Animal Model

Heme Oxygenase-1 Modulates Early Inflammatory Responses

Evidence from the Heme Oxygenase-1-Deficient Mouse

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Induction of heme oxygenase-1 (HO-1) is protective in tissue injury in models of allograft rejection and vascular inflammation through either prevention of oxidative damage or via immunomodulatory effects. To examine the specific role of HO-1 in modulating the immune response, we examined the differences in immune phenotype between HO-1 knockout (HO-1^{-/-}) and wild-type (HO-1^{+/+}) mice. Consistent with previous findings, marked splenomegaly and fibrosis were observed in HO-1^{-/-} mice. The lymph nodes of HO-1-deficient mice demonstrated a relative paucity of CD3- and B220-positive cells, but no such abnormalities were observed in the thymus. Flow cytometric analysis of isolated splenocytes demonstrated no differences in the proportions of T lymphocytes, B lymphocytes or monocytes/macrophages between the HO-1^{-/-} and HO-1^{+/+} mice. Significantly higher baseline serum IgM levels were observed in HO-1^{-/-} versus HO-1^{+/+} mice. Under mitogen stimulation with either lipopolysaccharide or anti-CD3/anti-CD28, HO-1^{-/-} splenocytes secreted disproportionately higher levels of pro-inflammatory Th1 cytokines as compared to those from HO-1^{+/+} mice. These findings demonstrate significant differences in the immune phenotype between the HO-1^{-/-} and the HO-1^{+/+} mice. The absence of HO-1 correlates with a Th1-weighted shift in cytokine responses suggesting a general pro-inflammatory tendency associated with HO-1 deficiency. (*Am J Pathol* 2004, 165:1045–1053)

A growing body of evidence suggests that overexpression of heme oxygenase-1 (HO-1) may protect organs/tissues from immune-mediated injury either through prevention of oxidative damage or via a local immunomodulatory influence on infiltrating inflammatory cells.^{1,2} This protective property has been observed both in models of organ transplant rejection and tissue inflammation (for review, see references^{1,3,4}). In contrast, the hallmark of HO-1 deficiency appears to be development of chronic non-specific inflammatory changes as demonstrated in studies using HO-1 knockout mice⁵ as well as in a human patient with HO-1 deficiency.⁶ Despite these observations, very little is known about the specific mechanisms involved in HO-1-mediated regulation of the immune response.

Previous studies have used chemical inhibitors or inducers of HO-1 to evaluate the immunomodulatory functions of HO-1. However, both chemical inducers (such as hemin) and inhibitors (such as tin or zinc protoporphyrin) of HO-1 have effects beyond altering HO-1 enzyme activity *per se*.⁷ In addition to inducing HO-1, hemin induces other genes as well (eg, adhesion molecules), many of which influence important cellular processes.⁸ Hemin also exhibits pro-oxidant properties and recent studies have shown that it causes mitochondrial injury.^{9,10} The metalloporphyrin inhibitors of HO-1 affect other enzyme systems such as NO synthase and guanylate cyclase, in addition to a variety of nonspecific effects.^{11,12} For such reasons, the use of transgenic mice either genetically deficient or overexpressing HO-1 will provide further insight for a role of HO-1 in the mediating its immune effects.

The development of the HO-1 knockout mouse and the description of the patient with HO-1 deficiency have sig-

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nificantly advanced our knowledge pertaining to the role of HO-1 in disease pathophysiology.¹³ Using HO-1 knockout mice, we and others have demonstrated a functional role for HO-1 in several models of tissue injury.^{7,14,15} However, the general role of HO-1 in forming an immune response has not yet been investigated in these mice. The purpose of the present study was to examine the immune phenotype associated with HO-1 deficiency as well as the influence of HO-1 activity on the immune response following mitogen stimulation.

Materials and Methods

Animals

Male HO-1^{-/-} mice (8 to 12 weeks of age, C57BL/6xFVB) carrying a targeted deletion of a large portion of the HO-1 gene, were selected from offspring of heterozygous/homozygous mating by PCR using tail DNA as previously described.^{5,14} Age-matched wild-type (+/+) littermates were used as controls. The original breeding colony of the HO-1^{+/-} mice was obtained from Dr. Susumu Tonegawa's laboratory (Massachusetts Institute of Technology, Cambridge, MA) and was of a predominantly C57BL/6 background. The yield of homozygous mice from this colony was very low (average of about 1 to 2 homozygotes per 160 to 200 pups from heterozygote breeding pairs) as was reported in the original publication by Poss and Tonegawa.⁵ We backcrossed C57BL/6 HO-1^{+/-} mice with wild-type FVB mice. The HO-1^{+/-} offspring of these mice was backcrossed to wild-type FVB mice and these backcrosses were repeated four to five times, to derive a strain of HO-1^{+/-} mice with a predominant FVB background. These mice were used in future matings, which resulted in an increased yield of HO-1^{-/-} mice to about 1 in 20 pups. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Florida, Gainesville, FL.

Histology and Immunohistochemistry

Animals were sacrificed and lymphoid organs including the thymus, spleen, and lymph nodes collected. The tissue was fixed with either 10% formalin for hematoxylin and eosin staining (H&E) or in paraformaldehyde-lysine-periodate for immunohistochemistry. Ferric iron was detected in tissues using a Prussian blue stain. In brief, paraffin sections were deparaffinized and hydrated. Slides were heated in 2% potassium ferrocyanide/2% hydrochloric acid for 20 minutes at 60°C. Slides were washed in water and counterstained in nuclear-fast red for 5 minutes before mounting. The immunohistochemical staining for HO-1 was performed using a polyclonal rabbit anti-rat HO-1 antibody (1:300, catalog no. SPA-895, Stressgen Biotechnologies, Victoria, BC), and the binding was visualized with 3, 3'-diaminobenzidine substrate using an ABC-peroxidase kit (Rabbit Elite HRP kit, Vector Laboratories Inc., Burlingame, CA) with hematoxylin (DAKO Corp., Carpinteria, CA) counter stain. Spleen, thymus, and lymph nodes were stained using monoclonal

rat antibodies against B cell (anti-mouse B-220, 1:50, catalog no. 550286, BD Biosciences Pharmingen, San Diego, CA), T cell (anti-human CD3, 1:500, catalog no. MCA1477, Serotec Inc., Raleigh, NC), and macrophage (anti-mouse CD11b, 1:200, catalog no. MCA74G, Serotec) antigens detected with a rat alkaline phosphatase kit (Rat AP kit, Vector Laboratories) containing biotinylated anti-rat secondary and ABC-AP conjugate. Blue chromogen with nuclear-fast red was used as a counterstain (Vector Laboratories).

Western Blot Analysis

For HO-1 and HO-2 immunoblots, spleens were washed twice with ice-cold PBS and lysed in a buffer containing a broad spectrum mixture of protease inhibitors consisting of 10 µg/ml aprotinin, 5 mmol/L EDTA, 1 µg/ml leupeptin, 0.7 µg/ml pepstatin A, 1 mmol/L phenylmethanesulfonyl fluoride, and Triton X-100. Protein concentration of lysates was assessed by the bicinchoninic acid assay (Pierce, Rockford, IL). Samples were separated in a 10% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1.5 hours with the anti-HO-1 antibody (1:500 dilution, catalog no. SPA-895, Stressgen) followed by incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000 dilution) for 1 hour. Labeled protein bands were examined by using a chemiluminescence method according to the manufacturer's recommendation (Amersham, Piscataway, NJ). The membranes were then stripped and re-probed with anti-HO-2 (1:1000) (SPA-897, Stressgen) and anti-actin (1:1000) (Sigma, St. Louis, MO) antibodies.

Flow Cytometry

Splenocytes were isolated in a standard fashion and suspended cells were stained with monoclonal antibodies against B cell (B-220), T cell (CD3, CD4, and CD8), and macrophage (CD11b) antigens (BD Biosciences Pharmingen), coupled with various chromogens (phycoerythrin or FITC) and then fixed with BD Cytofix (BD Biosciences Pharmingen). Appropriate controls and gating were used to analyze the staining on a flow cytometer (FACS Calibur; BD Biosciences).

Splenocyte Stimulation Assays

Isolated splenocytes were cultured under respective stimulation conditions at 1×10^5 cells/well in 200 µl of RPMI 1640 medium with 10% FBS (Cambrex, East Rutherford, NJ) in 96-well round-bottom microculture plates (Fisher Scientific, Pittsburgh, PA). The supernatants were collected at 48 hours for cytokine analysis after stimulation with LPS (1 µg/ml), anti-CD3 and anti-CD28 antibodies (1 µg/ml), or medium alone (unstimulated). Cytokine measurements of supernatants were performed using a commercially available multiplexed kit (Beadlyte Mouse Multi-Cytokine Detection System, Upstate, Lake Placid, NY) and the Luminex¹⁰⁰ LabMAP System (Luminex

Figure 1. Histological evaluation of tissues from heme oxygenase-1-deficient ($\text{HO-1}^{-/-}$) mice. **A:** Hematoxylin-eosin staining of the spleen from the wild-type ($\text{HO-1}^{+/+}$, **left**) and $\text{HO-1}^{-/-}$ (**right**) mice (age 8 to 12 weeks). **Insets** in both panels represent staining for HO-1 in the spleen using a polyclonal rabbit anti-rat HO-1 antibody (brown color). **B:** Iron staining with Prussian blue (**arrows**) of kidney (K) and liver (L) tissue from $\text{HO-1}^{-/-}$ (**left**) and $\text{HO-1}^{+/+}$ (**right**) mice (age 24 weeks). **Bar**, 100 μm .

Corp., Austin, TX). Measurement of 10 cytokines included: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), TNF- α , IFN- γ , and GM-CSF. The assay was performed according to the manufacturer's protocol.

Serum Immunoglobulin Measurement

Serum samples from animals were collected at the time of sacrifice and levels of IgA, IgM, IgE, IgG2a, and IgG3 assayed using Beadlyte Mouse Immunoglobulin Isotyping Kit (Upstate) and Luminex¹⁰⁰ LabMAP System (Luminex Corp).

Statistical Analysis

Data are presented as the mean $\pm$ SEM. All results are derived from 3 to 7 animals per group. Two-tailed Student's *t*-test were used for analyses comparing the different groups, with statistical significance considered if $P < 0.05$.

Results

HO-1 Deficiency Is Associated with Abnormalities of Lymphoid Tissue

To assess the phenotypic characteristics of the newly derived genetic background in comparison to the original

description of the murine HO-1 knockout,⁵ we performed a series of morphological studies. Consistent with previous studies,^{5,7} the mean body weights of the $\text{HO-1}^{+/+}$ and $\text{HO-1}^{-/-}$ mice were not significantly different (26.7 ± 1.4 versus 25.4 ± 2.6 g, respectively, $P = \text{NS}$) in the age matched group of mice studied (8 to 12 weeks). The $\text{HO-1}^{-/-}$ mice showed significant splenomegaly in comparison to the $\text{HO-1}^{+/+}$ littermates (220.6 ± 35.4 versus 68.3 ± 7.9 mg, respectively, $P < 0.001$), findings similar to the observations of Poss and Tonegawa.⁵ As shown in Figure 1A, histological examination of the spleen from the $\text{HO-1}^{-/-}$ mice revealed abnormal architecture associated with significant fibrosis. The absence of HO-1 protein in the $\text{HO-1}^{-/-}$ mice was confirmed by immunohistochemistry (Figure 1A, inset). While significant tissue iron deposition was noted in the kidneys and livers of $\text{HO-1}^{-/-}$ animals over 20 weeks of age (Figure 1B), no iron deposition was detectable by Prussian blue staining in the age group of animals used in our studies (data not shown). We also performed Western blot analysis for HO-1 and HO-2 proteins on spleens from $\text{HO-1}^{-/-}$ and $\text{HO-1}^{+/+}$ mice to evaluate for possible compensatory changes of HO-2 levels in the $\text{HO-1}^{-/-}$ mice. As shown in Figure 2, despite absolute lack of HO-1, no increase of HO-2 protein was observed in spleens from $\text{HO-1}^{-/-}$ mice as compared to $\text{HO-1}^{+/+}$ animals.

No significant structural differences were observed in lymph nodes or thymus obtained from $\text{HO-1}^{-/-}$ and $\text{HO-1}^{+/+}$ mice as judged by H&E staining. However, immu-

Figure 2. Expression of HO-1 and HO-2 protein in spleens from HO-1^{+/+} and HO-1^{-/-} mice. Western blot analysis of HO-1 and HO-2 protein in spleen extracts from HO-1^{-/-} and HO-1^{+/+} mice using anti-HO-1 and anti-HO-2 antibodies as described in Materials and Methods. The blots were stripped and re-probed with an anti-actin antibody to control for loading and transfer. HO-1, HO-2, and actin are identified as positive bands at ~32-kd, ~36-kd, and ~46 kd-sizes, respectively. Each **lane** represents protein from an individual animal.

nohistochemistry involving lymph nodes from HO-1^{-/-} mice demonstrated a relative paucity of both CD3- and B220-positive cells; a phenomenon not observed in the thymus, where both the distribution as well as intensity of CD3 and B220 staining was similar (Figure 3, A and B). Immunohistological evaluation of the spleen from HO-1^{-/-} mice revealed an abnormal distribution of CD3- and B220-positive cells within individual follicles (Figure 3C). CD3-positive cells were normally concentrated in the central region and B220 in the marginal zone of the follicle in HO-1^{+/+} mice, whereas this pattern was disturbed in HO-1^{-/-} mice (Figure 3C). Since the number of CD11b-positive cells appeared to be increased in HO-1^{-/-} spleens (Figure 3C), we performed a flow-cytometric analysis of various splenic cell populations. As demonstrated in Figure 3D, no significant differences were observed in the numbers of CD3-, CD4-, CD8-, B220-, and CD11b-positive cells in the HO-1^{-/-} compared to HO-1^{+/+} mice.

Selective Elevation of Serum IgM in HO-1-Deficient Mice

To assess the immunoglobulin profile in the HO-1^{-/-} and HO-1^{+/+} mice, we measured baseline serum levels of IgA, IgG2a, IgG3, IgE, and IgM. As shown in Figure 4, IgM levels were significantly elevated (~3-fold) in the HO-1^{-/-} compared to the HO-1^{+/+} mice, while no differences were noted for the other immunoglobulins tested.

HO-1^{-/-} Splenocytes Secrete Disproportionately High Levels of Pro-Inflammatory Cytokines on Stimulation

To assess whether there were any significant differences in immune responses between the HO-1^{-/-} and HO-1^{+/+} phenotypes, we performed splenocyte stimulation studies followed by cytokine measurements in supernatants.

The stimulation was performed either with LPS (for primarily monocyte/macrophage stimulation) or with anti-CD3/anti-CD-28 antibodies (for T-cell stimulation). The levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), TNF- α , IFN- γ , and GM-CSF were measured. Under baseline, unstimulated conditions, the levels of all cytokines measured were undetectable in both HO-1^{-/-} and HO-1^{+/+} splenocytes. As shown in Figure 5A (left panel), LPS stimulation resulted in a significant increase of IL-1, IL-6, IL-10, IFN- γ , and TNF- α in HO-1^{-/-} compared to the HO-1^{+/+} splenocytes. The most significant changes were observed with IL-1, IL-6, and TNF- α (Figure 5A, right panel) where the fold increase was 27, 37, and 19, respectively, in HO-1^{-/-} over HO-1^{+/+} mice.

Following stimulation with anti-CD3/anti-CD-28 antibodies (Figure 5B, left panel), splenocytes from the HO-1^{-/-} mice showed a significantly higher secretion of all cytokines, except IL-1 and IL-5. In these studies, the fold increase was disproportionately higher for GM-CSF (239-fold) and IL-6 (47-fold) in the HO-1^{-/-} over HO-1^{+/+} mice (Figure 5B, right panel). Collectively, these data suggest that HO-1 deficiency is associated with an exaggerated monocyte/macrophage and T cell activation yielding a predominantly proinflammatory state.

Discussion

Recent studies suggest a pivotal role for HO-1 expression as a cytoprotective response in diverse pathological conditions including organ transplant rejection, sepsis, atherosclerosis, ischemia-reperfusion injury, as well as others.^{2,3,16} Evidence supporting these observations has been derived from both *in vitro* and *in vivo* studies using pharmacological and/or genetic manipulation involving HO-1 inhibition or overexpression. HO-1 catalyzes the rate-limiting step in the degradation of cellular heme, producing equimolar quantities of iron, carbon monoxide (CO), and biliverdin. The beneficial effects of HO-1 induction have been attributed to several factors including the degradation of pro-oxidant heme,^{10,17} formation of biliverdin and bilirubin with their antioxidant properties,^{18–20} as well as release of CO, which has anti-apoptotic and anti-inflammatory properties.^{21,22} Co-induction of ferritin²³ leading to safe sequestration of the released iron molecule and p21,^{24,25} the cell cycle regulatory protein, represent additional mechanisms that have been attributed to the protective effects of HO-1 expression.

It is becoming more apparent that HO-1 activity not only provides protection in oxidant injury, but is also involved in direct modulation of the immune response.^{2,26} The mouse model of HO-1 deficiency provides the unique opportunity to study this involvement. In short, our studies provide evidence indicating a significant difference in the immune phenotype between HO-1^{-/-} and HO-1^{+/+} mice. The studies indicate that a deficiency of HO-1 may predispose to generally exaggerated inflammatory responses, suggesting that its activity is necessary for timely resolution of early inflammation. Moreover, our data showing the predominance of Th1-type cyto-

Figure 3. Characterization of lymphoid tissue from HO-1^{-/-} mice. Immunohistochemical staining (blue color) of lymph nodes (A), thymus (B), and spleen (C) from the HO-1^{+/+} (left) and HO-1^{-/-} (right) mice using monoclonal rat antibodies against human CD3, murine B220 (A, B, and C) and murine CD11b (in C). Bar, 100 μ m. D: Representative flow cytometric analysis of isolated splenocytes (black, HO-1^{+/+}; red, HO-1^{-/-}). The analysis was performed using antibodies against CD3, CD4, CD8, B220, and CD11b.

kines (eg, IL-1, IFN- γ , TNF- α , IL-6) following splenocyte stimulation suggest that HO-1 activity is also important in more downstream stages of the immune response (eg, modulation of lymphocyte activation). The latter activity is of particular interest given the recent findings that HO-1 is constitutively expressed in a subpopulation of T regulatory cells (CD4+CD25+), and that the HO-1 level increases even further following T cell stimulation.²⁷

The morphological data presented in this report demonstrate that the altered genetic background of our HO-1 knockout colony (C57Bl/6 $\times$ FVB) yields a very similar phenotype to the one originally reported. Specifically, the initial description of HO-1 knockout mice by Poss and

Tonegawa⁵ has involved animals on a C57Bl/6 background. They have noted that animals lacking HO-1 developed progressive inflammatory disease characterized by splenomegaly, lymphadenopathy, leukocytosis, and hepatic and renal inflammation. The animals display increased iron accumulation⁵ and are more sensitive to oxidative stress.²⁸ Since a full spectrum of the phenotypic changes associated with HO-1 deficiency is apparent in animals older than 20 to 24 weeks, we have restricted our functional studies to younger mice aged 8 to 12 weeks. At this point, the HO-1-deficient mice, although exhibiting marked splenomegaly, do not have any significant differences in their body weights, tissue iron deposition, or in

Figure 4. Serum immunoglobulin levels in HO-1^{-/-} mice. Baseline serum immunoglobulin profile of the HO-1^{+/+} (solid bars) and HO-1^{-/-} (open bars) mice. Serum levels of IgA, IgM, IgE, IgG2a, and IgG3 were assayed using Bedyte Mouse Immunoglobulin Isotyping Kit and Luminex¹⁰⁰ Lab-MAP System (Values are mean $\pm$ SEM, $n = 3$; *, $P < 0.05$).

relative numbers of various spleen cell subtypes minimizing thereby a potential influence of age-related phenomena.

Although it has been known that the phagocytosis of erythrocytes induces HO activity in rodent and porcine macrophages,^{29,30} Clerget and Polla³¹ have first proposed that the up-regulation of HO-1 might have an immunomodulatory function in addition to its obvious role in heme metabolism. The role of HO-1 in inflammation has been further explored by Willis et al³² in a model of pleural inflammation. In that system, prior induction of HO-1 (with heme) results in a significant decrease in inflammatory cell infiltration and exudates whereas inhibition of HO-1 (by tin protoporphyrin) significantly worsens inflammatory exudates, suggesting that HO-1 activity modulates the inflammatory response. In the context of inflammation, our findings of a dramatic increase in pro-inflammatory cytokines following macrophage stimulation in the HO-1 knockout provide additional evidence for the importance of HO-1 in macrophage function. These studies correlate with the recent report that HO-1 knockout mice show a significant up-regulation of monocyte chemoattractant protein (MCP-1) in the kidney following repeated exposure to heme as well as higher serum levels of MCP-1 at baseline.^{33,34}

The exact mechanisms involved in the broad range of anti-inflammatory and cytoprotective abilities of the HO-1 system have not been fully elucidated. One or more of the HO-1 reaction products have been evaluated as possible factors. For example, CO has been shown to exert significant anti-inflammatory and anti-apoptotic effects in several models of inflammatory tissue injury. CO has been shown to decrease IL-6 production from LPS-stimulated macrophages by interfering with AP-1 binding to the IL-6 promoter via a JNK-dependent pathway.³⁵ Our results demonstrate a significantly higher IL-6 secretion following LPS stimulation from the HO-1^{-/-} splenocytes. One could speculate that exposure of HO-1^{-/-} splenocytes to CO should potentially attenuate the increase in IL-6 release. Otterbein et al²² have demonstrated that CO also exerts anti-inflammatory effects, in part, by increas-

ing macrophage IL-10 production. Inoue et al³⁶ have corroborated these findings by providing evidence that the overexpression of exogenous HO-1 in macrophages leads to a significant increase in macrophage-derived IL-10 levels. In contrast, Lee and Chau³⁷ have reported that IL-10 induces HO-1 in macrophages and is responsible for its anti-inflammatory properties.

It should be noted that biliverdin and/or bilirubin are also capable of blocking key events in inflammation. Up-regulation of HO-1 activity has been shown to interfere with leukocyte adhesion to vascular endothelium via changes in expression of various adhesion molecules,^{38–40} a phenomenon that has been attributed to biliverdin and/or bilirubin, rather than CO.^{38,39} Elevated HO-1 levels correlate with a reduction in monocyte chemotaxis in response to oxidized LDL, an effect that is reversed by biliverdin and bilirubin.⁴¹ Our findings in HO-1 knockout mice provide a basis for the evaluation of the individual components of the HO-1 system in mediating its anti-inflammatory effects.

In our studies, we have observed significant differences in the serum immunoglobulin profile between the HO-1^{-/-} and HO-1^{+/+} mice. The results show that HO-1 deficiency is associated with significantly higher baseline levels of IgM. This finding suggests a possible abnormality in B cell activation with impaired immunoglobulin isotype switching and is consistent with the work of Coito et al,⁴² who has reported that HO-1 induction following blockade of selectin-P-selectin glycoprotein ligand-1 interaction is associated with a decrease in IgM levels in a cardiac allograft model. A direct causal effect of increased HO-1 activity on B cell function has, however, not been established and will require further investigation.

The induction of HO-1 clearly plays an important role in the immune processes associated with transplant rejection where it positively correlates with transplant survival. Using a xenotransplant model, Soares et al¹⁵ has reported that cardiac transplants from HO-1^{-/-} mice into rats have been rejected within 3 days whereas those from wild-type or heterozygous donors have survived for up to 60 days. Further evidence for the graft-protective properties of HO-1 have been provided in heart,^{42–45} liver,^{46,47} kidney,⁴⁸ thyroid,⁴⁹ and pancreatic islet⁵⁰ allografts as well. Ke and associates⁴⁶ have shown a correlation between high and low HO-1 levels in liver allografts and changes in cytokine profile within the infiltrated allograft toward the predominance of Th2-like molecules (IL-4 and IL-10) versus Th1-associated ones (IFN- γ and IL-2). Here also, high levels of HO-1 have been associated with increased allograft survival. These authors have also demonstrated a similar cytokine profile change in liver allograft recipients treated with a CO donor, methylene chloride, suggesting that CO might be responsible for the immunomodulatory properties of HO-1.⁴⁷

In a model of kidney transplantation, Tullius et al⁴⁸ have observed that the induction of HO-1 results in a significant reduction of graft infiltration with monocytes/macrophages and CD8-positive T cells. This has also been associated with a decrease in graft tissue TNF- α

Figure 5. Cytokine levels following splenocyte stimulation in HO-1^{-/-} mice. Profiles of cytokines secreted at 48-hour by isolated splenocytes from the HO-1^{+/+} (solid bars) and HO-1^{-/-} (open bars) mice following stimulation with LPS (A) and antibodies against CD3 and CD28 (B). Levels of 10 cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), TNF- α , IFN- γ , and GM-CSF) were measured using a multiplexed kit (Beadlyte Mouse Multi-Cytokine Detection System) and the Luminex¹⁰⁰ LabMAP System. Values are mean $\pm$ SEM, $n = 3$; *, $P < 0.05$. The hatched bars on the right of each panel represent HO-1^{-/-} to HO-1^{+/+} ratios for each cytokine.

mRNA levels as well as a reduction of IFN- γ mRNA. Here the mRNA levels of IL-2, IL-6, and IL-10 have been unaffected. The predominance of Th2-type cytokine production pattern in association with heightened HO-1 activity has been confirmed in other inflammatory conditions. Minamino and colleagues⁵¹ have demonstrated that hypoxic lungs of HO-1-overexpressing transgenic mice express significantly attenuate levels of proinflammatory cytokines like IL-1 β , IL-6, MCP-1, and macrophage inflammatory protein-2 as compared with those of wild-type animals. Sarady et al⁵² have studied isolated LPS-stimu-

lated macrophages and noted that both up-regulation of HO-1 and low concentrations of exogenously administered CO significantly decrease the release of GM-CSF. In our study, LPS stimulation of both HO-1^{-/-} and HO-1^{+/+} splenocytes has not resulted in increased GM-CSF levels, however, anti-CD3/anti-CD28 exposure has led to a dramatic increase in GM-CSF in the HO-1^{-/-} compared to HO-1^{+/+} splenocytes.

Taken together, the aforementioned findings suggest that the HO-1 system plays a pivotal role in the early phases of immune response. Its level of activity, espe-

cially in the acute phase period, seems to determine the fate of downstream events including the profile of lymphocyte maturation (Th1 *versus* Th2). The utilization of the HO-1 knockout mouse model will provide a unique opportunity for evaluation and design of mechanistic studies to further our understanding of the role of HO-1 in inflammation in general and the process of transplant rejection in particular.

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