

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

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## Running title page

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HO, heme oxygenase; MLR, mixed lymphocyte reaction; MNC, mononuclear cell; NO, nitric oxide; NOS, nitric oxide synthase; *P. acnes*, *Propionibacterium acnes*; Treg, regulatory T cell; ZnPP, zinc protoporphyrin;

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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* (*P. acnes*) and LPS. The expression of HO-1 mRNA and of protein in the liver was increased after repeated administrations of 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, administrations of 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 of protein in dendritic cells (DCs) was increased after administration of cobalt protoporphyrin IX. HO-1 decreased the mature markers MHCII 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<sup>+</sup> T cell activation and proliferation. Consequently, HO-1 inhibited the activation, proliferation, and Th1 polarization of liver infiltrating CD4<sup>+</sup> T cells and reduced the production of serum alanine aminotransferase and pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$ . 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* (*P. acnes*) and LPS comprise a commonly used animal model of FHF. This model can be pathophysiologically 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<sup>+</sup> T cells, leading to Th1 polarization. These events are essential to complete the priming phase and to develop severe hepatocyte damage after 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).

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 pro-inflammatory cytokines (Applegate et al., 1991; Otterbein and

Choi, 2000). HO-1 has been shown to display potent anti-oxidant, anti-inflammatory and anti-viral functions (Lee et al., 2003; Kruger et al., 2006; Benallaoua 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; Benallaoua et al., 2007; Pamplona et al., 2007). The mechanisms of action of HO-1 are diverse. HO-1 can function as a suppressor of TNF- $\alpha$  signaling in TNF- $\alpha$ -mediated airway inflammation (Lee et al., 2009). In recent years, the immunomodulatory capacity of HO-1 in adaptive responses has received more attention. It has been reported that HO-1 can induce graft tolerance by increasing CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) or by 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 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<sup>+</sup> T cells. HO-1 induction inhibits the maturation of DCs and subsequent suppression of activation, proliferation and Th1

polarization of CD4<sup>+</sup> 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, 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 (2009006).

### Reagents.

Heat-killed *P. acnes* was obtained from American Type Culture Collection [ATCC] (Manassas, VA). LPS derived from *Escherichia coli* 055:B4 was purchased from Sigma-Aldrich (St. Louis, MO). CoPPIX [Cobalt 7,12-diethenyl-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-dipropionic acid] and zinc protoporphyrin IX (ZnPPIX) [8,13-Bis(vinyl)-3,7,12,17-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, 3 after *P. acnes* priming. For the in vivo proliferation assay, mice were given an intraperitoneal injection of 1 mg BrdU (BD Pharmingen, San Diego, CA). 24 h later, mice were killed.



NuclearBrdU was detected 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 were paraffin embedded. Deparaffinized sections were stained with hematoxylin and eosin and analyzed by light microscopy.

### **Measurement of Serum Alanine Aminotransferase (ALT).**

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. Following two washes in PBS, the liver cells were resuspended in PBS and added to a 33% Percoll (GE Healthcare, Chalfont, Bucks, 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 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  $\mu\text{g/ml}$  DNase (Sigma) at 37°C for 30 min with constant stirring. Cell suspensions were layered onto 16.8% Nycodenz (Sigma). After centrifugation, the interface was collected. CD11c<sup>+</sup> cells were then positively selected using MACS (Miltenyi Biotec, Germany). These cells were further sorted using a FACS Aria instrument (Becton Dickinson, CA).

#### **Bone Marrow (BM)-Derived DC Preparation.**

Mature BMDCs were prepared, as previously described (Zhang et al., 1998). Briefly, c-Kit<sup>+</sup> hematopoietic cells were isolated by FACS from C57BL/6 BM and cultured for 6 days in RPMI 1640 containing 10% FBS, stem cell factor (10 ng/ml), GM-CSF (25 ng/ml), and IL-4 (10 ng/ml). CD11c<sup>+</sup> cells were magnetically sorted from this 6-day culture using MACS and stimulated with GM-CSF (10 ng/ml) plus LPS (2  $\mu\text{g/ml}$ ) for an additional 2-3 days to induce mature BMDCs. BMDCs were then treated with 50  $\mu\text{M}$  CoPPIX for 6 h.

#### **CD4<sup>+</sup> T Cell Isolation.**

CD4<sup>+</sup> T cells were isolated from the spleen of BALB/c mice using MACS according to the manufacturer's instructions.

#### **FACS Analysis.**

All labeling antibodies are from BD Pharmingen unless otherwise indicated. Cells were incubated with anti-CD16/32 Ab 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<sup>b</sup>-PE (AF6-120.1), CD62L-FITC (MEL-14), CD44-APC (IM7), B220-FITC (RA3-6B2), B220-PE (RA3-6B2) CD11c-Biotin (HL3), TNF- $\alpha$ -FITC (MP6-XT22), IFN- $\gamma$ -PE (XMG1.2). For Biotin-conjugated antibodies, incubation with APC-conjugated streptavidin was then performed. The labeled cells were analyzed using a FACS Aria instrument (Becton Dickinson).

### **Western Blot Analysis.**

Protein extracts were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane, blocked with 5% nonfat milk, and probed with antibodies specific for HO-1 (Stressgene Biotechnologies, Victoria, Canada), STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated STAT-1 (Cellular Signaling Technology, Beverly, MA), and  $\beta$ -actin (Sigma). Signals were detected with horseradish peroxidase-labeled secondary antibodies using chemiluminescence labeling.

### **HO-1 Activity Assay.**

HO-1 activity in mouse liver was quantified by evaluating bilirubin generation as previously described (Taille et al., 2001). In brief, livers from naïve 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, G6P, G6PDH, 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 using TRIzol (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions.  $\beta$ -actin was used as control. Real time PCR were performed using a SYBR green PCR mix and conducted with the ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). 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  $\beta$ -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  $\beta$ -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 ( $\Delta$ CT) between the CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control ( $\beta$ -actin). Relative expression was calculated as the difference ( $\Delta\Delta$ CT) between the  $\Delta$ CT values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as  $2^{-\Delta\Delta$ CT}. The primer sequences were as follows (Table 1).

### **Mixed Lymphocyte Reaction (MLR) Assay.**

MLR was performed by addition of B220<sup>+</sup>CD11c<sup>+</sup> DCs ( $3 \times 10^2$  to  $3 \times 10^4$  cells/well) to allogeneic CD4<sup>+</sup> T cells ( $2 \times 10^5$  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  $\mu$ Ci of [ $^3$ H]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 using ANOVA 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. On day 7, LPS was injected to induce lethal liver injury. When mice were treated with PBS or ZnPPiX, over 50% of *P. acnes*-primed mice (n = 10) died within 6 h in response to subsequent LPS injection, and over 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) as compared with those treated with PBS or ZnPPiX. Treatment with ZnPPiX increased serum ALT level as compared with PBS-treated group (Fig. 1B). HO-1 level in the livers was confirmed by real time PCR and Western blot 7 days after *P. acnes* priming. CoPPIX injection increased the expression of HO-1 mRNA and of protein in the liver as compared with PBS or ZnPPiX-treated groups (Fig. 2, A and B). HO-1 activity was also significantly increased in livers of CoPPIX-treated mice as compared with those from PBS or ZnPPiX-treated groups (Fig. 2C). All these results indicate that

HO-1 provides a protective effect for mice with liver injury caused by *P. acnes* and LPS. Interestingly, compared with PBS-treated group, ZnPIX 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 ZnPIX can induce HO-1 expression while inhibiting its activity (Maines et al., 1992; Rodgers et al., 1996).

### **HO-1 Inhibits the Infiltration of CD4<sup>+</sup> T Cells in Livers of *P. acnes*-Primed Mice**

It has been reported that *P. acnes*-induced liver injury is a Th1-polarized response and that the infiltration of CD4<sup>+</sup> 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. CoPIX treatment decreased sharply the total number of liver MNCs as well as the absolute number of CD4<sup>+</sup> T cells as compared with PBS or ZnPIX-treated groups. ZnPIX treatment increased the absolute number of CD4<sup>+</sup> T cells as compared with PBS-treated group (Fig. 3A). In addition, 7 days after *P. acnes* priming, the concentration of Th1 cytokines (TNF- $\alpha$ , IFN- $\gamma$ ) in serum increased sharply, whereas levels of the Th2 cytokine IL-4 in serum showed no significant difference. CoPIX reduced the concentration of TNF- $\alpha$  and IFN- $\gamma$  in serum significantly with no influence on IL-4 production (Fig. 3B). Compared with PBS-treated group, ZnPIX treatment increased the concentration of IFN- $\gamma$  significantly with no influence on TNF- $\alpha$  and IL-4 production (Fig. 3B). These results indicate that HO-1 alleviates *P. acnes*-induced liver injury through inhibition of CD4<sup>+</sup> T cell infiltration and Th1 cytokine production.

## HO-1 Inhibits the Activation and Proliferation of CD4<sup>+</sup> T Cells in Livers of *P. acnes*-Primed Mice

To further analyze the mechanism of reduced CD4<sup>+</sup> T cell infiltration in the liver after HO-1 induction, we examined the effect of HO-1 on CD4<sup>+</sup> 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<sup>+</sup> T cells in the liver as compared with those treated with PBS or ZnPPIX (CoPPIX: 20%  $\pm$  2%; PBS: 33%  $\pm$  1%; ZnPPIX: 37%  $\pm$  3%;  $p < 0.01$ ) (Fig. 4A). The level of CD44 expression was also reduced in CoPPIX-treated mice as compared with those treated with PBS or ZnPPIX (CoPPIX: 19%  $\pm$  2%; PBS: 47%  $\pm$  3%; ZnPPIX: 42%  $\pm$  3%;  $p < 0.01$ ) (Fig. 4A). BrdU staining showed that the percentage of CD4<sup>+</sup> T cells undergoing cell cycle progression was decreased sharply in CoPPIX-treated mice as compared with those treated with PBS or ZnPPIX (CoPPIX: 5%  $\pm$  1%; PBS: 12%  $\pm$  1%; ZnPPIX: 15%  $\pm$  1%;  $p < 0.01$ ) (Fig. 4B). Moreover, CoPPIX treatment reduced the percentage of CD4<sup>+</sup> T cells expressing intracellular IFN- $\gamma$  (CoPPIX: 5%  $\pm$  1%; PBS: 8%  $\pm$  1%; ZnPPIX: 11%  $\pm$  1%;  $p < 0.01$ ), whereas there was almost no effect on the intracellular expression of TNF- $\alpha$  after CoPPIX treatment (CoPPIX: 12%  $\pm$  1%; PBS: 14%  $\pm$  1%; ZnPPIX: 13%  $\pm$  1%;  $p > 0.05$ ) (Fig. 4C). These results collectively indicate that HO-1 inhibits the activation and proliferation of CD4<sup>+</sup> T cells.



## **HO-1 Reduces the Potency of DC to Stimulate CD4<sup>+</sup> T Cell Activation and Proliferation in Livers of *P. acnes*-Primed Mice**

We have reported previously that the recruitment of B220<sup>+</sup>CD11c<sup>+</sup> DCs from blood is important for ongoing liver injury in this *P. acnes*-induced liver injury model (Yoneyama et al., 2001). These DCs can activate *P. acnes*-specific CD4<sup>+</sup> T cells, leading to Th1 polarization. To analyze the mechanism of inhibited activation and proliferation of CD4<sup>+</sup> 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<sup>+</sup>CD11c<sup>+</sup> DCs increased markedly at day 7 after *P. acnes* priming. The absolute number of liver B220<sup>+</sup>CD11c<sup>+</sup> 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<sup>+</sup>CD11c<sup>+</sup> 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 as 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- $\alpha$  and IFN- $\gamma$  were decreased in CoPPIX-treated mice as 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- $\gamma$  while had no significant effects on the level of IL-2, TNF- $\alpha$  and IL-4 as compared with PBS-treated group (Fig. 5C). Further investigation showed that CoPPIX injection increased the expression of HO-1 mRNA and of protein in DCs as 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<sup>+</sup> T cells.

## **Maturation of DCs is Inhibited by CoPPIX Treatment in Livers of *P. acnes*-Primed Mice**

To understand the mechanism of inhibited potency of DCs to stimulate CD4<sup>+</sup> 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 as compared with PBS-treated groups. Although CoPPIX treatment did not show significant effect on the expression of CD86 on DCs, the expression of CD80 was reduced moderately (Fig. 6A). These results indicate that CoPPIX treatment inhibits the maturation of liver DCs. ZnPPIX treatment showed no significant difference on the expression of MHCII, CD80 and CD86 as compared with PBS-treated group (Fig. 6A). During maturation, DCs inhabit different microenvironments of chemokines and chemokine receptors. Immature DCs express CCR1, CCR2, CCR5 and CXCR1, while 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 the chemokine and chemokine receptor expression in liver DCs. In CoPPIX-treated mice, CCR7 was markedly downregulated in liver DCs as compared with PBS-treated groups (Fig. 6B), whereas CCR1 and CCR5 showed no significant difference (data not shown). CoPPIX treatment also reduced the expression of CCL2 and CCL22 as compared with PBS-treated groups (Fig. 6B), while the expression of CXCL10 and CCL5 showed no difference (data not shown). These results indicate that HO-1 inhibits the phenotypic and

functional maturation of DCs, and thus reduces the potency of DCs to stimulate CD4<sup>+</sup> T cells.

MHC classII transcription activator (CIITA) expression and STAT-1 phosphorylation play critical roles in the transcriptional regulation of MHCII expression in DCs (Chang et al., 1996). We examined the mRNA expression of CIITA in liver DCs on day 7 after *P. acnes* priming. The results showed that CoPPIX treatment inhibited the expression of CIITA in DCs (Fig. 6C). We next prepared BMDCs, and cultured with PBS, CoPPIX or ZnPPIX in vitro. CoPPIX treatment significantly increased the expression of HO-1, and inhibited the phosphorylation of STAT-1 in BMDCs (Fig. 6D). These data suggest that HO-1 may suppress MHCII expression through the inhibition of CIITA expression and STAT-1 phosphorylation.

## 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 *P. acnes*/LPS. We found that HO-1 induced in vivo by CoPPiX inhibited the infiltration of CD4<sup>+</sup> T cells into the liver after *P. acnes* priming. Moreover, the activation and proliferation of liver-infiltrating CD4<sup>+</sup> T cells were also impaired as indicated by the down-regulation of expression of activation markers CD62L and CD44 and by 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 *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 by 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 *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<sup>+</sup> 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<sup>+</sup> T cells in the liver. Activated CD4<sup>+</sup> T cells can produce high levels of TNF- $\alpha$  and IFN- $\gamma$  in the liver. Th1 polarization in the liver is essential to complete the priming process and to develop subsequent severe liver injury. In this study, HO-1 suppressed the expression of activation markers CD62L and CD44. Moreover, the remaining CD4<sup>+</sup> T cells showed inhibited BrdU incorporation, and lower IFN- $\gamma$  expression, suggesting that the activation, proliferation and Th1 polarization of liver-infiltrating CD4<sup>+</sup> 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<sup>+</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> Tregs (Xia et al., 2006; Yamashita 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<sup>+</sup> T cells is not achieved by increasing Tregs. These results demonstrate that HO-1 inhibits activation, proliferation and Th1 polarization of CD4<sup>+</sup> T cells through other mediators. CO is one of the endproducts 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, while others showed that increased NO can potentiate liver injury. NO can be produced by nitric oxide 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 didn't find significant change in

NO production after CoPPIX treatment (data not shown), which suggested that NO may not participate in the protective effect of HO-1 in this model. It has been reported that exogenous CO required the iNOS induction/NO production and activation of HO-1 expression, which suggested that the protective effects of CO or NO are dependent on HO-1. However, induction of HO-1 was able to significantly prevent liver injury in *inos<sup>-/-</sup>* mice (Zuckerbraun et al., 2003). These findings indicated that the protective effects of HO-1 are independently of iNOS/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 cluster in the liver, in which CD4<sup>+</sup> 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<sup>+</sup>CD11c<sup>+</sup> DCs in the liver showed no difference in CoPPIX treated mice as 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<sup>+</sup> T cell activation and proliferation after CoPPIX treatment, as indicated by MLR. The levels of TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 in the supernatant were decreased in CoPPIX-treated mice as 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 stimulation of T cells is related to the maturation of DCs, and it has been reported that HO-1 inhibits DC maturation and pro-inflammatory and allogeneic immune responses in vitro. In this study, HO-1 significantly reduced the expression of MHCII on B220<sup>+</sup>CD11c<sup>+</sup> 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 ROS could be involved in the inhibition of DC maturation (Chauveau et al., 2005), while 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. The latest research has shown that phosphorylation of I $\kappa$ B $\alpha$  is inhibited when HO-1 is induced and that HO-1 acts in DCs through downstream

mechanisms that could include STAT-3 (Mashreghi et al., 2008; Remy 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 as 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 downregulating DC maturation, inhibiting activation, proliferation, and Th1 polarization of liver-infiltrating CD4<sup>+</sup> T cells and reducing the production of serum ALT and pro-inflammatory cytokines.



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### **Authorship Contributions**

Participated in research design: Qiaoli Gu, Yanyun Zhang

Conducted experiments: Qiaoli Gu, Qiong Wu, Min Jin, Yichuan Xiao

Contributed new reagents or analytic tools: Jingwei Xu, Chaoming Mao, Fang Zhao, and Yi Zhang

Performed data analysis: Qiaoli Gu, and Min Jin

Wrote or contributed to the writing of the manuscript: Qiaoli Gu, Min Jin, and Yanyun Zhang.

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## Footnotes

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## Figure Legends

Figure 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. 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. Results are shown as means  $\pm$  SEM (n = 8 per group). \* $p$  < 0.05, \*\* $p$  < 0.01. (C) Histological analysis of hepatic lesion. Sections of livers were obtained from naïve mice or mice 7 days after *P. acnes* priming with or without LPS injection. PBS, CoPPIX or ZnPPiX was injected as described in “Materials and Methods”. (Original magnification  $\times$  200).

Figure 2. Induction of HO-1 by CoPPIX in the liver of *P. acnes*-primed mice. C57BL/6 mice were injected intraperitoneally with PBS, CoPPIX or ZnPPiX on days 0, 1, and 3 after *P. acnes* priming. Mice were sacrificed on day 7. (A) Expression of HO-1 mRNA in liver was detected by real time PCR. (B) Expression of HO-1 protein in liver was detected by Western blot. (C) HO-1 activity was assayed as described in “Materials and Methods”. Data in (A) and (C) are shown as means  $\pm$  SEM (n = 8 per group). \* $p$  < 0.05,

\*\* $p$  < 0.01.

Figure 3. HO-1 inhibits the infiltration of CD4<sup>+</sup> T cells in liver after *P. acnes* priming.

(A) Liver MNCs were prepared from naïve mice or *P. acnes*-primed mice treated with PBS, CoPPIX or ZnPPiX. The absolute numbers of CD4<sup>+</sup> T cells were determined by

multiplying the total MNC number by the percentage of CD4<sup>+</sup> population. (B) Concentrations of TNF- $\alpha$ , IFN- $\gamma$ , and IL-4 in serum from naïve mice or *P. acnes*-primed mice treated with PBS, CoPPIX or ZnPPIX were measured using a cytometric bead array kit. Data in (A) and (B) are shown as means  $\pm$  SEM (n = 8-9 per group). \* $p$  < 0.05; \*\* $p$  < 0.01.

Figure 4. HO-1 inhibits the activation and proliferation of CD4<sup>+</sup> T cells after *P. acnes* priming. (A) The expression of CD62L and CD44 on CD4<sup>+</sup> T cells in livers of *P. acnes*-primed mice treated with PBS, CoPPIX or ZnPPIX was analyzed by flow cytometry. (B) Mice were injected intraperitoneally with BrdU as described in “Materials and Methods”. The percentage of CD4<sup>+</sup> T cells expressing intracellular BrdU was analyzed by flow cytometry. (C) Percentage of CD4<sup>+</sup> T cells expressing intracellular IFN- $\gamma$  and TNF- $\alpha$  was analyzed by flow cytometry.

Figure 5. HO-1 induced in DCs inhibits the activation and proliferation of CD4<sup>+</sup> T cells. (A) Mice were treated as described in “Materials and Methods”, and the numbers of B220<sup>+</sup>CD11c<sup>+</sup> DCs in the liver from PBS-, CoPPIX- or ZnPPIX-treated mice 7 days after *P. acnes* priming were analyzed through flow cytometry. Results are expressed as means  $\pm$  SEM (n = 7-8 per group). (B) In allogeneic MLR, B220<sup>+</sup>CD11c<sup>+</sup> DCs isolated from liver of *P. acnes*-primed mice treated with PBS, CoPPIX or ZnPPIX were used as stimulator cells. CD4<sup>+</sup> T cells isolated from the spleen of naïve BABL/C mice were used as effector cells. DCs and T cells were cultured at indicated ratios as described in

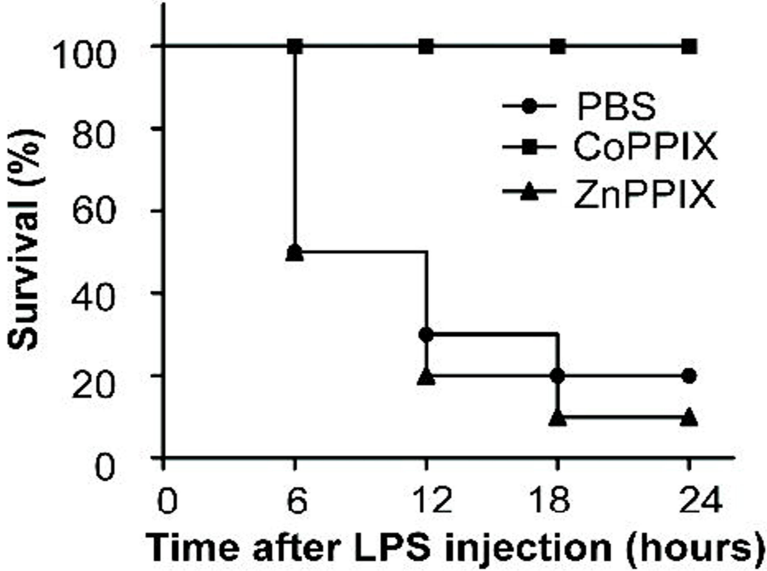
“Materials and Methods”, and the proliferation of T cells was assayed using [<sup>3</sup>H]thymidine. (C) Cytokines in the supernatant were examined using a cytometric bead array according to the manufacturer’s instructions. Results are expressed as means ± SEM (n = 7-8 per group). \**p* < 0.05, \*\**p* < 0.01. (D) HO-1 expression in liver DCs was measured using real time PCR. Results are expressed as means ± SEM (n = 7 per group). \*\**p* < 0.01. (E) Western blot analysis of HO-1 expression in liver DCs.

Figure 6. HO-1 inhibits DC maturation. (A) Flow cytometry analysis of liver B220<sup>+</sup> CD11c<sup>+</sup> DCs in *P. acnes*-primed mice treated with PBS, CoPPIX or ZnPPIX. \*\**p* < 0.01. (B) The mRNA expression of chemokines and chemokine receptors in liver DCs was measured using real time PCR. Results are expressed as means ± SEM (n = 7-9 per group). \*\**p* < 0.01. (C) The mRNA expression of CIITA in liver DCs was measured using real time PCR. Results are expressed as means ± SEM (n = 7-9 per group). \*\**p* < 0.01. (D) BMDCs were prepared as described in “Materials and Methods”. Western blot analysis of HO-1 and STAT-1 phosphorylation in BMDCs treated with PBS, CoPPIX or ZnPPIX.

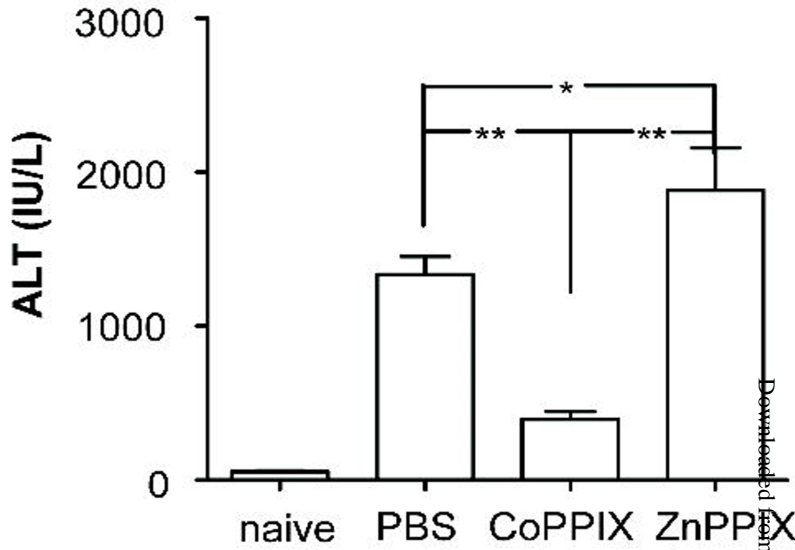
**Table 1. Oligonucleotides used in quantitative real-time PCR**

Gene		Primer (5 to 3 )
CCR7	Forward	CTACAGCGGCCTCCAGAAGA
	Reverse	CCATCTGGGCCACTTGGAT
CCL2	Forward	GTGATGGAGGGGGTCAGGA
	Reverse	GGGATGGGACAGCCTAAACT
CCL22	Forward	AGGTCCCTATGGTGCCAATGT
	Reverse	CGGCAGGATTTTGAGGTCCA
HO-1	Forward	CAGAAGAGGCTAAGACCGCCTT
	Reverse	TCTGGTCTTTGTGTTTCCTCTGTCA
CIITA	Forward	CAGGCTCCCACGGTAGAGA
	Reverse	GGTAGAGATGTAGGGGGTCCG
$\beta$ -actin	Forward	GTATGGAATCCTGTGGCATC
	Reverse	AAGCACTTGCGGTGCACGAT

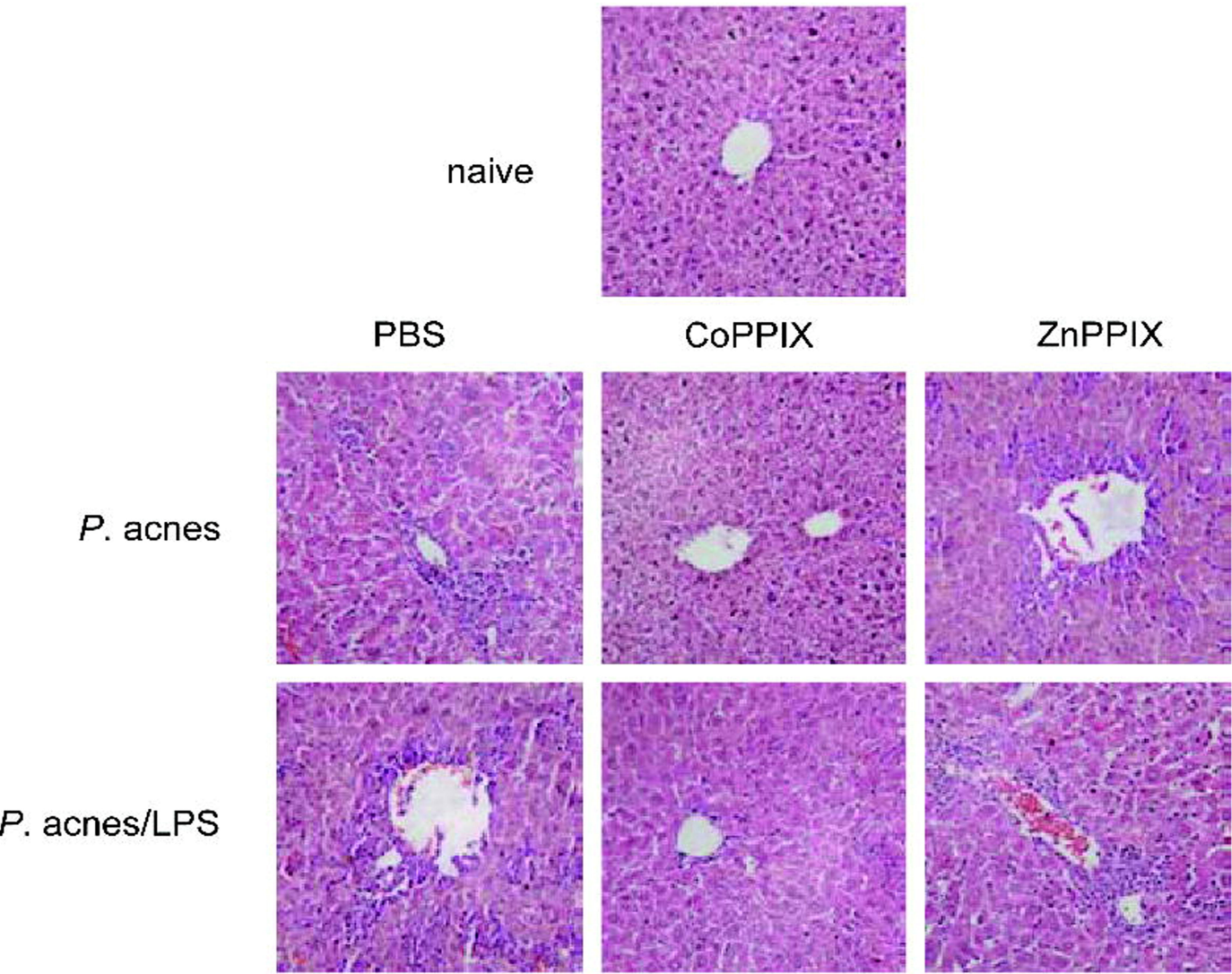
A



B

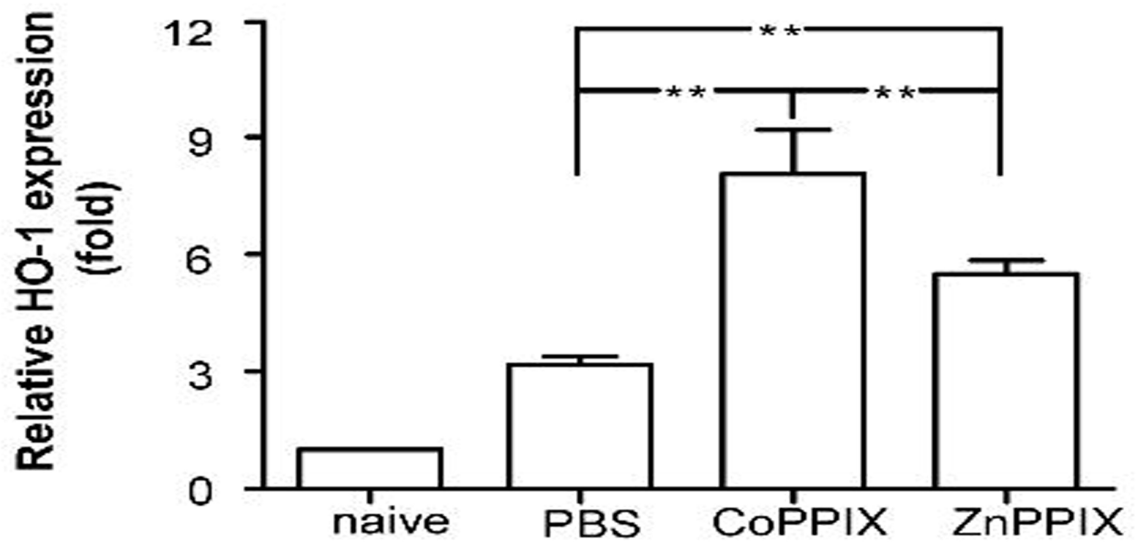


C

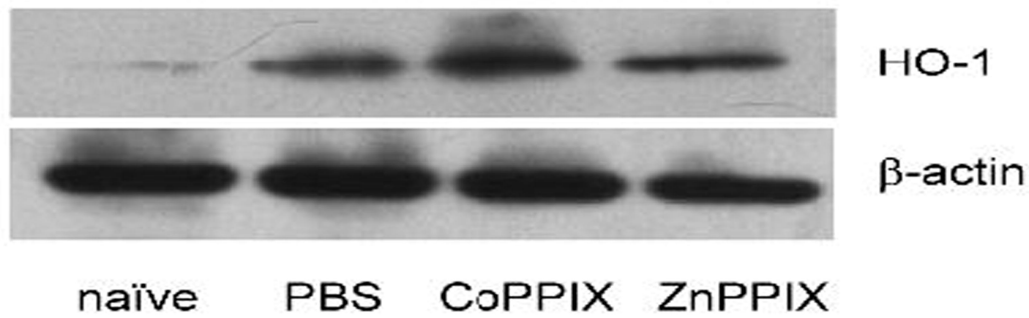


## Figure 2

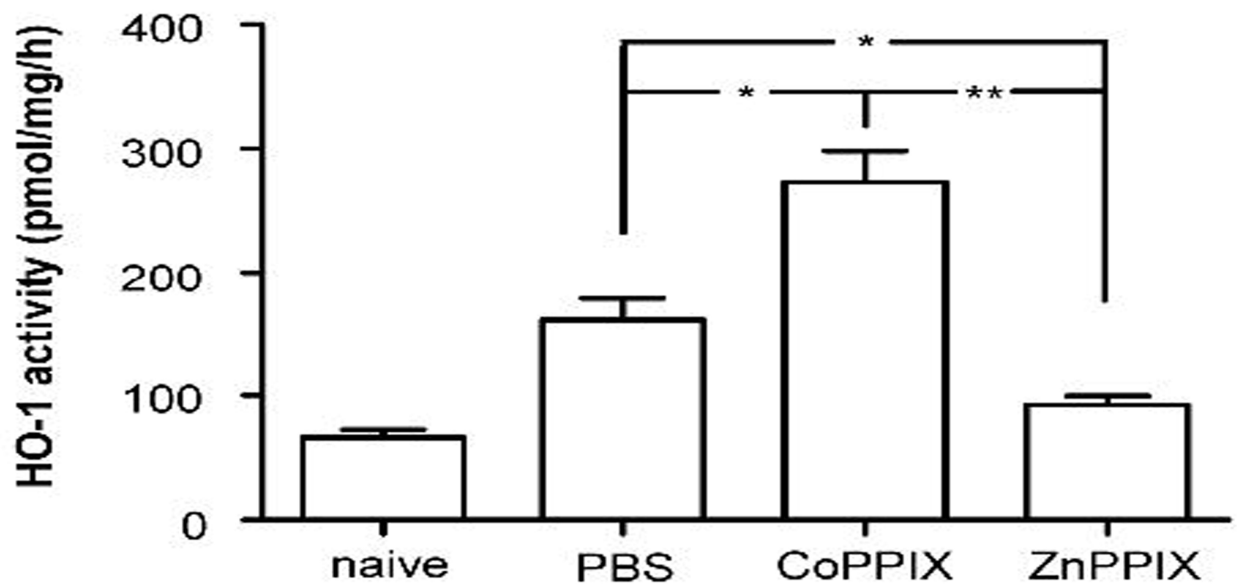
**A**



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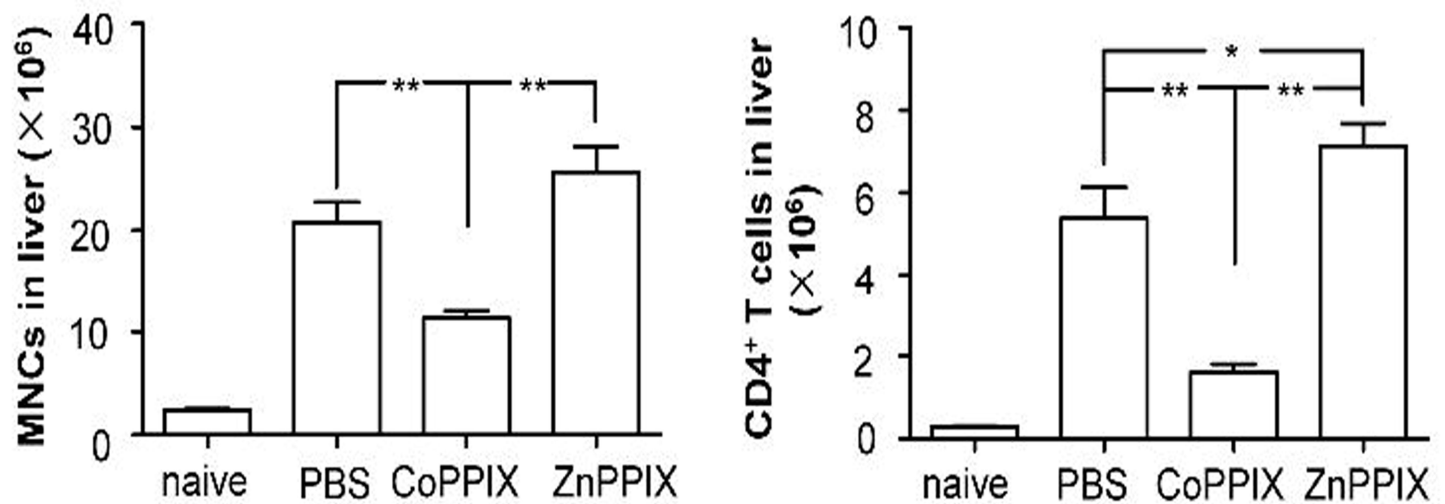


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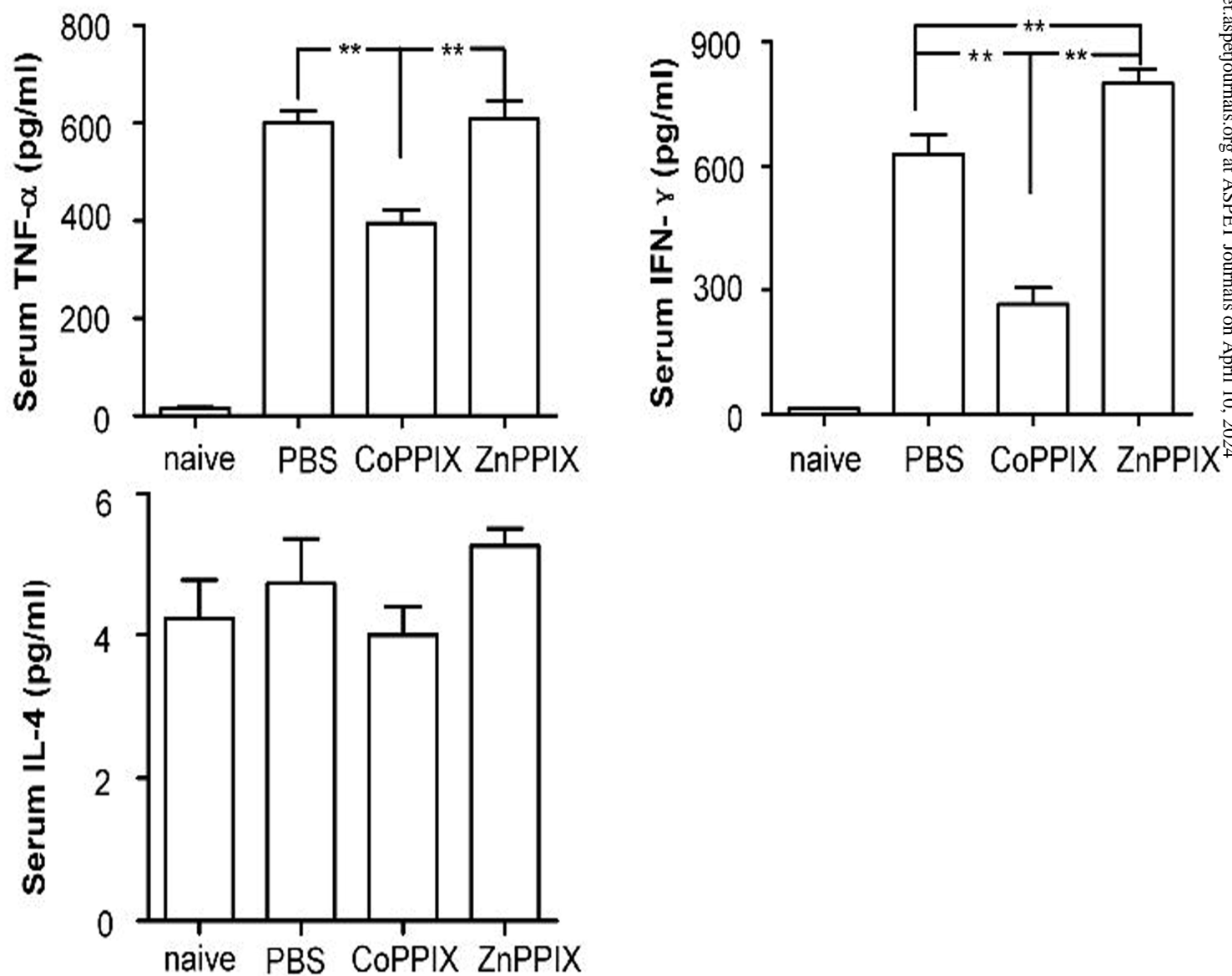


**Figure 3**

**A**

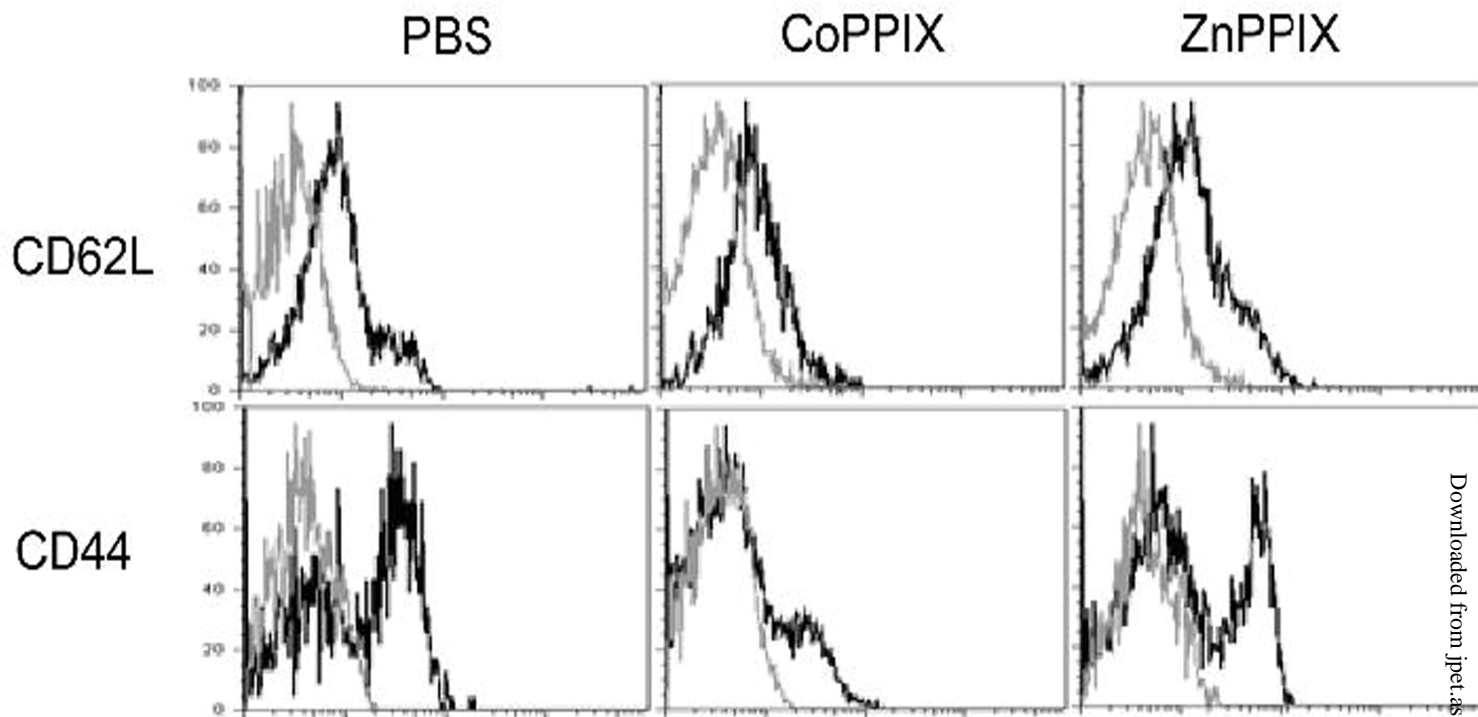


**B**

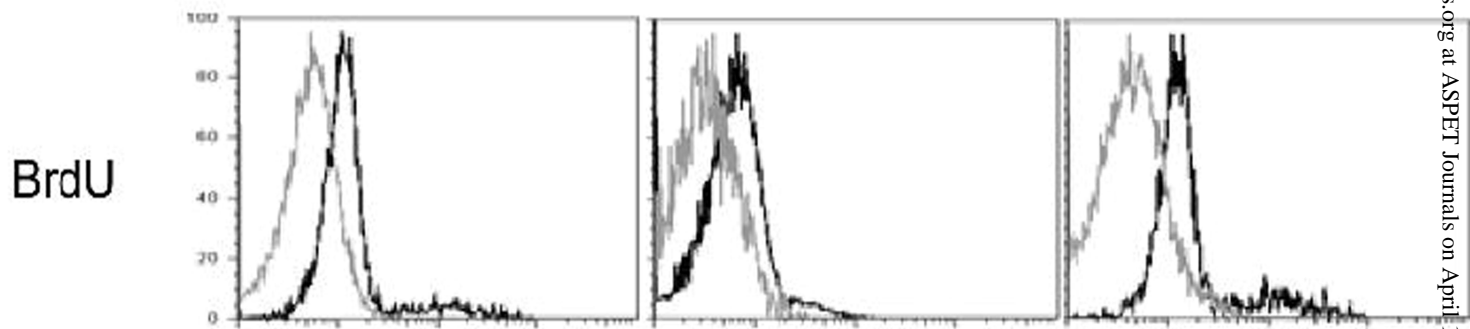




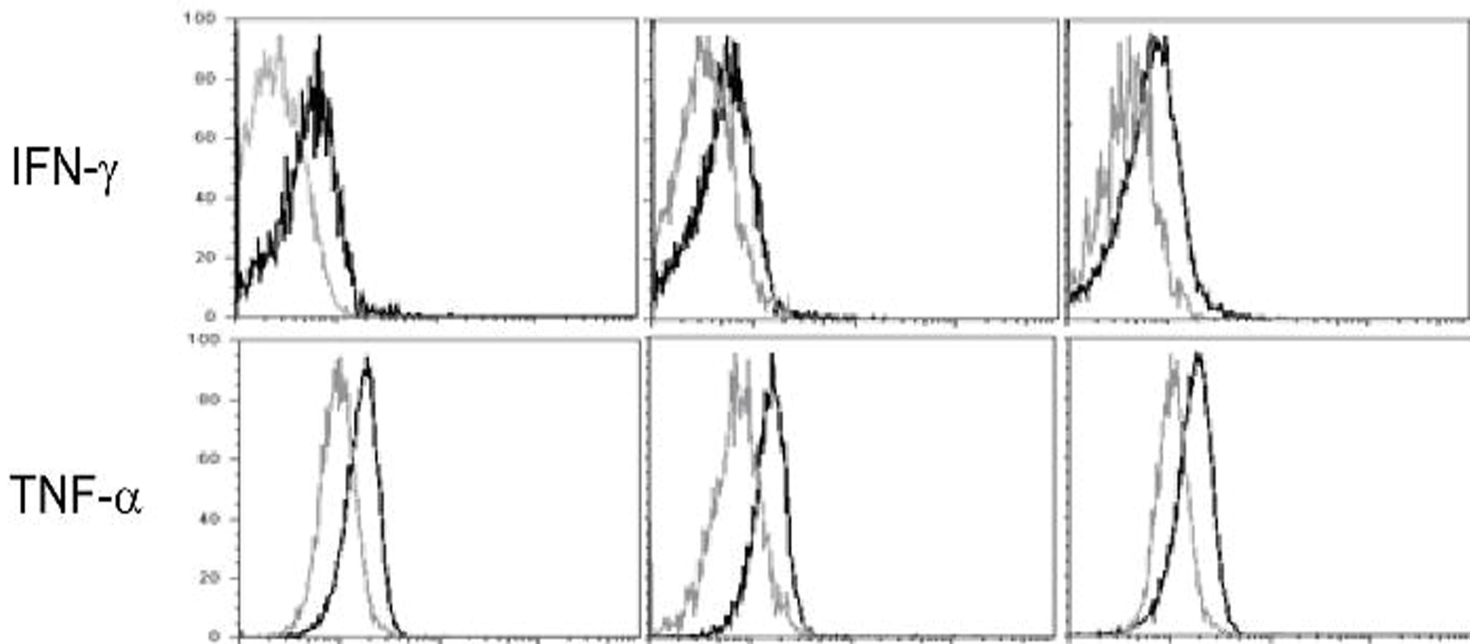
**A**



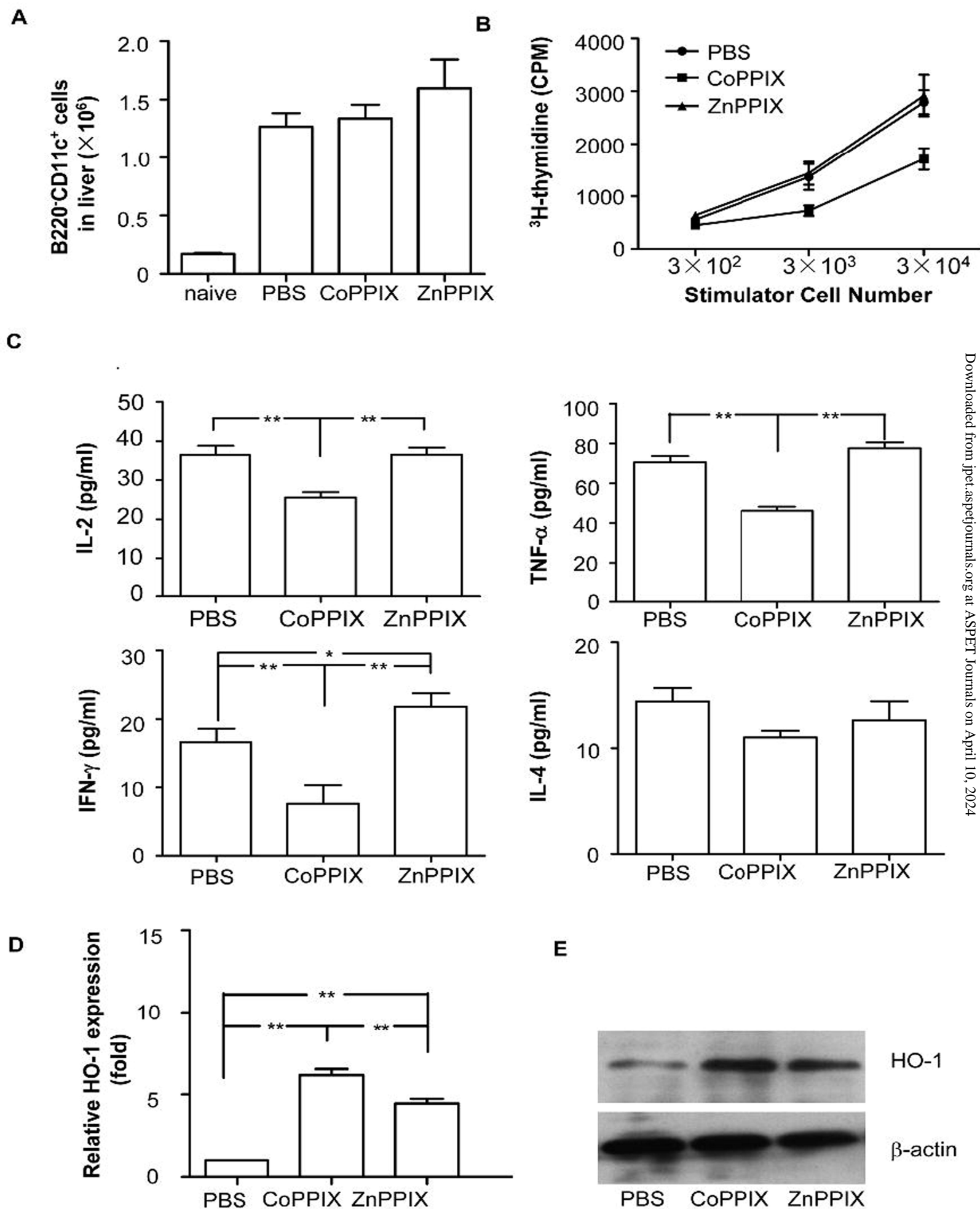
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**C**

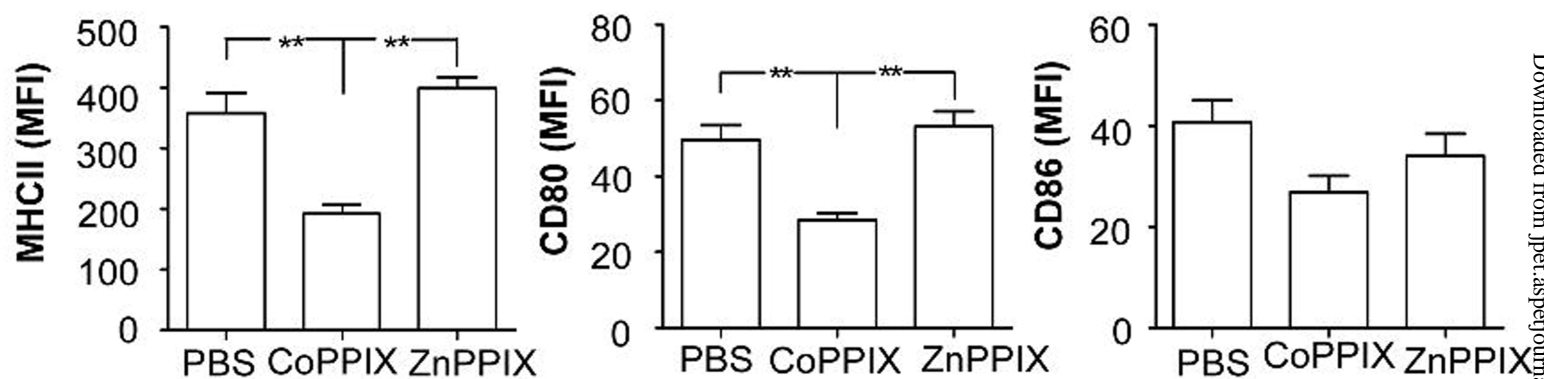
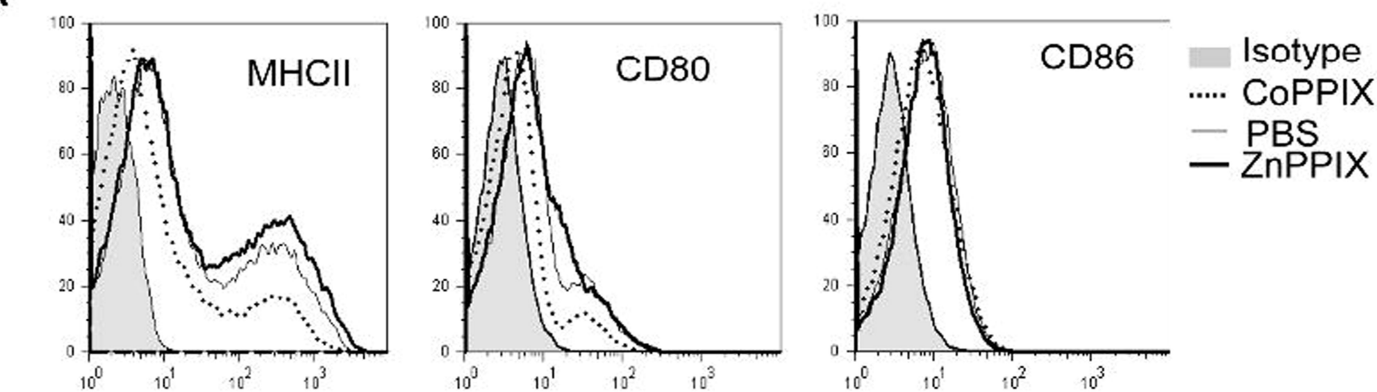


**Figure 5**

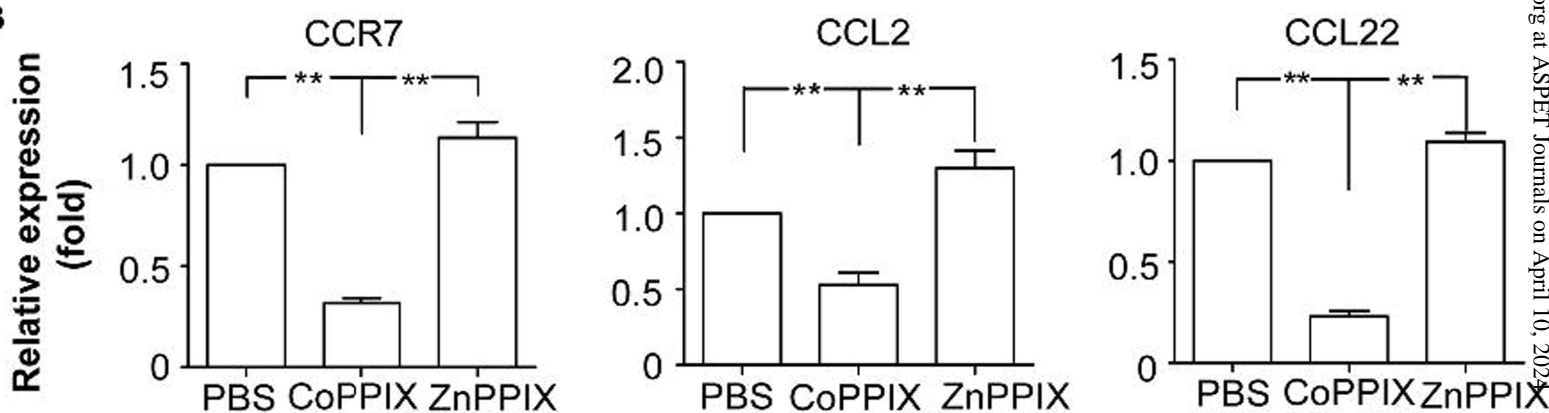


**Figure 6**

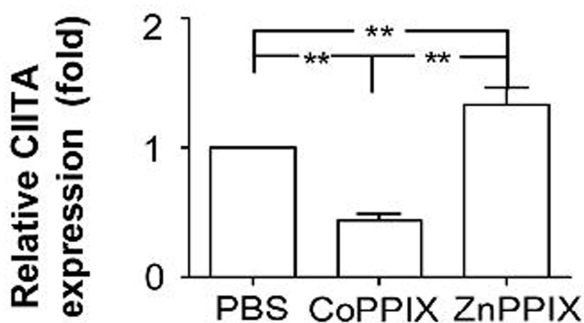
**A**



**B**



**C**



**D**

