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# Induction of indoleamine 2,3-dioxygenase expression via heme oxygenase-1-dependant pathway during murine dendritic cell maturation

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ABSTRACTS

Heme oxygenase (HO)-1 is expressed in a variety of conditions involved in the regulation of immune responses. In this study, we examined the role of HO-1 in dendritic cell (DC) maturation and expression of indoleamine 2,3-dioxygenase (IDO), a key enzyme that catalyzes the initial, rate-limiting step in tryptophan degradation. IDO deficiency led to diminished phenotypic and functional maturation of DCs *in vitro* and *in vivo*. IDO expression and DC maturation was abrogated by the HO inhibitor zinc protoporphrin, but increased by hemin, a potent inducer of HO-1. Moreover, LPS-induced HO-1 expression was mediated by an NF-κB-dependent pathway. Our findings provide additional insight into the immunological functions of IDO and HO-1, and suggest possible therapeutic adjuvants for the treatment of DC-related acute and chronic diseases.



## 1. Introduction

The biology of bone marrow-derived dendritic cells (BMDCs) and their potential use in the treatment of chronic infections and cancers are the focus of considerable research. DCs, which are potent antigen-presenting cells (APCs), have the unique ability to enhance T and B cell responses and potentiate immune tolerance [1,2]. Under steady state conditions, DCs exist as immature cells in peripheral tissue and function to protect against invading antigens [1,2]. During pathogen invasion or following inflammatory cytokine exposure, DCs undergo phenotypic changes associated with functional maturation [3]. To initiate adaptive immunity in response to signals from peptide/MHC complexes and co-stimulatory molecules, mature DCs migrate into the afferent lymphatics and move to T cell areas in the draining lymph nodes [3,4].

Indoleamine 2,3-dioxygenase (IDO), an enzyme that mediates the initial and rate-limiting step in tryptophan catabolism along the kynurenine pathway [5], is inducible in DCs in response to lipopolysaccharide (LPS) and interferon (IFN) stimulation [6]. Although IDO is critical to host defense against pathogens, it is now clear that this enzyme plays multiple roles in the immune system [7]. Studies have shown that human DCs exhibiting significant IDO activity inhibit T cell proliferation through tryptophan degradation [8]. In addition, IDO expression is critical for allogeneic fetal tolerance [9], tumor tolerance [10], and downregulation of autoimmunity [11], making it an interesting target for transplantation applications. The mechanisms that regulate the overall immunosuppressive effects of IDO remain incompletely understood, and may vary according to cell type [12]. In sharp contrast to the immunosuppressive effects of IFN-γ-activated DCs, Toll-like receptor (TLR) agonists such as LPS induce high IDO expression, suggesting a possible role for this enzyme in promoting DC maturation. For instance, pharmacological inhibition of IDO activity by 1-Methyl-D-tryptophan (1-MT) diminishes the expression of co-stimulatory molecules in LPS-stimulated human monocyte-derived DCs and murine BMDCs [13,14]. These observations prompted us to examine whether the manner of stimulation,

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LPS or IFN-γ, affects DC maturation and T cell proliferation differentially in IDO-expressing cells. We previously reported that IDO expression in mature mouse BMDCs is enhanced by LPS via an IFN-γ-independent pathway [6]. However, the precise regulatory mechanisms underlying LPS-induced IDO expression in BMDCs remains unclear.

Heme oxygenases (HOs) are rate-limiting intracellular enzymes that cleave heme through oxidation, thus producing biliverdin, carbon monoxide, and iron [15]. Three isoforms of HO (HO-1, HO-2 and HO-3) have been identified [15]. HO-1 is known for its cytoprotective effect against oxidative injuries and inflammation [16]. HO-1 protein is upregulated by numerous stress signals, including endotoxin, cytokines, and heavy metals, as well as by its substrate, heme [15]. Induction of HO-1 expression has therapeutic effects in a variety of immune-related conditions and disorders, including transplantation and inflammatory diseases [17–22]. HO-1 expression is abrogated during human and rat DC maturation [23]. Furthermore, this enzyme is decreased during monocyte-derived dendritic cell maturation in mouse spleen [24]. In contrast, LPS stimulation enhances expression of HO-1 during mouse BMDC

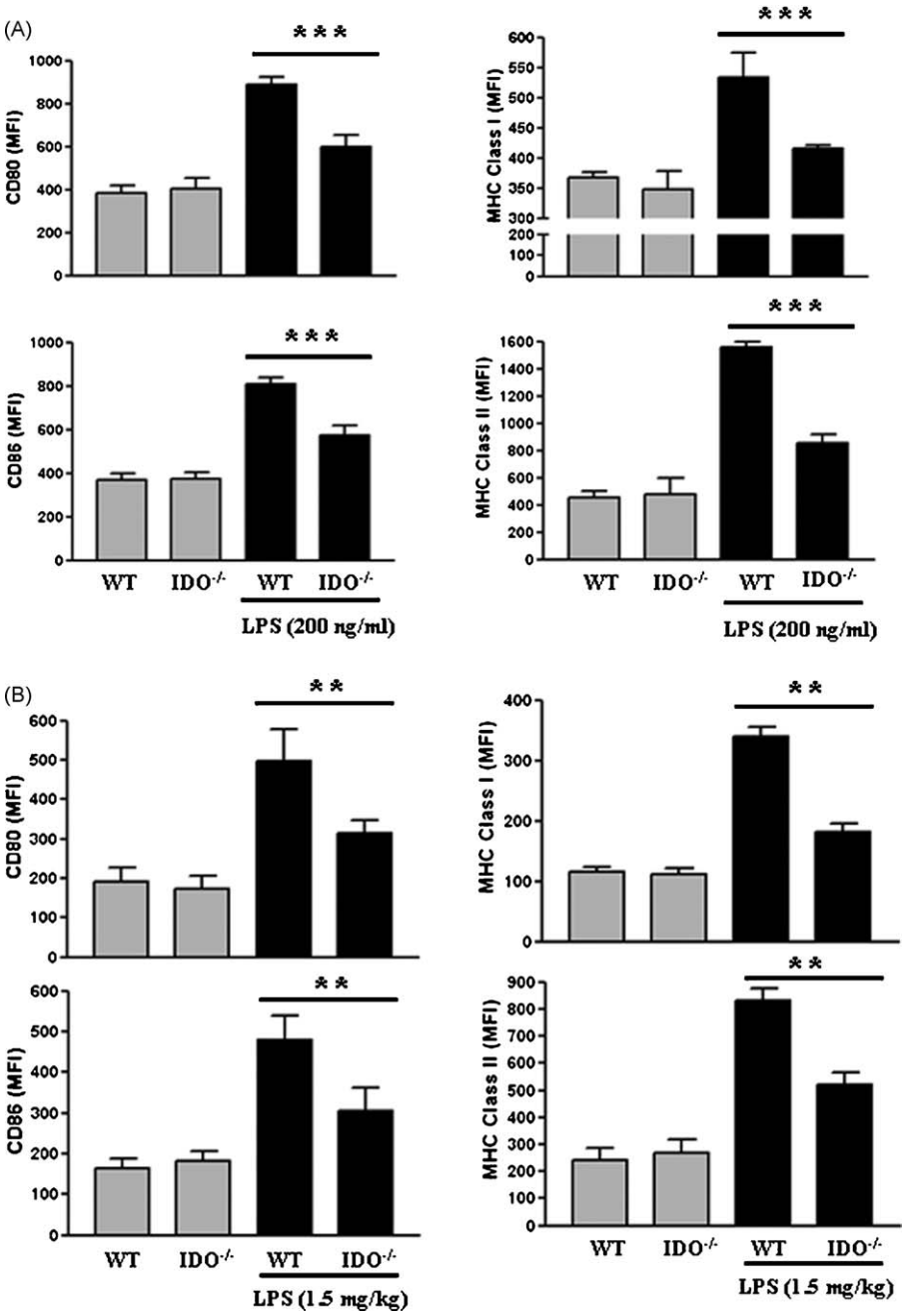


**Fig. 1.** Inhibition of IDO inhibits LPS-induced surface molecules and production of proinflammatory cytokines in murine DCs. BMDCs derived from WT and IDO$^{-/-}$ mice were stimulated with or without LPS (200 ng/ml) for 24 h. (A) Surface molecules were analyzed by flow cytometry in cells were gated on CD11c$^{+}$. The mean fluorescence intensity (MFI) values are mean ± SEM ($n$ = 3). (B) WT and IDO$^{-/-}$ mice were injected with PBS or the indicated dose of LPS; 24 h later, spleens were removed and stained with antibodies for CD11c-PE and IDO-FITC. Costimulatory molecules were analyzed by flow cytometry in the CD11c$^{+}$-gated cell population. The mean fluorescence intensity (MFI) values are presented as the mean ± SEM ($n$ = 3). $^{**}P < 0.001$. (C) The concentration of IL-12 p70 and TNF-α were measured by ELISA. Data are presented as mean ± SEM ($n$ = 3). $^{***}P < 0.001$. (D and E) Monocytes derived from WT and IDO$^{-/-}$ mice cultured with complete medium contained GM-CSF and IL-4 for 6 days. (D) The population of CD11c$^{+}$ cells was analyzed by flow cytometry. The mean fluorescence intensity (MFI) value within each histogram represents the incidence of CD11c$^{+}$ cells. The results are representative of four separate experiments. (E) Morphological shape was analyzed by an inverted phase contrast microscope. The results are representative of four separate experiments.

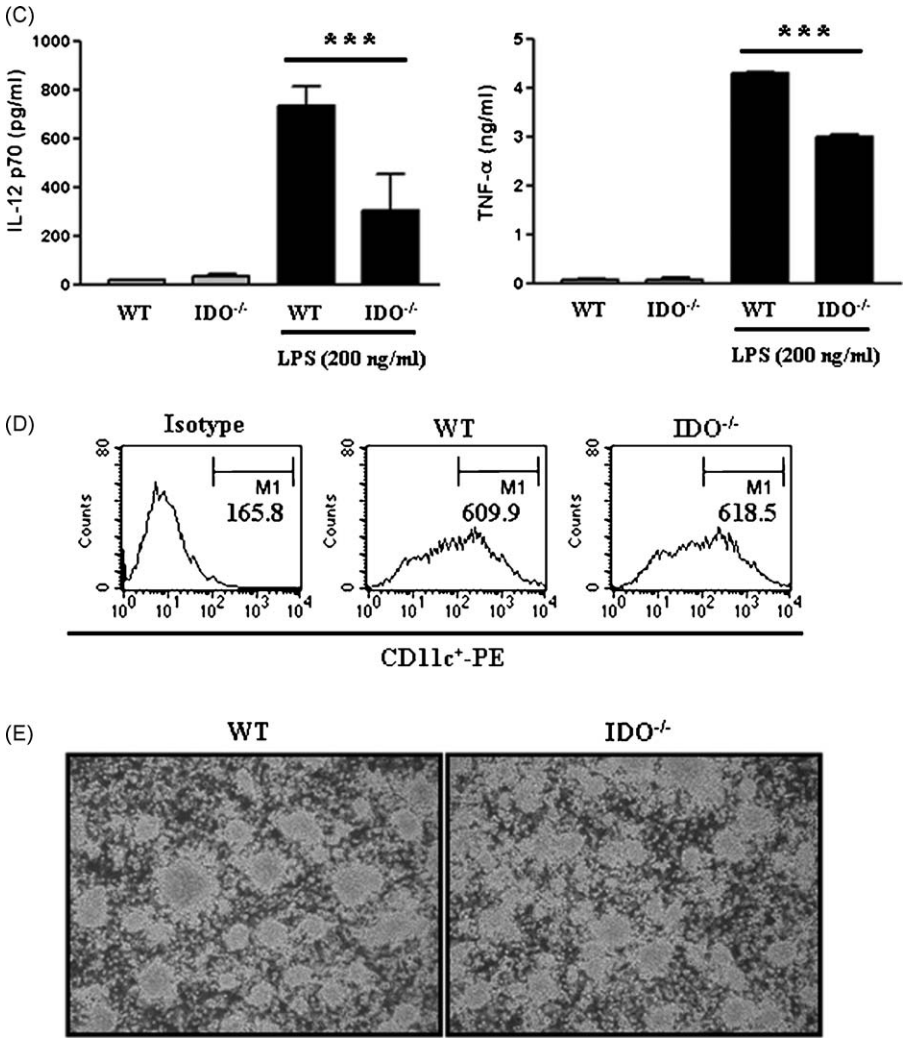


Fig. 1. (*Continued*).

maturation [25], suggesting a possible role for HO-1 in IDO expression during this biological process.

In this study, we examined the involvement of HO-1 in regulating LPS-induced IDO expression in mouse BMDCs. Our data show that HO-1-mediated IDO expression is dependent on the NF-κB pathway and constitutes an intermediate step in the DC maturation pathway. These studies provide new insight into the mechanism by which LPS induces IDO gene expression in murine BMDCs.

## 2. Materials and methods

### 2.1. Mice and materials

Male 6–8 weeks-old C57BL/6 (H-2K$^b$ and I-A$^b$) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). IDO$^{-/-}$ C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and mated with wild-type C57BL/6 mice to produce IDO$^{+/-}$ heterozygotes. Offspring of heterozygote crosses were genotyped by PCR of tail DNA to identify individuals that were homozygous null (−/−) for targeted disruption of the IDO gene; age-matched wild-type (+/+) littermates were used as controls. Mice were housed in an isolated pathogen-free environment within our animal facility for at least one week before use. All experiments were performed in accordance with the guidelines of the Ethics of Animal Experiments committee, Pusan National University. LPS from *Escherichia coli* (serotype 055:B5), BAY-11-7085 and tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$, CORM2) were obtained from Sigma (St. Louis, MO, USA). Recombinant mouse (rm) GM-CSF and rmIL-4 were obtained from R&D Systems (Minneapolis, MN, USA). The anti-IDO antibody was obtained from Alexis (San Diego, CA, USA). Phycoerythrin (PE)-conjugated anti-H-2Kb [major histocompatibility complex (MHC) class I], anti-I-Ab (MHC class II), anti-CD80, anti-CD86, and fluorescein isothiocyanate (FITC)-conjugated anti-CD11c Abs were obtained from ebioscience Inc. (San Diego, CA, USA). Anti-α-tubulin, anti-p65, anti-HO-1 and anti-Topo I antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). ZnPP and hemin were obtained from Porphyrin products (Philadelphia, PA, USA).

### 2.2. Generation and culture of DCs

DCs were generated from murine bone marrow monocytes according to the procedure of Inaba et al. [26] with minor modification. Briefly, bone marrow was flushed from the tibiae and femurs of 6–8-week-old male C57BL/6 mice and depleted of red blood cells with Red Blood cell Lysing buffer (Sigma, St. Louis, MO, USA). The cells were plated in 6-well culture plates ($1 \times 10^6$ cells/ml; 2 ml/well) in RPMI supplemented with 10% heat-inactivated FBS, 100 U/ml penicilline, 100 μg/ml stereptomycin, 20 ng/ml rmGM-CSF and 10 ng/ml rmIL-4 at 37 °C in a humidified 5% $CO_2$ atmosphere. On day 3 and 5 of the culture, floating cells were

gently removed and fresh medium was added. On day 6 of the culture, non-adherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation. On day 7, 80% or more of the non-adherent cells expressed CD11c.

### 2.3. In vivo treatment with LPS and surface molecules staining of splenocytes

The mice (20–25 g) were injected intraperitoneally (i.p.) with saline or LPS (1.5 mg/kg), dissolved in saline for 24 h. For surface molecules staining of splenic DCs, their spleens were disrupted and the cells were centrifuged at 400 × *g* for 5 min and removed red blood cells from mouse splenocytes by treatment of red blood cell lysing buffer (Sigma, St. Louis, MO, USA). And then the cells were harvested and washed twice with PBS containing with 2% fetal bovine serum and 0.1% sodium azide. Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and stained with PE-conjugated anti-H-2Kb [major histocompatibility complex (MHC) class I], anti-I-Ab (MHC class II), anti-CD80, anti-CD86, and FITC-conjugated anti-CD11c for 30 min at 4 °C. The stained cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

### 2.4. In vivo treatment with ZnPP and intracellular staining of IDO

Mice (20–25 g) were injected with saline or LPS (1.5 mg/kg, i.p.), dissolved in saline. ZnPP (5 mg/kg, i.p.) was injected 6 h before LPS administration, and after a 24-h LPS challenge, spleens were removed. Intracellular staining of IDO in splenic DCs, was assayed by flow cytometry as previously described [27]. Spleens were disrupted and cells were centrifuged at 400 × *g* for 5 min. After removing red blood cells by treatment with Red Blood Cell Lysing Buffer (Sigma, St. Louis, MO, USA), splenocytes were harvested and washed twice with PBS containing 2% fetal bovine serum (Thermo

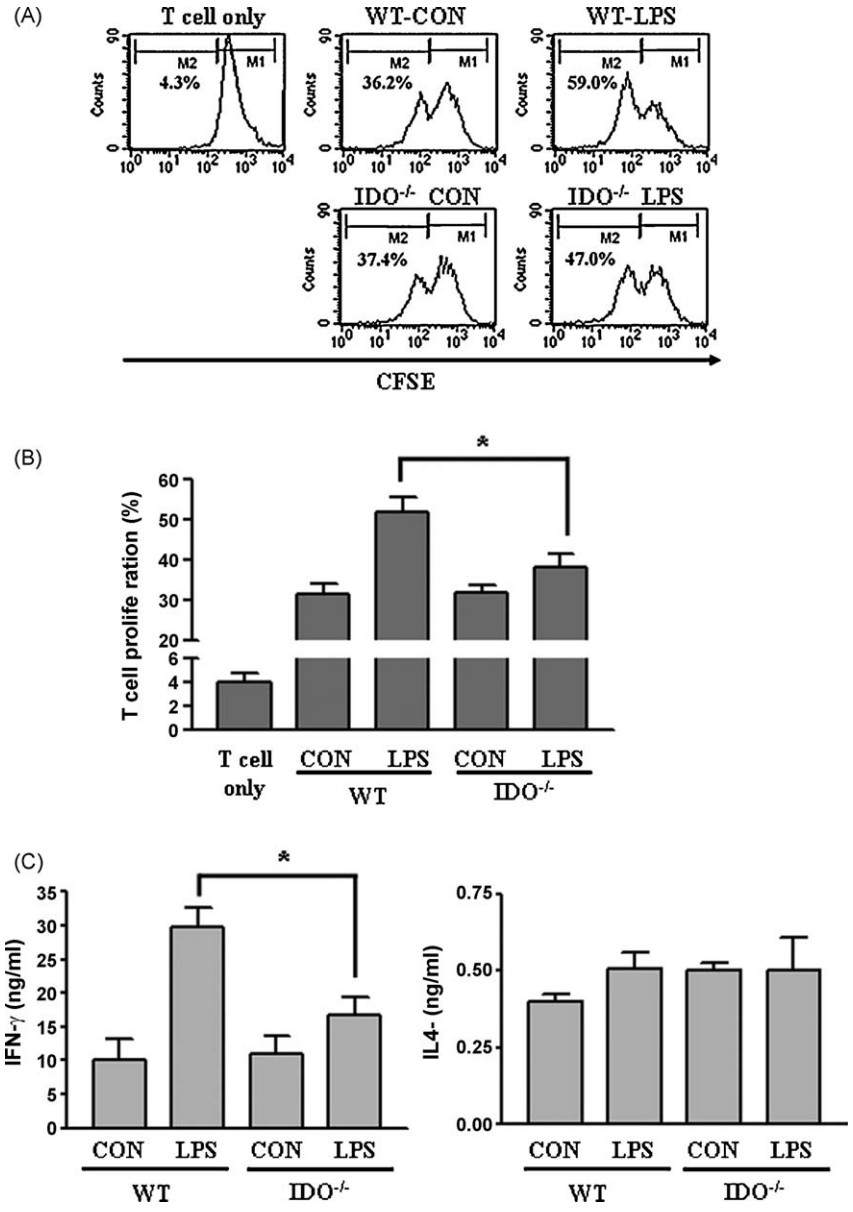


**Fig. 2.** Inhibition of IDO display impaired to induce the proliferation of allogeneic T cells and initiate Th1 responses *in vitro*. BMDCs derived from WT and IDO$^{-/-}$ mice were stimulated with or without LPS (200 ng/ml) for 24 h. DCs were incubated for 24 h with or without LPS (200 ng/ml). The DCs were then washed and cocultured with allogeneic T cells for 4 days. (A) Percentage of T-cell proliferation as determined by CFSE flow cytometry. The results are representative of four separate experiments. (B) Bar graphs show the mean ± SEM ($n$ = 4) of percentage of T cell proliferation in (A). Data are presented as the mean ± SEM $^{*}P < 0.05$. (C) Results for cells examined for cytokine release after 48 h. IL-4 and IFN-γ concentrations in culture supernatants were measured by ELISA. Data are presented as mean ± SEM ($n$ = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Fisher Scientific Inc. Waltham, MA, USA) and 0.1% sodium azide (Sigma, St. Louis, MO, USA). Cells were stained with anti-CD11c (N418) (ebioscience Inc. San Diego, CA, USA) and anti-IDO (Alexis, San Diego, CA, USA) antibodies, and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

### 2.5. Western blot analysis

DCs were washed twice with Tris-buffered saline and lysed by the addition of ice-cold lysis buffer, containing 0.5% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM $Na_3VO_4$, 10 μg/ml aprotinin and pepstatin A, and 5 μg/ml leupeptin. Lysates were incubated on ice for 20 min and microcentrifuged for 30 min at 12,000 × *g* at 4 °C to remove nuclei. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Pall Corporation, Port Washington, NY, USA). Membranes were blocked with 5% non-fat dried milk in T-PBS (0.2% Tween 20 in PBS) and incubated with a primary antibody against IDO (1:2000), anti-p65 (1:1000), anti-HO-1 (1:2000); an anti-α-tubulin (1:2000) and anti-Topo I (1:500) antibodies were used as a control for equal loading. Membranes were subsequently washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. Immunolabeling was detected using an ECL detection system (Millipore Corporation, Billerica, MA, USA).

### 2.6. Cytokine measurements

Plasma and cell culture supernatants were analyzed for TNF-α and IL-12 p70 content in triplicate using an ELISA kit as described by the manufacturer (R&D Systems, Minneapolis, MN, USA).

### 2.7. Transfection of siRNA

HO-1 siRNA (Catalog no. sc 35555), NF-κB p65 siRNA (Catalog no. sc29411) and non-specific control siRNA were obtained from Santa Cruz Biotechnology and used to transiently transfect BMDCs in 6-well plates as described by the manufacturer. After first determining optimal confluence conditions for transfection, cells were transfected with HO-1 siRNA (10–100 nM), NF-κB siRNA (10–100 nM) or non-specific control siRNA (100 nM); transfections were performed 48 h prior to experiments.

### 2.8. Flow cytometry

On day 7, BMDCs were harvested, washed with phosphate buffered saline (PBS) and resuspended in fluorescence activated cell sorter (FACS) washing buffer (2% fetal bovine serum and 0.1% sodium azide in PBS). Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and stained with phycoerythrin (PE)-conjugated anti-H-2Kb [major histocompatibility complex (MHC) class I], anti-I-Ab (MHC class II), anti-CD80, and anti-CD86 with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c for 30 min at 4 °C. The stained cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

### 2.9. Heme oxygenase activity assay

The HO activity was determined by measuring the amount of bilirubin produced from hemin added as the substrate as described [28]. The formed bilirubin was extracted with chloroform and the absorption measured as the difference in 464 and 530 nm (extinction coefficient, 40 $mM^{-1}$ $cm^{-1}$ for bilirubin). HO activity is expressed as pmol of bilirubin formed per mg protein per hr.

### 2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out using a ChIP assay kit (Millipore Corporation, Billerica, MA) according to a modification of the manufacturer's instructions. $2 \times 10^6$ cells were fixed with 1% formaldehyde for 10 min at 37 °C to cross-link the protein-DNA complexes. Then they were harvested and washed twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). For the remaining steps of the protein isolation, all buffers used to isolate the proteins contained phenylmethylsulfonyl fluoride and protease inhibitor mixture. Cells were added to SDS lysis buffer and incubated on ice for 10 min. Cell lysates were sonicated to shear the DNA to lengths between 200 and 1000 base pairs and then

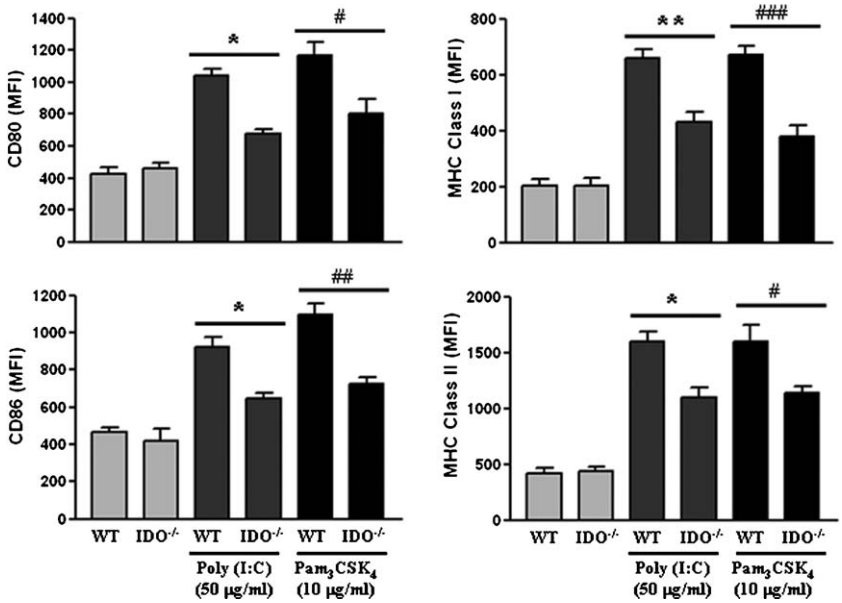


**Fig. 3.** Inhibition of IDO decreases Poly(I:C)- and Pam3CSK-induced surface molecules in BMDCs. BMDCs derived from WT and $IDO^{-/-}$ mice were stimulated with or without Poly(I:C) (50 μg/ml) and Pam3CSK (10 μg/ml) for 24 h. The population of $CD11c^+$ cells was analyzed by flow cytometry. The mean fluorescence intensity (MFI) values are mean ± SEM ($n = 4$).

centrifuged at 14,000 rpm for 10 min at 4 °C. The sonicated cell supernatants were diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, and 167 mM NaCl) and precleared with a 27% suspension of protein A-agarose/salmon sperm DNA for 30 min at 4 °C with agitation. The supernatant was recovered after pelleting the agarose by centrifugation and then incubated with 5 μg of a rabbit-specific Ab against p65 overnight at 4 °C. The Ab-protein-DNA complexes were collected by adding protein A-agarose/salmon sperm DNA for 1 h at 4 °C with agitation. Immunoprecipitated Ab-protein-DNA complexes were washed three times with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl) followed by three additional washes in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Chromatin complexes were eluted with 250 μl of freshly prepared extraction buffer (1% SDS, 0.1 M $NaHCO_3$). To reverse cross-links, 5 M NaCl was added to each combined elute to a final concentration of 0.3 M, then the solution was heated to 65 °C for 5 h. Proteins were digested with 100 μg/ml proteinase K for 1 h at 45 °C, and DNA was extracted with a QIAquick PCR purification kit (Qiagen). Precipitated DNA fragments were amplified by PCR. The sequences of GAS site of *IRF-1* promoter primer are as follows: forward primer 5′-CTTTCCAACA-CAGGCAAG-3′, reverse primer 5′-ACTGTGAAAGCACGTAC-3′.

### 2.11. Statistical analysis

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the

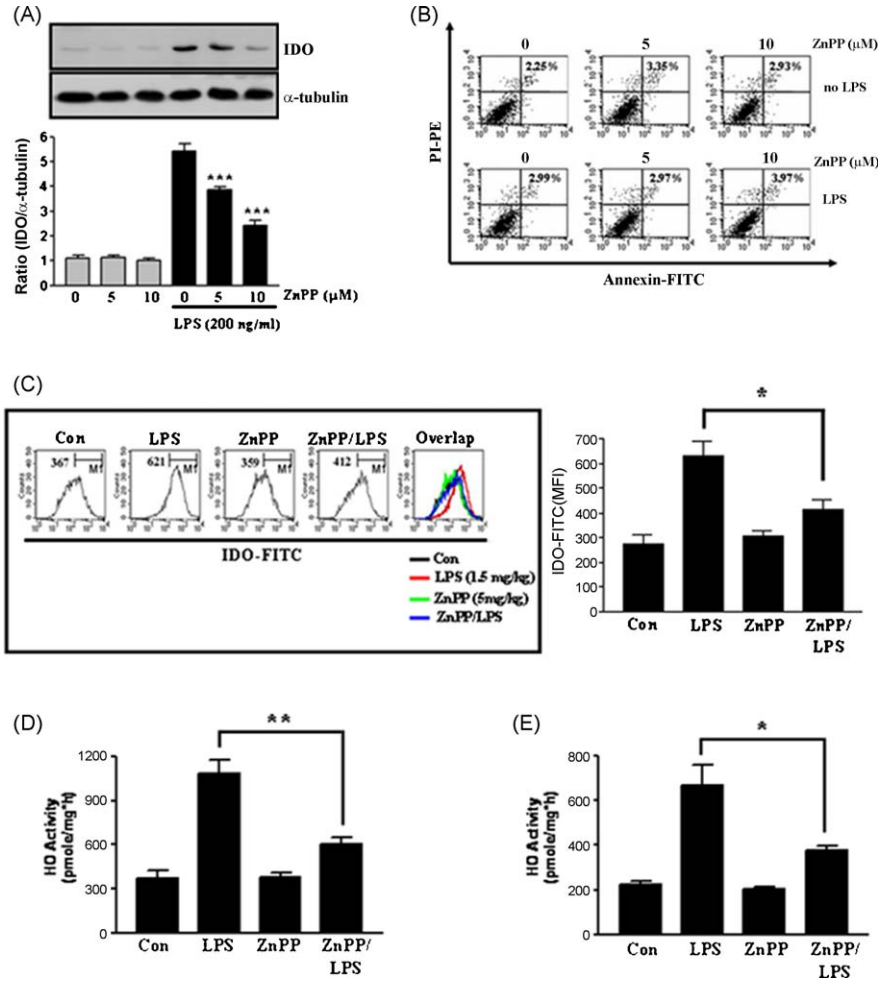


**Fig. 4.** HO-1 downregulation decreases LPS-induced IDO expression in murine DCs. (A and B) BMDCs were pre-treated with ZnPP (5 or 10 μM) for 1 h prior to stimulating with 200 ng/ml LPS for 24 h. (A) IDO and α-tubulin levels were determined by immunoblotting with anti-IDO and anti-α-tubulin antibodies. Top panel shows a representative blot stained with anti-IDO and anti-α-tubulin antibodies as indicated. The mean values ± SEM obtained from three separate experiments shown in the bottom panel, $^{***}P < 0.001$. vs. responses in the absence of ZnPP with LPS stimulation. (B) DCs were stained with annexin-V and PI. The percentage within each histogram represents the incidence of annexin-$V^+PI^+$. The results are representative of five separate experiments. (C) C57BL/6J mice were injected with ZnPP (5 mg/kg, i.p.) 6 h prior to LPS challenge (1.5 mg/kg, i.p.); 24 h later, spleens were removed and stained with antibodies for CD11c-PE and IDO-FITC. Histogram shows expression of intracellular IDO on cells gated for CD11c expression. Numbers indicate the mean fluorescence intensity (MFI) values of IDO expression in $CD11c^+$ DCs. The mean values ± SEM obtained from three separate experiments shown in the bottom panel. (D) BMDCs were pre-treated with ZnPP (10 μM) for 1 h prior to stimulating with 200 ng/ml LPS for 24 h. A microsomal fraction of BMDCs was isolated and HO activity was determined by the production of bilirubie ($n = 3$). Data are presented as the mean ± SEM $^{**}P < 0.01$. (E) C57BL/6J mice were injected with ZnPP (5 mg/kg, i.p.) 6 h prior to LPS challenge (1.5 mg/kg, i.p.); 24 h later, spleens were removed. A microsomal fraction of splenic DCs was isolated and HO activity was determined by the production of bilirubie ($n = 3$). Data are presented as the mean ± SEM $^{*}P < 0.05$. (F and G) BMDCs were transfected with HO-1 siRNA (10, 25, 50 or 100 nM) or control siRNA (100 nM) for 24 h, and then further incubated with 200 ng/ml LPS for 24 h. (F) HO-1, HO-2, IDO and α-tubulin levels were determined by immunoblotting with anti-HO-1, anti-HO-2, anti-IDO and anti-α-tubulin antibodies. Top panel shows a representative blot stained with anti-HO-1, anti-HO-2, anti-IDO and anti-α-tubulin antibodies as indicated. The mean values ± SEM obtained from three separate experiments are shown in bar graph. Asterisks indicate significant differences in the ratio of HO-1 (*) and IDO (#) compared to the values at control siRNA transfected cells at $^{**}P < 0.05$, $^{***}P < 0.001$ and $^{\#\#\#}P < 0.001$. (G) DCs were stained with annexin-V and PI. The percentage within each histogram represents the incidence of annexin-$V^+PI^+$. The results are representative of five separate experiments. (H) BMDCs derived from WT and $IDO^{-/-}$ mice were incubated with or without LPS (200 ng/ml) for 24 h. HO-1, HO-2, IDO and α-tubulin levels were determined by immunoblotting with anti-HO-1, anti-HO-2, anti-IDO and anti-α-tubulin antibodies. Lane 1 and 2; WT w/o LPS, lane 3 and 4; WT with LPS, lane 5 and 6; $IDO^{-/-}$ w/o LPS, and lane 7 and 8; $IDO^{-/-}$ with LPS. One representative experiment of three is shown.

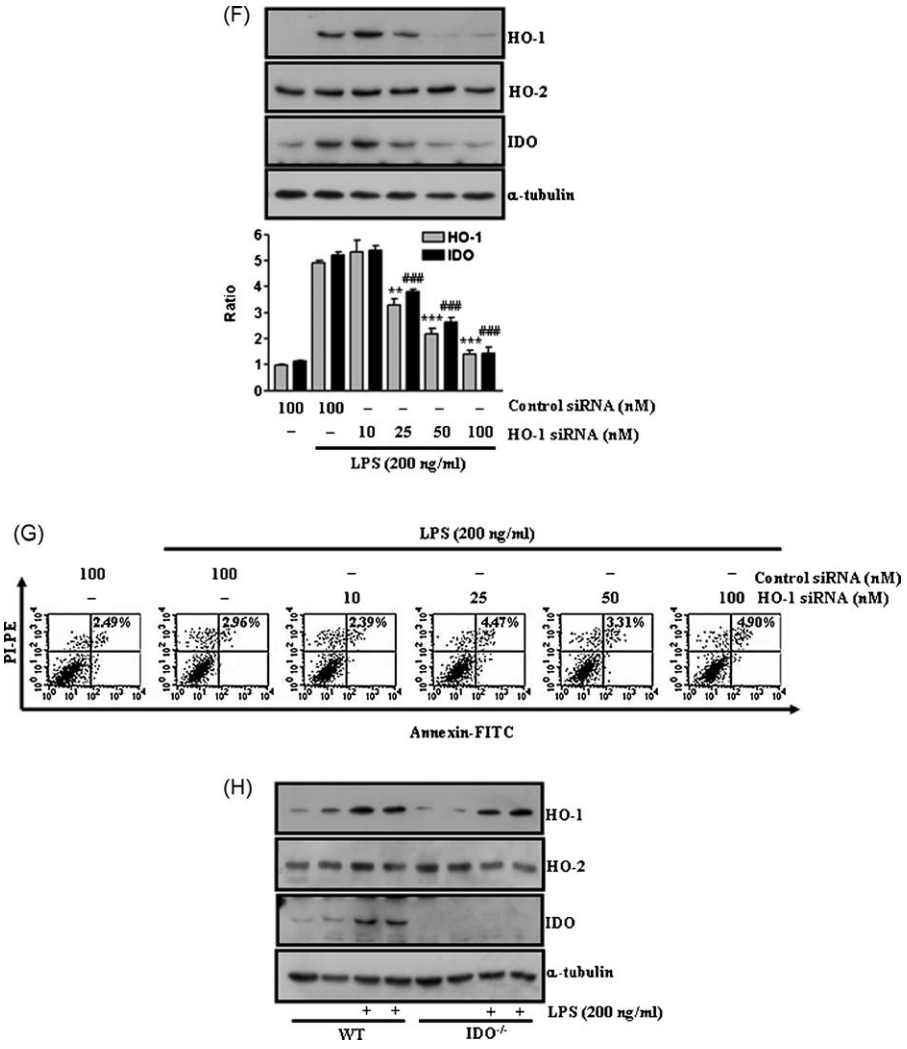


Fig. 4. (*Continued*).

mean ± SEM. ANOVA was used to compare experimental groups to control values, and Tukey's Multiple Comparison test was used to compare between multiple groups. $P$ values $<0.05$ were considered statistically significant.

## 3. Results

### 3.1. IDO deficiency leads to suppression of LPS-induced maturation of BMDCs and splenic DCs

To investigate the role of IDO in DC maturation, we evaluated the expression of cell surface molecules that indicate a DC phenotype, namely CD80, CD86, and MHC class I and II, in LPS-stimulated BMDCs derived from IDO knockout ($IDO^{-/-}$) and wildtype (WT) mice. As shown in Fig. 1A, 24-h LPS stimulation of WT cells cultured for 6 days resulted in upregulation of all the cell surface molecules tested. In contrast, expression of these receptors was reduced significantly in LPS-stimulated cells derived from $IDO^{-/-}$ mice, indicating that BMDC maturation was at least partially inhibited in the absence of IDO. We also evaluated the role of IDO in the maturation of splenic DCs *in vivo*. As observed, the levels of cell surface molecules in splenic DCs were lower in $IDO^{-/-}$ mice than in WT mice (Fig. 1B). Taken together, these data suggest that lack of IDO expression suppresses the ability of DCs to undergo maturation in response to LPS *in vitro* and *in vivo*.

In addition to these DC cell surface markers, we also examined the effect of IDO deficiency on pro-inflammatory cytokine production. A major attribute of mature DCs is the synthesis and release of cytokines with important modulatory functions in inflammation and T cell differentiation. Among these are the pro-inflammatory cytokines, IL-1β, IL-6, IL-12 and TNF-α [29]. IL-12 production is an especially important marker of DC maturation and can also be used as a Th1-inducing adjuvant [30]. We found that IL-12 p70 and TNF-α were produced at high levels in BMDCs derived from WT mice after stimulation with LPS for 24 h. However, this effect of LPS was diminished in the absence of IDO (Fig. 1C).

Next, we assessed the role of IDO in DC differentiation by monocytes. Bone marrow-derived monocytes were isolated from WT and $IDO^{-/-}$ mice, and changes in morphology and CD11c expression were measured. In these experiments, mouse monocytes were purified from bone marrow isolated from WT and $IDO^{-/-}$ mice and then cultured in complete medium containing GM-CSF and IL-4 for 6 days. These cells differentiated to become immature DCs, which are characterized by increased CD11c expression. Interestingly, no significant differences in the percentage of $CD11c^{+}$ DCs (Fig. 1D) and morpholocal shape (Fig. 1E) were observed between WT and $IDO^{-/-}$ mice. These results indicate that IDO is not involved in the differentiation of DCs from monocytes.

### 3.2. Impairment of the allostimulatory capacity of DCs in the absence of IDO

DCs have the unique ability to enhance a primary immune response by activating naïve T cells to differentiate into Th1 or Th2 cells. However, the abrogation of cell surface molecules expression in LPS-stimulated IDO$^{-/-}$ DCs would be decreases antigen presentation to T cells, leading to inhibition of T cell activation. To determine whether IDO deficiency in DCs also results in reduced T cell proliferation, WT and IDO$^{-/-}$ DCs were stimulated with LPS for 24 h and then analyzed in an allogeneic mixed lymphocyte reaction. As shown in Fig. 2A and B, the rate of proliferation of allogeneic T cells induced by LPS-treated WT DCs (WT-LPS) was greater than that of untreated WT DCs (WT-CON). In contrast, IDO deficiency led to decreased proliferation (IDO$^{-/-}$ LPS).

In addition, we investigated the cytokine production profile of T cells stimulated with LPS-treated WT and IDO$^{-/-}$ DCs. Allogeneic T cells primed with LPS-treated WT DCs produced a Th1 cytokine profile that included high levels of IFN-γ and low levels of IL-4. In contrast, T lymphocytes primed with LPS-treated IDO$^{-/-}$ DCs exhibited reduced IFN-γ production (Fig. 2C). However, IL-4 production was not affected by IDO deficiency. These results show that the majority of the effects of IDO on the ability of DCs to promote T cell differentiation.

### 3.3. Lack of IDO suppresses the induction of BMDC maturation by other TLR agonists

Next, we sought to investigate whether IDO plays a critical role in the induction of DC maturation by other TLR agonists. WT and IDO$^{-/-}$ DCs were stimulated for 24 h with $Pam_3CSK_4$ and poly(I:C), agonists for TLR1/2 and TLR3, respectively, and then expression of cell surface molecules was analyzed. IDO$^{-/-}$ DCs displayed diminished cell surface molecule expression following stimulation with $Pam_3CSK_4$ and poly(I:C) compared to WT DCs (Fig. 3). These data strongly demonstrate that IDO may be a critical and potent mediator of DC maturation.

### 3.4. The HO-1 inhibitor, ZnPP, inhibits LPS-induced IDO expression in BMDCs and splenic DCs

To examine the involvement of heme oxygenases (HOs) in the induction of IDO expression by LPS, we first measured HO-1 and HO-2 expression in mouse BMDCs after LPS stimulation. Expression of HO-1, but not HO-2, was enhanced by LPS stimulation in a dose- and time-dependent manner (data not shown). Next, to evaluate the influence of HO-1 on LPS-induced IDO expression, cells were pretreated with ZnPP, an inhibitor of HO-1 activity. The level of IDO protein induced *in vitro* by LPS stimulation was significantly decreased in the presence of ZnPP (Fig. 4A). To exclude the possibility that the effect of ZnPP was due to a reduction in the

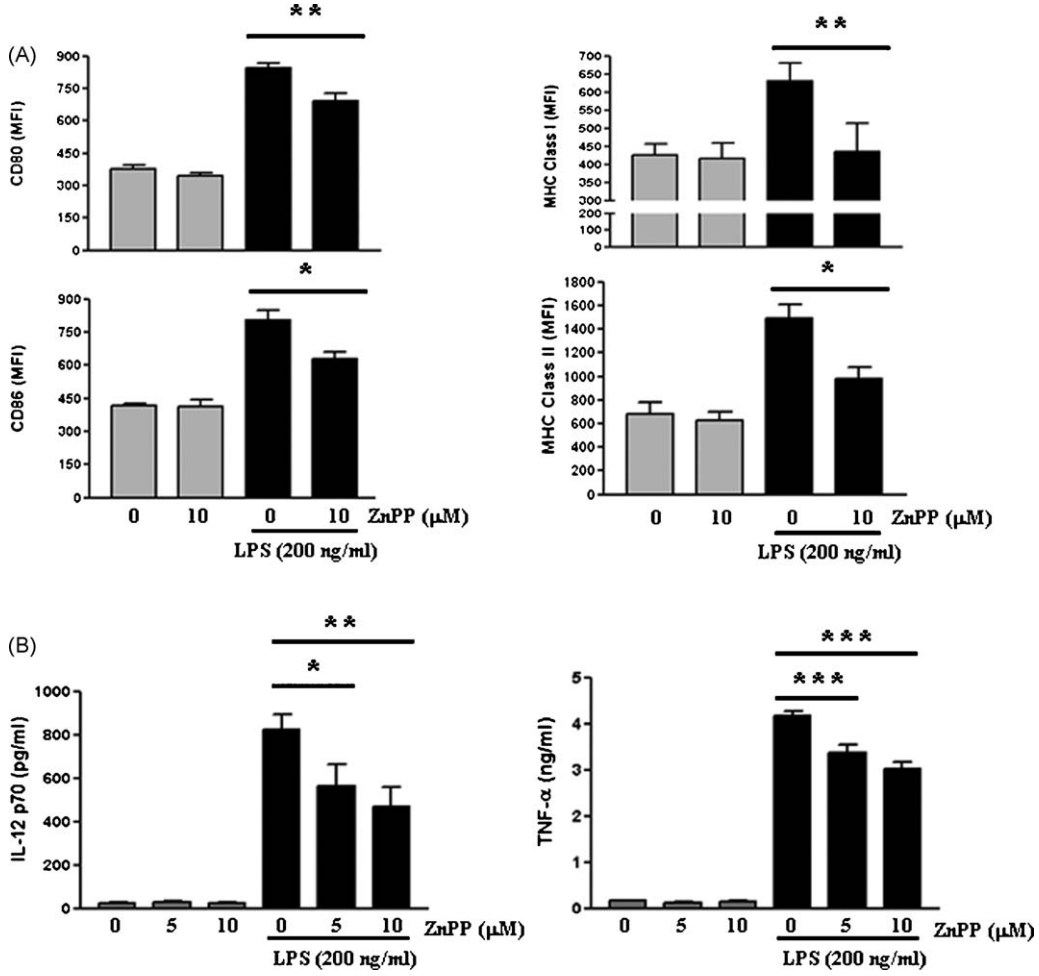


**Fig. 5.** HO-1 downregulation reduces LPS-induced surface molecules and pro-inflammatory cytokines. (A and B) BMDCs were pre-treated with ZnPP (10 μM) for 1 h prior to stimulating with 200 ng/ml LPS for 24 h. (A) Surface molecules were then analyzed by flow cytometry in cells were gated on CD11c$^+$. The mean fluorescence intensity (MFI) values are mean ± SEM ($n$ = 3). (B) IL-12 p70 and TNF-α concentrations were measured by ELISA. Data are presented as mean ± SEM ($n$ = 3). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

number of DCs via apoptosis, we used Annexin V and propidium iodide staining to assess apoptosis in CD11c$^+$ cells treated with the inhibitor. DCs were preincubated with ZnPP (5 and 10 μM) for 1 h and then either stimulated with LPS (200 ng/ml) for 24 h or left untreated. No significant differences were observed below 10 μM (Fig. 4B).

To confirm the effect of HO-1 on IDO expression, we assessed IDO expression in mouse splenic DCs challenged with LPS *in vivo*. Inbred C57BL/6J mice were injected with ZnPP (5 mg/kg, i.p.) 6 h prior to a 24-h LPS (1.5 mg/kg, i.p) challenge, and IDO expression in splenic DCs was assessed by flow cytometry. Similar to the effect of ZnPP on IDO expression *in vitro*, the inhibitor abrogated IDO expression in splenic CD11c$^+$ DCs induced by LPS *in vivo* (Fig. 4C). In addition, to test whether ZnPP prevent the HO activity, we measured the HO activity in BMDCs (Fig. 4D) and splenic DCs (Fig. 4E). As expected, ZnPP was strongly inhibited the LPS-induced HC activity in both the *in vitro* and *in vivo*. These data indicate that LPS-induced IDO expression in DCs may be regulated by an HO-1-dependent pathway. To determine whether HO-1 expression acts upstream of IDO in LPS-activated BMDCs, we evaluated IDO expression in cells transfected with an HO-1-specific siRNA. As shown in Fig. 4F, LPS-induced HO-1 expression decreased with increasing concentrations (10–100 nM) of HO-1 siRNA, whereas transfection with a control siRNA had no effect. As expected, siRNA-mediated HO-1 knockdown impaired LPS-induced IDO expression significantly. In addition, siRNA transfection did not affect cell viability (Fig. 4G). To further confirm that HO-1 acts as an upstream modulator of IDO expression in LPS-activated BMDCs, we measured HO-1 and IDO expression in LPS-activated IDO$^{-/-}$ and WT BMDCs. As shown in Fig. 4H, although IDO expression was absent in LPS-activated IDO$^{-/-}$ BMDCs, HO-1 expression remained unaffected. Collectively, these data show that HO-1 acts as an upstream mediator of IDO expression in LPS-activated BMDCs.

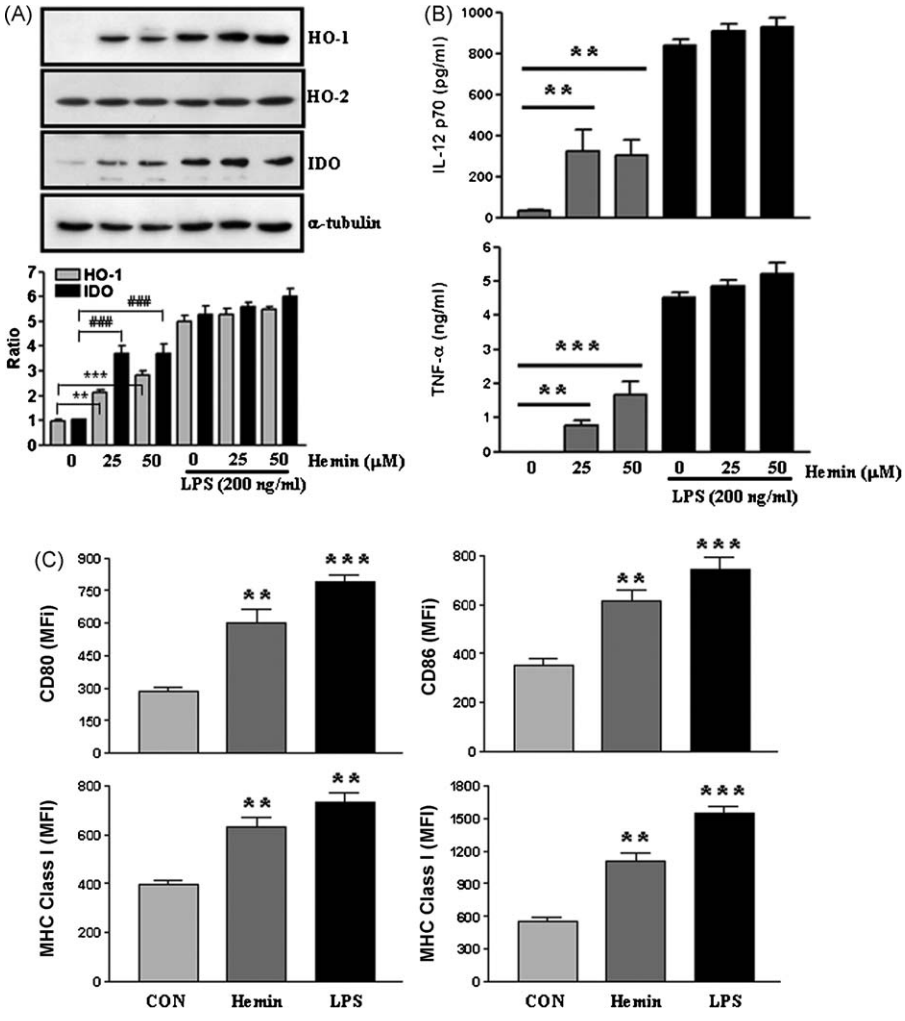


**Fig. 6.** Hemin increases IDO expression and pro-inflammatory cytokines in BMDCs. (A and B) BMDCs were pre-treated with hemin (25 and 50 μM) for 6 h prior to stimulating with 200 ng/ml LPS for 24 h. (*A*) HO-1, HO-2, IDO and α-tubulin levels were determined by immunoblotting with anti-HO-1, anti-HO-2, anti-IDO and anti-α-tubulin antibodies. Top panel shows a representative blot stained with anti-HO-1, anti-HO-2, anti-IDO and anti-α-tubulin antibodies as indicated. The mean values ± SEM obtained from three separate experiments are shown in the bottom panel. Asterisks indicate significant differences in the ratio of HO-1 (*) and IDO (#) compared to the values at control cells at $^{**}P < 0.05$, $^{***}P < 0.001$ and $^{\#\#\#}P < 0.001$. (B) IL-12 p70 and TNF-α concentrations were measured by ELISA. Data are presented as mean ± SEM ($n = 3$). $^{**}P < 0.01$. (C) BMDCs were pre-treated with hemin (50 μM) and LPS (200 ng/ml) for 24 h. Surface molecules were then analyzed by flow cytometry in cells were gated on CD11c$^+$. The mean fluorescence intensity (MFI) values are mean ± SEM ($n = 3$). $^{**}P < 0.01$ and $^{***}P < 0.001$. vs. control. (D) BMDCs were pre-treated with ZnPP (10 μM) for 1 h prior to stimulating with 50 μM hemin for 24 h. Surface molecules were then analyzed by flow cytometry in cells were gated on CD11c$^+$. The mean fluorescence intensity (MFI) values are mean ± SEM ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$. (E) BMDCs derived from WT and IDO$^{-/-}$ mice were stimulated with or without hemin (50 μM) for 24 h. Surface molecules were analyzed by flow cytometry in cells were gated on CD11c$^+$. The mean fluorescence intensity (MFI) values are mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (F and G) BMDCs were treated with CORM2 (30 μM) and 200 ng/ml LPS for 24 h. (F) Surface molecules were then analyzed by flow cytometry in cells were gated on CD11c$^+$. The mean fluorescence intensity (MFI) values are mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. vs no stimulation. (B) IDO and α-tubulin levels were determined by immunoblotting with anti-IDO and anti-α-tubulin antibodies. One representative experiment of three is shown.

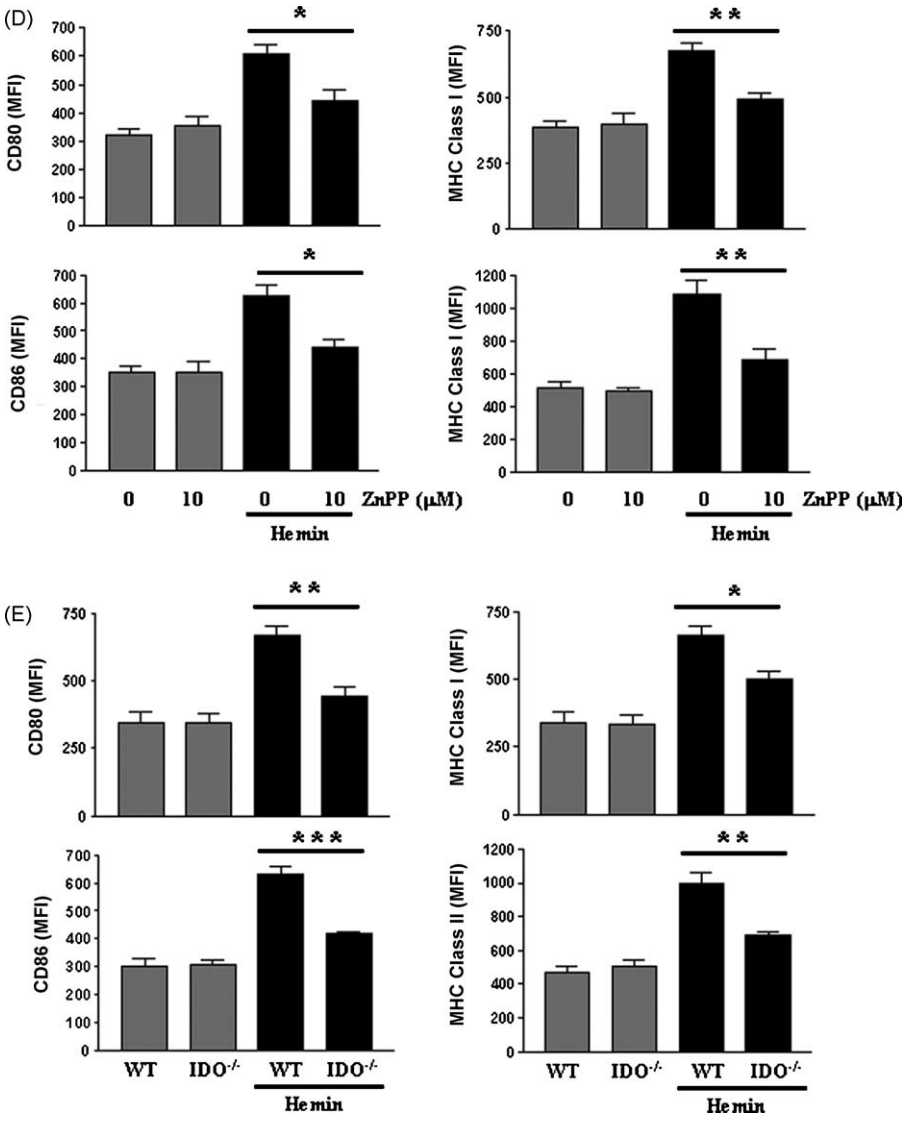


Fig. 6. (*Continued*).

### 3.5. ZnPP suppresses the expression of surface molecules and pro-inflammatory cytokines in LPS-stimulated BMDCs

To investigate the role of HO-1 in DC maturation *in vitro*, we measured CD80, CD86, and MHC class I and II expression in LPS-stimulated BMDCs in the presence and absence of ZnPP. As shown in Fig. 5A, 24-h LPS stimulation of cells cultured for 6 days induced the expression of these surface molecules. ZnPP-treated DCs consistently expressed lower levels of these surface markers compared to untreated cells, indicating that ZnPP treatment impaired BMDC phenotypic maturation. In addition, we tested whether ZnPP affected the production of IL-12 p70 and TNF-α following LPS-induced DC maturation. Similar to IDO$^{-/-}$ BMDCs, the levels of IL-12 p70 and TNF-α were decreased significantly in the presence of ZnPP (Fig. 5B). Thus, these results suggest that ZnPP-mediated blockade of HO-1 suppresses LPS-induced maturation of DCs via inhibition of IDO expression.

### 3.6. Hemin increases IDO expression and pro-inflammatory cytokine production in BMDCs

Hemin plays a major role in inducing HO-1 expression in various cell types [31,32]. Therefore, we proceeded to determine whether hemin is also involved in inducing IDO expression and production of pro-inflammatory cytokines in BMDCs. Pre-treatment of BMDCs with hemin for 6 h prior to LPS stimulation increased the expression of HO-1 and IDO, inducing an increase that was comparable to that produced by LPS stimulation (Fig. 6A). Furthermore, the levels of IL-12 p70 and TNF-α (Fig. 6B) were also enhanced significantly in hemin-stimulated BMDCs, indicating that IDO expression, which is involved in DC maturation, is tightly regulated by HO-1 expression in BMDCs. In addition, to confirm whether hemin promote the maturation of BMDCs, we measured the expression of surface molecules in BMDCs treated with hemin and LPS. As shown in Fig. 6C, hemin also significantly enhanced cell surface molecules as well as LPS treatment. Next, to investigate whether inhibition of HO activity by ZnPP reverses the effects of hemin, we measured the expression of surface molecules in hemin-stimulated BMDCs in the presence and absence of ZnPP. As shown in Fig. 6D, hemin-induced the expression of surface molecules was also diminished in ZnPP-treated BMDCs. Finally, we sought to investigate whether IDO plays a critical role in the induction of DC maturation by hemin. We evaluated the expression of cell surface molecules in hemin-stimulated BMDCs derived from IDO knockout (IDO$^{-/-}$) and wildtype (WT) mice. As shown in Fig. 6E, the expression of surface molecules was reduced

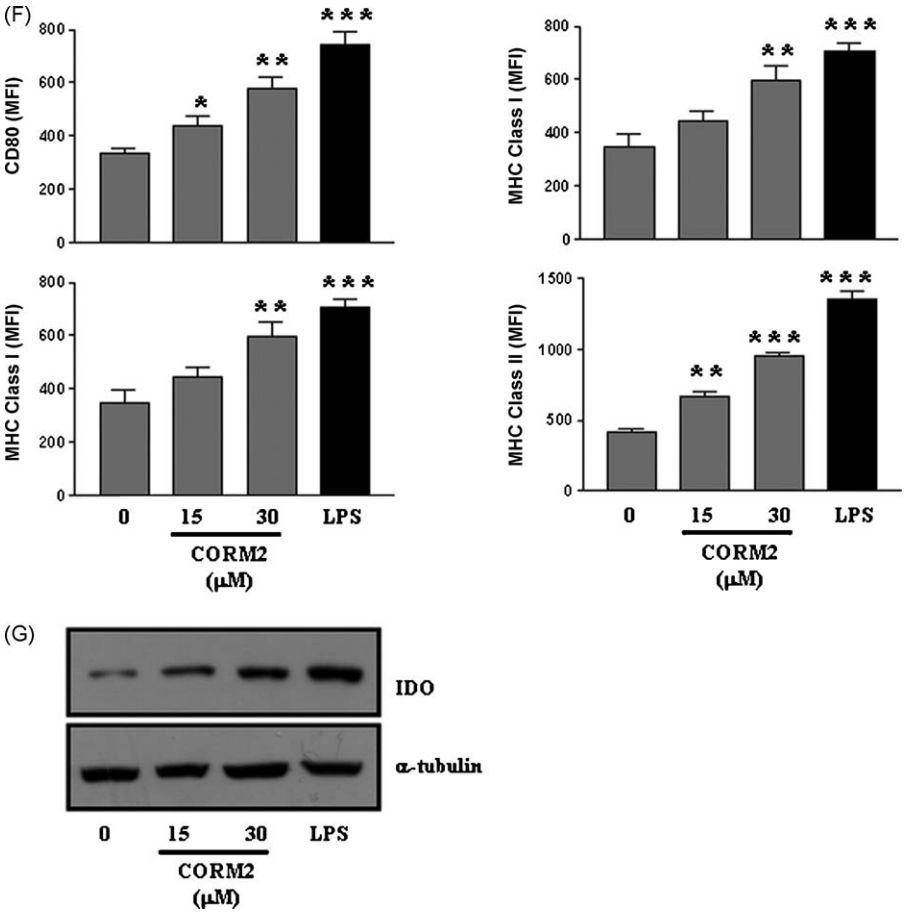


Fig. 6. (*Continued*).

significantly in hemin-stimulated cells derived from IDO$^{-/-}$ mice compared that in hemin-stimulated derived from WT mice, indicating that ZnPP-mediated blockade of HO-1 suppresses maturation of DCs by hemin via impairing IDO expression.

Carbon monoxide (CO) mimics the effects of HO-1, indicating that HO-1 acts through the production of CO [33]. To investigate whether CO by hemin affects to the maturation DCs and IDO expression, BMDCs were treated with tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$, CORM2; 15 and 30 μM) or LPS (200 ng/ml) for 24 h. As for hemin-treated DCs, the expression of cell surface molecules (Fig. 6F) and IDO expression (Fig. 6G) was significantly enhanced in CORM2-treated DCs. Altogether, these results demonstrate that exogenous CO mimicked the effect of HO-1 induction on DC maturation, suggesting that CO generated by hemin largely contribute to the actions of HO-1 on DCs.

### 3.7. LPS regulates HO-1 expression in BMDCs via a NFκB-dependent pathway

LPS stimulation has been shown to activate the NF-κB signaling pathway in DCs [34,35], and recent evidence indicates that NF-κB activation is an important underlying event in DC maturation [36]. To determine the involvement of NF-κB in HO-1-mediated IDO expression in BMDCs, we measured HO-1 expression in LPS-stimulated BMDCs in the presence of BAY-11-7085, an NF-κB inhibitor. Treatment of LPS-induced BMDCs with BAY-11-7085 resulted in dramatic, dose-dependent inhibition of HO-1 expression (Fig. 7A). To exclude the possibility that this effect of BAY-11-7085 was due to a reduction in the number of DCs via apoptosis, we tested the apoptotic sensitivity of DCs to BAY-11-7085. According to Annexin V and propidium iodide staining of CD11c$^+$ cells, no significant differences were observed below 20 μM (Fig. 7B). Next, to confirm the role of NF-κB in LPS-induced HO-1 expression, we assessed expression of this enzyme in DCs transfected with siRNA against the NF-κB p65 subunit. As shown in Fig. 7C, LPS-induced expression of HO-1, but not HO-2, was diminished in siRNA-transfected DCs in a dose-dependent manner. These results indicate that LPS modulates HO-1 expression via an NF-κB-dependent pathway in BMDCs.

### 3.8. BAY-11-7085 inhibits IDO expression and pro-inflammatory cytokine production in LPS-induced BMDCs

To determine whether the NF-κB pathway is involved in IDO expression and the production of pro-inflammatory cytokines in BMDCs, we pretreated cells with BAY-11-7085 for 2 h prior to LPS stimulation. Like ZnPP-treated cells, LPS-induced IDO expression (Fig. 8A) and pro-inflammatory cytokine production (Fig. 8B) were decreased in BAY-11-7085-treated BMDCs in a dose-dependent manner. The binding of transcription factors such as STAT1 and NF-κB to gamma activated sequence (GAS) site of the IRF-1 is essential for the induction of IDO by IFN-γ [37]. Moreover, LPS also induced the expression IRF-1 in macrophage [38]. To determine whether LPS treatment enhanced p65 subunit of NF-κB binding to the GAS site of the IRF-1 in BMDCs, we assessed ChIP assay using p65-specific Ab and PCR. As shown in Fig. 8C, we observed that the binding of p65 to GAS region of IRF-1 severely enhanced in LPS-activated BMDCs. Taken together, these results suggest that IDO expression is regulated by a NF-κB-dependent pathway in LPS-activated BMDCs.

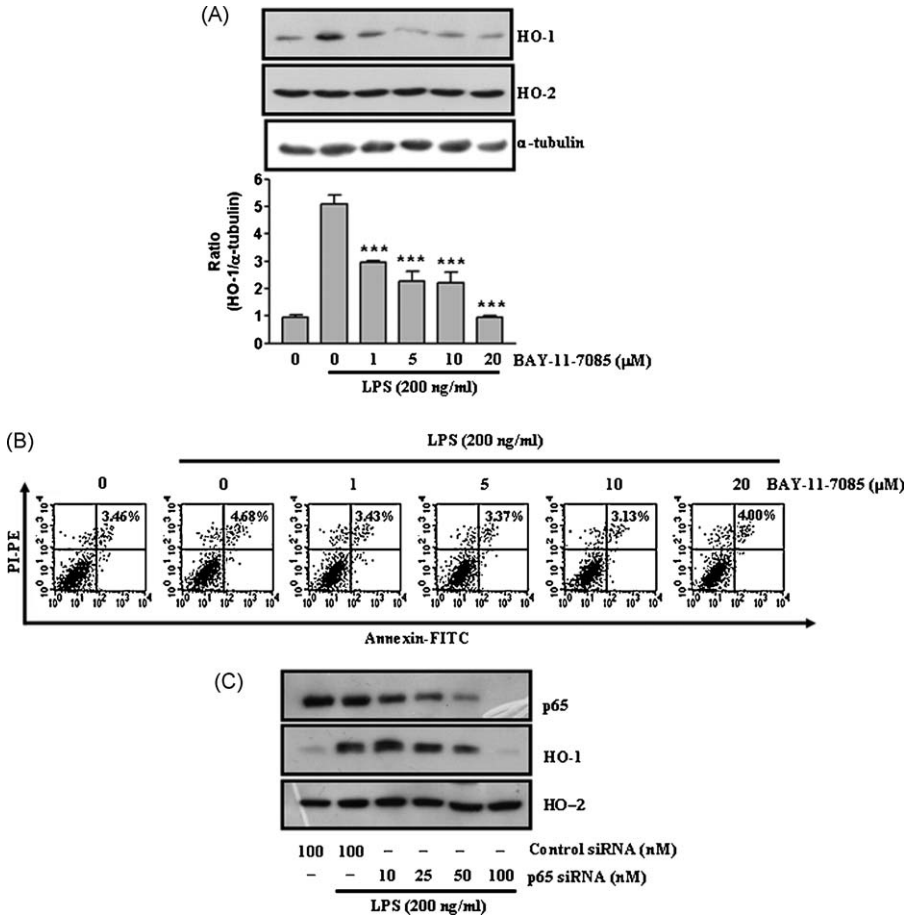


**Fig. 7.** BAY-11-7085 inhibits LPS-induced HO-1 expression in BMDCs. (A and B) BMDCs were pre-incubated without or with BAY-11-7085 (1, 5, 10 or 20 μM) for 2 h, and then further incubated with or without 200 ng/ml LPS for 24 h. (A) HO-1, HO-2 and α-tubulin levels were determined by immunoblotting with anti-HO-1, anti-HO-2 and anti-α-tubulin antibodies. Top panel shows a representative blot stained with anti-HO-1, anti-HO-2 and anti-α-tubulin antibodies as indicated. The mean values ± SEM obtained from three separate experiments are shown in the bottom panel. $^{***}P < 0.001$. vs. responses in the absence of BAY-11-7085 with LPS stimulation. (B) DCs were stained with annexin-V and PI. The percentage within each histogram represents the incidence of annexin-$V^+PI^+$. The results are representative of five separate experiments. (C) BMDCs were transfected with HO-1 siRNA (10, 25, 50 or 100 nM) or control siRNA (100 nM) for 24 h, and then further incubated with 200 ng/ml LPS for 24 h. HO-1, HO-2, IDO and α-tubulin levels were determined by immunoblotting with anti-HO-1, anti-HO-2, anti-IDO and anti-α-tubulin antibodies. One representative experiment of three is shown.

## 4. Discussion

To the best of our knowledge, this is the first report describing the role of HO-1 on LPS-induced IDO expression and the effect of IDO deficiency on DC maturation. DCs have a unique ability to enhance or suppress T cell immune responses. IDO expression in these cells is critical to the suppressive mechanisms. IDO is a key immunomodulatory enzyme that promotes peripheral immune tolerance by inhibiting T cell proliferation and activation by regulating tryptophan catabolism [39,40]. IDO contains heme as the sole prosthetic group and catabolizes tryptophan into kynurenie, which leads to tryptophan depletion. These events result in the suppression of proliferation or induction of apoptosis in activated T cells *in vivo* and *in vitro* [8,41]. Alterations in this mechanism can lead to autoimmune dysfunction, including the suppression of tumor cell targeting by T cells [42,43]. IDO is also an important regulator of immunity in infections and transplantation [7]. We have recently demonstrated that IDO play a critical role in severe sepsis by endotoxin via the IL-12 and IL-10 balance on IDO [44]. Furthermore, it has been recently reported that genetically disrupted of IDO inhibited the DC phenotype and function *in vitro* but not *in vivo* [45].

By assessing the effect of IDO deficiency, we showed that IDO regulates DC maturation and that HO-1 initiates a specific Th1 immune response by regulation of IDO expression. The expression of IDO was increased in DCs in response to different stimuli, including LPS and IFN-γ. LPS-mediated TLR4 signaling induces IDO expression to promote DC maturation in humans and mice. [13,14]. In addition, IFN-γ-treated murine BMDCs inhibit T cell proliferation via induction of IDO [40,46]. Therefore, we examined the role of IDO in the maturation of BMDCs and splenic DCs isolated from WT and $IDO^{-/-}$ mice and found that IDO is indeed a potent mediator of DC maturation in response to LPS. The LPS-induced increase in expression of CD80, CD86, MHC class I, and MHC class II exhibited by WT BMDCs and splenic DCs was attenuated in $IDO^{-/-}$ cells, suggesting that IDO regulates the phenotypic and functional maturation of murine DCs.

DCs can prime T helper cells to a Th1 immune response, leading to the production of cytokines, namely IL-12, IL-6, IL-1β, and TNF-α, that play a critical role in inflammation (e.g., sepsis). We found that the production of IL-12 p70 and TNF-α was downregulated in IDO knockout BMDCs. These data show that IDO is necessary for the generation of IL-12 p70 and TNF-α in response to LPS stimulation, which is consistent with a previous report that demonstrated that the IDO inhibitor, 1-MT, inhibits production of IL-12 p70 by human DCs [14]. In addition, we elucidated the signaling mechanism involved in mediating IDO expression in murine BMDCs in response to LPS stimulation. We and other groups reported that IDO induction by LPS in human monocytes and murine BMDCs is independent of IFN-γ and occurs via the p38

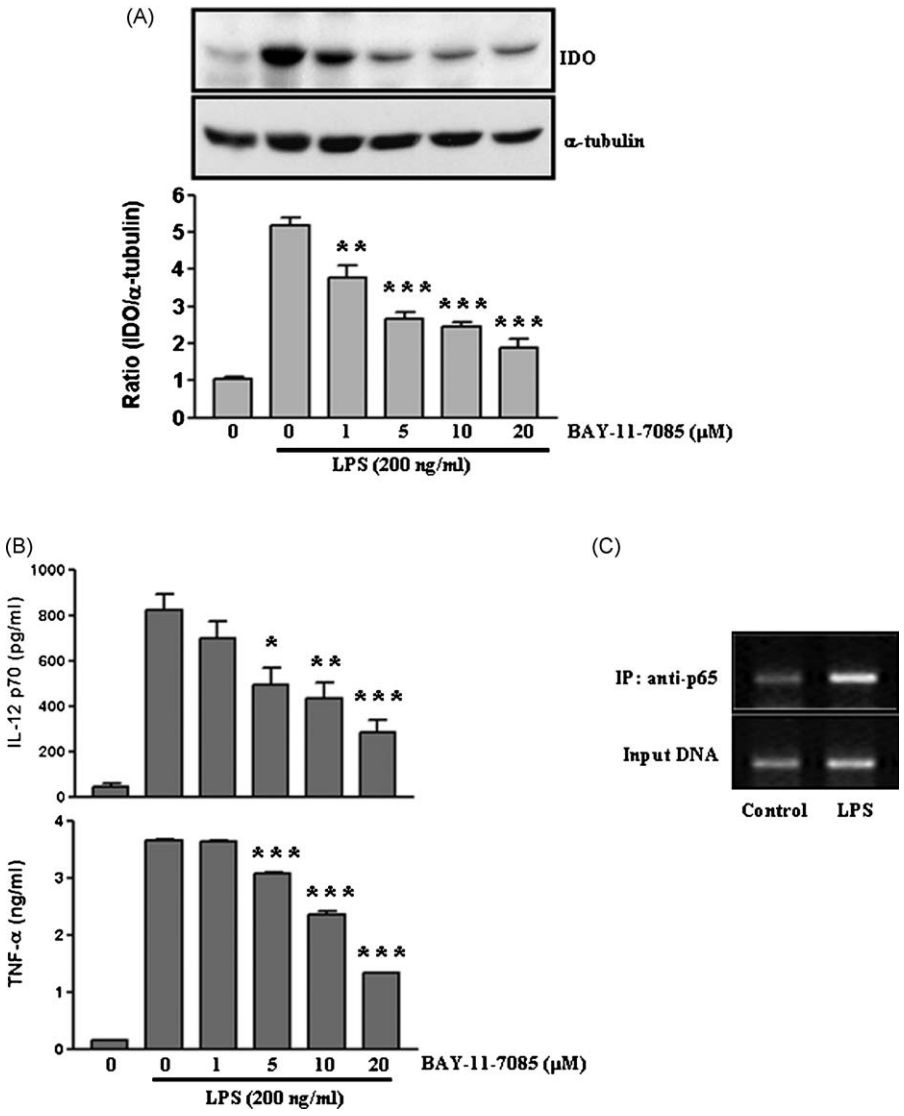


**Fig. 8.** BAY-11-7085 inhibits LPS-induced IDO expression and production of pro-inflammatory cytokines in BMDCs. (A and B) BMDCs were pre-treated with the indicated concentrations of BAY-11-7085 for 2 h prior to stimulating with 200 ng/ml LPS for 24 h. (A) IDO and α-tubulin levels were determined by immunoblotting with anti-IDO and anti-α-tubulin antibodies. Top panel shows a representative blot stained with anti-IDO and anti-α-tubulin antibodies as indicated. The mean values ± SEM obtained from three separate experiments are shown in the bottom panel. $^{**}P < 0.01$ and $^{***}P < 0.001$. vs. responses in the absence of BAY-11-7085 with LPS stimulation. (B) IL-12 p70 and TNF-α concentrations were measured by ELISA. Data are presented as mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. responses in the absence of BAY-11-7085 with LPS stimulation. (C) BMDCs were treated with LPS (200 ng/ml) for 1 h. ChIP assays were performed after immunoprecipitation (IP) of chromatin with anti-p65 Ab. Immunoprecipitated chromatin and 1% of input chromatin used for each immunoprecipitation were subjected to PCR with primers specific for the GAS site of IRF-1 promoter.

MAPK, NF-κB, and STAT pathways [6,47]. Furthermore, several upstream signaling pathways are known to increase HO-1 expression. Park et al. [48] reported that up-regulation of HO-1 expression by rottlerin is regulated by reactive oxygen species (ROS), p38 MAPK and ERK in human colon cancer HT29 cells. Here we demonstrate that the NF-κB pathway plays a role in the regulation of IDO expression and HO-1 is an important mediator of this regulatory process. In addition, we found that the induction of HO-1 expression by LPS is inhibited by SP600125 (a specific inhibitor of JNK) (Supplementary Figure 1A) but not by SB203580 (a specific inhibitor of p38 MAPK) (Supplementary Figure 1B), indicating that HO-1 expression is upregulated by LPS dependant on JNK pathway.

Recent studies show that HO-1 is critical for transplantation tolerance as well as for the prevention of different immune-related diseases [49]. This enzyme is also known for its cytoprotective effect against oxidative inflammation. HO-1 catalyzes heme degradation, resulting in local production of carbon monoxide, biliverdin, and free iron, which induces the expression of heavy chain ferritin, an iron-binding protein. All three metabolites produced have been reported to have immune protective effects [49]. In addition, recent evidence suggests that expression of HO-1 in DCs may be involved in some tolerogenic effects [23]. HO-1 expression in T cells is not required for Treg function, supporting the fact that the tolerogenic effect of HO-1 may occur via its expression in the graft or by DCs rather than in infiltrating T cells and Tregs. Other studies have shown that HO-1 expression is lower in unstimulated immature DCs and in DCs treated with IL-10 or LPS [23]. Induction of HO-1 expression also increases the activity and expression of IDO in LPS-activated murine macrophages [50]. However the exact role of HO-1 in this process remains to be elucidated.

To investigate the mechanism by which HO-1 can affect IDO expression in mouse DCs, we analyzed the expression of cell surface molecules and proinflammatory cytokines in these cells. The HO antagonist ZnPP inhibited LPS-induced IDO expression in

BMDCs and splenic DCs, and also suppressed the expression of cell surface molecules and pro-inflammatory cytokines. These data suggest that HO-1 is involved in modulating IDO expression, which in turn induces LPS-mediated DC maturation. Moreover, siRNA-mediated knockdown of HO-1 also inhibited LPS-induced IDO expression, whereas IDO deficiency did not affect the induction of HO-1 by LPS. Heme also induced the expression of IDO and IL-12 p70 and TNF-α. Together, these results indicate that heme and HO-1 may act as potent pro-inflammatory mediators of DC maturation via the IDO pathway in murine BMDCs. The NF-κB signaling pathway is involved in regulating IDO gene expression in various cell types [47,51,52]. Consistent with this, we found that the NF-κB inhibitor, BAY-11-7085, inhibited LPS-induced HO-1 and IDO expression significantly and attenuated production of the pro-inflammatory cytokines IL-12 p70 and TNF-α.

In conclusion, the data presented here indicate that endotoxin-induced HO-1 and IDO expression elicits a pro-inflammatory response in DCs. In addition, HO-1-mediated IDO expression may regulate these inflammatory mechanisms and act as an effective target for modulating inflammatory immune responses by endotoxin.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.04.025.

## References

[1] Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol 2003;21:685–711.
[2] Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767–811.
[3] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245–52.
[4] Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol 2005;5(8):617–28.
[5] Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol Today 1999;20(10):469–73.
[6] Jung ID, Lee CM, Jeong YI, Lee JS, Park WS, Han J, et al. Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells. FEBS Lett 2007;581(7):1449–56.
[7] Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004;4(10):762–74.
[8] Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol 2000;164(7):3596–9.
[9] Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281(5380):1191–3.
[10] Mellor A. Indoleamine 2,3 dioxygenase and regulation of T cell immunity. Biochem Biophys Res Commun 2005;338(1):20–4.
[11] Ueno A, Cho S, Cheng L, Wang J, Hou S, Nakano H, et al. Transient upregulation of indoleamine 2,3-dioxygenase in dendritic cells by human chorionic gonadotropin downregulates autoimmune diabetes. Diabetes 2007;56(6):1686–93.
[12] Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 1999;189(9):1363–72.
[13] Hwang SL, Chung NP, Chan JK, Lin CL. Indoleamine 2, 3-dioxygenase (IDO) is essential for dendritic cell activation and chemotactic responsiveness to chemokines. Cell Res 2005;15(3):167–75.
[14] Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, et al. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. Eur J Immunol 2007;37(11):3054–62.
[15] Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–54.
[16] Otterbein LE, Choi AM. Heme oxygenase: colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol 2000;279(6):L1029–37.
[17] Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat Med 1996;2(1):87–90.
[18] Hancock WW, Gao W, Shemmeri N, Shen XD, Gao F, Busuttil RW, et al. Immunopathogenesis of accelerated allograft rejection in sensitized recipients: humoral and nonhumoral mechanisms. Transplantation 2002;73(9):1392–7.
[19] Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, et al. Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med 1998;4(9):1073–7.
[20] Chauveau C, Bouchet D, Roussel JC, Mathieu P, Braudeau C, Renaudin K, et al. Gene transfer of heme oxygenase-1 and carbon monoxide delivery inhibit chronic rejection. Am J Transplant 2002;2(7):581–92.
[21] Braudeau C, Bouchet D, Tesson L, Iyer S, Remy S, Buelow R, et al. Induction of long-term cardiac allograft survival by heme oxygenase-1 gene transfer. Gene Ther 2004;11(8):701–10.
[22] Araujo JA, Meng L, Tward AD, Hancock WW, Zhai Y, Lee A, et al. Systemic rather than local heme oxygenase-1 overexpression improves cardiac allograft outcomes in a new transgenic mouse. J Immunol 2003;171(3):1572–80.
[23] Chauveau C, Remy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert FX, et al. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. Blood 2005;106(5):1694–702.
[24] Listopad J, Asadullah K, Sievers C, Ritter T, Meisel C, Sabat R, et al. Heme oxygenase-1 inhibits T cell-dependent skin inflammation and differentiation and function of antigen-presenting cells. Exp Dermatol 2007;16(8):661–70.
[25] Mashreghi MF, Klemz R, Knosalla IS, Gerstmayer B, Janssen U, Buelow R, et al. Inhibition of dendritic cell maturation and function is independent of heme oxygenase 1 but requires the activation of STAT3. J Immunol 2008;180(12):7919–30.
[26] Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992;176(6):1693–702.
[27] Wang Q, Zhang W, Ding G, Sun L, Chen G, Cao X. Dendritic cells support hematopoiesis of bone marrow cells. Transplantation 2001;72(5):891–9.
[28] Srisook K, Cha YN. Biphasic induction of heme oxygenase-1 expression in macrophages stimulated with lipopolysaccharide. Biochem Pharmacol 2004;68(9):1709–20.
[29] Blanco P, Palucka AK, Pascual V, Banchereau J. Dendritic cells and cytokines in human inflammatory and autoimmune diseases. Cytokine Growth Factor Rev 2008;19(1):41–52.
[30] Kranzer K, Bauer M, Lipford GB, Heeg K, Wagner H, Lang R. CpG-oligodeoxynucleotides enhance T-cell receptor-triggered interferon-gamma production and up-regulation of CD69 via induction of antigen-presenting cell-derived interferon type I and interleukin-12. Immunology 2000;99(2):170–8.
[31] Xia ZW, Xu LQ, Zhong WW, Wei JJ, Li NL, Shao J, et al. Heme oxygenase-1 attenuates ovalbumin-induced airway inflammation by up-regulation of foxp3 T-regulatory cells, interleukin-10, and membrane-bound transforming growth factor- 1. Am J Pathol 2007;171(6):1904–14.
[32] Hayashi S, Takamiya R, Yamaguchi T, Matsumoto K, Tojo SJ, Tamatani T, et al. Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. Circ Res 1999;85(8):663–71.
[33] Kim HP, Ryter SW, Choi AM. CO as a cellular signaling molecule. Annu Rev Pharmacol Toxicol 2006;46:411–49.
[34] Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. J Immunol 2001;166(6):3837–45.
[35] An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, et al. Involvement of ERK, p38 and NF-kappaB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 2002;106(1):38–45.
[36] Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. J Exp Med 1998;188(11):2175–80.
[37] Darnell Jr JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264(5164):1415–21.
[38] Ohmori Y, Hamilton TA. Requirement for STAT1 in LPS-induced gene expression in macrophages. J Leukoc Biol 2001;69(4):598–604.
[39] Katz JB, Muller AJ, Prendergast GC. Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. Immunol Rev 2008;222:206–21.
[40] Lee HJ, Jeong YI, Lee TH, Jung ID, Lee JS, Lee CM, et al. Rosmarinic acid inhibits indoleamine 2,3-dioxygenase expression in murine dendritic cells. Biochem Pharmacol 2007;73(9):1412–21.
[41] Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T cell apoptosis by tryptophan catabolism. Cell Death Differ 2002;9(10):1069–77.
[42] Grohmann U, Fallarino F, Bianchi R, Orabona C, Vacca C, Fioretti MC, et al. A defect in tryptophan catabolism impairs tolerance in nonobese diabetic mice. J Exp Med 2003;198(1):153–60.
[43] Friberg M, Jennings R, Alsarraj M, Dessureault S, Cantor A, Extermann M, et al. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. Int J Cancer 2002;101(2):151–5.

[44] Jung ID, Lee MG, Chang JH, Lee JS, Jeong YI, Lee CM, et al. Blockade of indoleamine 2,3-dioxygenase protects mice against lipopolysaccharide-induced endotoxin shock. J Immunol 2009;182(5):3146–54.
[45] de Faudeur G, de Trez C, Muraille E, Leo O. Normal development and function of dendritic cells in mice lacking IDO-1 expression. Immunol Lett 2008;118(1): 21–9.
[46] Jeong YI, Jung ID, Lee JS, Lee CM, Lee JD, Park YM. (−)-Epigallocatechin gallate suppresses indoleamine 2,3-dioxygenase expression in murine dendritic cells: evidences for the COX-2 and STAT1 as potential targets. Biochem Biophys Res Commun 2007;354(4):1004–9.
[47] Fujigaki H, Saito K, Fujigaki S, Takemura M, Sudo K, Ishiguro H, et al. The signal transducer and activator of transcription 1alpha and interferon regulatory factor 1 are not essential for the induction of indoleamine 2,3-dioxygenase by lipopolysaccharide: involvement of p38 mitogen-activated protein kinase and nuclear factor-kappaB pathways, and synergistic effect of several proinflammatory cytokines. J Biochem 2006;139(4):655–62.
[48] Park EJ, Lim JH, Nam SI, Park JW, Kwon TK. Rottlerin induces heme oxygenase-1 (HO-1) up-regulation through reactive oxygen species (ROS) dependent and PKC delta-independent pathway in human colon cancer HT29 cells. Biochimie 92(1): 110–5.
[49] Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;24(8): 449–55.
[50] Oh GS, Pae HO, Choi BM, Chae SC, Lee HS, Ryu DG, et al. 3-Hydroxyanthranilic acid, one of metabolites of tryptophan via indoleamine 2,3-dioxygenase pathway, suppresses inducible nitric oxide synthase expression by enhancing heme oxygenase-1 expression. Biochem Biophys Res Commun 2004;320(4): 1156–62.
[51] Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11(3):312–9.
[52] Robinson CM, Hale PT, Carlin JM. NF-kappaB activation contributes to indoleamine dioxygenase transcriptional synergy induced by IFN-gamma and tumor necrosis factor-alpha. Cytokine 2006;35(1–2):53–61.