Carbon Monoxide Inhibits TLR-Induced Dendritic Cell Immunogenicity[1]

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Heme oxygenase-1 (HO-1) exerts its functions via the catabolism of heme into carbon monoxide (CO), Fe^{2+}, and biliverdin, as well as by depletion of free heme. We have recently described that overexpression of HO-1 is associated with the tolerogenic capacity to dendritic cells (DCs) stimulated by LPS. In this study, we demonstrate that treatment of human monocyte-derived DCs with CO blocks TLR3 and 4-induced phenotypic maturation, secretion of proinflammatory cytokines, and alloreactive T cell proliferation, while preserving IL-10 production. Treatment of DCs with biliverdin, bilirubin, and deferoxamine or replenishing intracellular heme stores had no effect on DC maturation. HO-1 and CO inhibited LPS-induced activation of the IFN regulatory factor 3 pathway and their effects were independent of p38, ERK, and JNK MAPK. HO-1 and CO treatment also inhibited mouse DC maturation in vitro and mouse DC immunogenic properties in vivo, as shown by adoptive cell transfer in a transgenic model of induced diabetes. Thus, for the first time, our data show that CO treatment inhibits DC immunogenicity induced by TLR ligands and that blockade of IFN regulatory factor 3 is associated with this effect. *The Journal of Immunology,* **2009, 182: 1877–1884.**

Heme oxygenases are the rate-limiting enzymes in the catabolism of heme, yielding equimolar amounts or carbon monoxide (CO),[5] free iron, and biliverdin (BV) (1), which is subsequently reduced into bilirubin (BL). Heme oxygenase 1 (HO-1), the inducible form of heme oxygenases, has protective effects in a variety of experimental inflammatory models (reviewed in Ref. 2). The physiological importance of HO-1 has been demonstrated in both mice and humans, where HO-1 deficiency resulted in a progressive and chronic inflammation and a reduced cellular resistance to oxidative stress (3–5). Induction of HO-1 expression by pharmacological activators or gene transfer have therapeutic effects in a variety of conditions or disorders involving inflammation and immune responses, including organ transplantation and autoimmunity (6–12). In several models, CO mimics the effects of HO-1 (reviewed in Ref. 13), indicating that HO-1 acts via the generation of CO. However, other end products of HO-1 activity, such as BV (14), free iron depletion by increased H chain ferritin expression (15), or cellular efflux pumps (16), or heme depletion (17) can also mediate the effects of HO-1.

Dendritic cells (DCs) play a major role in the initiation and regulation of the immune response. They have distinct stages of cell development, activation, and maturation and have the potential to induce both immunity and tolerance (reviewed in Ref. 18). In the absence of inflammation, immature DCs (iDCs) located in peripheral tissues continuously capture innocuous and cell-associated self-Ags and migrate to draining lymph nodes where they can induce tolerance (19). In the presence of danger and TLR signals, DCs mature, acquiring the ability to stimulate differentiation of naive T cells into effector cells. In certain conditions, phenotypically mature DCs have tolerogenic functions (18).

We previously showed that human and rat iDCs express HO-1, that this expression is restricted to certain DC populations, and that HO-1 expression drastically decreases upon DC maturation (20). We and others have demonstrated that overexpression of HO-1 in DCs inhibits their LPS-induced maturation and proinflammatory functions (20, 21), and it has been recently demonstrated that HO-1 expression in mouse DCs modulates the suppressive capacity of regulatory T cells (22). However, nothing is known regarding the role of the end products of heme degradation on DC functions.

In this study, we demonstrate that CO is the only end product capable of inhibiting LPS-induced DC phenotypic maturation, secretion of proinflammatory cytokines, and induction of alloreactive T cell proliferation, while preserving their production of IL-10. HO-1 and CO also inhibit proinflammatory cytokine production by

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[5] Abbreviations used in this paper: CO, carbon monoxide; DC, dendritic cell; iDC, immature DC; HO-1, heme oxygenase 1; IRF-3, IFN regulatory factor 3; HA, hemagglutinin; CoPP, cobalt protoporphyrin; MnPP, manganese protoporphyrin; CORM2, tricarbonyldichlororuthenium(II) dimer; iCORM, inactive form of CORM2; BV, biliverdin; BL, bilirubin; DFO, deferoxamine; poly(I:C), polyinosinic:polycytidylic acid; [^{3}H]Td, [^{3}H]thymidine; TRIF, TIR domain adaptor-inducing IFN.

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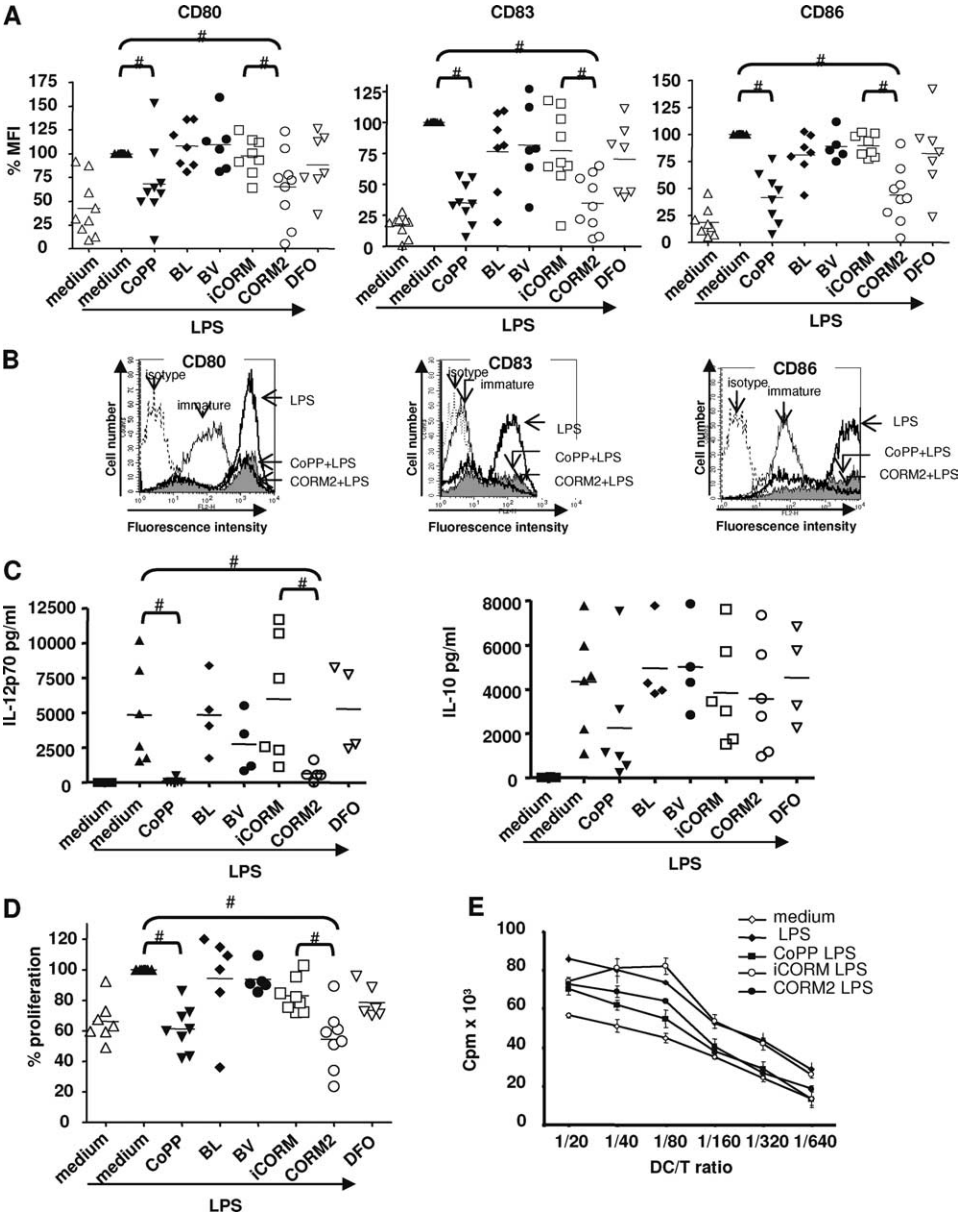

FIGURE 1. CO renders DCs refractory to LPS-induced maturation. *A*, FACS analysis showing the phenotype of human DCs treated or not with CoPP, BV, BL, iCORM, CORM2, or DFO and stimulated with LPS. Expression of cell markers was considered to be maximal (100%) for LPS treatment. Each point corresponds to an individual experiment and the horizontal bar corresponds to the mean. #, $p < 0.05$. *B*, Histograms for FACS analysis of CD80, CD83, and CD86 expression for one of six to nine representative experiments. *C*, Production of IL-12 p70 and IL-10 in the supernatants was assessed by ELISA. #, $p < 0.05$. *D*, Proliferation of allogeneic T cells induced by 4 days of culture with human DCs (ratio 1:20). Proliferation induced by LPS-treated DCs was considered to be maximal (from 19,500 to 73,000 cpm). #, $p < 0.05$. *E*, One representative MLR experiment of five to eight experiments is shown. Results are expressed as means ± SD of triplicate values after subtraction of spontaneous [^{3}H]Td. #, $p < 0.05$ compared with LPS-treated DCs.

DCs induced by the TLR3 ligand. An analysis of the intracellular signaling pathways showed that HO-1 and CO inhibit the phosphorylation of IFN regulatory factor 3 (IRF-3). Importantly, transgenic mice expressing influenza virus hemagglutinin (HA) in pancreatic β cells and adoptively transferred with transgenic anti-HA $CD8^+$ T cells did not develop diabetes when immunized with HO-1-overexpressing or CO-treated HA peptide-loaded DCs, whereas mice immunized with control HA peptide-loaded DCs did. These provided evidence that HO-1-overexpressing or CO-treated HA peptide-loaded DCs display impaired immunogenic functions in vivo.

Materials and Methods

Mice

The Ins-HA-transgenic mice (23) express HA of the influenza virus in pancreatic islets and the TCR-$HA_{512–520}$ transgenic mice (24) express a TCR-specific for the H-$2K^d$-restricted (IYSTVASSL) epitope of HA (Département de Cryopréservation, Typage, et Archivage Animal Orléans, France). All animal experiments were performed under specific pathogen-free conditions in accordance with the European Union Guidelines.

Cell preparation, culture, and treatments

Mouse DCs. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10 ng/ml of supernatant from COS cells transfected with murine GM-CSF cDNA, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 mM 2-ME (all from Sigma-Aldrich). At day 8, a positive selection of iDCs (purity >95%) was performed by magnetic cell sorting using mouse CD11c microbeads (Miltenyi Biotec).

Human monocyte-derived DCs. Human PBMC were isolated following standard procedures from leukapheresis samples of healthy donors following institutional-approved protocols (Etablissement Français du Sang, Nantes, France). Positive selection of monocytes (purity >90%) was performed by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec). Monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from Sigma-Aldrich), IL-4 (40 ng/ml; R&D systems), and GM-CSF (1000 IU/ml; Gentaur). At day 5,

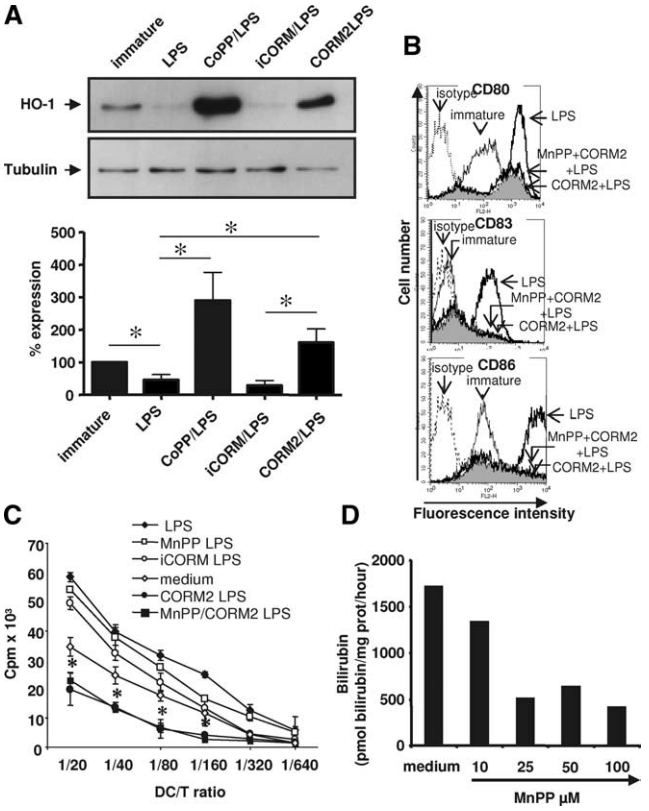

FIGURE 2. CO renders DCs refractory to LPS-induced maturation independently of HO-1 induction. *A*, *upper*, Western blot analysis showing HO-1 expression in human DCs treated or not with CoPP, iCORM, or CORM2 and stimulated or not with LPS. Anti-β tubulin Ab was used as a loading control. *A*, *lower*, Bar graphs show densitometry analysis ± SD of HO-1 Western blot signals after normalization with β-tubulin. Data are the mean ± SD of six independent experiments. *, $p < 0.05$. *B*, FACS analysis showing the phenotype of human DCs treated or not with CORM2 and/or MnPP and stimulated with LPS. Similar results were obtained in three independent experiments. *C*, DCs were treated or not with iCORM, CORM2, and/or MnPP and stimulated or not with LPS. Thereafter, DCs were cultured with allogeneic T cells at different ratios for 4 days and proliferating T cells were labeled with [^{3}H]Td. Results are shown as means ± SD of triplicate values after subtraction of spontaneous [^{3}H]Td. *, $p < 0.005$ compared with iCORM plus LPS-treated DCs. Similar results were obtained in three independent experiments. *D*, The concentrations of MnPP used in this study inhibit HO-1 activity. Rat NR8383 macrophages which spontaneously express a high level of HO-1 were treated or not with different doses of MnPP for 24 h. HO activity was assessed by BL production in microsomal pellets of the cells. BL production was measured spectrophotometrically and expressed as picomoles of BL per milligram of protein per hour (453 nm = 40 mM to1 cm^{-1}).

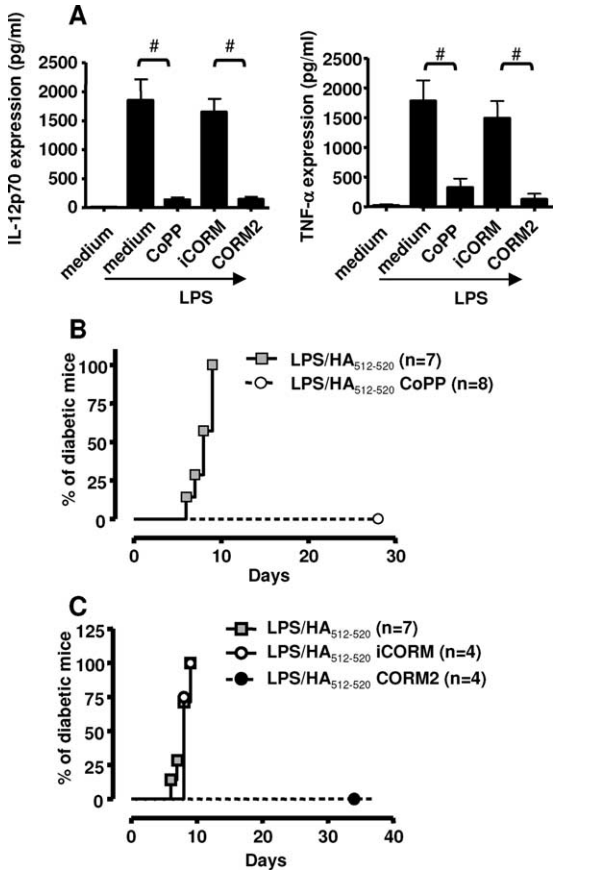

FIGURE 3. HO-1 overexpression and CO treatment inhibit maturation of mouse DCs in vitro and immunogenicity in vivo. *A*, Production of IL-12 p70 and TNF-α in the supernatants of mouse bone marrow-derived DCs, treated or not with CoPP, iCORM, or CORM2 and then stimulated with LPS for 24 h, was assessed by ELISA. Data presented are mean ± SD of 4–13 independent experiments for IL-12p70 and of three independent experiments for TNF-α. #, $p < 0.05$. *B* and *C*, Diabetes incidence after transfer of LPS-treated, LPS plus CoPP-treated, and after transfer of LPS plus iCORM-treated and LPS plus CORM2-treated HA-loaded DCs. $n = 4–8$ for each group from two experiments performed separately.

iDCs were harvested and cultured using the above described medium.

Treatment of DCs. iDCs were treated with cobalt protoporphyrin (CoPP), an inducer of HO-1, as previously described (20). Briefly, iDCs were pulsed for 2 h with 50 μM CoPP, washed, and then cultured with TLR ligands. iDCs were also treated 2 h before addition of TLR ligands with the HO-1 inhibitor manganese protoporphyrin (MnPP; 50 μM, also during TLR stimulation) (all porphyrins were protected from light at all times and were from Frontier Scientific), tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$, CORM2; 30 μM for human DCs, 100 μM for mouse DCs; Sigma-Aldrich), and the inactive form of CORM2 (iCORM, $(Ru(DMSO)_4Cl_2)$) with the only substitution of carbonyl groups (released as CO) by DMSO (25), bilirubin (BL, 10 μM; Calbiochem), BV (10 μM; MP Biomedical), the iron chelator deferoxamine mesylate (DFO, 400 μM; Sigma-Aldrich), JNK inhibitor SP600125 (10 μM), p38 MAPK inhibitor pyridinyl imidazole SB203580, (20 μM), and ERK inhibitor PD98059 (10 μM) (all from Calbiochem). Treated DCs were then cultured for 24 h with LPS (1 μg/ml, *Escherichia coli* 0111:B4; Sigma-Aldrich), or polyinosinic:polycytidylic acid (poly(I:C), 100 μg/ml; Sigma-Aldrich). For diabetes induction, DCs were pulsed for 2 h with $HA_{512–520}$ peptide (5 μM; Sigma-Aldrich) and LPS (1 μg/ml; Sigma-Aldrich).

Mixed Lymphocyte Reaction

DCs were cultured in triplicate with 10^5 allogeneic PBL as previously described (20). Proliferation was determined 4 days later by uptake of [^{3}H]thymidine ([^{3}H]Td, 0.5 μCi/well; Amersham Biosciences) during the last 8 h of culture.

Cell extracts and Western blot analysis

Western blot analysis was performed as previously described (7). Briefly, cell protein extracts were electrophoresed on a SDS-polyacrylamide gel and blotted. Membranes were blocked and incubated with Abs. HO-1 was detected using a rabbit anti-HO-1 Ab (StressGen). Total and activated/phosphorylated forms of extracellular signal-regulated kinases (ERK1 and

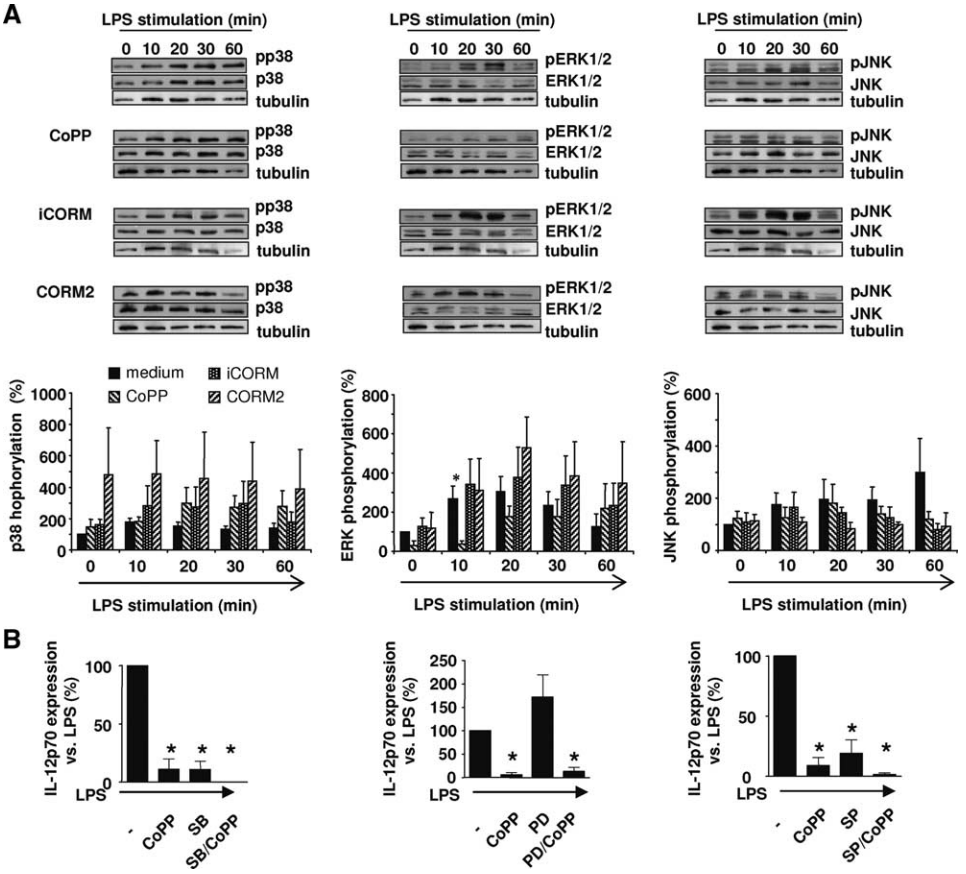

FIGURE 4. HO-1 and CO render DCs refractory to LPS-induced maturation independently of the p38 MAPK, ERK, and JNK pathways. *A*, Human DCs were treated or not with CoPP, iCORM, or CORM2 and stimulated with LPS for 10, 20, 30, and 60 min. Western blots show representative experiments from three to five experiments for p38, ERK, and JNK MAPK phosphorylated or not. Anti-β- tubulin Ab was used as a loading control. Bar graphs show densitometry analysis $\pm$ SD of MAPK phosphorylation signals after normalization with the total expression of JNK and ERK for the respective phosphorylated forms and with β-tubulin for phosphorylated p38 since we observed increased expression of total p38 following LPS treatment. Data are the mean $\pm$ SD of three to five independent experiments. *, $p < 0.05$. *B*, Human DCs were treated or not with CoPP and/or with SB203580 (SB), PD98059 (PD), or SP600125 (SP) inhibitors of p38 MAPK, ERK, and JNK, respectively, and then stimulated with LPS for 24 h. Secretion of IL-12p70 was considered to be maximal for LPS treatment (from 1,743 to 26,100 pg/ml). Data presented are the mean $\pm$ SD of three to five independent experiments. *, $p < 0.05$.

2), c-Jun NH_2 -terminal kinases (JNK1–3), and p38 MAPK and the phosphorylated forms of IκBα and IRF-3 were detected using rabbit Abs. Cell β-tubulin was detected using a mouse anti-β-tubulin Ab (Calbiochem). Membranes were then incubated with HRP-labeled secondary Abs (Jackson ImmunoResearch Laboratories) and detection was performed by ECL (Amersham Biosciences). Blots were analyzed by densitometry following nonsaturating exposure.

NF-κB luciferase reporter assay

Human DCs were transduced at 400:1 multiplicity of infection with a recombinant replication-deficient adenovirus containing a NF-κB promoter luciferase reporter gene provided by Dr. P. McCray (University of Iowa). DCs were cultured for 24 h before being treated or not with CoPP, iCORM, or CORM2 and LPS stimulated for 6 h. Then, the cells were washed and lysed in luciferase cell lysis buffer (Promega). Luciferase activity was measured from cell lysates with VICTOR2 (PerkinElmer) using a luciferase assay system (Promega).

Flow cytometry

DCs were stained with anti-CD80, anti-CD83, and anti-CD86 PE-conjugated mAbs (Immunotech). Staining was assessed on viable DCs excluding TO-PRO-3 iodide or 7-actinoaminomycin D using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). PE-labeled mouse anti-IgG1 Ab (Immunotech) was used as a negative control.

Cytokine measurement

Human ELISA kits were used for IL-12p70, p40, IL-23, TNF-α, IL-10, and IFN-inducible protein 10 (IP-10; BD Pharmingen), and IFN-β (PBL Biomedical Laboratories) and mouse ELISA kits were used for IL-12p70, TNF-α, and IP-10 (BD Pharmingen).

Diabetes induction

Six- to 8-wk-old Ins-HA mice were injected i.v. with 0.5×10^6 $CD8^+$ T cells (purity >95%) isolated from $HA_{512–520}$ TCR-transgenic mice (24) (Miltenyi Biotec). Twenty-four hours later, mice were injected i.v. with 15,000 HA-loaded LPS-matured DCs. Diabetes was monitored using Clinistix urinalysis strips (Bayer). Mice were considered diabetic when the glucose concentration was above 5.5 mmol/L.

Statistical analysis

Statistical significance was assessed using the nonparametric one-way ANOVA test with a Tukey post test. Differences were considered significant when $p < 0.05$.

Results

CO inhibits DC maturation and function in vitro

We previously showed that HO-1 induction in human DCs partially inhibits LPS-induced maturation (20). In this study, we investigated the role of the heme degradation products in this effect. Human iDCs were treated with CORM2 (25), BL, BV, or DFO. We induced HO-1 in DCs using CoPP, an inducer of HO-1 (2), and we used an inactive form of CORM2 (iCORM) (25) as a negative control of CO treatment. Treatments were adapted to avoid toxicity on DCs (data not shown). As for CoPP-treated cells or HO-1 gene transfer-treated DCs, CORM2-treated DCs were refractory to LPS-induced phenotypic and functional maturation (Fig. 1 and supplemental Fig. 1[6]). A significant inhibition of CD80, CD83, and CD86 cell surface expression was observed in CORM2-treated DCs compared with untreated or iCORM-treated cells incubated with LPS, whereas BL, BV, and DFO had no effect on DC maturation (Fig. 1, *A* and *B*). CORM2 treatment significantly decreased LPS induction of the proinflammatory cytokine IL-12p70 (Fig. 1*C*), IL-12p40, and IL-23, but had almost no effect on TNF-α (supplemental Fig. 2). The expression of the anti-inflammatory

[6] The online version of this article contains supplemental material.

cytokine IL-10 was preserved (Fig. 1*C*). BL, BV, and DFO treatments, on the other hand, did not influence the LPS-induced expression of IL-12p70 or IL-10 (Fig. 1*C*). Treatment with CoPP, iCORM, CORM2, BL, BV, and DFO alone (no LPS) did not induce DCs to secrete cytokines (data not shown). CORM2 plus LPS-treated DCs were weaker stimulators of allogeneic T cells compared with control LPS-treated DCs and iCORM plus LPS-treated DCs (Fig. 1, *D* and *E*). BL, BV, and DFO treatments had no effect on T cell proliferation induced by allogeneic DCs (Fig. 1*D*).

Given that CO is known to up-regulate HO-1 expression not only when administered as a gas (26, 27), but also as CORM2 (28–30), we assessed whether CORM2 could induce HO-1 expression in human DCs. Compared with LPS, CORM2 moderately increased HO-1 levels, whereas iCORM had no effect (Fig. 2*A*). The reduction on CD80, CD83, and CD86 expression (Fig. 2*B*) and on the allostimulatory capacity of CORM2-treated DCs (Fig. 2*C*) were preserved in the presence of the selective HO-1 inhibitors MnPP (at concentrations that inhibited HO-1 activity, Fig. 2*D*) and tin protoporphyrin (supplemental Fig. 3). Altogether, these results demonstrate that exogenous CO mimicked the effect of HO-1 induction on DC maturation and function, independently of HO-1 induction, suggesting that CO generated by HO-1 activity largely contributes to the actions of HO-1 on DCs.

HO-1 expression and CO treatment inhibit DC immunogenicity in a murine in vivo model

To investigate whether HO-1 overexpression and CO treatment inhibit DC functions in vivo, we used a transgenic mouse model in which diabetes is induced by DCs. We first determined whether maturation of mouse DCs was also inhibited by induction of HO-1 or CO treatment. Treatments were used at concentrations nontoxic to mouse DCs as determined by flow cytometric analysis of physical parameters and 7-aminoactinomycin D staining (data not shown). In contrast to human and rat DCs (Ref. 20 and this manuscript), CoPP-induced HO-1 overexpression or CORM2 treatment had no effect on the LPS-induced CD80, CD86, and CD40 surface expression in mouse BALB/c and C57BL/6 DCs compared with untreated or iCORM-treated cells incubated with LPS (supplemental Fig. 4). In contrast, LPS-induced expression of the proinflammatory cytokines IL-12p70 and TNF-α was significantly decreased by CoPP and CORM2 treatment (Fig. 3*A*).

To analyze the effect of HO-1 overexpression and CO treatment on Ag presentation in vivo, we used a model of induced autoimmune diabetes in transgenic mice. Ins-HA transgenic mice adoptively transferred with naive anti-HA $CD8^+$ T cells developed diabetes in 6–9 days, only when immunized with both matured and HA peptide-loaded DCs and not when DCs were either immature or not loaded with HA peptide (supplemental Fig. 5). Immature mouse DCs were treated with CoPP, iCORM, CORM2, or medium alone, then stimulated with LPS, loaded with HA peptides, and adoptively transferred to recipient mice. Our results show that CoPP- and CORM2-treated DCs (Fig. 3, *B* and *C*) or after HO-1 gene transfer (supplemental Fig. 6) are not capable of inducing diabetes, whereas mice injected with HA-loaded control DCs or DCs treated with control iCORM develop diabetes 6–9 days after DC transfer. Altogether, these results show that in vitro treatment of mouse DCs with HO-1 or CO inhibits not only LPS-induced maturation in vitro but also their immunogenic capacities in vivo.

HO-1 and CO inhibition of human DC maturation is independent of the MAPK signaling pathways

LPS has been shown to activate multiple signaling pathways in DCs, including the ERK, the JNK, and the p38 MAPK pathways (31) and this was confirmed in four of five of our experiments (Fig. 4*A*). Phosphorylation of p38 MAPK and JNK were not significantly modified following HO-1 induction or CORM2 treatment compared with control untreated or iCORM-treated conditions (Fig. 4*A*). ERK phosphorylation was significantly inhibited by CoPP only at 10 min but not by CORM2 (Fig. 4*A*). To further analyze the implication of these pathways in the effects of HO-1 on DC maturation, DCs were pretreated with inhibitors of P38 MAPK, ERK, and JNK, followed by CoPP and LPS treatment. Inhibition of the p38 MAPK or of the JNK pathway largely inhibited IL-12p70 secretion, as already described (31), and when associated to HO-1 induction IL-12p70 secretion was totally abolished (Fig. 4*B*), showing an additive effect of both treatments and confirming that the p38 or JNK MAPK pathways are not involved in the effects of HO-1 on DC maturation. Inhibition of the ERK pathway (confirmed by Western blot analysis, data not shown) slightly increased the LPS induced IL-12p70 secretion, as previously described (31), and, when combined with HO-1 expression, did not reverse the inhibition of IL-12p70 secretion observed with HO-1 alone (Fig. 4B), indicating that the ERK pathway is not implicated in the inhibitory effects of HO-1 on DC maturation.

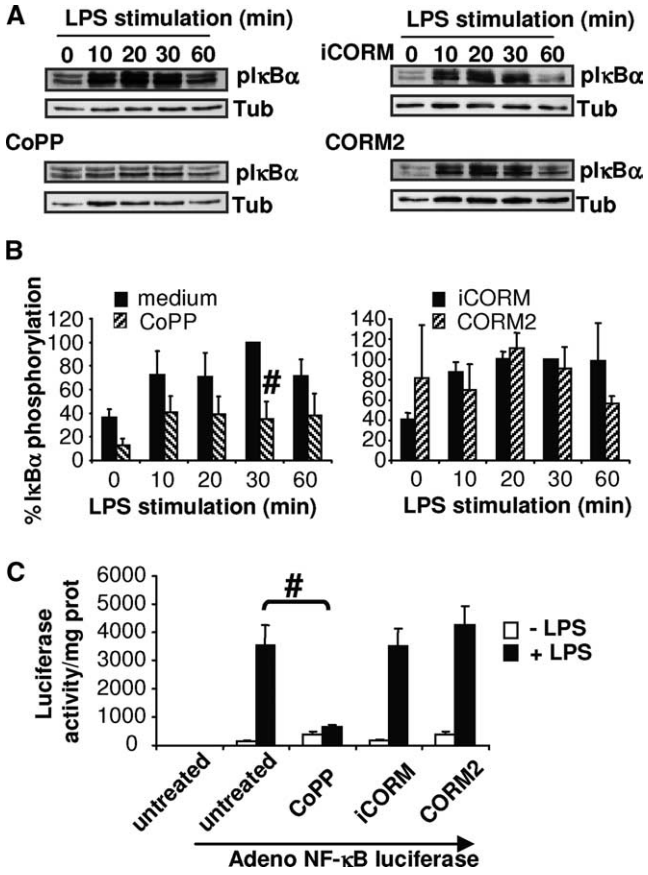

FIGURE 5. Action of HO-1 and CO on the NF-κB signaling pathway. *A*, Western blot analysis showing the phosphorylation of IκBα in human DCs treated or not with CoPP, iCORM, or CORM2 and then stimulated with LPS for 10, 20, 30, and 60 min. Anti-β-tubulin Ab was used as a loading control. Similar results were obtained in three independent experiments. *B*, Bar graphs show densitometry analysis $\pm$ SD of IκBα phosphorylation signals after normalization with β-tubulin expression. Data presented are the mean $\pm$ SD of three independent experiments. $*$, $p < 0.05$. *C*, Human DCs were transduced with a recombinant adenovirus containing a NF-κB luciferase reporter gene, cultured for 24 h, treated or not with CoPP, iCORM, or CORM2 and stimulated with LPS for 6 h. Cells were lysed and luciferase activity was measured. Similar results were obtained in three independent experiments. #, $p < 0.05$.

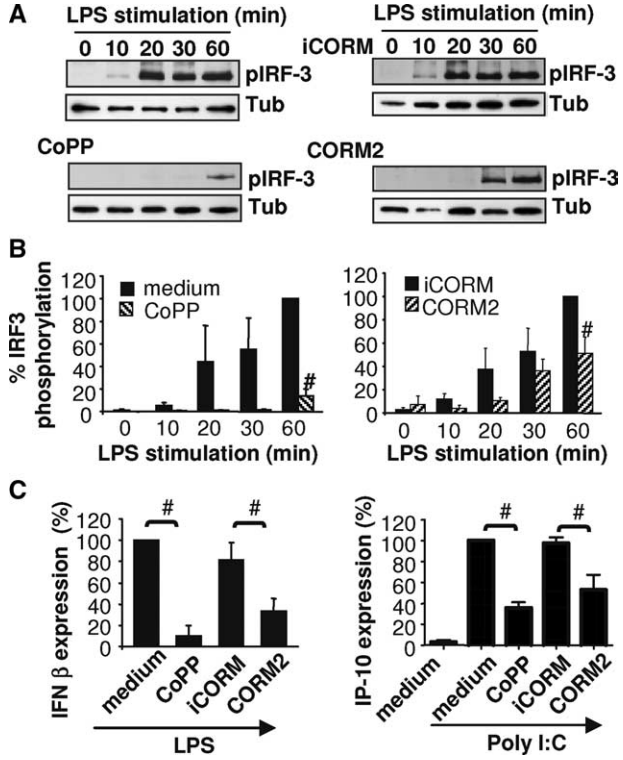

FIGURE 6. HO-1 and CO inhibit IRF-3 phosphorylation. *A*, Western blot analysis showing IRF-3 phosphorylation in human DCs treated or not with CoPP, iCORM, or CORM2 and then stimulated with LPS for 10, 20, 30, and 60 min. Anti-β-tubulin Ab was used as a loading control. Similar results were obtained in four independent experiments. *B*, Bar graphs show densitometry analysis ± SD of IRF-3 phosphorylation signals after normalization with tubulin. Phosphorylation of IRF-3 was considered to be maximal (100%) for 60 min of LPS treatment and 60 min of iCORM plus LPS treatment. *C*, IFN-β and IP-10 secretion by human DCs treated or not with CoPP, iCORM, or CORM2 and then stimulated with LPS or poly(I:C) for 24 h. Secretion was considered to be maximal for LPS (from 9 to 131 pg/ml IFN-β) and for poly(I:C) treatment (from 16,500 to 27,400 pg/ml IP-10). Data presented are the mean ± SD of seven independent experiments. #, $p < 0.05$.

HO-1 but not CO inhibits LPS-induced NF-κB activation in human DCs

The transcription factor NF-κB plays a central role in the LPS-induced maturation of human DCs (32). NF-κB is present in the cytosol as an inactive form bound to the inhibitory IκBα protein. Phosphorylation and subsequent degradation of IκBα result in the release and nuclear translocation of NF-κB (33). CoPP-induced HO-1 overexpression in DCs significantly inhibited the LPS-induced phosphorylation of IκBα. In contrast, CORM2 did not inhibit LPS-induced IκBα phosphorylation (Fig. 5, *A* and *B*). These results were confirmed by experiments using an NF-κB luciferase reporter assay in which the activation of NF-κB is blocked by induction of HO-1 expression but not by CORM2 treatment (Fig. 5*C*).

HO-1 and CO inhibits LPS-induced IRF-3 phosphorylation in human DCs

LPS-induced TLR4 activation is also associated with TIR domain-containing adaptor-inducing IFN (TRIF) recruitment, which results in nuclear translocation of IRF-3, a critical transcription factor for IFN-α and β gene expression (34, 35). The IRF-3 pathway is also activated by TLR3 and TLR3 is unique in that it does not use the MyD88 pathway but only recruits TRIF (36). IRF-3 is found in the cytoplasm as an inactive monomer and becomes activated by phosphorylation, followed by dimerization and translocation to the nucleus (37). Western blot analysis showed that LPS treatment induced an increase in phosphorylated IRF-3 (Fig. 6, *A* and *B*), which was significantly inhibited by CoPP-induced HO-1 expression and by CORM2 treatment (Fig. 6, *A* and *B*). To confirm the effect of HO-1 and CO on the IRF-3 pathway, we analyzed the expression of IFN-β and of the chemokine IP-10, which are induced upon IRF-3 activation by LPS treatment or TLR3 activation with poly(I:C). LPS-induced IFN-β and IP-10 production was significantly reduced with CoPP-induced HO-1 expression or CORM2 treatment (Fig. 6*C*). Altogether, these results demonstrate that HO-1 and CO inhibit LPS and poly(I:C)-mediated IRF-3 activation in DCs.

Discussion

The actions of HO-1 are mediated by the end products of heme metabolism. BL and BV are antioxidant (38) and also inhibit complement activation (39), T cell proliferation, IL-2 production, and cellular cytotoxicity (40). Free iron, despite its prooxidant effects, induces an over-expression of ferritin, which in turn has strong antioxidant effects through depletion of free iron and also by other less-characterized effects that result in induction of tolerogenic DCs (41). CO inhibits the production of TNF-α, IL-1β, and MIP-1β by LPS-activated mouse macrophages and increases IL-10 expression (42). In addition, CO inhibits T cell proliferation (43). Moreover, we (17) and others (44) have shown that heme depletion due to HO-1 activity also mediates the actions of HO-1.

We (20) and others (21) demonstrated that CoPP-induced HO-1 and HO-1 gene transfer in human and rat DCs inhibited LPS-induced phenotypic maturation and proinflammatory cytokine expression, whereas the expression of IL-10 was preserved. These DCs overexpressing HO-1 also displayed a diminished ability to stimulate allogeneic T cell proliferation (20, 21). HO-1 expression in mouse DCs was recently described to modulate the suppressive capacity of $CD4^+CD25^+$ regulatory T cells (22). We now show that CO treatment blocked the LPS-induced phenotypic maturation of human DCs in addition to reducing the expression of the proinflammatory cytokines and the capacity of DCs to stimulate alloreactive T cell proliferation. In contrast, treatment of human DCs with BL, BV, or DFO or replenishing heme cellular stores (data not shown) had no effect on DC phenotype or cytokine production. However, our results do not exclude the possibility that BL, BV, or iron metabolism has additive or synergistic effects with CO on DCs. On mouse DCs, CoPP-induced HO-1 expression and CO treatment inhibited the expression of proinflammatory cytokines but, as recently described (12) and in contrast with human and rat DCs, we could not detect a significant effect of HO-1 on the expression of phenotypic maturation markers.

Other groups have also analyzed the effect of HO-1 on DC biology using CoPP (12, 21, 45), a classical compound to induce HO-1 in many other cell types (46). It has recently been described that DCs treated with CoPP up-regulate HO-1 and Stat-3 and that is the last molecule, the one responsible for the effect of CoPP using mouse DCs, but unfortunately this point was not analyzed using human and rat DCs and therefore it is difficult to conclude definitively (47). We confirmed the results obtained with CoPP in human but also mouse DCs using HO-1 gene transfer and other groups did it in DCs using gene transfer of HO-1 (21) or HO-1-deficient mice (12). Therefore, HO-1 and eventually CO act in DCs through downstream mechanisms that could include Stat-3, as has been described in HO-1 in endothelial cells (48). To add a level of complexity, Stat-3 has been described as inducing HO-1 (49); therefore, a positive feedback mechanism may exist between Stat-3 and HO-1.

To determine whether in vitro HO-1 overexpression in DCs or CO treatment of DCs acts on DC functions in vivo, we used a transgenic mouse diabetes model in which cytotoxic T cell effector functions were induced by HA-loaded DCs leading to diabetes in Ins-HA-transgenic mice. In contrast, mice receiving DCs that had been treated with CoPP or CO before being matured and loaded with HA did not develop diabetes. These results demonstrate that HO-1 overexpression and CO treatment inhibited the immunogenicity of matured DCs responsible for the activation of $CD8^+$ T effector cells and final induction of diabetes.

Previously published data described that overexpression of HO-1 or in vivo systemic CO treatment attenuated the progression of diabetes in spontaneously autoimmune diabetic NOD mice (10, 11). These results and ours in the model of induced autoimmune diabetes support the use of ex vivo CO-treated DCs loaded with β cell autoantigens to induce tolerance in NOD mice. The inhibition of $CD8^+$ T cell responses in our induced diabetes model is in agreement with the inhibition of $CD8^+$ T cell responses by HO-1 and CO systemic treatment very recently reported in models of experimental autoimmune encephalomyelitis and cerebral malaria (12, 50).

We demonstrate that HO-1 (20) and CO inhibit the production of cytokines critical for T cell responses. Secretion of IL-12p70 and IL-12p40 are strongly inhibited, whereas IL-10 was preserved. A decrease of IL-12 production and concomitant secretion of IL-10 has been shown to be involved in the induction of tolerance and anergy in T cells (51). Increased expression of HO-1 by mouse macrophages or treatment of these cells with CO inhibits the production of proinflammatory cytokines induced by LPS stimulation while preserving IL-10 secretion (42, 52), although IL-10 did not mediate the anti-inflammatory effects of HO-1 (42). Our results show that HO-1 and CO preserve IL-10 secretion in DCs but treatment with an anti-IL-10 Ab did not significantly reverse the inhibitory effect of HO-1 and CO on IL-12p70 secretion (supplemental Fig. 7), suggesting that HO-1 and CO inhibit DC maturation and function independently of IL-10.

ERK, JNK, and p38 MAPK signaling pathways have distinct roles in the maturation process of DCs and, in agreement with reported data obtained with LPS- or TNF-α-treated DCs (for review, see Ref. 31), we found that these pathways were rapidly and transiently activated in DCs by LPS. HO-1 and CO are known to increase p38 MAPK phosphorylation in macrophages and endothelial cells and to inhibit JNK phosphorylation in macrophages and ERK1/2 phosphorylation in vascular smooth muscle cells and T cells (for review, see Ref. 13). We did not detect any effect of HO-1 overexpression or CO on LPS-induced phosphorylation of p38 MAPK and JNK in DCs. In contrast, the LPS-induced phosphorylation of ERK was modestly inhibited by HO-1 overexpression in DCs, but the inhibition of the ERK pathway alone did not inhibit IL-12p70 secretion and did not reverse the inhibitory effect of HO-1 overexpression on the LPS-induced IL-12p70 secretion, suggesting that although the ERK pathway was inhibited by HO-1, it does not mediate the effect of HO-1 and HO-1-derived CO on DC maturation and functions. Increased cGMP have been described as a signaling transduction pathway induced by CO (13), but we did not observe an effect of this pathway in the inhibition of LPS-induced maturation of DCs treated with HO-1 or CO (data not shown).

Signaling through TLRs can be broadly categorized into two pathways, the MyD88- and the TRIF-dependant pathways. All TLRs, except TLR3, activate the MyD88 pathway, whereas TLR3 and TLR4 activate the TRIF pathway (53). The MyD88 and TRIF pathway commonly activate NF-κB (34). IRF-3 activation by TRIF signaling is critical for LPS induction of IFN-β, IP-10, and other chemokine genes (54). We noted that HO-1 inhibited the IκBα phosphorylation and, thus, the subsequent activation of NF-κB, whereas CO has no significant effect on the activation of the NF-κB pathway. However, we observed a consistent and significant decrease in LPS-induced IL-12p70 and IL-12p40 secretion when DCs were treated with CO. Generation of the active IL-12p70 required the expression of both IL-12p40 and IL-12p35 subunits. TLR3 and TLR 4-induced IL-12p35 (55) and IL-12p40 (56) expression have been shown to be dependent on IRF-3 phosphorylation. In mouse macrophages, HO-1 and CO have been described to inhibit the LPS-induced phosphorylation of IRF-3, whereas TLR3-induced IRF3 activation was inhibited by HO-1 but not by CO (57). In human DCs, both HO-1 and CO inhibited not only LPS-induced IRF-3 activation but also TLR3-induced IRF-3 activation, as demonstrated by the inhibition of IP-10 secretion activated by poly(I:C). These effects on human DCs explain the inhibition of LPS-induced secretion of IL-12p70, reflecting the inhibition of IL12p35 and IL-12p40 expression. Whereas HO-1 inhibits TLR4-mediated IRF-3 activation in both human and mouse DCs, CO inhibits TLR3-induced IRF-3 activation in human but not mouse DCs. Other species differences were observed since HO-1 and CO inhibited human and rat DC phenotypic maturation but had no effect on that of mouse DCs and, additionally, LPS-induced TNF-α production was drastically inhibited in rat (20) and mouse DCs as well as in mouse macrophages (42, 57), whereas TNF-α production by human DCs was much less efficiently inhibited. Collectively, all of these observations suggest that HO-1 and CO have different inhibitory effect on rat, mouse, and human DCs.

In conclusion, the present study demonstrates that CO is capable of modulating DC functions in vitro, and that ex vivo treatment can inhibit immunogenicity in vivo in an induced autoimmune diabetes model. Although additional studies are needed to investigate the precise mechanism of CO action, our results demonstrate that CO ex vivo treatment could promote the tolerogenic capacities of DCs.

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Disclosures

The authors have no financial conflict of interest.

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