Heme Oxygenase-1 Alleviates Mouse Hepatic Failure through Suppression of Adaptive Immune Responses

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ABSTRACT

Heme oxygenase-1 (HO-1) has protective effects on liver damage induced by noxious stimuli. The mechanism of action of HO-1 is not well understood. In the present study, we investigate the effect of HO-1 in a model of fulminant hepatic failure induced by Propionibacterium acnes and lipopolysaccharide (LPS). The expression of HO-1 mRNA and protein in the liver was increased after repeated administration of the HO-1 inducer cobalt protoporphyrin IX. We found that HO-1 protected mice from acute liver damage induced by P. acnes/LPS and prolonged survival. On the contrary, administration of the HO-1 inhibitor zinc protoporphyrin IX increased liver damage induced by P. acnes/LPS. Subsequently, to investigate the underlying mechanisms of HO-1 in the acute liver injury model, we primed mice with P. acnes only. We found that the expression of HO-1 mRNA and protein in dendritic cells (DCs) was increased after the administration of cobalt protoporphyrin IX. HO-1 decreased the mature markers major histocompatibility complex II and CD80 on liver DCs. The expression of CCR7, CCL2, and CCL22 mRNA, which are expressed by mature DCs, was also reduced. These liver DCs could not efficiently stimulate CD4+ T cell activation and proliferation. Consequently, HO-1 inhibited the activation, proliferation, and T helper 1 polarization of liver-infiltrating CD4+ T cells and reduced the production of serum alanine aminotransferase and proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α. Taken together, our data suggest that HO-1 alleviates P. acnes/LPS-induced fulminant hepatic failure, probably by inhibiting DC-induced adaptive responses.

Introduction

Fulminant hepatic failure (FHF) is a clinical complication characterized by sudden and severe impairment of liver function. Mice with acute and massive liver injury induced by heat-killed Propionibacterium acnes and LPS comprise a commonly used animal model of FHF. This model can be pathophysiological classified into two different phases: a priming phase with the injection of P. acnes resulting in mononuclear cell (MNC) infiltration into liver and granuloma formation and an eliciting phase induced by LPS, with increased inflammatory infiltrates, leading to massive liver damage (Yoneyama et al., 1998). We and others have reported that peripheral blood dendritic cell (DC) precursors migrate to liver and mature into functional DCs (Yoneyama et al., 2001). Matured DCs can activate P. acnes-specific CD4+ T cells, leading to Th1 polarization. These events are essential to complete the priming phase and develop severe hepatocyte damage after the injection of LPS (Yoneyama et al., 1998) and indicate that DC-induced adaptive responses are important in the pathogenesis of FHF caused by P. acnes/LPS. CTLA-4Ig can increase indoleamine 2,3-dioxygenase expression in DCs, an event involved in alleviating FHF, suggesting that DCs may present a useful target for immune intervention (Nakayama et al., 2005).

ABBREVIATIONS: FHF, fulminant hepatic failure; ALT, alanine aminotransferase; BM, bone marrow; BrdU, 5-bromo-2′-deoxyuridine; CO, carbon monoxide; CoPPIX, cobalt protoporphyrin IX; CIITA, major histocompatibility complex class II transcription activator; CT, cycle threshold; DC, dendritic cell; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HO, heme oxygenase; IFN-1, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; MACS, magnetic cell sorting; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MNC, mononuclear cell; NO, nitric oxide; NOS, NO synthase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; STAT-1, signal transducer and activator of transcription 1; Th1, T helper 1; TNF-α, tumor necrosis factor-α; Treg, regulatory T cell; ZnPPIX, zinc protoporphyrin IX.
Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of heme to biliverdin, carbon monoxide (CO), and free iron (Maines et al., 1986; Maines, 1988). Three isoforms of HO (HO-1, HO-2, and HO-3) have been identified. HO-1 is a stress-response enzyme whose expression is induced by a variety of stimuli including oxidative stress, hypoxia, and proinflammatory cytokines (Applegate et al., 1991; Otterbein and Choi, 2000). HO-1 has been shown to display potent antioxidant, anti-inflammatory, and antiviral functions (Lee et al., 2003; Kruger et al., 2006; Benaloua et al., 2007; Protzer et al., 2007; Tsuchihashi et al., 2007). Induction of HO-1 by metal protoporphyrins or gene transfer can exert beneficial effects in a variety of conditions such as liver injury, diabetes, and cerebral malaria (Sass et al., 2004; Kruger et al., 2006; Benaloua et al., 2007; Pampolina et al., 2007). The mechanisms of action of HO-1 are diverse. HO-1 can function as a suppressor of TNF-α signaling in TNF-α-mediated airway inflammation (Lee et al., 2009). In recent years, the immunomodulatory capacity of HO-1 has received more attention. It has been reported that HO-1 can induce graft tolerance by increasing CD4+ CD25+ regulatory T cells (Tregs) or promoting activation-induced cell death of T cells (McDaid et al., 2005; Xia et al., 2006; Lee et al., 2007). HO-1 in DCs also contributes to the function of Tregs (George et al., 2008). In a model of multiple sclerosis, HO-1 inhibits T cell proliferation through inhibiting MHCII expression by DCs (Chora et al., 2007). Although much evidence has proven the immunomodulatory capacity of HO-1, the mechanisms underlying it have not been clearly elucidated.

In the present study, we focus on the immunomodulatory function of HO-1 in adaptive responses to study the mechanism of HO-1 by using a model of FHF. We show that induction of HO-1 by cobalt protoporphyrin IX (CoPPIX) protects mice from P. acnes/LPS-induced liver injury and prolongs survival. The suppression of liver injury is associated with decreased liver-infiltrating CD4+ T cells. HO-1 induction inhibits the maturation of DCs and subsequent suppression of activation, proliferation, and Th1 polarization of CD4+ T cells, all of which contribute to the alleviation of P. acnes/LPS-induced FHF.

Materials and Methods

Mice. Female C57BL/6 (7–9 weeks old) mice and BALB/c mice were purchased from the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences and maintained under pathogen-free conditions. All animal experiments complied with the protocols approved by the Laboratory Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine, Shanghai, China (20090006).

Reagents. Heat-killed P. acnes was obtained from the American Type Culture Collection (Manassas, VA). LPS derived from Escherichia coli 055:B4 was purchased from Sigma (St. Louis, MO). CoPPIX [cobalt 7,12-diethyl-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-diopropanoic acid] and zinc protoporphyrin IX (ZnPPIX) [8,13-bis(vinyl)-5,7,12,17-tetramethyl-21H,23H-porphine-2,18-dipropionic acid zincII] (Frontier Scientific Inc., Logan, UT) were dissolved in 0.2 N NaOH, neutralized with 0.2 N HCl, diluted to 1 mg/ml with PBS, and sterilized by filtration.

Liver Injury Induction and In Vivo Treatment. C57BL/6 mice were injected with P. acnes (1 mg/100 µl in PBS) via the tail vein. Seven days later, they were given an intravenous injection of LPS (1 µg/100 µl in PBS). PBS, CoPPIX, or ZnPPIX was administered intraperitoneally on days 0, 1, and 3 after P. acnes priming. For the in vivo proliferation assay, mice were given an intraperitoneal injection of 1 mg of BrdU (BD Pharmingen, San Diego, CA). Twenty four hours later, the mice were killed. Nuclear BrdU was detected by using the FITC-labeled anti-BrdU Flow Kit according to the manufacturer’s instructions (BD Pharmingen).

Histology. Liver tissues were fixed in 10% neutral buffered formalin and paraffin-embedded. Deparaffinized sections were stained with hematoxylin and eosin and analyzed by light microscopy.

Measurement of Serum Alanine Aminotransferase. Serum ALT levels were measured with a Reflotron GPT (Roche Diagnostics, Basel, Switzerland).

MNC Isolation. Livers were excised, minced, and passed through a nylon mesh screen. After two washes in PBS, the liver cells were suspended in PBS and added to a 33% Percoll (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) gradient. After centrifugation at room temperature, the pellet was recovered, treated with red blood cell lysis solution, washed twice, and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS).

DC Isolation. DCs were isolated as described previously (De Creus et al., 2005) with minor modifications. In brief, mice were injected with collagenase solution (1 mg/ml in PBS; Sigma) through the portal vein at 37°C for 5 min at a flow rate of 4 ml/min. The liver then was excised and incubated in collagenase solution containing 20 µg/ml DNase (Sigma) at 37°C for 30 min with constant stirring. Cell suspensions were layered onto 16.8% Nycodenz (Sigma) and centrifuged. The cells were then collected and incubated with anti-CD4+ anti-CD11c+ cells were then positively selected by using MACS (Miltenyi Biotec Inc., Auburn, CA). These cells were further sorted by using a FACSaria instrument (BD Biosciences, San Jose, CA).

Bone Marrow-Derived DC Preparation. Mature BMDCs were prepared as described previously (Zhang et al., 1998). In brief, c-Kit hematopoietic cells were isolated by FACS from C57BL/6 BM and cultured for 6 days in RPMI 1640 medium containing 10% FBS, stem cell factor (10 ng/ml), granulocyte macrophage– colony-stimulating factor (25 ng/ml), and IL-4 (10 ng/ml). CD11c+ cells were magnetically sorted from this day 6 culture by using MACS and stimulated with granulocyte macrophage– colony-stimulating factor (10 ng/ml) plus LPS (2 µg/ml) for an additional 2 to 3 days to induce mature BMDCs. BMDCs were then treated with 50 µM CoPPIX for 6 h.

CD4+ T Cell Isolation. CD4+ T cells were isolated from the spleen of BALB/c mice by using MACS according to the manufacturer’s instructions (Miltenyi Biotec Inc.).

FACS Analysis. All labeling antibodies are from BD Pharmingen unless otherwise indicated. Cells were incubated with anti-CD16/32 antibody to block Fc receptor, then labeled with a panel of fluorescently conjugated antibodies: anti-mouse CD4–FITC (RM4-5), CD4–PE (RM4-5), CD80–FITC (16-10A1), C86–FITC (GL1), I-A–PE (AF6-120.1), CD62L–FITC (MEL-14), CD44–allophycocyanin (IM7), B220–FITC (RA3–6B2), B220–PE (RA3–6B2), CD11c–Biotin (HL3), TNF-α–FITC (MP6-XT22), and IFN-γ–PE (XM1.G2). For biotin-conjugated antibodies, incubation with allophycocyanin-conjugated streptavidin was then performed. The labeled cells were analyzed by using a FACSaria instrument (BD Biosciences).

Western Blot Analysis. Protein extracts were resolved on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, blocked with 5% nonfat milk, and probed with antibodies specific for HO-1 (AKELA Pharma Inc., Montreal, QC, Canada), STAT-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), phosphorilated STAT-1 (Cell Signaling Technology, Danvers, MA), and β-actin (Sigma). Signals were detected with horseradish peroxidase-labeled secondary antibodies by using chemiluminescence labeling.

HO-1 Activity Assay. HO-1 activity in mouse liver was quantified by evaluating bilirubin generation as described previously (Taaile et al., 2001). In brief, livers from naive mice were first rinsed in ice-cold 1.15% KCl-20 mM Tris-HCl buffer, pH 7.4. After centrifugation, the supernatant (source of biliverdin reductase) was incubated at 37°C for 30 min, in a reaction mixture containing hemin, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase,
and liver protein extracts. Bilirubin generation was measured spectrophotometrically and expressed as picomoles of bilirubin per milligram of protein per hour.

Real-Time PCR. Total RNA was extracted by using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. β-Actin was used as control. Real-time PCR were performed by using a SYBR green PCR mix and conducted with the ABI Prism 7900HT system (Applied Biosystems, Foster City, CA). Thermocycler conditions included an initial holding at 50°C for 2 min, then 95°C for 10 min; this was followed by a two-step PCR program: 95°C for 15 s and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). The β-actin gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of β-actin. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (CT; cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔCT) between the CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control (β-actin). Relative expression was calculated as the difference (ΔΔCT) between the ΔCT values of the test sample and the control sample. Relative expression of genes of interest was calculated and expressed as $2^{-ΔΔCT}$. The primer sequences used are in Table 1.

Mixed Lymphocyte Reaction Assay. MLR was performed by the addition of B220$^+$ CD11c$^+$ DCs (3 × 10$^5$ to 3 × 10$^6$ cells/well) to allogeneic CD4$^+$ T cells (2 × 10$^5$ cells/well) in RPMI 1640 medium supplemented with 10% FBS in 96-well, U-bottom microtest tissue culture plates. Triplicate wells were cultured for each group in MLR. Cultures were pulsed with 1 μCi of [3H]thymidine/well for the last 16 h of culture. Proliferative responses in each independent experiment were determined by averaging the cpm of each triplicate assay. The cytokines in the supernatant were measured by a cytometric bead array (BD Pharmingen) according to the manufacturer’s instructions.

Statistical Analysis. All statistical analyses were performed by using analysis of variance for multiple comparisons. p < 0.05 was considered significant in all tests.

Results

HO-1 Alleviates the Mortality and Severity of Acute Liver Damage. The mouse model of acute liver damage was induced with P. acnes and LPS. We first investigated whether pharmacologic induction of HO-1 using CoPPIX, a protoporphyrin that induces HO-1 expression and activity, could influence the development of acute liver injury induced by P. acnes and LPS. C57BL/6 mice were injected intraperitoneally with PBS, CoPPIX, or ZnPPIX, an inhibitor of HO enzymatic activity, on days 0, 1, and 3 after P. acnes priming.

Fig. 1. Protective effect of CoPPIX on lethal liver injury induced by P. acnes and LPS. A, cumulative survival rate of mice after LPS injection in P. acnes-primed mice is shown. C57BL/6 mice were injected intraperitoneally with PBS, CoPPIX, or ZnPPIX on days 0, 1, and 3 after P. acnes priming. On day 7, LPS was injected. B, serum ALT levels after LPS injection in P. acnes-primed mice treated with PBS, CoPPIX, or ZnPPIX are shown. Results are shown as means ± S.E.M. (n = 8 per group). *, p < 0.05; **, p < 0.01. C, histological analysis of hepatic lesion. Sections of livers were obtained from naive mice or mice 7 days after P. acnes priming with or without LPS injection. PBS, CoPPIX, or ZnPPIX was injected as described under Materials and Methods. Original magnification, 200×.
On day 7, LPS was injected to induce lethal liver injury. When mice were treated with PBS or ZnPPIX, more than 50% of P. acnes-primed mice (n = 10) died within 6 h in response to subsequent LPS injection, and more than 70% of the mice died within 12 h (Fig. 1A). In contrast, intraperitoneal injection with CoPPIX significantly improved the survival rate. All mice (n = 10) survived for 24 h (Fig. 1A), and no mice died in the subsequent time observed (data not shown). Moreover, at 6 h after subsequent LPS challenge, multilobular hepatic injury was observed in livers from P. acnes-primed mice treated with PBS or ZnPPIX, accompanied by a high level of ALT (Fig. 1, B and C). Treatment with CoPPIX caused a marked reduction in liver injury and serum ALT level (Fig. 1, B and C) compared with those treated with PBS or ZnPPIX. Treatment with ZnPPIX increased serum ALT level compared with the PBS-treated group (Fig. 1B). HO-1 level in the livers was confirmed by real-time PCR and Western blot 7 days after CoPPIX treatment (CoPPIX, 12 ± 2%; PBS, 35 ± 1%; ZnPPIX, 37 ± 3%; p < 0.01) (Fig. 2B). The level of CD44 expression was also significantly increased in the livers of CoPPIX-treated mice compared with those from PBS- or ZnPPIX-treated groups (Fig. 2C). All of these results indicate that HO-1 provides a protective effect for mice with liver injury caused by P. acnes and LPS. It is noteworthy that compared with the PBS-treated group, ZnPPIX treatment increased the expression of HO-1 mRNA on the one hand and inhibited HO-1 activity on the other hand (Fig. 2, A and C). These results are consistent with previous reports that ZnPPIX can induce HO-1 expression while inhibiting its activity (Maines and Trakshel, 1992; Rodgers et al., 1996).

**HO-1 Inhibits the Infiltration of CD4+ T Cells in the Livers of P. acnes-Primed Mice.** It has been reported that P. acnes-induced liver injury is a Th1-polarized response and the infiltration of CD4+ T cells into the liver is the main response of liver injury (Yoneyama et al., 2001; Nakayama et al., 2005). We found that 7 days after P. acnes priming, significant numbers of intrahepatic MNCs were detected in mice. CoPPIX treatment sharply decreased the total number of liver MNCs as well as the absolute number of CD4+ T cells compared with the PBS- or ZnPPIX-treated groups. ZnPPIX treatment increased the absolute number of CD4+ T cells compared with the PBS-treated group (Fig. 3A). In addition, 7 days after P. acnes priming, the concentration of Th1 cytokines (TNF-α, IFN-γ) in serum increased sharply, whereas levels of the Th2 cytokine IL-4 in serum showed no significant difference. CoPPIX reduced the concentration of TNF-α and IFN-γ in serum significantly with no influence on IL-4 production (Fig. 3B). Compared with the PBS-treated group, ZnPPIX treatment increased the concentration of IFN-γ significantly with no influence on TNF-α and IL-4 production (Fig. 3B). These results indicate that HO-1 alleviates P. acnes-induced liver injury through the inhibition of CD4+ T cell infiltration and Th1 cytokine production.

**HO-1 Inhibits the Activation and Proliferation of CD4+ T Cells in the Livers of P. acnes-Primed Mice.** To further analyze the mechanism of reduced CD4+ T cell infiltration in the liver after HO-1 induction, we examined the effect of HO-1 on CD4+ T cell activation and proliferation. Liver infiltrating MNCs were isolated 7 days after P. acnes priming. Flow cytometric analysis showed that CoPPIX treatment reduced the level of CD62L expression on CD4+ T cells in the liver compared with those treated with PBS or ZnPPIX (CoPPIX, 20 ± 2%; PBS, 35 ± 1%; ZnPPIX, 37 ± 3%; p < 0.01) (Fig. 4A). The level of CD44 expression was also reduced in CoPPIX-treated mice compared with those treated with PBS or ZnPPIX (CoPPIX, 19 ± 2%; PBS, 47 ± 3%; ZnPPIX, 42 ± 3%; p < 0.01) (Fig. 4A). BrdU staining showed that the percentage of CD4+ T cells undergoing cell cycle progression was decreased sharply in CoPPIX-treated mice compared with those treated with PBS or ZnPPIX (CoPPIX, 5 ± 1%; PBS, 12 ± 1%; ZnPPIX, 15 ± 1%; p < 0.01) (Fig. 4B). Moreover, CoPPIX treatment reduced the percentage of CD4+ T cells expressing intracellular IFN-γ (CoPPIX, 5 ± 1%; PBS, 8 ± 1%; ZnPPIX, 11 ± 1%; p < 0.01), whereas there was almost no effect on the intracellular expression of TNF-α after CoPPIX treatment (CoPPIX, 12 ± 1%; PBS, 14 ± 1%; ZnPPIX, 13 ± 1%; p > 0.05) (Fig. 4C). These results collectively indicate that HO-1 inhibits the activation and proliferation of CD4+ T cells.

**HO-1 Reduces the Potency of DC to Stimulate CD4+ T Cell Activation and Proliferation in the Livers of P. acnes-Primed Mice.** We have reported previously that the recruitment of B220−CD11c+ DCs from blood is important for ongoing liver injury in this P. acnes-induced liver injury. Materials and Methods.
model (Yoneyama et al., 2001). These DCs can activate *P. acnes*-specific CD4⁺ T cells, leading to Th1 polarization. To analyze the mechanism of inhibited activation and proliferation of CD4⁺ T cells after HO-1 induction, we then examined the effect of HO-1 on the recruitment of DCs from blood. As we found previously, B220⁻CD11c⁺ DCs increased markedly at day 7 after *P. acnes* priming. The absolute number of liver B220⁻CD11c⁺ DCs showed no significant difference among PBS-, CoPPIX-, or ZnPPIX-treated groups (Fig. 5A), suggesting that HO-1 has no significant effect on the recruitment of B220⁻CD11c⁺ DCs from blood. We next examined the potency of DCs to stimulate allogeneic T cells in the MLR in vitro. In allogeneic MLR, DCs from CoPPIX-treated mice showed reduced potency to stimulate T cell proliferation compared with DCs from PBS- or ZnPPIX-treated mice (Fig. 5B). Cytokine analysis of the T cell culture stimulated with DCs showed that the levels of IL-2, TNF-α, and IFN-γ were decreased in CoPPIX-treated mice compared with those in PBS-treated mice. However, the level of IL-4 showed no difference among the three groups. ZnPPIX treatment increased the level of IFN-γ, whereas it had no significant effects on the levels of IL-2, TNF-α, and IL-4 compared with the PBS-treated group (Fig. 5C). Further investigation showed that CoPPIX injection increased the expression of HO-1 mRNA and protein in DCs compared with PBS- or ZnPPIX-treated groups (Fig. 5, D and E). These results indicate that HO-1 reduces the potency of DCs to stimulate the activation, proliferation, and Th1 polarization of CD4⁺ T cells.

**Maturation of DCs Is Inhibited by CoPPIX Treatment in the Livers of *P. acnes*-Primed Mice.** To understand the mechanism of inhibited potency of DCs to stimulate CD4⁺ T cells after HO-1 induction, we investigated the characteristics of liver DCs on day 7 after *P. acnes* priming. Flow cytometry showed that CoPPIX treatment significantly reduced the level of MHCII expression on liver DCs compared with the PBS-treated groups. Although CoPPIX treatment did not show a significant effect on the expression of MHCII, CD80, and CD86 compared with the PBS-treated group (Fig. 6A). During maturation DCs inhabit different microenvironments of chemokines and chemokine receptors. Immature DCs express CCR1, CCR2, CCR5, and CXCR1, whereas mature DCs express high levels of CCR7, CCL22, CCL5, CCL2, and CXCL10 (Sallusto et al., 1998). To further investigate the effects of HO-1 on the maturation of DCs, we next examined chemokine and chemokine receptor expression in liver DCs. In CoPPIX-treated mice, CCR7 was markedly downregulated in liver DCs compared with the PBS-treated groups (Fig. 6B), whereas CCR1 and CCR5 showed no sig-
HO-1 has beneficial effects in tissue injury. However, the mechanism of function of HO-1 remains unknown. In the present study, we provide experimental evidence that pharmacologically induced HO-1 can protect mice from lethal liver injury in a model of FHF induced by \textit{P. acnes}/LPS. We found that HO-1 induced in vivo by CoPPIX inhibited the infiltration of CD4$^+$ T cells into the liver after \textit{P. acnes} priming. Moreover, the activation and proliferation of liver-infiltrating CD4$^+$ T cells were also impaired as indicated by the down-regulation of expression of activation markers CD62L and CD44 and BrdU incorporation. Further investigation showed that HO-1 decreased the mature markers MHCII and CD80 on liver DCs. These DCs cannot efficiently stimulate T cell proliferation in vitro. These data suggest that HO-1 alleviates \textit{P. acnes}/LPS-induced FHF, probably by inhibiting DC-induced adaptive responses.

It has been reported that HO-1 can be induced by pharmacologic means or gene transfer. In this study, after treatment with CoPPIX, a potent inducer of HO-1 (Smith et al., 1993; Shan et al., 2006), the expression of HO-1 was increased in the liver in a model of FHF caused by \textit{P. acnes}/LPS. HO-1 protected mice from acute liver damage and prolonged survival. On the other hand, treatment with ZnPPIX, an inhibitor of HO-1 activity (Drummond and Kappas, 1981; Roach et al., 2009), failed to protect mice from liver damage. ZnPPIX can also induce HO-1 expression. The accumulation of heme from inhibition of HO-1 activity may result in increased HO-1 expression because heme is a strong inducer of HO-1. Previous evidence indicates that liver-infiltrating CD4$^+$ T cells are critical in the priming phase of this liver injury model. Our results demonstrate that induction of HO-1 by CoPPIX sharply reduces the number of CD4$^+$ T cells in the liver. Activated CD4$^+$ T cells can produce high levels of TNF-α and IFN-γ in the liver. Th1 polarization in the liver is essential to complete the priming process and develop subsequent severe liver injury. In this study, HO-1 suppressed the expression of activation markers CD62L and CD44. Moreover, the remaining CD4$^+$ T cells showed inhibited BrdU incorporation and lower IFN-γ expression, suggesting that the activation, proliferation, and Th1 polarization of liver-infiltrating CD4$^+$ T cells were suppressed. These data indicate that HO-1 can suppress immune responses and alleviate liver injury through the inhibition of activation, proliferation, and Th1 polarization of CD4$^+$ T cells. CD4$^+$/CD25$^+$ Tregs are regarded as an important mediator of immunosuppression. It has been reported that HO-1 leads to a significant increase in the percentage of CD4$^+$/CD25$^+$ Tregs (Xia et al., 2006). However, our results show that HO-1 does not increase the number of Tregs (data not shown), indicating that inhibited activation, proliferation, and Th1 polarization of CD4$^+$ T cells is not achieved by increasing Tregs. These results demonstrate that HO-1 inhibits activation, proliferation, and Th1 polarization of CD4$^+$ T cells through other mediators. CO is one of the end products of heme degradation and confers the protective effects of HO-1 (Pae et al., 2004). Another gas, nitric oxide (NO), plays a crucial role in the maintenance of liver function. Some studies reported that NO can inhibit the production of proinflammatory cytokine and prolonged animal survival, whereas others showed that increased NO can potentiate liver injury. NO can be produced by NO synthase (NOS). NO/NOS and CO/HO-1 are closely linked. It has been reported that induction of HO-1 is likely to modulate NO production (Maines 1997). However, we did not find significant change in NO production after CoPPIX treatment (data not shown), which suggested that NO may

**Discussion**

HO-1 has beneficial effects in tissue injury. However, the mechanism of function of HO-1 remains unknown. In the present study, we provide experimental evidence that pharmacologically induced HO-1 can protect mice from lethal liver injury in a model of FHF induced by \textit{P. acnes}/LPS. We found that HO-1 induced in vivo by CoPPIX inhibited the infiltration of CD4$^+$ T cells into the liver after \textit{P. acnes} priming. Moreover, the activation and proliferation of liver-infiltrating CD4$^+$ T cells were also impaired as indicated by the down-regulation of expression of activation markers CD62L and CD44 and BrdU incorporation. Further investigation showed that HO-1 decreased the mature markers MHCII and CD80 on liver DCs. These DCs cannot efficiently stimulate T cell proliferation in vitro. These data suggest that HO-1 alleviates \textit{P. acnes}/LPS-induced FHF, probably by inhibiting DC-induced adaptive responses.

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**Fig. 4.** HO-1 inhibits the activation and proliferation of CD4$^+$ T cells after \textit{P. acnes} priming. A, the expression of CD62L and CD44 on CD4$^+$ T cells in the livers of \textit{P. acnes}-primed mice treated with PBS, CoPPIX, or ZnPPIX was analyzed by flow cytometry. B, mice were injected intraperitoneally with BrdU as described under Materials and Methods. The percentage of CD4$^+$ T cells expressing intracellular BrdU was analyzed by flow cytometry. C, the percentage of CD4$^+$ T cells expressing intracellular IFN-γ and TNF-α was analyzed by flow cytometry.
It has been reported that exogenous CO required the inducible NOS induction/NO production and activation of HO-1 expression, which suggested that the protective effects of CO or NO depend on HO-1. However, induction of HO-1 was able to significantly prevent liver injury in inos/ mice (Zuckerbraun et al., 2003). These findings indicated that the protective effects of HO-1 are independent of inducible NOS/NO activity.

DCs play important roles in inflammation and immune responses (Banchereau et al., 2000). We have previously found that DC-induced adaptive response participates in P. acnes/LPS-induced lethal liver injury (Yoneyama et al., 2001). In the priming phase of P. acnes/LPS-induced FHF, DC-T cells cluster in the liver, in which CD4⁺ T cells can further proliferate and complete polarization. The recruitment of DCs in the liver is an initial event and is a prerequisite for liver injury in this model. In the present study, the number of B220⁺CD11c⁺ DCs in the liver showed no difference in CoPPIX-treated mice compared with control groups, indicating that HO-1 does not affect the recruitment of DCs in liver. However, liver DCs showed reduced potency as stimulators of CD4⁺ T cell activation and proliferation after CoPPIX treatment, as indicated by MLR. The levels of TNF-α, IFN-γ, and IL-2 in the supernatant were decreased in CoPPIX-treated mice compared with control groups. These results suggest that HO-1 inhibits the DC-induced adaptive response, which in turn limits Th1 polarization and disease progression.

The potency of DCs in the stimulation of T cells is related to the maturation of DCs, and it has been reported that HO-1 inhibits DC maturation and proinflammatory and allogeneic immune responses in vitro (Chauveau et al., 2005). In this study, HO-1 significantly reduced the expression of MHCII on B220⁺CD11c⁺ DCs. The expression of CD80 on DCs was also decreased, although moderately. These data clearly
demonstrate that HO-1 suppresses the maturation of DCs in P. acnes/LPS-treated mice. During maturation, DCs undergo a switch in expression of chemokine receptors and secrete different chemokines. Further investigation showed that HO-1 suppressed the expression of CCR7, CCL2, and CCL22 in DCs. CXCL10 and CCL5 also showed a tendency to decrease. These findings lend further credence to the notion that HO-1 inhibits phenotypic and functional maturation of DCs in liver.

Although HO-1 contributed to inhibition of DC maturation both in vitro and in vivo (Martins et al., 2005; Kotsch et al., 2007), the mechanism underlying this inhibition remains undetermined. Previous data have shown that decreased levels of reactive oxygen species could be involved in the inhibition of DC maturation (Chauveau et al., 2005), whereas others reported that suppressed STAT-1 phosphorylation and CIITA expression contribute to the protective effect of HO-1 (Chora et al., 2007). It is well known that CIITA and STAT-1 phosphorylation are critical for MHCII expression in DCs. In our study, we observed the inhibition of CIITA expression and STAT-1 phosphorylation in DCs after CoPPIX treatment, providing further evidence that HO-1 exerts an inhibitory effect on the maturation of DCs. Other research has shown that phosphorylation of inhibitor of nuclear factor-kB is inhibited when HO-1 is induced and HO-1 acts in DCs through downstream mechanisms that could include STAT-3 (Mashreghi et al., 2008; Rémy et al., 2009). In our study, there is a significant increase in the expression of STAT-3 in the liver after CoPPIX injection. However, the expression of STAT-3 and SCOS-3 (a surrogate marker for STAT-3 activation) in DCs shows no difference in CoPPIX-treated mice compared with control groups (data not shown). These results suggest that HO-1 may act in DCs through multiple pathways, elucidation of which will require further investigation.

In conclusion, the present study provides convincing evidence to support existence of an immunomodulation function of HO-1 during the pathogenesis of fulminant hepatitis in a mouse model induced by P. acnes and LPS. The immunomodulation mechanism of HO-1 most likely functions through down-regulating DC matu-
ration, inhibiting activation, proliferation, and Th1 polarization of liver-infiltrating CD4+ T cells, and reducing the production of serum ALT and proinflammatory cytokines.

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Authorship Contributions

Participated in research design: Gu and Yanyun Zhang.
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