The American Journal of Pathology, Vol. 173, No. 1, July 2008

DOI: 10.2353/ajpath.2008.070963

Immunopathology and Infectious Disease

Suppression by $CD4^+CD25^+$ Regulatory T Cells Is Dependent on Expression of Heme Oxygenase-1 in Antigen-Presenting Cells

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Heme oxygenase-1 (HO-1) has been viewed as a cytoprotective protein, ameliorating the effects of inflammatory cellular damage, and as beneficial in allograft protection from acute and chronic rejection, suggesting important functions in both innate and adaptive immune responses. Mice deficient in HO-1 exhibit defective immune regulation characterized by a proinflammatory phenotype. We examined if impaired regulatory T cell (Treg) function contributes to the immunoregulatory defects observed in *HO-1*$^{-/-}$ mice. *HO-1*$^{-/-}$ mice exhibited a significantly higher proportion of Foxp3-expressing cells among total $CD4^+$ and $CD4^+CD25^+$ cells in comparison to *HO-1*$^{+/+}$ mice, and *HO-1*$^{-/-}$ Treg cells were at least as effective as *HO-1*$^{+/+}$ Treg cells in suppressing proliferation of effector T cells *in vitro* from either *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ mice. However, the absence of HO-1 in antigen-presenting cells abolished the suppressive activity of Treg cells on effector T cells. These findings demonstrate that HO-1 activity in antigen-presenting cells is important for Treg-mediated suppression, providing an explanation for the apparent defect in immune regulation in *HO-1*$^{-/-}$ mice. *(Am J Pathol 2008, 173:154–160; DOI: 10.2353/ajpath.2008.070963)*

Historically, heme oxygenase-1 (HO-1) has been viewed as a cytoprotective protein, ameliorating the effects of inflammatory cellular damage. The demonstration, however, of a beneficial role for HO-1 in allograft protection from acute and chronic rejection,[1–3] strongly suggests an important function of this enzyme in both innate and adaptive immune responses. In the original description of a mouse model of HO-1 deficiency, Poss and Tonegawa[4] noted an age-related overgrowth of the $CD4^+$ T-cell population, suggesting impaired regulation of T-cell proliferation. Our previous work assessing immune function in *HO-1*$^{-/-}$ mice supported this notion because it showed a predominance of Th1-type cytokine secretion [eg, interleukin (IL)-1, interferon-γ, tumor necrosis factor-α, IL-6] from splenocytes after polyclonal stimulation of T cells, implying that HO-1 activity is important in modulation of lymphocyte activation.[5] This is of particular interest given reports that HO-1 is constitutively expressed in the $CD4^+CD25^+$ subset of Treg cells,[6] and that HO-1 levels increase even further after T-cell stimulation.[7] Furthermore, the report by Song and colleagues[8] demonstrated that carbon monoxide (CO), a byproduct of HO activity, has a very strong inhibitory effect on CD3-activated T-cell proliferation. Previously, we proposed a hypothetical model for the immunomodulatory effects of HO-1 in the maintenance of peripheral tolerance based on then available evidence that CO produced in regulatory T (Treg) cells may be an integral component of the suppression of T-cell activation by Treg cells in the presence of effector T (Teff) cells and antigen-presenting cells (APCs).[9]

The purpose of the present study was to analyze the role of HO-1 in Treg-mediated suppression. As a first step, we performed a phenotypic analysis of lymphocytes obtained from the peripheral lymphoid organs of *HO-1*$^{-/-}$ mice for potential abnormalities. In the second step, we explored the functional significance of these findings in a series of T-cell proliferation/suppression assays. Finally, we examined the possibility that HO-1 ex-

Supported by the National Institutes of Health (grants K08 AI 57362 to M.H.K., and R01 DK 075532 and R01 HL068157 to A.A).

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Accepted for publication March 27, 2008.

A guest editor acted as editor-in-chief for this manuscript. No person at the University of Alabama at Birmingham was involved in the peer review process or final disposition for this article.

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pression in the APCs may modulate the suppressive capacity of Treg cells. We found that Treg cells from HO-1-deficient mice functioned normally in the presence of wild-type APCs and Teff cells, but suppression by both HO-1-deficient and *HO-1*$^{+/+}$ Treg cells was abolished in the presence of HO-1-deficient APCs.

Materials and Methods

Animals

Male and female *HO-1*$^{-/-}$ mice (8 to 12 weeks of age, C57BL/6 × FVB) 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 mating.[10] Age-matched wild-type (*HO-1*$^{+/+}$) littermates were used as controls. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Immunofluorescence Staining and Flow Cytometry

Spleens were harvested and single cell suspensions prepared according to standard protocols. The suspended cells were stained with monoclonal antibodies against B cells (CD45R/B-220), T cells (CD3, CD4, CD8), and several markers of Treg cells (CD25, Foxp3, CTLA-4, GITR, LAG-3), coupled with biotin (CD25) or various chromogens (fluorescein isothiocyanate, phycoerythrin, PerCP, APC, and Alexa 647) and immediately analyzed. Cells were pretreated with antibodies specific for CD16/32 to inhibit nonspecific binding of phycoerythrin to FCγ receptors. For the detection of intracellular antigens, the cells were first surface stained with antibodies, then fixed, permeabilized, and stained with antibodies against Foxp3 and CTLA-4. Isotype-matched antibodies were used as negative controls. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed with Winlist analysis software (Verity Software House, Topsham, MA).

Suppression Assays

$CD4^+CD25^+$ (Treg) and $CD4^+CD25^-$ (Teff) were isolated from total splenocytes using magnetic bead separation kits according to the manufacturer's instructions (Miltenyi, Auburn, CA). This methodology yields a population of $CD4^+$-depleted cells (APCs), $CD4^+CD25^+$ cells (Treg), and $CD4^+CD25^-$ cells (Teff). APCs were irradiated (3300 rad) and plated onto 96-well plates (10^5 cells/well). Teff cells at a constant number (10^4 cells/well) were then added with a varying number of Treg cells to provide Teff/Treg ratios of 1:0, 1:2, 1:1, 2:1, 4:1, and 8:1. A combination of 1 μg/ml of soluble anti-CD3 and 1 μg/ml of soluble anti-CD28 (eBioscience, San Diego, CA) provided the polyclonal stimulus for proliferation. In experiments in which purified dendritic cells (DCs) were used, stimulation was provided by incubation with soluble anti-CD3 at 250 ng/ml. Cells were incubated in RPMI with 10% fetal bovine serum in a total volume of 200 μl. At 5 days of culture, 1 μCi of ^{3}H-thymidine (Amersham Biosciences, Piscataway, NJ) was added for the final 16 hours to assess proliferation. Suppression was determined by ^{3}H-thymidine incorporation, with the percent suppression = [1 − (mean cpm Treg + Teff)/(mean cpm Teff) × 100%]. In some experiments, incorporation was measured by BrdU incorporation using a commercial cell proliferation enzyme-linked immunosorbent assay kit (Roche Diagnostics, Indianapolis, IN).

Isolation of Bone Marrow-Derived Dendritic Cells (BMDCs)

BMDCs were isolated using a modification of the method described by Lutz and colleagues.[11] Nonadherent bone marrow cells were cultured in complete Dulbecco's modified Eagle's medium containing 400 U/ml GM-CSF (Peprotech, Rocky Hill, NJ). The medium was changed every third day, and the nonadherent cells were removed on day 9 and used in suppression assays as noted.

HO Enzyme Activity Assay

HO activity assays were performed in spleen microsomes as previously described.[12,13] Briefly, spleen microsomes from *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ mice were incubated with rat liver cytosol (3 mg), a source of biliverdin reductase, hemin (20 μmol/L), glucose-6-phosphate (2 mmol/L), glucose-6-phosphate dehydrogenase (0.2 U), and NADPH (0.8 mmol/L) for 1 hour at 37°C in the dark. The formed bilirubin was extracted with chloroform, change in optical density from 464 to 530 nm was measured and enzyme activity expressed as nmol of bilirubin formed per 60 minutes per mg protein.

Western Blots

Samples were prepared in Laemmli buffer and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. The gels were subsequently transferred to a Hybond-P polyvinylidene difluoride membrane. The membrane was blocked in a 5% nonfat dry milk and 1% bovine serum albumin solution for 1 hour at room temperature before addition of antibodies specific for HO-1 and HO-2 (SPA-896 and SPA-897, respectively; Stressgen Biotechnologies, Victoria, Canada). The membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG antibody for 1 hour (Jackson ImmunoResearch, West Grove, PA). Peroxidase antibodies on the membranes were detected using the ECL chemiluminescent detection system (Amersham Biosciences).

Statistical Analysis

The statistical analysis was performed using the two-tailed unpaired *t*-test, and a *P* value less than 0.05 was considered significant. Data are presented as mean ± SEM.

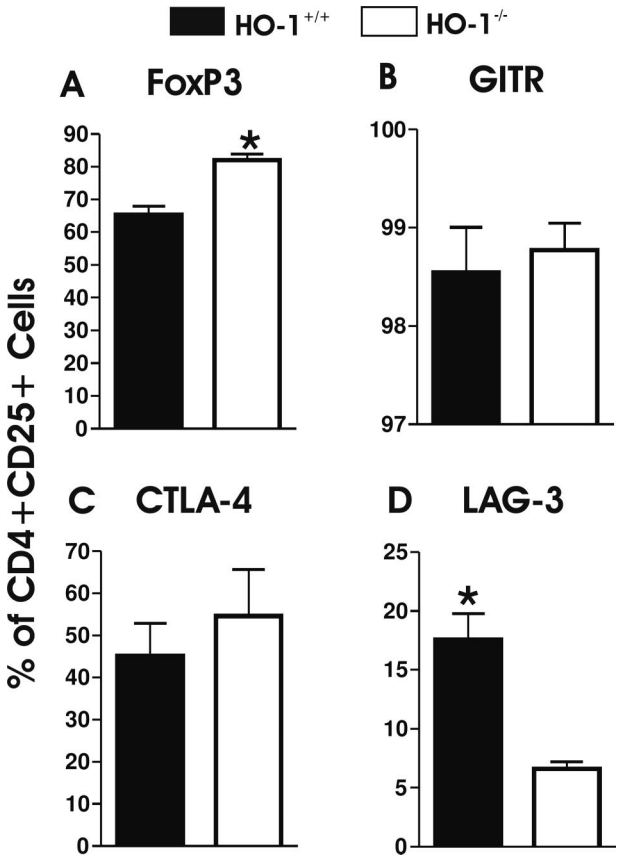

Figure 1. Flow cytometric analysis of gated $CD4^+CD25^+$ mouse splenocytes expressing FoxP3 (**A**), GITR (**B**), CTLA-4 (**C**), and LAG-3 (**D**) ($n = 4$ for *HO-1*$^{-/-}$ and $n = 3$ for *HO-1*$^{+/+}$; all analyses were gated on live cells). *P* values were calculated using the Student's *t*-test. $^*P < 0.05$ versus *HO-1*$^{+/+}$.

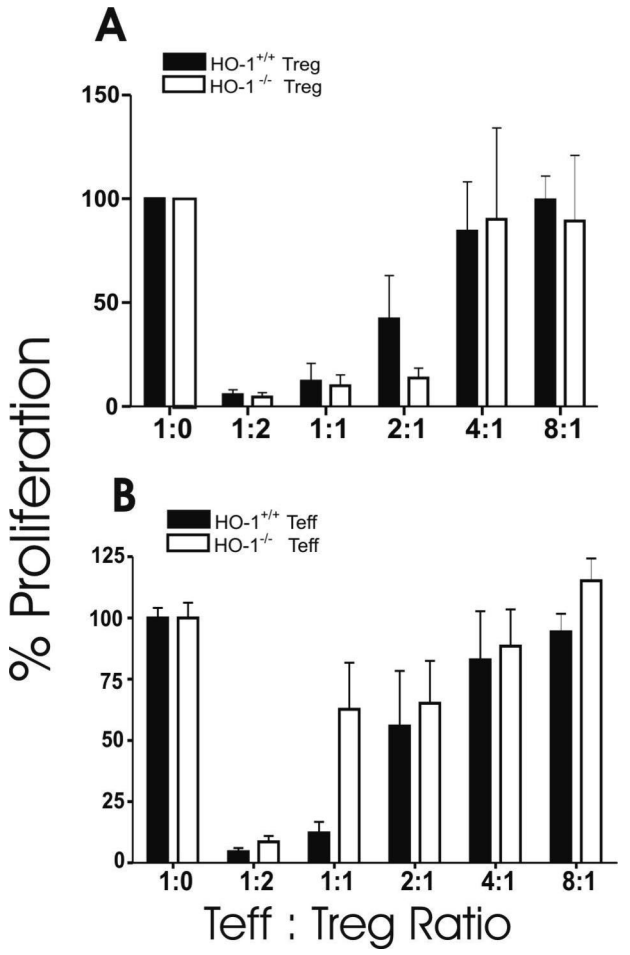

Figure 2. Results of *in vitro* suppression assays as a function of the presence or absence of HO-1 expression by Treg in the presence of HO-$1^{+/+}$ Teff cells and APCs (**A**); the presence or absence of HO-1 expression by Teff cells in the presence of *HO-1*$^{+/+}$ Treg cells and APCs (**B**). In both panels, solid bars designate wild-type cells, and open bars designate HO-1-deficient cells (Treg cells in **A** and Teff cells in **B**). At least three separate experiments were performed for each set of conditions.

Results

HO-1 Deficiency Is Associated with Abnormalities in Treg Phenotype

Previous work from our laboratory and others clearly demonstrated that proinflammatory tendencies are associated with HO-1 deficiency.[5] Based on these observations, we characterized the phenotype of Treg cells in the spleens of HO-1-deficient mice, and examined the frequency of cells bearing a variety of putative Treg markers. We found that the overall frequency of splenic $CD4^+CD25^+$ cells was the same in both *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ mice (2.12 ± 0.12, $n = 3$, and $2.27 \pm 0.32\%$, $n = 4$, of gated lymphoid cells, respectively; $P = 0.497$, Student's *t*-test). However, among these cells, the proportion of $FoxP3^+$ cells was significantly higher in *HO-1*$^{-/-}$ animals (Figure 1A, $P = 0.005$). The proportion of cells expressing glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) among $FoxP3^+$ cells was not significantly different in *HO-1*$^{-/-}$ animals in comparison to *HO-1*$^{+/+}$ animals (Figure 1, B and C, respectively). Both GITR and CTLA-4 are involved in inhibition of the suppressive activity of Treg cells. Interestingly, however, the expression of LAG-3 (Figure 1D), a marker previously associated with Treg-suppressive function,[14,15] was significantly lower in *HO-1*$^{-/-}$ mice ($P = 0.010$) suggesting that, in fact, *HO-1*$^{-/-}$ Treg function might be impaired.

The Absence of HO-1 Expression in Treg Cells Does Not Impair Their in Vitro *Suppressive Function*

Based on the aforementioned findings, we examined the suppressive capacity of Treg cells obtained from either *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ mice on polyclonal $CD4^+$ T-cell proliferation in response to crosslinking of CD3 and CD28 in the presence of *HO-1*$^{+/+}$ APCs. As depicted in Figure 2A, *HO-1*$^{-/-}$ Treg cells suppressed the proliferation of Teff cells in a dose-dependent manner, at least as efficiently as those from *HO-1*$^{+/+}$ mice. Of note, the origin of Teff cells from either *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ animals had no bearing on the degree of suppression (Figure 2B). These findings imply that the presence or absence of HO-1 within Treg cells does not influence their suppressive capacity.

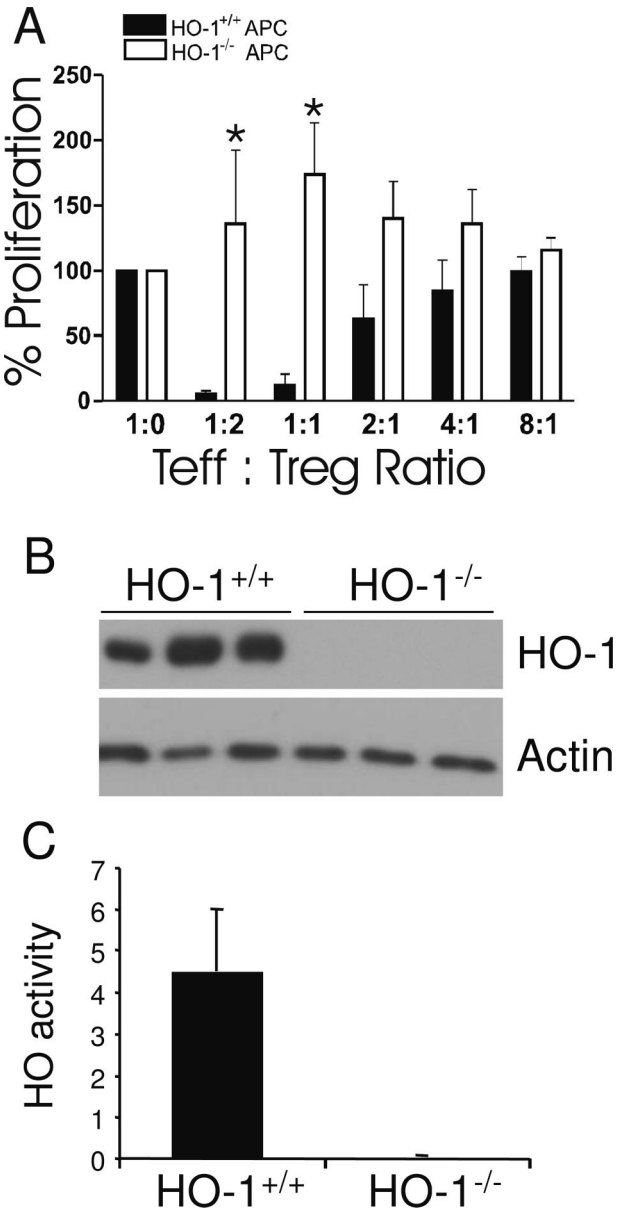

Figure 3. A: Suppression of T-cell proliferation as a function of the presence or absence of HO-1 in APCs. Solid bars designate *HO-1*$^{+/+}$ APCs, and open bars designate HO-1-deficient APCs. At least three separate experiments were performed for each set of conditions. *$P < 0.05$ versus *HO-1*$^{+/+}$. **B:** HO-1 protein expression in splenic microsomes isolated from *HO-1*$^{+/+}$ ($n = 3$) and *HO-1*$^{-/-}$ ($n = 3$) mice. **C:** HO enzyme activity in splenic microsomes from *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ mice ($n = 3$ for each genotype). HO activity was measured by bilirubin generation (nmol/hour/mg protein).

The Absence of HO-1 Expression in APCs Impairs Suppressive Function of Treg Cells

Next we evaluated the potential importance of HO-1 in APCs on the modulation of Treg function. In experiments similar to those described above, polyclonally stimulated Teff cells were co-cultured with various quantities of Treg cells in the presence of APCs obtained from either *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ mice. As demonstrated in Figure 3A, the absence of HO-1 in APCs abolished the suppressive influence of Treg cells on Teff cell proliferation. Here also, the HO-1 genotype of Teff and Treg cells played no role

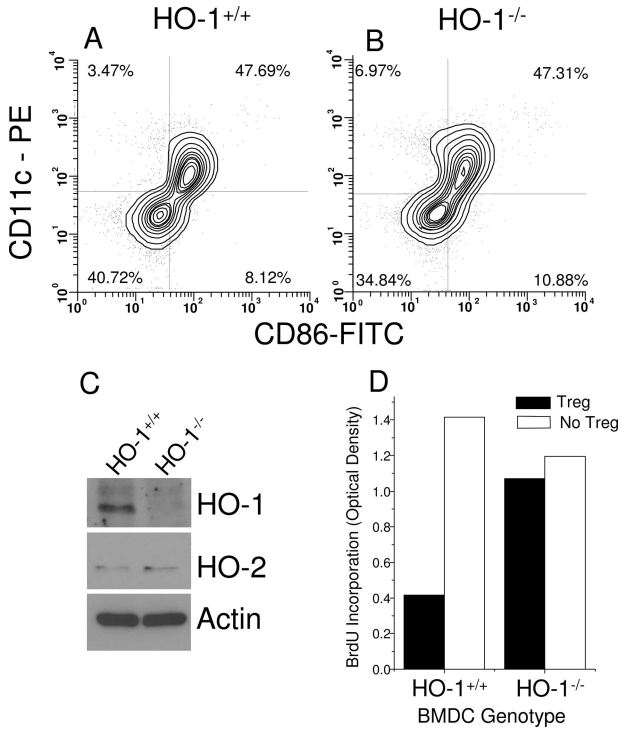

Figure 4. Surface phenotype of HO-1$^{+/+}$ (**A**) and HO-1$^{-/-}$ (**B**) BMDCs at 5 days of culture just before addition to suppression assays. The phenotype was defined by staining with anti-CD11c-phycoerythrin and anti-CD86-fluoresecin isothiocyanate. **C:** Western blot of HO-1 and HO-2 expression in BMDCs on day +9 of culture. The HO-1 genotype is indicated above each lane. Actin served as a loading control. **D:** CD4^{+}CD25^{+} Treg function is contingent on expression of HO-1 in dendritic cells. This plot depicts BrdU incorporation after 5 days of stimulation under conditions specified in the Materials and Methods. Results are derived from pooled BMDCs generated from two to three mice from each genotype.

(data not shown). Western blots of microsomal fractions of splenocytes confirmed that the mice were deficient in expression of HO-1 (Figure 3B). Splenic HO enzyme activity in *HO-1*$^{-/-}$ mice also showed that HO activity was undetectable (Figure 3C). In these initial experiments, the APC population was heterogeneous, consisting of monocytes, B cells, and DCs. To determine whether HO-1 expression in DCs could be important, Teff cells were co-cultured with Treg cells in the presence of BMDCs grown in culture from *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ mice. The surface phenotype of the BMDCs isolated from *HO-1*$^{-/-}$ mouse bone marrow was the same as immature *HO-1*$^{-/-}$ DCs, with positive CD11c expression and low levels of expression of CD86 (Figure 4, A and B) and MHC class II (data not shown). Low levels of HO-1 expression were observed in DCs from *HO-1*$^{+/+}$ mice, but not from *HO-1*$^{-/-}$ mice. HO-2 levels were low and unchanged (Figure 4C). The absence of HO-1 expression in the dendritic cells resulted in a loss of suppression by the Treg cells (Figure 4D), consistent with the results seen with APCs isolated from the spleen (Figure 3A). Treg cells proliferated readily when incubated without Teff cells in the presence of *HO-1*$^{-/-}$ APCs, whereas they did not proliferate in the presence of *HO-1*$^{+/+}$ APCs (Figure 5). Collectively, these data suggest that the presence of HO-1 in APCs is essential for maintaining the suppressive function of Treg cells.

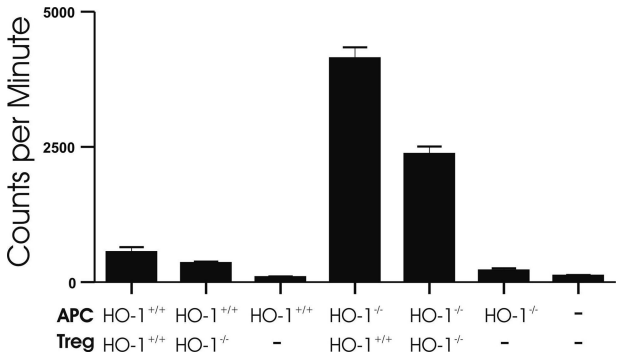

Figure 5. The lack of HO-1 in APCs is associated with proliferation of $CD4^+CD25^+$ Treg cells under stimulation with anti-CD3 and anti-CD28 antibodies. The plot depicts [H^3]thymidine uptake results after 5 days of incubation under stated conditions. Measurements were compiled from at least three separate experiments.

Discussion

The results of this study provide insights into the nature of the immune abnormalities associated with HO-1 deficiency. We demonstrate that *HO-1*$^{-/-}$ mice exhibit significantly higher frequencies of $FoxP3^+$ cells among $CD4^+CD25^+$ T-cell populations. The expression of lymphocyte activation gene-3 (LAG-3), a protein suggested to correlate with increased suppression by Treg cells[15–17] was significantly lower in *HO-1*$^{-/-}$ animals. These differences suggest that Treg function could be impaired in *HO-1*$^{-/-}$ mice, which would provide an explanation for the immunoregulatory defects observed in these mice. However, our *in vitro* studies demonstrate that the suppressive function of Treg cells was normal in the presence of wild-type Teff cells and APCs, indicating that the immune dysregulation in *HO-1*$^{-/-}$ mice was not attributable to an intrinsic defect in Treg function. Given that Treg function is known to depend on the activity of APCs, we examined the role of HO-1 in these cells to influence Treg function as determined by *in vitro* suppression assays. Our results clearly demonstrate that a lack of HO-1 in APCs significantly impairs the suppressive function of Treg cells under conditions of APC excess.

Previous characterizations of *HO-1*$^{-/-}$ mice indicate that they develop chronic inflammatory changes with time, with a relative overgrowth of the $CD4^+$ T-cell subset.[4] Studies by Pae and colleagues[7] have suggested that up-regulation of HO-1 in Treg cells may be related to the suppressive function of these cells. On the other hand, others have demonstrated that $CD4^+CD25^+$ cells from HO-1-deficient BALB/c mice retain their suppressive capacity both *in vitro* and *in vivo*.[18] DCs actively participate in modulation of regulatory T-cell activity.[19,20] As shown here and by others, immature DCs express HO-1, but apparently reduce or lose this expression as they mature.[21] Furthermore, the protective effects of HO-1 induction against the development of diabetes coincides with reduced DC infiltration into islets.[22,23] Therefore, it is possible that induction of HO-1 or its products can inhibit DC maturation. Our current study supports this concept in that we did not observe any defect in the *in vitro* suppressive activity by Treg cells from *HO-1*$^{-/-}$ mice in the presence of wild-type APCs and Teff cells. However, the absence of HO-1 in APCs resulted in a substantial loss of suppressive function by the Treg cells, indicating that the regulatory abnormality in *HO-1*$^{-/-}$ mice could be related to the induction and maintenance of Treg function by APCs rather than to an intrinsic defect of the Treg cells alone. Recent observations suggest that the up-regulation of HO-1 in APCs is essential for abolishing pathological findings in models of neuroinflammation. The protective effect of HO-1 was associated with down-regulation of MHC class II molecules, but not with changes in the infiltration of Treg cells into the lesions.[24,25] HO-1 mediated alteration of the expression of either MHC molecules or co-stimulatory molecules and subsequent changes in APC/Treg interactions may be the underlying explanation for the differences in Treg suppressive activity and could explain findings reported in other experimental systems.[26–28]

It is well established that the expression of MHC and co-stimulatory molecules, as well as the cytokine milieu affect lymphocyte stimulation.[29] We have previously demonstrated that HO-1 deficiency favors Th1 polarization of the profile of cytokines released by activated splenocytes.[5] This effect was observed when lymphocytic populations were stimulated with anti-CD3/CD28 antibodies or when APCs were also activated by exposure to the Toll ligand, bacterial lipopolysaccharide. In addition, large amounts of IL-6 were released from stimulated *HO-1*$^{-/-}$ splenocytes in comparison to wild-type controls. IL-6 released by fully activated mature DCs is capable of reversing Treg-mediated suppression, likely by decreasing the susceptibility of $CD4^+CD25^-$ cells to Treg influence.[30] Moreover, such activated DCs can reverse the anergy of Treg,[30,31] a phenomenon seen in our study in the presence of HO-1-deficient APCs. Thus, the functional status of APCs, particularly DCs, can affect both the proliferation of Teff cells as well as the function of Treg cells.[32] It is conceivable that the crucial role of HO-1 activity is to modulate antigen presentation and other ancillary functions of APCs. Furthermore, these findings highlight a more general role for tolerogenic APCs in reinforcing the function of Treg. A prime example is the existence of DCs with a so-called tolerogenic phenotype that can be achieved by interference with the process of maturation.[33,34] These tolerogenic DCs both suppress the activation of Teff cells and promote T-cell regulatory mechanisms. It would therefore be informative if the observations in this experimental system were dependent on cell to cell contact, soluble factors, or both. Experiments are currently underway to determine how HO-1 affects DC and T-cell interactions.

Interference with NF-κB activation in the presence of antigen is one of the described means by which DCs can acquire the tolerogenic phenotype.[35,36] Interestingly, both HO-1 as well as the product of its activity, CO, have been shown to inhibit activation of the NF-κB pathway,[37–39] and CO has been shown to inhibit polyclonal T-cell proliferation in response to anti-CD3 and anti-CD28.[40] It is therefore possible that the induction of HO-1 during the early steps of DC activation may aid in the acquisition of a tolerogenic phenotype. Moreover, tolero-

genic DCs, unlike mature DCs, secrete large amounts of Th2 cytokines, such as IL-10. Interestingly, there is a very close correlation between HO-1 activity and IL-10 secretion. HO-1 activity can result in increased IL-10 production.[41] On the other hand, the anti-inflammatory properties of IL-10 require the expression of HO-1 in immune cells.[42] The latter was demonstrated in our recent work showing that IL-10 prevented the immune processes associated with chronic allograft rejection, an effect dependent on systemic expression of HO-1.[3]

We conclude that the activity of HO-1 is an important regulatory mechanism affecting multiple levels of the immune response. The elucidation of its effects on specific immune cell populations will aid the development of therapeutic strategies for a variety of inflammatory disorders, including autoimmune diseases and transplant rejection. Conversely, inhibition of HO activity may serve an adjuvant effect to increase Teff responses in cases of cancer or infection by persistent pathogens.

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