Heme Oxygenase-1 Expression in Murine Dendritic Cell Subpopulations

Effect on CD8⁺ Dendritic Cell Differentiation in Vivo

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Heme oxygenase-1 (HO-1) is a microsomal enzyme with antioxidant, antiapoptotic, and immunoregulatory functions. We studied the expression of HO-1 by bone marrow-derived dendritic cells (BMDCs) and splenic DC subpopulations under quiescent conditions or following lipopolysaccharide (LPS) stimulation. The kinetics of HO-1 expression by BMDCs depended on the conditions under which they were propagated. Expression of HO-1 in mouse BMDCs in 100 U/ml GM-CSF peaked at 16 hours after LPS treatment and maintained expression for at least 48 hours. But cultures in 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) showed peak expression by 16 hours that disappeared by 48 hours after LPS stimulation, similar to BMDCs cultured in both 100 U/ml GM-CSF and IL-4 (10 ng/ml). By flow cytometry, a large proportion of CD8⁺ splenic DCs strongly expressed HO-1, and this population significantly increased following LPS administration in vivo. In *HO-1*^{-/-} mice, the proportion of splenic CD8⁺ DCs was significantly decreased in comparison with *HO-1*^{+/+} mice. In addition, a unique subpopulation of MHC II⁻CD11b⁺CD11c⁺ cells was prominent in *HO-1*^{-/-} spleens. Injection of GFP-labeled *HO-1*^{+/+} splenic DC precursors into *HO-1*^{+/+} mice resulted in the generation of GFP⁺CD8⁺ DCs in the spleen after 5 days, but GFP⁺CD8⁺ DCs failed to appear in *HO-1*^{-/-} spleens. Conversely, GFP⁺*HO-1*^{-/-} splenic cells also generated GFP⁺CD8⁺ DCs in *HO-1*^{+/+} mice. These results show that HO-1 is involved in splenic DC differentiation, and/or the homing of CD8⁺ splenic DC precursors appears to be dependent on HO-1 expression by

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Heme oxygenase (HO) is an enzyme that catalyzes the rate-limiting step in the degradation of heme to biliverdin, carbon monoxide, and iron.^{1–3} Recently, we and others^{4–11} have shown that, in addition to its well-known cytoprotective effects, HO-1 (inducible isoform) is also involved in immune regulation. Analyses of HO-1-deficient mice and a single reported clinical case of HO-1 deficiency show that a systemic lack of HO-1 results in profound immune dysregulation, characterized by a number of autoimmune lesions, as well as age-related overgrowth of CD4⁺ T cells and premature death.^{12,13} Stimulation of *HO-1*^{-/-} splenocytes with anti-CD3 and anti-CD28 results in secretion of a preponderance of Th1-type cytokines in comparison with splenocytes from normal wild-type littermates,¹² and the function of regulatory T cells is dependent on expression of HO-1 by the antigen-presenting cells (APCs), especially dendritic cells (DCs),¹⁴ suggesting that APCs in general, and DCs in particular, could be a key component of the immunoregulatory effects of HO-1. If true, this hypothesis would predict that perturbation of HO-1 expression will affect DC function. Conversely, it is also possible that DCs in

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different functional states or DC subpopulations associated with immune responses would exhibit differential regulation of HO-1 expression in both the quiescent, immature state and the activated mature state. This is an important question, because DCs are not a monolithic cell lineage but are composed of a heterogeneous array of subsets that arise from a variety of precursors (for review, see Ref. 15) and are associated with diverse immune functions including T cell activation, tolerance induction and maintenance, as well as integration of signals arising from innate immune responses.

A series of elegant studies has shown coupling between expression of HO-1 and DC maturation, suggesting that HO-1 can directly regulate DC maturation.^{10,16–18} Although specific details of this finding remain controversial, a question that remains unanswered is how HO-1 expression is regulated with respect to DC subpopulations *in vivo* and *in vitro*. The purpose of these studies was to analyze the expression of HO-1 within splenic resident DCs in the mouse because, relative to other experimental systems, murine splenic DC subpopulations are relatively well characterized, therefore allowing us to glean more specific information regarding how HO-1 affects DC differentiation in the context of physiological immune responses.

In this study, we found that expression of HO-1 by DCs *in vivo* is differentially regulated among DC subpopulations in mice. We showed that CD8⁺ splenic DCs express high levels of HO-1 and that differentiation or homing of CD8⁺ DCs in the murine spleen is dependent on HO-1 expression of the host.

Materials and Methods

Animals

Male and female *HO-1*^{−/−} mice (8 to 12 weeks of age) carrying a targeted deletion of a large portion of the HO-1 gene were selected by genotyping using tail DNA as previously described from offspring of heterozygous/homozygous matings.¹⁹ Sex- and age-matched wild-type (*HO-1*^{+/+}) littermates were used as controls. GFP⁺ C57BL/6 mice expressing a transgene coding for green fluorescent protein (GFP) under control of the human ubiquitin C promoter have been described previously.²⁰ We purchased these mice from The Jackson Laboratory (Bar Harbor, ME; strain: C57BL/6-Tg(UBC-GFP)30Scha/J). GFP⁺ mice were bred with *HO-1*^{+/−} mice to generate GFP⁺*HO-1*^{+/+} and GFP⁺*HO-1*^{−/−} mice. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (Birmingham, AL).

In Vivo and in Vitro Study Design

For the analysis of HO-1 expression of splenic DCs *in vivo*, we divided *HO-1*^{+/+} mice into LPS treated (L2654, 2 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) and untreated groups (*n* = 3/group). HO-1 knockout mice were used as negative controls for flow cytometric measurements of HO-1 expression in splenic cells or in splenic DC sub-

populations. For the adoptive transfer of splenocytes from GFP⁺ mice, we injected 10⁷ GFP⁺ splenocytes into the tail vein of *HO-1*^{+/+} mice or *HO-1*^{−/−} mice (*n* = 3/group). After 5 days, the spleen was removed and prepared for flow cytometry. Carbon monoxide-releasing molecule CORM-2 (10 mg/kg; Sigma-Aldrich, St. Louis, MO) was i.v. injected into the tail vein of *HO-1*^{−/−} mice 2 hours before and 1, 2, and 4 days after injection of GFP⁺ splenocytes. Biliverdin (30 mg/kg; Aldrich Chemical, Milwaukee, WI) was i.p. injected into *HO-1*^{−/−} mice 2 hours before and daily for 4 consecutive days after injection of GFP⁺ splenocytes. Zinc protoporphyrin (25 mg/kg; Frontier Scientific, Logan, UT) was injected i.p. into *HO-1*^{+/+} mice 2 hours before and daily for 4 consecutive days. Cultured bone marrow-derived DCs (BMDCs), prepared as described below, were harvested at 0, 4, 8, 16, 24, and 48 hours after LPS treatment (final concentration, 500 ng/ml).

Splenic DC Preparation

Spleens were harvested, cut into small fragments, minced, and digested with 100 U/ml collagenase D (Roche Diagnostics, Indianapolis, IN)-containing Dulbecco's PBS (Invitrogen, Grand Island, NY) solution and incubated for 20 minutes at room temperature. EDTA was added (final concentration, 0.1 M) to disrupt DC-T cell complexes and incubated for 5 minutes. Cells were filtered through a 70- μ m stainless steel sieve (BD Falcon; BD Biosciences, Bedford, MA) for removing undigested fibrogenous materials and centrifuged at 300 $\times$ *g* for 10 minutes. Supernatants were removed, and red blood cells were lysed using buffered ammonium chloride lysis solution. After cells were washed in PBS, they were used for experiments.

BMDC Preparation and Culture

Murine BMDCs were generated as previously described²¹ with some modifications. In brief, BM was collected from tibias and femurs of mice and flushed into plates with a syringe. Ammonium chloride lysis solution was used to lyse red blood cells for 5 minutes. At day 0, 2 $\times$ 10⁶ BM cells were seeded into 6-well plates. Dulbecco's modified Eagle's medium/F-12 (Thermo Scientific, Logan, UT) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L, Sigma-Aldrich), penicillin-streptomycin (100 U/ml and 100 μ g/ml, respectively; Sigma-Aldrich), and 2-mercaptoethanol (50 μ mol/L; Chemicon International, Temecula, CA) was used as culture medium. Cultures were divided into three groups: i) low-dose granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ) (100 U/ml), ii) high-dose GM-CSF (800 U/ml), and iii) low-dose GM-CSF plus IL-4 (PeproTech) (10 ng/ml). At day 3, 5 ml of medium was added to the plates without removing any medium. On day 6, nonadherent cells were harvested with gentle pipetting, and 1.5 million cells were redistributed into new 6-well plates with fresh cytokine-free me-

dium and cultured for 24 hours. At day 7, these cells were used for *in vitro* experiments.

Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was used for synthesis of cDNA using the SuperScript III first-strand synthesis system (Invitrogen), according to the manufacturer's instructions. In brief, the first-strand cDNA synthesis reaction was primed using random hexamers (50 ng/ μ l) and 2'-deoxynucleoside 5'-triphosphates (10 mmol/L). The reaction was incubated at 65°C for 5 minutes and placed on ice for 1 minute. A cDNA synthesis mix containing 2 μ l of 10 $\times$ reverse transcriptase buffer, 4 μ l of 25 mmol/L MgCl₂, 2 μ l of 0.1 M DTT, 1 μ l of RNaSeOUT (40 U/ μ l), and 1 μ l of SuperScript III reverse transcriptase (200 U/ μ l) was added to the tube containing RNA. Reverse transcription quantitative PCRs were performed using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR was performed in a final volume of 20 μ l containing 3 μ l of cDNA, 10 μ l of Brilliant SYBR Green ER (Invitrogen), 3 μ l of primer (10 pM), and 4 μ l of distilled water. The PCR protocol was as follows: i) uracil-*N*-glycosylase step (50°C for 2 minutes); ii) initial activation step (95°C for 10 minutes); and iii) two-step cycling including denaturation (95°C for 15 seconds), and annealing/detection (60°C for 1 minute), for 40 cycles. HO-1 gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase by using the formula $2^{-\Delta Ct}$ (where Ct is threshold cycle). The average Ct values for the HO-1 and the housekeeping gene were calculated from triplicate samples. Fold expression of HO-1 mRNA is defined as the fold change in mRNA levels at each time relative to levels at an initial "0" time. The primers used for HO-1 were 5'-AGGTACACATCCAAGCCGAGAA-3' and 5'-CTCTGGACACTGACCCTTCTG-3' and for glyceraldehyde-3-phosphate dehydrogenase were 5'-TCCCAC-TCTCCACCTTCGA-3' and 5'-AGTTGGGATAGGGCC-TCTCTTG-3'.

Western Blot Analysis

BMDCs were prepared for Western blot analysis as follows: 1.5 million cells were harvested and lysed in radioimmunoprecipitation assay solution (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% deoxycholic acid, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, and 1 mmol/L sodium fluoride) with protease inhibitors (Roche Applied Science, Indianapolis, IN). The lysates were sonicated several times for 1 minute and centrifuged at 300 $\times$ g for 10 minutes at 4°C. The protein concentration of each lysate was determined by using the BCA assay (Thermo Scientific, Rockford, IL). Equal amounts of protein (10 μ g) were loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane (Hybond C-Extra; Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% nonfat dried milk in PBS containing

0.1% Tween-20 and probed with anti-HO-1 antibodies (Abs) (SPA 896, 1/5000; StressGen Biotechnologies, Victoria, British Columbia, Canada). Membranes were stripped and reprobed with actin as a loading control. Immunoreactive bands were detected using horseradish peroxidase-linked Ab against rabbit IgG and visualized with enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA), to the manufacturer's instructions.

Flow Cytometry

Cells were washed once with cold PBS and were incubated for 10 minutes at room temperature with anti-mouse CD16/32 (eBioscience, San Diego, CA) to block nonspecific binding to FC γ 3 receptors. Cells were initially stained with fluorescein isothiocyanate-conjugated (FITC) anti-mouse CD86 (clone GL-1), phycoerythrin (PE)-conjugated anti-mouse CD19 (clone 1D3), anti-mouse CD11c biotin (clone HL3), and APC-conjugated anti-mouse major histocompatibility complex class II (MHC II) (clone M5/114.15.2) for 30 minutes on ice. Cells were washed and stained with (PerCP)-conjugated anti-mouse streptavidin for additional 30 minutes on ice. PE-, FITC-, and APC-conjugated isotype matched Abs of irrelevant specificity were used as controls. All Abs were purchased from BD Pharmingen (San Diego, CA) or eBioscience. For intracellular HO-1 staining in splenic DCs, cells were fixed using 2% formaldehyde (Polysciences, Warrington, PA) for 10 minutes at room temperature and centrifuged for 10 minutes at 300 $\times$ g. Ice-cold methanol was added to the cells and incubated on ice for an additional 10 minutes. Cells were washed twice with cold staining solution (PBS, 0.02% sodium azide, and 5% bovine serum albumin) and stained with unconjugated rabbit anti-mouse HO-1 Ab (SPA 895; StressGen Biotechnologies), PerCP-conjugated anti-mouse CD4 (clone L3T4) or CD8 Ab (clone 53-6.7), PE-conjugated anti-mouse CD11c Ab (clone HL3), and Alexa 647-conjugated CD8 Ab (clone 53-6.7) or APC-conjugated anti-mouse CD11b Ab (clone M1/70) for 30 minutes on ice. After washing, cells were stained with FITC-conjugated goat anti-rabbit IgG secondary Ab for 30 minutes on ice. PE-, PerCP-, and APC-conjugated Abs and rabbit Abs of irrelevant specificity were used as isotype controls. DC subsets in HO-1^{+/+} mice and HO-1^{-/-} mice were identified by incubation with FITC-conjugated, PE-conjugated, PerCP-conjugated and APC-conjugated mAb specific for CD11c, CD4, CD8, and MHC II, respectively. Data acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences), and results were analyzed using Winlist software (Verity Software House, Topsham, MA).

Statistical Analysis

Values are expressed as mean $\pm$ SE. Analyses were performed using analysis of variance and the Student-Newman-Keuls test. Differences were considered statistically significant when the *P* value was <0.05.

Figure 1. Kinetic analyses of HO-1 protein expression in BMDCs with respect to culture conditions after LPS-induced maturation. BMDCs were cultured in GM-CSF at 100 U/ml (A), GM-CSF at 800 U/ml (B), and GM-CSF (100 U/ml) plus IL-4 (10 ng/ml) (C) for the indicated times. The blots shown in this figure are representative of three separate experiments with similar results. Actin was used as a loading control. The histograms represent densitometry analysis of HO-1 bands normalized to actin and are expressed as the fold change relative to time 0 (* $P < 0.05$ versus "0" time value).

Results

HO-1 Expression by BMDCs in Vitro

Figure 1A shows expression of HO-1 as measured by Western blot analysis in BMDC cultures propagated for 6 days in 100 U/ml GM-CSF. Under these conditions, HO-1 expression was readily detectable for at least 48 hours following stimulation by LPS. In contrast to previous reports,¹⁷ we did not observe a diminution of HO-1 expression with respect to maturation, as determined by expression of increased levels of the costimulatory molecule, CD86, and surface MHC II molecules (Figure 2A). However, increasing the concentration of GM-CSF used to propagate the BMDCs from 100 to 800 U/ml resulted in a change in this behavior, as shown in Figure 1B. HO-1 expression peaked by 16 hours poststimulation and was nearly absent by 48 hours poststimulation. Again, we observed a concomitant increase in the expression of both CD86 and MHC II, indicating that the LPS treatment promoted DCs to maturation (Figure 2A). The inclusion of IL-4 with the GM-CSF resulted in lower levels of HO-1 in comparison with the other two conditions, but by 24 hours, the levels of HO-1 also began to drop and were nearly undetectable by 48 hours (Figure 1C). The proportion of CD11c⁺ cells was similar in low GM-CSF and high

GM-CSF culture conditions, whereas the addition of IL-4 increased this proportion (Supplemental Figure 1, see <http://ajp.amjpathol.org>). The highest proportion of mature BMDCs, as determined by increased expression of CD86 and MHC II, also occurred in these cultures. It is interesting to note that the most rapid and complete transition to CD86^{hi}/MHC II^{hi} phenotype was achieved in the cultures propagated in low-dose GM-CSF and IL-4 (Figure 2A). HO-1 mRNA levels measured by real-time PCR (Figure 2B) appeared to reflect the trends observed using western blots. Cultures containing 100 U/ml GM-CSF expressed the highest levels of HO-1 mRNA, achieving peak expression by 16 hours after stimulation with LPS (12-fold versus time 0; $P < 0.05$). HO-1 expression was not detectable in BM cells immediately following isolation in the presence or absence of GM-CSF alone or GM-CSF plus IL-4 for 16 hours (data not shown), suggesting that in the progenitor state these growth factors do not induce HO-1.

HO-1 Expression in Splenic DCs in the Steady-State in Vivo Following LPS Administration

Steady-state murine CD11c⁺/MHC II⁺ resident splenic DCs can be subdivided into three subpopulations based

Figure 2. Expression of MHC II, CD86, and HO-1 mRNA by BMDCs propagated in different culture conditions in the presence of LPS. **A:** BMDCs were cultured in GM-CSF at 100 U/ml (white bar), GM-CSF at 800 U/ml (black bar), and GM-CSF (100 U/ml) plus IL-4 (10 ng/ml) (gray bar). The histogram depicts the mean proportion of cells positive for both CD86 and MHC II in each culture condition ($n = 5$). * $P < 0.05$: low or high GM-CSF versus IL-4 plus GM-CSF at 0 and 24 hours. ** $P < 0.05$: low GM versus high GM-CSF at 48 hours. *** $P < 0.05$: low GM-CSF versus IL-4 plus GM-CSF at 48 hours. **B:** BMDCs were cultured in GM-CSF at 100 U/ml (closed diamond), GM-CSF at 800 U/ml (closed box), and GM-CSF (100 U/ml) plus IL-4 (10 ng/ml) (open triangle) for the indicated time. Data shown represent the mean expression relative to time 0 $\pm$ SEM for three independent experiments. * $P < 0.05$ versus "0" time value.

Figure 3. DCs from HO-1 knockout mice are deficient in the CD8⁺ DC subset. Splenic cells from both wild-type mice and HO-1 knockout mice were harvested as described in *Materials and Methods*. CD8 and CD4 expression is shown on CD11c and MHC II-gated cells from knockout mice (A) and wild-type mice (B). Figures are representative of three separate experiments. Numbers within graph quadrants depict the percentage of CD11c⁺MHC II⁺ positive cells. The histograms shown depict the average from three animals in the percentage of CD4⁺CD8⁺ DC (C) and CD4⁻CD8⁻ cells (D) in wild-type mice and knockout mice ($n = 3$; * $P < 0.05$).

on their expression of CD4 and CD8: CD4⁻CD8⁻, CD4⁺CD8⁻, and CD4⁺CD8⁺.^{15,22} Figure 3A–D represents the distribution of CD4 and CD8 staining on splenic DCs, defined as CD11c⁺/MHC II⁺ cells isolated after collagenase digestion of spleens to release CD8⁺ DCs from the periarteriolar sheaths. As previously reported,^{15,22,23} the majority of cells was CD4⁺, with significant CD4⁻CD8⁺ and CD4⁻CD8⁻ subpopulations. Typically, these subpopulations are enumerated after Ab and complement or immunomagnetic bead depletion.^{22,23} However, these procedures were avoided because *in vitro* manipulations activate the HO-1 gene and also drive DCs to mature.^{24,25} To reduce contamination of the signal by intact, viable, autofluorescent cells that appear to be monocytes/macrophages, selective gating of DCs on the basis of CD11c and MHC II expression was done. We first determined the distribution of DC subpopulations in the spleens of HO-1-deficient mice in comparison with wild-type littermates and then determined whether there were differences in the expression of HO-1 in these cells. As negative controls, we used cells from HO-1-deficient animals to determine background staining. The distribution of these subpopulations is significantly changed in mice lacking HO-1 (Figure 3). Most notably, the proportion of CD8⁺ DCs is greatly reduced in HO-1-deficient mice (HO-1^{+/+} = 12.05 ± 1.42 versus HO-1^{-/-} = 3.79 ± 0.46; $n = 3$; $P = 0.016$), and the proportion of CD4⁻CD8⁻ DC is significantly increased (HO-1^{+/+} = 28.32 ± 0.81 versus HO-1^{-/-} = 45.68 ± 0.55; $n = 3$; $P = 0.003$) relative to the wild-type littermates. When these same

Figure 4. CD8⁺ splenic DCs express HO-1 protein *in vivo*. Coexpression of CD8 and HO-1 in splenic DCs from HO-1^{-/-} (A), PBS-injected wild-type mice (B), and LPS-treated wild-type mice (C). Figures are representative of three separate experiments. Numbers within graph quadrants are the percentage of CD11c⁺MHC II⁺ positive cells. The histogram (D) shows the average of three experiments expressed as the proportion of CD8⁺ cells expressing HO-1 divided by total CD8⁺ cells in HO-1 wild-type mice 24 hours after PBS and LPS administration ($n = 3$ in each group; * $P < 0.05$).

subpopulations were evaluated for expression of HO-1 by flow cytometry, we found that most DCs stain weakly or are negative for expression of HO-1 *in vivo*, with the exception of the CD8⁺ subpopulation, among which the majority exhibited strong expression of HO-1 (Figure 4, A and B).

To determine whether the expression of HO-1 changed among these subpopulations *in vivo* relative to LPS-induced maturation, the expression of HO-1 in DC subpopulations was determined after LPS administration *in vivo*. Wild-type mice were injected with LPS, and the splenocytes were then analyzed for HO-1 expression at 24 hours. As in the steady-state animals, CD8⁺ DCs remained the predominant HO-1-expressing subpopulation. The proportion of CD8⁺ DCs expressing HO-1 increased from 52.53 ± 4.67 to 76.59 ± 4.76 (Figure 4, C and D). Further examination of CD11c⁺ subpopulations revealed the existence of apparently unique CD11b⁺/CD11c⁺ cells in HO-1-deficient mice (7.70 ± 1.74 versus 1.25 ± 0.41 in wild-type; $P = 0.0002$) (Figure 5, A–C). Phenotypic analysis showed that the majority of these cells is CD11b⁺, MHC II^{lo}, CD24⁺, CD45RA^{int}, and Ly6c⁺ (Figure 5, D–F).

Differentiation of CD8⁺ Splenic DCs from Late Precursors in HO-1-Deficient Mice

CD8⁺ DCs are short-lived with a life span of <3 days, and they arise from intrasplenic CD8⁻ precursors.^{15,23,26} To determine whether the development of CD8⁺ DCs or

Figure 5. A unique splenic DC subpopulation in HO-1 knockout mice. Splenic cells from both HO-1 knockout mice (A) and wild-type mice (B) were gated on MHC II and plotted with respect to CD11c and CD11b staining. Cells gated as shown by the dotted line in A were analyzed for expression of CD24 (D), CD45RA (E), and Ly6c (F). Figures are representative of five separate experiments. The histogram (C) depicts the mean percentage of total gated cells in knockout mice (HO-1^{-/-}) and wild-type mice (HO-1^{+/+}), respectively ($n = 5$; * $P < 0.05$). Dotted line: isotype control.

their precursors are dependent on HO-1, we used a modification of a previously described¹⁵ late precursor assay to show that steady-state splenic CD8⁺ DCs arise from a nonmonocyte splenic precursor. We adoptively transferred splenocytes from GFP⁺ transgenic mice²⁰ to HO-1-deficient mice or their wild-type littermates. Five days later, the spleens of the recipient mice were analyzed for the presence of GFP-labeled DCs. Because CD8⁺ DCs already present in the GFP-labeled spleen cells live <3 days, it was presumed that GFP⁺ CD8⁺ DCs observed in the recipient mouse at 5 days postinfusion arose from the transferred GFP⁺ precursors.

Flow cytometry analysis of splenocytes from wild-type or HO-1^{-/-} mice injected 5 days previously with GFP-labeled wild-type splenocytes showed significant differences in the distribution of GFP-labeled DC subpopulations. Figure 6B shows that GFP⁺-labeled CD8⁺ DCs can be found in HO-1^{+/+} mice injected with GFP⁺ splenocytes. In contrast, GFP-labeled CD8⁺ DCs were detected with a much lower frequency in the spleens of HO-1^{-/-} mice injected with cells prepared from the same GFP⁺ splenocyte preparations (Figure 6, A and C). To determine whether late precursors for CD8⁺ DCs were present in the spleens of HO-1^{-/-} mice, we performed adoptive transfer of splenocytes from GFP⁺ HO-1^{-/-} mice into both GFP⁻HO-1^{+/+} and HO-1^{-/-} mice. Few GFP-labeled CD8⁺ DCs were found in HO-1^{-/-} mice, whereas GFP⁺-labeled CD8⁺ DCs were found in HO-1^{+/+} mice injected with GFP⁺ splenocytes in numbers comparable with mice injected with HO-1^{+/+} cells (Figure 6, D–F). These results indicate that absence of GFP-labeled CD8⁺ DCs in HO-1^{-/-} mice is not caused by a lack of precursor cells but may result from a failure of splenic CD8⁺ DC

Figure 6. Development of late DC precursors into CD8⁺ DCs in HO-1-deficient mice. Splenocytes (1×10^7) from GFP⁺ B6 mice and GFP⁺ HO-1^{-/-} mice were i.v. injected into HO-1^{-/-} and HO-1^{+/+} mice. Spleens from these mice were harvested 5 days after injection, and cells were used for analysis of data. Cells shown were gated on GFP⁺ and MHC II⁺ events. Plots show the appearance of CD11c⁺CD8⁺ DCs in the spleens of HO-1^{-/-} (A and D) and HO-1^{+/+} (B and E) mice receiving adoptively transferred splenocytes from GFP⁺ B6 (A–C) and GFP⁺ HO-1^{-/-} (D–F) mice. Numbers within each quadrant are the percentage of gated cells. C and F show the mean and SEM of CD11c⁺CD8⁺ splenocytes detected in recipient mice (genotype indicated on the x-axis) in three separate experiments ($n = 3$ /group; * $P < 0.05$).

precursors to home or differentiate into CD8⁺ DCs in the spleens of HO-1-deficient mice.

To determine whether the degradation products of heme could correct the effects of HO-1 deficiency, we treated HO-1^{-/-} mice with the carbon monoxide releasing molecule CORM-2 or biliverdin just before adoptive transfer of GFP⁺ splenocytes and then daily until spleen removal. Neither CORM-2 nor biliverdin was able to restore normal numbers of GFP⁺CD8⁺ DCs in HO-1^{-/-} recipient mice (2.38 ± 0.36 and 1.49 ± 0.03 in the CORM-2- and biliverdin-treated groups, respectively, versus 1.25 ± 0.41 in the untreated HO-1^{-/-} group, $n = 3$; $P = \text{ns}$). To determine whether there was a direct relationship between HO-1 expression and DC differentiation, we treated HO-1^{+/+} mice with the HO inhibitor zinc protoporphyrin but found no suppression of the splenic CD8⁺ DCs in HO-1^{+/+} mice (12.39 ± 0.52 in zinc protoporphyrin-treated group versus 12.05 ± 1.42 in the control group; $n = 3$; $P = \text{ns}$). These results suggest that HO-1 deficiency affects homing or differentiation of CD8⁺ DCs indirectly by altering the development or seeding of cells present within the splenic microenvironment.

Discussion

The results described in this paper have generated four key findings: i) the kinetics of HO-1 expression in BMDCs in response to LPS is dependent on the conditions under which the BMDCs are cultured; ii) as previously reported, BMDCs lose expression of HO-1 because they are driven to maturation by LPS,¹⁷ but only under specific conditions; iii) in the spleen under quiescent conditions and in the presence of LPS, CD8⁺ DCs very strongly express HO-1 relative to other subpopulations; and iv) HO-1 may

play a role in the differentiation of CD8⁺ DCs from precursors, or alternatively, may affect the homing of DC precursors. HO-1 is involved in recycling of heme components in the course of normal metabolism within the APCs but is also integral in the catabolism of heme-containing proteins from materials acquired by phagocytosis or pinocytosis, which are major modes of acquisition of moieties ultimately presented as antigens. Therefore, HO-1, an inducible gene product, would be expected to be relatively abundant in cells involved in antigen presentation. Previous studies have shown that HO-1 can be readily detected in monocytes/macrophages as well as DCs. However, recent evidence has suggested that HO-1 is involved in the differentiation, maturation, and function of DCs.

Our results provide a potential explanation for data obtained by different laboratories showing that inhibition of BMDC maturation may be HO-1 dependent¹⁷ or independent.¹⁸ It is common to propagate BMDCs under a variety of conditions ranging from 100 to 1000 U/ml GM-CSF with or without IL-4.^{22,27,28} We found that BMDCs propagated in lower concentrations of GM-CSF (100 U/ml) in the absence of IL-4 did not exhibit a detectable decrease in HO-1 expression in the presence of LPS. Higher concentrations of GM-CSF (ie, 800 U/ml) or the use of GM-CSF and IL-4 propagated BMDCs that down-regulated expression of HO-1 in response to LPS. These results illustrate the limitations of *in vitro* experimental systems that rely on the use of BMDC for making general conclusions regarding the role of HO-1 in DC function but do not detract from the strength of studies in which HO-1 levels or its products are manipulated in BMDCs for potential therapeutic purposes.^{16,17}

It has been suggested that murine BMDCs are able to promote antitumor activity,^{29, 30} and the maturation status of BMDCs is important in the induction of antitumor immunity.²⁹ Immature BMDCs may induce tolerance, whereas mature BMDCs are necessary for antigen processing and presentation.³⁰ Our studies show that HO-1 expression in response to LPS is dependent on culture conditions and that BMDCs grown in the presence of IL-4 contain a greater proportion of mature cells. Length polymorphisms in a (GT)_n repeat region in the proximal HO-1 promoter can also affect levels of HO-1 expression and modulate disease development.³¹ If, as suggested by the results of Chauveau et al,¹⁷ that HO-1 can also impact DC maturation, then proper control of HO-1 expression under specific culture conditions could result in BMDCs that more effectively induce antitumor activity. This could be of potential benefit in cancer patients where DCs are being increasingly considered as cell-based therapy.

To obtain information regarding the role of HO-1 in DCs in a physiological milieu, we examined patterns of expression in resident splenic DC subpopulations and found, in agreement with previous studies in the rat,¹⁷ not all DC subpopulations express equal quantities of HO-1 as detected by immunofluorescence. We found that only high levels of HO-1 could be reliably detected by flow cytometry using the SPA-895 polyclonal rabbit anti-HO-1 Ab. It is notable that the splenic DC subpopulations expressing the highest levels of HO-1 were CD8⁺ DCs. This

subpopulation resides within the periarteriolar sheaths and marginal zones of the spleen and is thought to participate in immunoregulatory pathways. Interestingly, CD8⁺ DCs appear to be largely absent in *HO-1*^{-/-} mice, which exhibit a variety of abnormalities, including inflammatory lesions with increasing age. This suggests that there could be a relationship between the observed abnormalities, the lack of HO-1, and the apparent absence of CD8⁺ DCs. This possibility is supported by the results of the late precursor assays in which GFP-labeled wild-type splenocytes infused into *HO-1*^{-/-} mice failed to generate GFP-labeled CD8⁺ DCs *in vivo* in comparison with the control wild-type mice.

Several lines of evidence support the idea that HO-1 is involved in both adaptive and innate immune responses. As noted above, *HO-1*^{-/-} mice have a number of immunological abnormalities, including age-related accumulation of CD4⁺ cells, and significantly enhanced production of proinflammatory cytokines by their splenocytes in response to LPS.¹² Up-regulation of HO-1 can prolong allograft survival.³²⁻³⁸ *HO-1*-deficient mice have a reduced life span, apparently resulting from immunological dysregulation as well as other issues, including the accumulation of iron in the tissues.^{12,13} Similar abnormalities were reported in a case of HO-1 deficiency in a human who, in addition, had marked atherosclerosis.³⁹⁻⁴¹ Given the results reported in this work as well as those discussed above, it is arguable that a significant reason for the observed immunological abnormalities is dysregulation of APC differentiation and function. In previous studies, we showed that HO-1-deficient splenic APCs as well as *HO-1*^{-/-} BMDCs fail to support regulatory T cell-mediated suppression *in vitro*.¹⁴ Here we report abnormalities in the distribution of DC subpopulations in *HO-1*^{-/-} mice, and that CD8⁺ DCs fail to appear in late precursor assays of *HO-1*^{-/-} mice *in vivo*, despite the fact that precursors of CD8⁺ DCs appear to be present in the spleens of *HO-1*^{-/-} mice. The data are consistent with findings in which induction of HO-1 inhibits LPS-induced DC maturation and cytokine production^{10,17,18,42} as well as reducing the ability to support T cell activation by polyclonal or allogeneic stimulation.^{12,17,42} However, the role of HO-1 in DC function and differentiation *in vivo* remains largely unexplored because the majority of data have been generated using DCs propagated from BM cells in the presence of GM-CSF with or without IL-4. Although this approach has been informative, it does not answer the question of why HO-1 deficiency results in such profound immunological abnormalities and what role HO-1 expression in the DC lineage plays in regulation of the immune response.

The DC lineage is markedly heterogeneous, with an array of subpopulations characterized by different surface antigens, phenotypes, and tissue distributions. Conventional mouse DCs can be delineated by expression of CD4 and CD8 as CD4⁻CD8⁻, CD4⁺CD8⁻, and CD4⁻CD8⁺ subsets.^{15,23,43-45} We found that, among DCs in the spleen, CD8⁺ DC exhibit prominent expression of HO-1. The functional significance of these high expression levels is unclear, although characterization of DC subsets in *HO-1*^{-/-} mice suggests that the differen-

tiation of CD8⁺ DCs or the homing of their precursors may be dependent on systemic expression of HO-1. In normal mice, late precursors for CD8⁺ DCs can be found in the spleen. Shortman and colleagues^{15,23} have characterized these precursors and found that they can be adoptively transferred by i.v. injection to host mice in which they give rise to splenic CD8⁺ DCs. Using splenocytes from GFP⁺ mice, we replicated these results in normal host mice but found that transfer of precursors into HO-1^{-/-} mice resulted in only very small number of GFP⁺ CD8⁺ DCs in the host spleen.

The decrease in the proportion of CD8⁺ DCs in the spleens of HO-1^{-/-} mice in the adoptive transfer experiments can be explained by several mechanisms. First, it is possible that HO-1 deficiency results in a defect in a later stage of differentiation of pre-DCs into DCs in the spleen but has no effect on homing capacity. A second possibility is that HO-1 deficiency in the recipient animal results in the death of the labeled precursors before arrival to the spleen, although a more likely scenario in the "failure to arrive" hypothesis would be sequestration of the cells in places other than the spleen—perhaps the liver, which is also one of the first places in which particulate materials tend to arrive and accumulate after intravenous injection. Our results support the idea that HO-1 deficiency in the host plays a major role in the appearance of labeled CD8⁺ DCs in spleen. The appearance of normal proportions of GFP⁺CD8⁺ DCs in the spleens of HO-1^{+/+} adoptive transfer recipients that received splenocytes from GFP⁺ HO-1^{-/-} mice indicates that the precursors to CD8⁺ DCs are present in the spleens of HO-1^{-/-} mice.

In conclusion, our studies show that the kinetics of HO-1 in BMDCs in response to LPS is dependent on culture conditions. CD8⁺ DCs express high levels of HO-1 *in vivo*; this subpopulation is greatly reduced in HO-1-deficient mice, and the differentiation of CD8⁺ DCs or homing of their precursors is dysregulated in HO-1 knockout mice. It is possible that the defect in expression of CD8⁺ DCs could be related to the immunological abnormalities observed in the absence of HO-1 expression.

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