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Palash Mandal, Sanjoy Roychowdhury, Pil-Hoon Park, Brian T. Pratt, Thierry Roger and Laura E. Nagy

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Adiponectin and Heme Oxygenase-1 Suppress TLR4/MyD88-Independent Signaling in Rat Kupffer Cells and in Mice after Chronic Ethanol Exposure

Palash Mandal,* Sanjoy Roychowdhury,* Pil-Hoon Park, † Brian T. Pratt,* Thierry Roger,‡§ and Laura E. Nagy*,¶

Alcoholic liver disease is mediated via activation of TLR4 signaling; MyD88-dependent and -independent signals are important contributors to injury in mouse models. Adiponectin, an anti-inflammatory adipokine, suppresses TLR4/MyD88-dependent responses via induction of heme oxygenase-1 (HO-1). Here we investigated the interactions between chronic ethanol, adiponectin, and HO-1 in regulation of TLR4/MyD88-independent signaling in macrophages and an in vivo mouse model. After chronic ethanol feeding, LPS-stimulated expression of IFN-β and CXCL10 mRNA was increased in primary cultures of Kupffer cells compared with pair-fed control mice. Treatment of Kupffer cells with globular adiponectin (gAcrp) normalized this response. LPS-stimulated IFN-β/CXCL10 mRNA and CXCL10 protein was also reduced in RAW 264.7 macrophages treated with gAcrp or full-length adiponectin. gAcrp and full-length adiponectin acted via adiponectin receptors 1 and 2, respectively. gAcrp decreased TLR4 expression in both Kupffer cells and RAW 264.7 macrophages. Small interfering RNA knockdown of HO-1 activity with zinc protoporphyrin blocked these effects of gAcrp. C57BL/6 mice were exposed to chronic ethanol feeding, with or without treatment with cobalt protoporphyrin, to induce HO-1. After chronic ethanol feeding, mice were sensitized to in vivo challenge with LPS, expressing increased IFN-β/CXCL10 mRNA and CXCL10 protein in liver compared with control mice. Pretreatment with cobalt protoporphyrin 24 h before LPS challenge normalized this effect of ethanol. Adiponectin and induction of HO-1 potently suppressed TLR4-dependent/MyD88-independent cytokine expression in primary Kupffer cells from rats and in mouse liver after chronic ethanol exposure. These data suggest that induction of HO-1 may be a useful therapeutic strategy in alcoholic liver disease. The Journal of Immunology, 2010, 185: 4928–4937.

The innate immune system is involved in many stages of an organism’s response to injury or stress. Components of the innate immune response, including NK and NKT cells (1), Kupffer cells (2), and the complement system (3, 4), are involved in the hepatic response to chronic alcohol exposure. Kupffer cells, the resident macrophages in the liver, are particularly critical to the onset of ethanol-induced liver injury. Ablation of Kupffer cells prevents the development of fatty liver and inflammation in rats chronically exposed to ethanol via intragastric feeding (5). Increased exposure of Kupffer cells to LPS during chronic ethanol consumption, associated with impaired barrier function of the intestine (6), results in activation of TLR4 and increased production of inflammatory mediators (7). Mice deficient in TLR4 or TNF-αR1 expression are protected from chronic ethanol-induced liver injury (7).

TLR4 signaling is mediated via MyD88-dependent and -independent pathways (8). Although the rapid LPS-stimulated expression of TNF-α is characteristic of MyD88-dependent signaling, MyD88-independent signals are more slowly activated and result in increased expression of type I IFN and IFN-dependent genes (8). Chronic ethanol exposure sensitizes Kupffer cells to TLR4-MyD88-dependent responses, such as rapid activation of MAPKs and NF-κB, as well as TNF-α expression (2). Treatment of Kupffer cells with adiponectin, an abundant 30-kDa adipokine with potent anti-inflammatory properties (9), normalizes these effects of chronic ethanol on LPS-induced MAPK and NF-κB activation, as well as TNF-α production in primary cultures of Kupffer cells (10). Treatment of mice with supraphysiologic concentrations of adiponectin during chronic ethanol exposure prevents the development of liver injury, decreasing both steatosis and TNF-α expression in the liver (11).

Despite the efficacy of adiponectin in decreasing LPS-stimulated responses in Kupffer cells and preventing liver injury in mice, the development of adiponectin for therapeutic interventions in patients with alcoholic liver disease (ALD) is likely of limited utility, because of the high concentration of adiponectin in the circulation, as well as the complex oligomeric structure of adiponectin. Therefore, recent studies have focused on the downstream molecular targets of adiponectin to identify anti-inflammatory targets that are more amenable to pharmacologic intervention. We recently identified an IL-10 and heme oxygenase-1 (HO-1)
dependent pathway in Kupffer cells that mediates the anti-inflammatory effects of globular adiponectin (gAcrp) on LPS-stimulated TNF-α expression (12). Similarly, induction of HO-1 expression in mice by treatment with cobalt protoporphyrin (CoPP) normalizes TNF-α expression in response to in vivo LPS challenge after chronic ethanol exposure (12).

A critical role for TLR4 has been identified in the progression of multiple forms of liver disease, including ALD, non-ALD, and viral hepatitis (13). More recently, data have suggested that the MyD88-independent/Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF)-dependent pathway of TLR4 signaling is also a key contributor to chronic ethanol-induced liver injury (14, 15) and acute ethanol (16) in mice. Little is known about the impact of chronic ethanol on the regulation of MyD88-independent signaling. Here we report that chronic ethanol exposure sensitizes Kupffer cells to LPS-stimulated expression of IFN-β and CXCL10 mRNA, two critical TLR4–MyD88-independent responses. This sensitization was also observed in an in vivo mouse model of LPS challenge after chronic ethanol exposure. Treatment of Kupffer cells with adiponectin decreased these MyD88-independent responses. Importantly, induction of HO-1 was sufficient to alleviate ethanol-induced sensitization of MyD88-independent responses in both Kupffer cells and mice.

Materials and Methods

Animals

Adult male Wistar rats weighing 140–150 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lieber-DeCarli ethanol diet (regular, no. 710260) was purchased from Dyets (Bethlehem, PA).

Materials

Recombinant human gAcrp expressed in Escherichia coli was purchased from Peprotech (Rocky Hill, NJ). Recombinant human full-length Acrp expressed in human embryonic kidney 293 cells were purchased from Biovender Research and Diagnostic Products (Candler, NC). Full-length TLR4-LUC (containing −2715 to +52 of the TLR4 promoter in a luciferase reporter plasmid) was previously described (17) and pRenilla/luciferase under the control of the thymidine kinase promoter, as a control for transfection efficiency. After 24 h, cells were treated with or without 100 ng/ml LPS, as indicated in the figure legends. Validated SiRNA sequences were purchased from Ambion/Research Laboratories–FBS at a concentration of 2 × 10⁶ cells/ml prewarmed DMEM per well. After nucleofection, gene expression was analyzed at different times, as indicated in the figure legends. Transfected cells were subcultured and seeded at 10⁴/cm² in 96-well plates for flow cytometry analysis and quantitative real-time PCR (QRT-PCR).

Chronic ethanol feeding and Kupffer cell isolation

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. Chronic ethanol feeding to rats and mice, as well as the isolation and culture of Kupffer cells, were performed as previously described (19, 20). In brief, rats were allowed free access to a Lieber–DeCarli high-fat complete liquid diet for 2 wk. Rats were then randomly assigned to pair- or ethanol-fed groups. Ethanol-fed rats were allowed free access to a liquid diet containing 17% of the calories (3.3% v/v) from ethanol for 2 d and then a liquid diet containing 35% of the calories (6.6% v/v) from ethanol for 4 wk (19). Control rats were pair-fed a liquid diet in which malitol dextrose were substituted isocalorically for ethanol over the entire feeding period. Kupffer cells were isolated and cultured as previously described (19, 21). In brief, ischemia-reperfused Kupffered (19, 21). In brief, ischemia-reperfused Kupffer cells were isolated and cultured as previously described (19, 21). In brief, ischemia-reperfused Kupffer cells were isolated and cultured as previously described (19, 21).

Mouse ethanol feeding

Ten- to 12-wk-old female C57BL/6 mice were randomly assigned to ethanol- or pair-fed groups. Ethanol-fed mice were allowed free access to a complete Lieber–DeCarli high-fat diet with increasing concentrations of ethanol 5.5% of calories [1% v/v] for 2 d, 11% of calories [2% v/v] for 2 d, 22% of calories [4% v/v] for 7 d, 27% of calories [5% v/v] for 7 d, and finally, 32% of calories [6% v/v] for 7 d (22). Control mice were pair-fed diets that isocalorically substituted maltose dextrose for ethanol. HO-1 induction was expressed using the following protocols. At the end of the feeding protocol, mice were treated with 5 mg/kg COOP or vehicle (saline) via i.p. injection. Twenty-four hours later, mice were injected i.p. with 0.7 μg LPS/g body weight or an equivalent volume of sterile, endotoxin-free saline (0.09%). Mice were anesthetized, blood was collected into lithium tubes, and plasma was isolated and stored at −80°C to terminate the experiment. Livers were perfused and excised as previously described (22). Portions of each liver were either fixed in formalin or frozen in optimal cutting temperature compound (Sakura Finetek U.S.A., Torrance, CA) for histology, preserved in RNA later (Ambion, Austin, TX), and stored at −80°C for RNA isolation, or flash frozen in liquid nitrogen and stored at −80°C until further analysis.

Transfection of RAW 264.7 macrophages

For transfection with DNA plasmids, RAW 264.7 macrophages were grown in six-well plates to 60–70% confluency and then transiently transfected with control and expression vectors using SuperFect transfection reagent (for DNA-only transfections) according to the manufacturer’s instructions. Transfected cells were subcultured and seeded at 10.2 × 10⁶/cm² in 96-well plates.

Nucleofection in RAW 264.7 macrophages

For small interfering RNA (siRNA) knockdown experiments, RAW 264.7 cells were transfected using the Amaxa Nucleofector apparatus (Lonzza). Transfected cells were seeded at 10.2 × 10⁶/cm² in 96-well plates and cultured for 24 h before experimental manipulation. A nuclesofection protocol for RAW 264.7 macrophages with siRNA. In brief, 2 × 10⁶ cells were resuspended in 105 μl Cell Line Nucleofector Solution V (Amaka Biosystems, Cologne, Germany) and were nucleofected with 100 nM specific or scrambled siRNA (siRNA sequences are shown later) in the Nucleofector device using the D-032 program, according to the instructions of the manufacturer. After nucleofection, 500 μl prewarmed DMEM was added to the transfection mix and cells were transferred to 12-well plates containing 1.5 ml prewarmed DMEM per well. After nucleofection, gene expression was analyzed at different times, as indicated in the figure legends. Validated Silencer Select siRNA predesigned sequences were purchased from Ambion/Applied Biosystems: mouse adiponectin receptor (adipoR1) sequences sense: 5′-GGCCUGAAGACA AGCA CUATT-3′ and antisense: 5′-UAG-UGGUUGUCUTUUCAGACGc-3′; mouse adipor2 sequences sense: 5′-GGCCCAUCCAUCCAUUCUACAtt-3′ and antisense: 5′-UUCACUAGCUU- GCGGC CGGc-3′; nonspecific siRNA scrambled duplex sense: 5′-GGC CGC UUU GUA GGA UUC G-3′ and antisense: 5′-CGA AUC CAA AGG ACG GGC C-3′. Efficiency of knockdown was determined by Western blot analysis and quantitative real-time PCR (QRT-PCR).
RNA isolation and QRT-PCR

Total RNA was isolated, reverse transcribed, and QRT-PCR amplification was performed. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S or β-actin. RNA was isolated from Kupffer cells and RAW 264.7 macrophages using the RNeasy Micro Kit (Qiagen), with on-column DNA digestion using the RNase-free DNase set (Qiagen) according to the manufacturer’s instructions. Total RNA (200–300 ng) was reverse transcribed using the RETROscript kit (Ambion/Applied Biosystems) with random decamers as primers. RT-PCR amplification was performed in a Mx3000p (Stratagene, La Jolla, CA) using SYBR Green PCR Core Reagents (Applied Biosystems, Warrington, U.K.). All primers used for RT-PCR analysis were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are shown in Supplemental Table 1. Statistical analysis of RT-PCR data were performed using ΔCt values.

Immunohistochemistry of HO-1

Formalin-fixed paraffin-embedded liver sections (4 μm) were deparaffinized in SafeClear II xylene substitute (three times 3 min each; Protocol, Kalamazoo, MI) and hydrated consecutively in 100% (two times), 70%, and 30% ethanol, followed by two washes in PBS to detect HO-1 protein in mouse liver. Sections were then blocked with 2% BSA (diluted in PBS) containing 0.1% Triton X-100 for 1 h, followed by overnight incubation with the primary Ab (1:300 dilution) at 4°C. All sections were then washed in PBS (three times 5 min each), incubated with the fluorescein-conjugated secondary Ab (Alexa Fluor 488-labeled goat anti-rabbit IgG, 1:300) for 2 h in the dark at room temperature, washed again in PBS, and mounted with VECTASHIELD containing DAPI and antifade reagent (Vector Laboratories, Burlingame, CA). No specific immunostaining was seen in sections incubated with PBS rather than the primary Ab.

Western blot analysis

After treatment of RAW 264.7 macrophages with gAcrp or CoPP for 18 h, cells were washed by cold PBS and lysed in radio immunoprecipitation assay lysis buffer containing 100 μM sodium orthovanadate. Total cellular extracts (20 μg) were separated in 10% SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. Membranes were first probed with HO-1 Ab, followed by secondary Ab conjugated with HRP, and then visualized by ECL detection reagents (Amersham Biosciences). The membranes were then stripped and reprobed with either total ERK1/2 or Hsc70 Ab for loading control. Western blot analysis was performed using ECL for signal detection. Signal intensities were

![FIGURE 1](http://www.jimmunol.org/)
quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD).

**Flow cytometry analysis**

After 18-h culture with or without gAcrp, RAW 264.7 cells were gently scraped and adjusted to 1 million/ml with culture media. Cells were >90% viable as determined by trypan blue exclusion. The cells were centrifuged at 100 × g for 10 min. The pellet was washed with PBS and resuspended in 100 μl PBS + 0.1% sodium azide and then blocked with 1.0 μg anti-mouse CD32/CD16 Fcγ Block Abs for 15 min at 4°C. Cells were then stained with 0.5 μg fluorochrome conjugated TLR4-MD2 (PE-conjugated TLR4-MD2) or isotype control (PE-conjugated IgG1) diluted in Dulbecco’s PBS containing 0.1% sodium azide for 30 min. Cells were washed twice with PBS and resuspended in 0.5 ml wash buffer (final concentration ∼10⁶ cells in 0.5 ml) and kept on ice until flow cytometric measurements were performed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data were acquired and processed using FlowJo software (Becton Dickinson).

**ELISA assays**

Cell culture supernatants from RAW 264.7 macrophages were collected after treatment with adiponectin and LPS, and CXCL10 concentration measured by ELISA according to the manufacturer’s instructions (R&D, Minneapolis, MN). The concentration of CXCL10 in mouse plasma and liver lysates was also assayed by ELISA.

**Statistical analysis**

Because of the limited number of Kupffer cells available from each animal, data from several feeding trials are presented in this study. All values are reported as means ± SEM. Data were analyzed by general linear models procedure (SAS Institute, Carey, NC). Data were log transformed, if needed, to obtain a normal distribution. Follow-up comparisons were made by least square means testing.

**Results**

Adiponectin alleviates the sensitization of Kupffer cells to TLR4–MyD88-independent cytokine expression after chronic ethanol

Chronic ethanol exposure sensitizes Kupffer cells to activation of TLR4/MyD88-dependent signaling by LPS, resulting in enhanced activation of MAPKs and NF-κB, and increased expression of TNF-α (2). Treatment of Kupffer cells with gAcrp normalizes these MyD88-dependent responses (10). Because recent studies have identified MyD88-independent/TRIF-dependent pathways as critical for the development of ethanol-induced liver injury in mice (14, 15), we investigated the interactions between chronic ethanol and gAcrp on the sensitivity of MyD88-independent signals in Kupffer cells. Expression of IFN-β and CXCL10 mRNA, considered a molecular signature for TRIF signaling, was increased in response to a 4-h challenge with LPS in Kupffer cells from both pair- and ethanol-fed rats. However, expression was 2-fold greater in Kupffer cells from ethanol-fed rats (Fig. 1A, 1B).

Treatment of Kupffer cells with gAcrp completely prevented LPS-stimulated IFN-β and CXCL10 mRNA expression (Fig. 1A, 1B).

**adipoRs and suppression of MyD88-independent responses in RAW 264.7 macrophages**

In RAW 264.7 macrophages, LPS increased expression of IFN-β and CXCL10 mRNA expression; both messages reached a maximal response at 4 h (Supplemental Fig. 1). LPS-induced increases in IFN-β and CXCL10 were dose-dependently decreased by gAcrp at concentrations from 0.1–1 μg/ml (Fig. 1C, 1D). Treatment of RAW 264.7 macrophages with 1–10 μg/ml full-length adiponectin also decreased LPS-induced IFN-β and CXCL10 mRNA expression, but only at greater concentrations (Fig. 1E, 1F). A similar suppression of CXCL10 expression was reported in human macrophages in response to 10 μg/ml full-length adiponectin (23). Pretreatment of RAW 264.7 macrophages with gAcrp and full-length adiponectin also suppressed LPS-stimulated accumulation of CXCL10 protein in the cell culture media after 8 h (Fig. 1G, 1H).

Adiponectin signals primarily via two receptors, adipoR1 and adipoR2 (24). RAW 264.7 macrophages were transfected or not with siRNA against adipoR1, adipoR2, or scrambled siRNA to identify the adipoR used by gAcrp and full-length adiponectin in...
macrophages. Transfection with specific siRNAs effectively decreased expression of adipor1 and adipor2 (Supplemental Fig. 2A, 2B). In cells not transfected with siRNA or transfected with a scrambled siRNA control, both Gacrp and full-length adiponectin suppressed LPS-stimulated IFN-β and CXCL10 mRNA expression (Fig. 2). siRNA knockdown of adipor1 prevented only the response to full-length adiponectin (Fig. 2). Therefore, Gacrp and full-length adiponectin suppressed TLR4-stimulated IFN-β and CXCL10 expression via adipor1 and adipor2, respectively, in line with the observation that Gacrp has a relatively higher affinity for adipor1, and full-length adiponectin has a higher affinity for adipor2 (24).

gacrp decreases expression of TLR4 in Kupffer cells and RAW 264.7 macrophages

Because adiponectin suppressed both TLR4–MyD88-dependent (10) and MyD88-independent/TRIF-dependent responses, we hypothesized that the anti-inflammatory effects of Gacrp may be mediated via decreased expression of TLR4 or CD14, components of the LPS-receptor complex required for LPS sensing by innate immune cells. TLR4 and CD14 mRNA expression was increased in Kupffer cells after ethanol feeding (Fig. 3A, 3B) (25). Treatment of Kupffer cells with increasing concentrations of Gacrp dose-dependently decreased TLR4 mRNA (Fig. 3A), with no effect on CD14 mRNA (Fig. 3B). Kupffer cells from ethanol-fed rats were more sensitive to the effects of Gacrp on TLR4 mRNA (Fig. 3A).

Using a luciferase reporter construct driven by the full-length TLR4 promoter, we assessed the effect of Gacrp on the transcription of TLR4 in RAW 264.7 macrophages. Treatment of RAW 264.7 macrophages with Gacrp decreased TLR4 promoter activity by ~45–55% (Fig. 3C). Gacrp decreased the expression of TLR4 mRNA by 60% (Fig. 3D) and had no effect on the mRNA stability of TLR4 (Supplemental Fig. 3). This decrease in TLR4 mRNA was associated with decreased surface expression of TLR4/MD2 complex, measured by flow cytometry (66 ± 14%; n = 4; p < 0.03) (Fig. 3E).

HO-1 is necessary and sufficient to decrease TLR4 expression and MyD88-independent responses in RAW 264.7 macrophages

In primary Kupffer cells from rats, the anti-inflammatory effects of Gacrp on LPS-stimulated TNF-α expression are mediated via a HO-1–dependent pathway (12). Therefore, we investigated whether HO-1 was involved in Gacrp-mediated decrease in TLR4 expression and reduction in LPS-stimulated IFN-β and CXCL10. Gacrp increased the expression of HO-1 mRNA and protein in RAW 264.7 macrophages (Fig. 4A, 4B). When HO-1 was overexpressed in RAW 264.7 macrophages, at expression levels comparable with cells treated with Gacrp (Supplemental Fig. 4),

**FIGURE 3.** Adiponectin suppressed TLR4 expression in Kupffer cells and RAW 264.7 macrophages. A and B, Kupffer cells from pair- and ethanol (EtOH)-fed rats were isolated and cultured overnight with 0–1000 ng/ml Gacrp for 18 h. Expression of TLR4 and CD14 mRNA was normalized to 18S mRNA. Values are expressed as fold increases relative to Kupffer cells from pair-fed rats not treated with Gacrp. n = 4; *p < 0.05 ethanol-fed compared with pair-fed; †p < 0.1 compared with cells not treated with Gacrp. C, TLR4 promoter activity was measured in RAW 264.7 macrophages transiently transfected with a full-length TLR4 promoter-luciferase reporter construct and a Renilla luciferase control plasmid. After 24 h, cells were treated with or without 1 μg/ml Gacrp for 18 h. Values represent relative luciferase activity. n = 4; ‡p < 0.05. D, RAW 264.7 macrophages were treated without or with 1 μg/ml Gacrp for 18 h, and expression of TLR4 mRNA measured and normalized to 18S mRNA. n = 4; ¶p < 0.05 compared with cells not treated with adiponectin. E, RAW 264.7 macrophages were cultured with or without 1 μg/ml Gacrp for 18 h, and cell surface expression of TLR4/MD2 (solid line), relative to isotype controls (dotted line), was measured by flow cytometry. Data are representative of four experiments.
TLR4 mRNA expression was reduced by \(\sim 50\%\) (Fig. 4C). Transfection with the empty vector had no effect on TLR4 mRNA expression (Fig. 4C). Further, overexpression of HO-1 alleviated LPS-stimulated IFN-\(\beta\) and CXCL10 mRNA to a similar extent as treatment with gAcrp (Fig. 4D,4E).

In macrophages, TLR4/MyD88-independent signals lead to increased IFN-\(\beta\) expression; IFN-\(\beta\) then activates IFN-inducible gene transcription, primarily via activation of the transcription factor STAT1 (26). LPS increased STAT1 phosphorylation at 1–2 h in RAW 264.7 macrophages (data not shown), consistent with the time courses for IFN-\(\beta\) and CXCL10 mRNA expression (Supplementary Fig. 1). LPS-activated STAT1 phosphorylation was completely blocked by treatment with gAcrp or HO-1 overexpression (Fig. 4F).

HO-1 mediates the inhibitory effects of gAcrp on LPS-stimulated MyD88-independent pathway

RAW 264.7 macrophages were transfected with siRNA against HO-1 or scrambled siRNA (Supplemental Fig. 4C). When HO-1 expression was knocked down, the anti-inflammatory effects of gAcrp on LPS-stimulated IFN-\(\beta\) and CXCL10 were attenuated (Fig. 5A,5B). Similarly, if the activity of HO-1 was inhibited by treating RAW 264.7 macrophages with zinc protoporphyrin, inhibition of LPS-stimulated IFN-\(\beta\) and CXCL10 mRNA expression was reduced (Fig. 5C, 5D). Taken together, these data demonstrate that increased expression of HO-1 is necessary and sufficient to reduce TLR4 expression, as well as TRIF-dependent signaling, in response to gAcrp.

Induction of HO-1 decreases LPS-stimulated IFN-\(\beta\) and CXCL10 expression in macrophages

We next tested the ability of two different small molecules to mimic the anti-inflammatory effects of gAcrp or overexpression of HO-1 in macrophages. Treatment of cells with the heme analog, CoPP, induced the expression of HO-1 in RAW 264.7 macrophages (Supplemental Fig. 4B). When RAW 264.7 macrophages were pretreated with CoPP for 18 h, LPS-stimulated expression of IFN-\(\beta\) and CXCL10 mRNA was suppressed to a similar extent as in cells pretreated with gAcrp (Fig. 6A,6B). The anti-inflammatory effects of HO-1 are primarily mediated by its enzymatic products, CO and/or biliverdin/bilirubin (27). Pretreatment of RAW 264.7 macrophages with the CO-releasing molecule-2 for 4 h before challenge with LPS was as effective as CoPP or gAcrp in alleviating the effects of LPS on IFN-\(\beta\) and CXCL10 expression (Fig. 6A,6B). Pretreatment of Kupffer cells with CoPP was equally effective at suppressing TLR4-stimulated IFN-\(\beta\) and CXCL10 mRNA expression (Fig. 6C,6D).

Treatment of mice with CoPP reduced chronic ethanol-induced sensitization to TLR4-MyD88–independent responses in mouse liver

Because CoPP effectively prevented TLR4–MyD88-independent responses in isolated Kupffer cells, we next asked whether chronic ethanol feeding would sensitize mice to LPS-stimulated IFN-\(\beta\) and CXCL10 expression, and whether treatment of mice with CoPP could alleviate this effect of chronic ethanol. C57BL/6 mice were allowed free access to an ethanol-containing liquid diet for...
28 d, at a final concentration of 6% ethanol (v/v), equivalent to 32% of total calories, or pair-fed control diets. Mice were then treated with a single injection of CoPP or vehicle. Twenty-four hours after the CoPP treatment, mice were challenged with LPS or saline. Body weights and food intake for all experimental groups are shown in Supplemental Table 2. Expression of HO-1 in liver was low in both pair- and ethanol-fed mice at baseline (Fig. 7A). Although challenge with LPS modestly increased expression of HO-1 in both treatment groups, treatment with CoPP robustly increased expression of HO-1 in livers from both pair- and ethanol-fed mice at 28 d. CO-releasing molecule-2 (CORM2) and zinc protoporphyrin (ZnPP) were used as HO-1 inducers to confirm the results. The expression of IFN-β and CXCL10 was normalized to 18S mRNA. Values with different superscripts (a, b, c) are significantly different from each other, \( p < 0.05 \).
ethanol-fed mice, in a predominantly sinusoidal distribution (Fig. 7A). LPS challenge increased expression of IFN-β and CXCL10 mRNA in both pair- and ethanol-fed mice at 4 h; this response was greater after chronic ethanol feeding (Fig. 7B, 7C). Similarly, the LPS-stimulated increase in CXCL10 protein in plasma (Fig. 7D) and liver lysates (Fig. 7E) was increased after chronic ethanol. Importantly, pretreatment with CoPP reduced the sensitivity of both pair- and ethanol-fed mice to LPS-stimulated responses (Figs. 7B–D, 8).

**Discussion**

The development of ALD is a complex process involving both the parenchymal and nonparenchymal cells in the liver. Enhanced inflammation in the liver during ethanol exposure is associated with liver injury; activation of TLR4 by LPS and signaling via MyD88-dependent and -independent pathways contribute to chronic ethanol-induced liver injury (7, 14, 15). Increased activation of TLR4 by LPS during chronic ethanol exposure is due to increased exposure to gut-derived endotoxins, as well as a sensitization of macrophages to stimulation by LPS (2). Here we report that, in addition to the sensitization of MyD88-dependent signaling reported in the past (2), chronic ethanol also enhances TLR4 signaling via MyD88-independent pathways, both in isolated Kupffer cells and in an in vivo mouse model of chronic ethanol exposure. Chronic ethanol exposure increased LPS-stimulated IFN-β mRNA expression, as well as the IFN-inducible gene, CXCL10; expression of these cytokines is a signature for TLR4–TRIF-dependent signaling. Moreover, here we have identified an adiponectin/HO-1 pathway that attenuates TLR4 signaling via both MyD88-dependent and -independent pathways (summarized in Fig. 8). Induction of HO-1 after chronic ethanol feeding to mice countered this sensitization, suppressing LPS-stimulated IFN-β/CXCL10 expression in liver within 24 h.

Recent studies have explored the potential utility of adiponectin as an anti-inflammatory agent to treat ALD (11). Importantly, we find that adiponectin treatment normalized LPS-induced TNF-α production (10, 12), as well as IFN-β and CXCL10 mRNA expression (Fig. 1), in primary cultures of Kupffer cells after chronic ethanol exposure. Interestingly, we found that both globular and full-length adiponectin suppressed LPS-stimulated expression of IFN-β and CXCL10 mRNA. The response to gAcrp was mediated by adipor1, whereas the response to full-length adiponectin was dependent on adipor2 (Fig. 2). These data are consistent with the differential affinities of gAcrp and full-length adiponectin for
adipoR1 and adipoR2, respectively (24). Studies investigating the specific downstream signaling events activated by adipoR1/R2 in macrophages are currently under investigation.

Chronic ethanol feeding increased expression of TLR4 mRNA in Kupffer cells isolated from rats (Fig. 3A) and in mouse liver after chronic ethanol exposure (28). In cellular models, changes in the expression of TLR4 mRNA influence TLR4-dependent responses (29). Therefore, increased expression of TLR4 likely contributes to ethanol-induced increases in TLR4–MyD88-dependent and -independent signaling in the liver. Treatment of macrophages with gAcrp or CoPP to induce HO-1 reduced expression of TLR4 mRNA and protein, likely via a decrease in TLR4 transcription.

We show that induction of HO-1 was sufficient to suppress TLR4–TRIF-dependent responses in macrophages and in mice after chronic ethanol exposure. Induction of HO-1 is also effective at the prevention of steatohepatitis induced by feeding a methylcholine-deficient diet, a model of nonalcoholic steatohepatitis (30), and protects against liver ischemia-reperfusion injury (31). However, it is not yet clear which cell(s) in the liver contribute to these cytoprotective effects of HO-1. In our studies, HO-1 expression is primarily sinusoidal, consistent with previous reports that HO-1 is typically expressed in Kupffer cells in the liver (32). However, in methylcholine-deficient diet-fed mice, HO-1 was observed in fatty hepatocytes (30). Similarly, whereas induction of HO-1 dampens TLR4–TRIF-dependent responses in Kupffer cells and RAW 264.7 macrophages (Fig. 1) (31), as well as in mice after chronic ethanol exposure (Fig. 7), other studies find that HO-1 upregulates IFN-β production in some disease models (33, 34). Taken together, these studies suggest that there are complex cell–cell interactions involving HO-1 within the liver that contribute to the protective effect of HO-1 and its enzymatic products in the liver in response to different insults.

Chronic ethanol-induced sensitization to the TLR4–TRIF-dependent pathway, associated with increased expression of TLR4 mRNA (28), likely contributes to ethanol-induced liver injury. TRIF-dependent signaling is critical to mediating the innate immune response to LPS, as the expression of TLR4 target genes is mediated primarily through MyD88-independent, rather than MyD88-dependent pathways (35). Moreover, recent studies have identified MyD88-independent/TRIF-dependent signaling as an essential element in chronic ethanol-induced liver injury in mice (14, 15). Here we find that chronic ethanol exposure sensitized mice to LPS-stimulated activation of typical MyD88-independent/TRIF-dependent responses, including expression of IFN-β mRNA and the IFN-inducible CXCL10 mRNA in liver, as well as CXCL10 protein in plasma and liver lysates. However, the specific contributions of type 1 IFNs to the pathophysiologic progression of ALD have not been well characterized. The protective effects of CoPP treatment in suppressing signature cytokines in both the MyD88 (12) and TRIF pathways (Fig. 8) in Kupffer cells and in mice after chronic ethanol exposure suggests that CoPP would likely be effective in preventing ethanol-induced liver injury, independent of the relative contributions of MyD88-dependent and -independent pathways in the progression of ethanol-induced liver injury.

The controlled and appropriate resolution of inflammation is an essential feature of the innate immune response; failure to terminate an inflammatory response likely contributes to a number of chronic inflammatory diseases, including ALD (36). Importantly, the resolution of inflammation is an active, highly coordinated response, with inflammatory signals initiating the induction of negative regulators (37). During ethanol exposure, despite high expression of inflammatory mediators, there appears to be a generalized failure in the resolution of inflammation. Here we have identified an adiponectin/HO-1–mediated anti-inflammatory pathway that, when activated, can effectively dampen the effects of chronic ethanol on TLR4-mediated signaling, resulting in decreased activation of MyD88-mediated signaling (10, 12), as well as MyD88-independent signaling. Induction of HO-1, either in Kupffer cells isolated from rats after chronic ethanol feeding or in an in vivo mouse model of chronic ethanol exposure, suppressed LPS-stimulated expression of IFN-β and CXCL10 mRNA expression. In summary, these data suggest that strategies, such as the induction of HO-1 identified in this study, aimed at decreasing TLR4 expression and/or TLR4-mediated signaling via both the MyD88-dependent and -independent pathways will likely be useful in treatment or prevention, or both, of ethanol-induced liver injury, as well as other types of liver injury, such as nonalcoholic steatohepatitis and ischemia-reperfusion injury (30, 31).

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Disclosures
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References


