fig. 2B). The suppression of the DME genes was completely blocked in TIRAP(-/-) mice. These results indicate that upon LTA injection, TLR2 does modulate the expression of DME genes via TIRAP.

TIRAP Is Involved in TLR2-Mediated Regulation of NR Gene Expression. The regulation of DME genes is under the control of basal transcription factors as well as the xenobiotic NRs PXR and CAR. Studies have shown that PXR and CAR heterodimerize with the central NR RXR α and bind to target sequences in the promoter regions of DME genes, thus inducing their expression (Xie, 2009). Our studies show that RNA levels of PXR and CAR were reduced ~60 to 70% by LTA treatment of TLR2(+/+) mice, whereas no such reduction was observed in the TLR2(-/-) mice (Fig. 3A). Likewise, LTA treatment resulted in the down-regulation of nuclear RXR α protein levels compared with saline controls after 4 h in TLR2(+/+) but not TLR2(-/-) mice (Fig. 3A). Our previous studies have shown that LTA does not affect RXR α RNA levels (Ghose et al., 2009). The resident nuclear protein RAR α was used as a loading control as described before (Ghose et al., 2004).

LTA treatment reduced PXR and CAR RNA and RXR α nuclear protein levels in TIRAP(+/+) mice but not in TIRAP(-/-) mice (Fig. 3B). This finding indicates that reduction of NRs by LTA is dependent on the first adaptor of the TLR2 signaling pathway, TIRAP.

Regulation of Cytokine Gene Expression by TLR2 and TIRAP.

It is known that cytokines produced by Kupffer cells act on hepatocytes to suppress gene expression in the liver (Su, 2002; Morgan et al., 2008; Seki and Brenner, 2008). As expected, RNA levels of the

proinflammatory cytokines, IL-1 β , TNF α , and IL-6, were significantly induced by LTA treatment of TLR2(+/+) mice (Fig. 4A). However, no such induction was detected in the TLR2(-/-) mice, indicating that TLR2 mediates the cytokine response in the liver upon treatment by LTA. Surprisingly, induction of cytokine RNA levels by LTA was detected in the livers of both TIRAP(+/+) and TIRAP(-/-) mice, indicating that LTA-mediated cytokine RNA induction in liver requires TLR2, but not TIRAP (Fig. 4B).

Role of TLR2 and TIRAP in Regulation of Cell-Signaling Pathways. It is known that NR-mediated regulation of DME gene expression in inflammation involves cross-talk with cell-signaling components (Li et al., 2002; Cheng et al., 2003; Pascucci et al., 2003; Oesch-Bartlomowicz and Oesch, 2005). The mitogen-activated protein kinase JNK is involved in phosphorylation and nuclear export of the central NR RXR α (Ghose et al., 2004; Zimmerman et al., 2006) and in the regulation of expression and activity of some phase I and phase II DMEs (Yu et al., 1999; Pascucci et al., 2003). JNK activation by LTA was markedly attenuated in TLR2(-/-) mice as demonstrated by a reduction in phospho-JNK levels compared with those in the TLR2(+/+) mice (Fig. 5A). It has been shown that there is a cross-talk between NRs, DMEs, and NF- κ B, a key regulator of inflammation and immune response (Ke et al., 2001; Zhou et al., 2006). LTA treatment of TLR2(+/+) mice led to NF- κ B activation as measured by degradation of I κ B α , whereas no such activation was observed in TLR2(-/-) mice (Fig. 5A).

The cell-signaling components, JNK and NF- κ B, were activated by LTA treatment of TIRAP(+/+) mice as expected (Fig. 5B). In the TIRAP(-/-) mice activation of JNK was attenuated. Expression of I κ B α was reduced 80% by LTA compared with the saline control in TIRAP(+/+) mice, whereas the reduction was 40% in the TIRAP(-/-) mice.

Induction of Serum Cytokine Levels Is Regulated by TIRAP.

The results show that the LTA receptor, TLR2, down-regulates hepatic DME genes and NRs by a TIRAP-dependent pathway (Figs. 2

FIG. 3. Regulation of NR expression by TLR2 and TIRAP. TLR2(+/+) and TLR2(-/-) mice (A) and TIRAP(+/+) and TIRAP(-/-) mice (B) were given intraperitoneal injections of 6 mg/kg LTA or saline (Sal), and livers were harvested after 8 h. RNA was isolated from the livers ($n = 6$ /group) and analyzed by TaqMan real-time PCR as described above. *, significant difference ($p < 0.05$). Nuclear extracts were prepared from saline and LTA-injected mouse livers, and RXR α protein levels were measured by Western blotting. Quantification of the blots by densitometry was done after RXR α levels were normalized over RAR α . Data are representative of four to five independent experiments. Error bars denote S.D.

FIG. 4. Regulation of hepatic cytokine gene expression by TLR2 and TIRAP. TLR2(+/+) and TLR2(-/-) mice (A) and TIRAP(+/+) and TIRAP(-/-) mice (B) were given intraperitoneal injections of 6 mg/kg LTA or saline (Sal), and livers were harvested after 1 h. $n = 6$ /group. RNA was isolated from the livers and analyzed by TaqMan real-time PCR as described above. *, significant difference ($p < 0.05$).

and 3). However, TIRAP is not involved in TLR2-mediated induction of cytokines (Fig. 4). So the next goal was to investigate whether LTA treatment affected secretion of cytokines in mouse serum in the TIRAP(-/-) mice compared with that in the corresponding wild-type mice. Serum levels of the proinflammatory cytokines, IL-1 β , IL-6, and TNF α , were induced in LTA-treated TIRAP(+/+) mice as expected (Fig. 6), whereas cytokine levels in the serum were not induced

in TIRAP(-/-) mice. This finding indicates that TIRAP is involved in regulating the levels of cytokines in the serum after LTA treatment; however, it is not involved in regulating the expression of cytokines in the liver.

TIRAP Is Involved in TLR2-Mediated Regulation of DME Gene Expression in Primary Mouse Hepatocytes. Our results show that hepatic DME genes are not down-regulated by LTA in

FIG. 5. Regulation of cell-signaling pathways by TLR2 and TIRAP. TLR2(+/+) and TLR2(-/-) mice (A) and TIRAP(+/+) and TIRAP(-/-) mice (B) were given intraperitoneal injections of 6 mg/kg LTA or saline, and livers were harvested after 1 h. Whole-cell extracts were prepared from the livers of saline and LTA-injected mice, and the samples were analyzed by immunoblotting. Phosphorylation of JNK (P-JNK) (top panel) and degradation of I κ B α (bottom panel) were measured as markers of JNK and NF- κ B activation, respectively. Quantification of the blots by densitometry was done after normalizing P-JNK levels over total JNK and I κ B α levels over β -actin. Data are representative of four to five independent experiments. Error bars denote S.D.

FIG. 6. Induction of serum cytokine levels is regulated by TIRAP. TIRAP(+/+) and TIRAP(-/-) mice were given intraperitoneal injections of 6 mg/kg LTA or saline, mice were sacrificed after 1 h, and blood was collected. Serum IL-1 β , IL-6, and TNF α levels were determined by enzyme-linked immunosorbent assay. $n = 6$ /group. Error bars denote S.D.

TIRAP(-/-) mice even though cytokine RNA levels are elevated in the liver. Although there is a role for Kupffer cell-derived cytokines in regulation of gene expression in hepatocytes, it is possible that LTA directly acts on hepatocytes to reduce DME genes. Several studies have shown that hepatocytes express TLR2 and are responsive to TLR2 ligands (Liu et al., 2002; Matsumura et al., 2003; Galloway et al., 2008).

Treatment of primary mouse hepatocytes with LTA (1 μ g/ml) resulted in ~40 to 50% reduction in Cyp3a11, Cyp2b10, and Sultn RNA levels compared with saline, in “control” or “no siRNA”-treated samples (Fig. 7). However, TLR2 or TIRAP siRNA treatment attenuated LTA-mediated down-regulation of these genes. There is no effect of TLR2 or TIRAP siRNA on expression of DME genes in saline-treated samples. The validity and specificity of siRNA was demonstrated by the reductions in TLR2 or TIRAP protein expressions by treatment of hepatocytes with TLR2 or TIRAP siRNA, respectively (Fig. 7). These results were confirmed by treating hepatocytes isolated from TLR2(-/-) or TIRAP(-/-) mice or their corresponding wild-type counterparts with saline or LTA (Fig. 8). Thus, TLR2-mediated down-regulation of DME genes in primary mouse hepatocytes is dependent on TIRAP.

Discussion

The liver responds to inflammation by down-regulating key hepatic genes involved in regulation of metabolic processes in the liver. This

FIG. 7. Regulation of DME gene expression by LTA in hepatocytes treated with TLR2 or TIRAP siRNA. Primary hepatocytes from C57BL/6 mice were preincubated with siRNA for 24 h, followed by saline or LTA treatment (1 μ g/ml) for 8 h. A, RNA was isolated, and real-time PCR was performed as described under *Materials and Methods*. $n = 6$ /group. Error bars denote S.D. B, total cell lysates were analyzed by immunoblotting to determine the inhibition of TLR2 or TIRAP by their respective siRNAs.

leads to disruption of drug metabolism and clearance and can cause drugs to become potentially ineffective or even toxic. To understand the mechanism of alteration of hepatic drug metabolism in inflammation, we are focusing on elucidating inflammation-induced signaling pathways in the liver. Cross-talk between the signaling components

FIG. 8. Regulation of DME gene expression by LTA in hepatocytes isolated from TLR2(-/-) or TIRAP(-/-) mice. Primary hepatocytes from TLR2(-/-) or TIRAP(-/-) and their corresponding wild-type mice were treated with saline or LTA (1 μ g/ml) for 8 h. RNA was isolated, and real-time PCR was performed as described under *Materials and Methods*. $n = 6$ /group. Error bars denote S.D.

and DME gene regulators contribute to the down-regulation of these genes. We find that the LTA-mediated regulation of hepatic DME genes and serum cytokines involves the LTA receptor TLR2 as expected. However, the first adaptor protein, TIRAP, mediates the effects of LTA on DME genes in vivo and in isolated primary hepatocytes but not on cytokine expression in the liver.

In the liver, cytokines released from Kupffer cells bind to their respective receptors on hepatocytes to initiate signaling events, leading to down-regulation of DME genes. Of interest, our results show that in LTA-treated TIRAP(−/−) mice, cytokine gene expression was still elevated, whereas expression of NR and DME genes was not reduced. Because TIRAP is not known to be involved in the cytokine signaling pathway, we anticipated that the cytokines induced by LTA in the liver would reduce NR and DME genes in TIRAP(−/−) mice. However, the liver is also exposed to the systemic cytokine pool generated by immune cells from extrahepatic tissues. We found that the LTA-mediated increase in serum cytokine levels seen in TIRAP(+/+) mice was markedly reduced in TIRAP(−/−) mice. Thus, it is plausible that although the expression of cytokines was induced in the liver, the effect of LTA on hepatic DME genes was attenuated because of reduced levels of serum cytokines.

In addition to cytokines acting on hepatocytes, several lines of evidence indicate that hepatocytes themselves express TLRs and are responsive to TLR ligands (Liu et al., 2002; Scott and Billiar, 2008; Scott et al., 2009). In support of these observations, we found that LTA treatment of isolated primary mouse hepatocytes down-regulated DME genes by a TLR2- and TIRAP-dependent mechanism. Thus, LTA treatment of mice probably activates TLR2 signaling in the hepatocytes, leading to down-regulation of DME genes by a TIRAP-dependent pathway.

Our results show that TIRAP is required for TLR2-mediated DME gene regulation in vivo and in isolated primary hepatocytes but is not necessary for cytokine production, probably by Kupffer cells. Although TIRAP is known to be involved in TLR2 signaling, recent studies have shown that TIRAP was not required for the induction of IL-6 or NF-κB activation at high concentrations of the TLR1/2 ligand Pam₃Cys-Ser-(Lys)₄ or the TLR2/6 ligand macrophage-activating lipopeptide-2 (Kenny et al., 2009). Likewise, induction of IL-6 by *Salmonella typhimurium*, which is sensed by TLR2, required TIRAP only at low levels of bacteria (Kenny et al., 2009). It is known that liver is the primary site for the clearance of bacterial components from the circulation, and several studies with the TLR4 ligand LPS had shown that Kupffer cells produce a strong and rapid cytokine response to LPS, although hepatocytes also play a role in LPS clearance (Scott and Billiar, 2008; Scott et al., 2009). However, the relative contribution of these two cell types in exposure to bacterial endotoxins in vivo is controversial. Because of the unique anatomical link between the liver and intestines, Kupffer cells encounter high levels of gut-derived toxins (Su, 2002; Seki and Brenner, 2008). We have previously shown that TIRAP was also not involved in TLR4-mediated induction of hepatic cytokines (Ghose et al., 2008). These results indicate that Kupffer cells may not require TIRAP to recruit MyD88 to initiate signaling, whereas hepatocytes may require TIRAP to recruit MyD88. Thus, the requirement of TIRAP for TLR2 signaling may vary within the cell types in the liver.

In further support of a dual role for TIRAP in the liver, LTA moderately activated NF-κB in TIRAP(−/−) mice, but activation of JNK was attenuated in the TIRAP(−/−) mice. Because NF-κB is involved in cytokine gene expression, it is plausible that LTA-mediated activation of NF-κB was still present in the Kupffer cells of TIRAP(−/−) mice. JNK is involved in regulation of NRs and DMEs,

and attenuation of the effects of LTA on these genes coincided with the lack of JNK activation in TIRAP(−/−) mice.

A novel aspect of our findings is that TIRAP is involved in TLR2-mediated DME regulation in vivo and in isolated primary hepatocytes but not in regulation of cytokine expression in the liver. These results indicate that TLR2 on hepatocytes is directly involved in regulation of DME genes. Furthermore, recruitment of TIRAP by TLR2 may be required to down-regulate DME genes but is not required for cytokine induction in the liver. The contribution of a hepatocyte-specific TLR signaling pathway in DME gene regulation in vivo remains to be further investigated.

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Authorship Contributions

Participated in research design: Ghose and Guo.

Conducted experiments: Guo and Gandhi.

Contributed new reagents or analytic tools: Vallejo.

Performed data analysis: Guo and Gandhi.

Wrote or contributed to the writing of the manuscript: Ghose and Guo.

Other: Ghose acquired funding for the research.

References

- Akira S and Takeda K (2004) Toll-like receptor signalling. *Nat Rev Immunol* 4:499–511.
- Ashino T, Oguro T, Shioda S, Horai R, Asano M, Sekikawa K, Iwakura Y, Numazawa S, and Yoshida T (2004) Involvement of interleukin-6 and tumor necrosis factor α in CYP3A11 and 2C29 down-regulation by bacillus Calmette-Guerin and lipopolysaccharide in mouse liver. *Drug Metab Dispos* 32:707–714.
- Assenat E, Gerbal-Chaloin S, Larrey D, Saric J, Fabre JM, Maurel P, Vilarem MJ, and Pascucci JM (2004) Interleukin 1 β inhibits CAR-induced expression of hepatic genes involved in drug and bilirubin clearance. *Hepatology* 40:951–960.
- Berry MN and Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol* 43:506–520.
- Cheng PY, Wang M, and Morgan ET (2003) Rapid transcriptional suppression of rat cytochrome P450 genes by endotoxin treatment and its inhibition by curcumin. *J Pharmacol Exp Ther* 307:1205–1212.
- Dunne A, Ejdebäck M, Ludidi PL, O'Neill LA, and Gay NJ (2003) Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. *J Biol Chem* 278:41443–41451.
- Dunne A and O'Neill LA (2003) The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE* 2003:RE3.
- Galloway E, Shin T, Huber N, Eismann T, Kuboki S, Schuster R, Blanchard J, Wong HR, and Lentsch AB (2008) Activation of hepatocytes by extracellular heat shock protein 72. *Am J Physiol Cell Physiol* 295:C514–C520.
- Ghose R, Guo T, and Haque N (2009) Regulation of gene expression of hepatic drug-metabolizing enzymes and transporters by the Toll-like receptor 2 ligand, lipoteichoic acid. *Arch Biochem Biophys* 481:123–130.
- Ghose R, White D, Guo T, Vallejo J, and Karpen SJ (2008) Regulation of hepatic drug-metabolizing enzyme genes by Toll-like receptor 4 signaling is independent of Toll-interleukin 1 receptor domain-containing adaptor protein. *Drug Metab Dispos* 36:95–101.
- Ghose R, Zimmerman TL, Thevananthar S, and Karpen SJ (2004) Endotoxin leads to rapid subcellular re-localization of hepatic RXR α : a novel mechanism for reduced hepatic gene expression in inflammation. *Nucl Recept* 2:4.
- Gu X, Ke S, Liu D, Sheng T, Thomas PE, Rabson AB, Gallo MA, Xie W, and Tian Y (2006) Role of NF-κB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J Biol Chem* 281:17882–17889.
- Hattori Y, Kasai K, Akimoto K, and Thiemermann C (1997) Induction of NO synthesis by lipoteichoic acid from *Staphylococcus aureus* in J774 macrophages: involvement of a CD14-dependent pathway. *Biochem Biophys Res Commun* 233:375–379.
- Hornig T, Barton GM, Flavell RA, and Medzhitov R (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 420:329–333.
- Kagan JC and Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125:943–955.
- Ke S, Rabson AB, Germino JF, Gallo MA, and Tian Y (2001) Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor- α and lipopolysaccharide. *J Biol Chem* 276:39638–39644.
- Kenny EF, Talbot S, Gong M, Golenbock DT, Bryant CE, and O'Neill LA (2009) MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. *J Immunol* 183:3642–3651.
- Li D, Zimmerman TL, Thevananthar S, Lee HY, Kurie JM, and Karpen SJ (2002) Interleukin-1 β -mediated suppression of RXR:RAR transactivation of the Ntcp promoter is JNK-dependent. *J Biol Chem* 277:31416–31422.
- Liu S, Gallo DJ, Green AM, Williams DL, Gong X, Shapiro RA, Gambotto AA, Humphris EL, Vodovotz Y, and Billiar TR (2002) Role of Toll-like receptors in changes in gene expression and NF-κB activation in mouse hepatocytes stimulated with lipopolysaccharide. *Infect Immun* 70:3433–3442.
- Martin GS, Mannino DM, Eaton S, and Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348:1546–1554.

- Matsumura T, Degawa T, Takii T, Hayashi H, Okamoto T, Inoue J, and Onozaki K (2003) TRAF6-NF- κ B pathway is essential for interleukin-1-induced TLR2 expression and its functional response to TLR2 ligand in murine hepatocytes. *Immunology* **109**:127–136.
- Morgan ET (2009) Impact of infectious and inflammatory disease on cytochrome P450-mediated drug metabolism and pharmacokinetics. *Clin Pharmacol Ther* **85**:434–438.
- Morgan ET, Goralski KB, Piquette-Miller M, Renton KW, Robertson GR, Chaluvari MR, Charles KA, Clarke SJ, Kacevska M, Liddle C, et al. (2008) Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos* **36**:205–216.
- Oesch-Bartlomowicz B and Oesch F (2005) Phosphorylation of cytochromes P450: first discovery of a posttranslational modification of a drug-metabolizing enzyme. *Biochem Biophys Res Commun* **338**:446–449.
- Pagani R, Portolés MT, Bosch MA, Díaz-Laviada I, and Municio AM (1987) Direct and mediated *Escherichia coli* lipopolysaccharide action in primary hepatocyte cultures. *Eur J Cell Biol* **43**:243–246.
- Pascucci JM, Dvorák Z, Gerbal-Chaloin S, Assenat E, Maurel P, and Vilarem MJ (2003) Pathophysiological factors affecting CAR gene expression. *Drug Metab Rev* **35**:255–268.
- Rochette-Egly C (2003) Nuclear receptors: integration of multiple signalling pathways through phosphorylation. *Cell Signal* **15**:355–366.
- Scott MJ and Billiar TR (2008) β 2-Integrin-induced p38 MAPK activation is a key mediator in the CD14/TLR4/MD2-dependent uptake of lipopolysaccharide by hepatocytes. *J Biol Chem* **283**:29433–29446.
- Scott MJ, Liu S, Shapiro RA, Vodovotz Y, and Billiar TR (2009) Endotoxin uptake in mouse liver is blocked by endotoxin pretreatment through a suppressor of cytokine signaling-1-dependent mechanism. *Hepatology* **49**:1695–1708.
- Seki E and Brenner DA (2008) Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* **48**:322–335.
- Sewer MB and Morgan ET (1997) Nitric oxide-independent suppression of P450 2C11 expression by interleukin-1 β and endotoxin in primary rat hepatocytes. *Biochem Pharmacol* **54**:729–737.
- Sheedy FJ and O'Neill LA (2007) The Toll in Toll: Mal and Tram as bridges for TLR2 and TLR4 signaling. *J Leukoc Biol* **82**:196–203.
- Su GL (2002) Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol* **283**:G256–G265.
- Takeuchi O and Akira S (2010) Pattern recognition receptors and inflammation. *Cell* **140**:805–820.
- Warren GW, Poloyac SM, Gary DS, Mattson MP, and Blouin RA (1999) Hepatic cytochrome P-450 expression in tumor necrosis factor- α receptor (p55/p75) knockout mice after endotoxin administration. *J Pharmacol Exp Ther* **288**:945–950.
- Warren GW, van Ess PJ, Watson AM, Mattson MP, and Blouin RA (2001) Cytochrome P450 and antioxidant activity in interleukin-6 knockout mice after induction of the acute-phase response. *J Interferon Cytokine Res* **21**:821–826.
- Xie W (2009) *Nuclear Receptors in Drug Metabolism*, John Wiley & Sons, Hoboken, NJ.
- Xu DX, Wei W, Sun MF, Wu CY, Wang JP, Wei LZ, and Zhou CF (2004) Kupffer cells and reactive oxygen species partially mediate lipopolysaccharide-induced downregulation of nuclear receptor pregnane X receptor and its target gene CYP3a in mouse liver. *Free Radic Biol Med* **37**:10–22.
- Yu R, Lei W, Mandelkar S, Weber MJ, Der CJ, Wu J, and Kong AN (1999) Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *J Biol Chem* **274**:27545–27552.
- Zhou C, Tabb MM, Nelson EL, Grün F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman BM, Thummel KE, et al. (2006) Mutual repression between steroid and xenobiotic receptor and NF- κ B signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**:2280–2289.
- Zimmerman TL, Thevananthar S, Ghose R, Burns AR, and Karpen SJ (2006) Nuclear export of retinoid X receptor α in response to interleukin-1 β -mediated cell signaling: roles for JNK and SER260. *J Biol Chem* **281**:15434–15440.

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