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Heme Oxygenase-1 As a Target for the Design of Gene and Pharmaceutical Therapies for Autoimmune Diseases

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Abstract: One of the major goals in the research of autoimmune diseases is to develop specific therapies to regulate the expression and function of gene products that could contribute to restoring tolerance to self-constituents and replace conventional systemic immunosuppression, which is associated with important undesired side effects. Although significant progress has been made on the understanding of the pathogenesis of autoimmunity, therapies for these ailments have not seen a change. During the last decade, different strategies such as pharmacologic or gene therapy modulation of heme oxygenase-1 (HO-1) and the administration of its metabolic product, carbon monoxide (CO), have been shown to display beneficial immunoregulatory and cytoprotective properties. In different experimental autoimmune conditions, such as Experimental autoimmune encephalomyelitis, type-1 diabetes and systemic lupus erythematosus, genetic or pharmacological modulation of HO-1, as well as delivery of CO have shown to ameliorate disease progression. Furthermore, it has been demonstrated that dendritic cell and monocyte function can be modulated by HO-1 and/or CO. In this article, recent data related to the immunoregulatory properties of HO-1/CO will be discussed, focusing on their potential therapeutic use to treat autoimmune diseases.

Keywords: Heme oxygenase-1, carbon monoxide, autoimmunity, tolerance, therapy.

1. INTRODUCTION

The development of autoimmunity is characterized by an auto-reactive immune response capable of injuring healthy host cells and tissues. The etiology of autoimmune diseases is unknown and associated with a confluence of genetic predisposition and environmental exposure [1]. During the last decade, important progress has been made in the characterization of the molecular mechanisms responsible for the onset and progression of autoimmunity, such as the involvement of Th17 related cytokines in multiple sclerosis [2, 3] and type I interferons in systemic lupus erythematosus (SLE) [4, 5]. Although this knowledge has promoted the development of anti-inflammatory drugs and biological agents to interfere with the immunepathogenesis, the current autoimmune therapies have not changed much in clinical practice with the advance of immunology. Thus, there is an urgent need for therapy improvement in terms of efficacy, adverse effects and specificity. Because cell-destruction and tissue damage during autoimmunity are mainly caused by immune cell-mediated inflammation [6-8], new therapies should be designed to target these inflammatory processes and suppress the damaging consequences of autoimmunity.

Heme oxygenase-1 (HO-1) is a powerful anti-inflammatory enzyme, which plays a critical role in stress conditions such as organ transplantation and hypertension [9-12]. We have previously reported that the restoration of HO-1 levels and administration of HO-1-derived metabolic product decreases inflammation in a mouse model of SLE [13]. Further, treatment with inducers of the HO-1/CO axis has also been shown to be effective at promoting tolerance in other pathologic conditions such as type 1 diabetes [14, 15] and neuroinflammation [16, 17]. We have recently described that patients with SLE, as well as lupus prone mice, show decreased levels of HO-1 in peripheral blood monocytes [13, 18]. In this context, HO-1 induction arises as a promising treatment to decrease inflammation in autoimmune diseases.

In this review, we highlight and discuss current knowledge about HO-1 as a potential target for the treatment of autoimmune diseases. In addition, we address recent *in vitro* results showing the decrease of inflammation mediated by myeloid immune cells caused by metabolic products derived from heme degradation by HO-1.

2. HO-1 BIOLOGY AND TISSUE EXPRESSION

Hemeoxygenases or haemoxygenases (HO) are enzymes that regulate cellular levels of the heme group [19]. A large

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proportion of heme transport and utilization is carried out by macrophages, either by defective erythrocyte phagocytosis or upon CD163-mediated uptake of hemoglobin–haptoglobin complexes [20]. These processes mainly occur in tissues enriched in macrophages, such as spleen and liver [19, 21]. The HO enzyme catabolyzes heme degradation into Fe^{2+}, biliverdin and carbon monoxide (CO) and is the rate-limiting enzyme in heme compounds degradation. Biliverdin is subsequently converted into bilirubin in a reaction catalyzed by the biliverdin reductase enzyme [22]. HO can modulate the function of some hemeproteins including nitric oxide synthase, soluble guanylatecyclase, cytochrome P450, peroxidase and catalase through the degradation of the heme group and CO release, which binds to this group, thereby inhibiting hemeproteins activity [19].

There are three isoenzymes of HO: an inducible isoform designated HO-1 and two constitutively expressed isoforms, known as HO-2 and HO-3 [23]. There is great diversity in the tissue distribution of HO-1 and HO-2 isoforms [22]. HO-1 is a heat shock and a stress protein induced by oxidative agents [24, 25], which is constitutively expressed in testis, brain, placenta and the vascular system [26-30]. On the other hand, HO-2 is highly expressed in epidermal cells, germ cells and neural tissues, playing a relevant role in the normal function of these organs [31, 32]. Moreover, HO-2 may protect neural tissues from oxidative stress by reducing lipid peroxidation through the catabolism of free heme [33]. In addition, it has been recently shown in mouse models that HO-2 is capable of protecting from renovascular hypertension and cardiac hypertrophy [29]. Thus, HO-2 is an important component of cells that constitutively participate in the regulation of physiological processes. In contrast, studies focusing on HO-3 enzyme are still rather limited [34].

The HO-1 isoform is mainly induced by heme, oxidative or free radical molecules during inflammatory conditions [9, 24, 25]. HO-1 is expressed in different cell types, such as hepatocytes [35], endothelial cells [36], myeloid cells [37, 38] and cells from the respiratory tract [39]. HO-1 expression can be induced through the activation of different signaling pathways, including cyclic adenosine-5′-monophosphate (cAMP)-dependent mechanisms [40], protein kinase C (PKC), phospholipase A2 (PLA2) [41] and by mitogen-activated protein kinases (ERK and P38) [42, 43]. In addition, fine tuning of HO-1 mRNA and protein levels is achieved by the activity of transcription factors Nrf2 and Bach1 [44]. It is thought that a protein known as Keap1 keeps Nrf2 in the cytoplasm preventing nuclear translocation. In response to oxidative stress conditions, ROS can react with Keap1 sulfhydryl groups, which promotes Nrf2 release and translocation into the nucleus [45]. Nrf2 binds to small Maf proteins to form heterodimers and transactivates the antioxidant response elements (ARE), which induces the expression of antioxidative gene products, such as HO-1, NADPH-generating enzymes, glutathione biosynthesis enzymes, chaperone proteins and ubiquitin-proteasomes [45]. These ARE gene products react with ROS derived from environmental and intracellular sources, limiting cellular damage. On the contrary, Bach1/2 with small Maf proteins binds to Maf response elements (MARE), including ARE, to inhibit HO-1 gene transcription and allow HO-1 transcription when heme-Bach1 interaction occurs (Fig. **1**) [46, 47].

Although the main function of HO-1 is to degrade free heme, several immunoregulatory features have been attributed to this enzyme. The anti-inflammatory role of HO-1 is thought to be mediated by the by-products generated after heme group degradation, which will be discussed in the following sections.

3. HO-1 PROMOTES CELL VIABILITY

Antioxidant Capacity of HO-1

Heme group degradation plays an important role in cellular defense and survival. The HO-1 cytoprotective effects may reduce pathogenesis during several oxidation-mediated inflammatory diseases by limiting both, oxidative stress and apoptosis. HO-1 contributes to regulate intracellular prooxidant systems by degrading heme group and releasing CO from hemeproteins and enzymes, such as iNOS, peroxidase and catalase [19]. These reactions also promote the binding of ferritin to the free iron released from HO-1 catalysis over hemeproteins, which prevents free radical formation [48]. Moreover, it has been shown that cultured astrocytes from rat forebrain increase HO-1 expression after hydrogen peroxide exposure, preventing oxidative stress-mediated injury [33]. Further, in a mouse model of ischemic stroke with middle cerebral artery occlusion, HO-1 overexpression protects from injury, showing an increased expression of ferritin, decreased iron deposits and reduced lipid peroxidation [49]. Likewise, in diabetic rats, HO-1 induction increases superoxide dismutase and catalase activity, decreasing reactive oxygen species(ROS) levels in aortic tissue [50].

Anti-apoptotic Properties of HO-1

The anti-apoptotic capacity of HO-1 has been demonstrated at different levels. In primary cultures of cerebrovascular endothelial cells from newborn pigs, the induction of HO-1 inhibited caspase 3 activity after glutamate-induced cell death [51]. After cerebral ischemia, transgenic mice that overexpress HO-1 showed neuroprotection by enhancing the expression of the antiapoptotic factor Bcl-2 [49]. Also, by using an *in vitro* model of anoxia-reoxygenation with rat pulmonary endothelial cells, it has been suggested that CO released by HO-1 catalysis activates p38 MAPK signaling pathway, limiting caspase-3 activity and inhibiting cell death [52]. In human neutrophils, it has been shown that heme decreases Bad degradation -a proapoptotic Bcl-2 member- and induces Bcl-xL expression, limiting the spontaneous mitochondrial apoptotic pathway [52, 53]. More importantly, Kahlo *et al.* showed that human trophoblasts from spontaneous abortions expressed low levels of HO-1 which were associated with a decreased expression of the anti-apoptotic molecule Bag-1 [54]. Furthermore, it has been shown that HO-1 overexpression by gene transfer increased Bcl-2 and Bag-1 levels in a model of liver transplantation [55]. In addition, HO-1 induction decreases cell death of PC12 epithelial cells by preventing the depolarization of mitochondrial transmembrane potential [56]. Furthermore, HO-1 induction and CO administration to mice improve kidney function following ischemia and protect against treatment with cytostatic drugs [57, 58]. Cisplatin is an anti-neoplastic drug, which leads to severe impairment of renal function by oxidative stress and caspase-mediated apoptosis. Interestingly, the

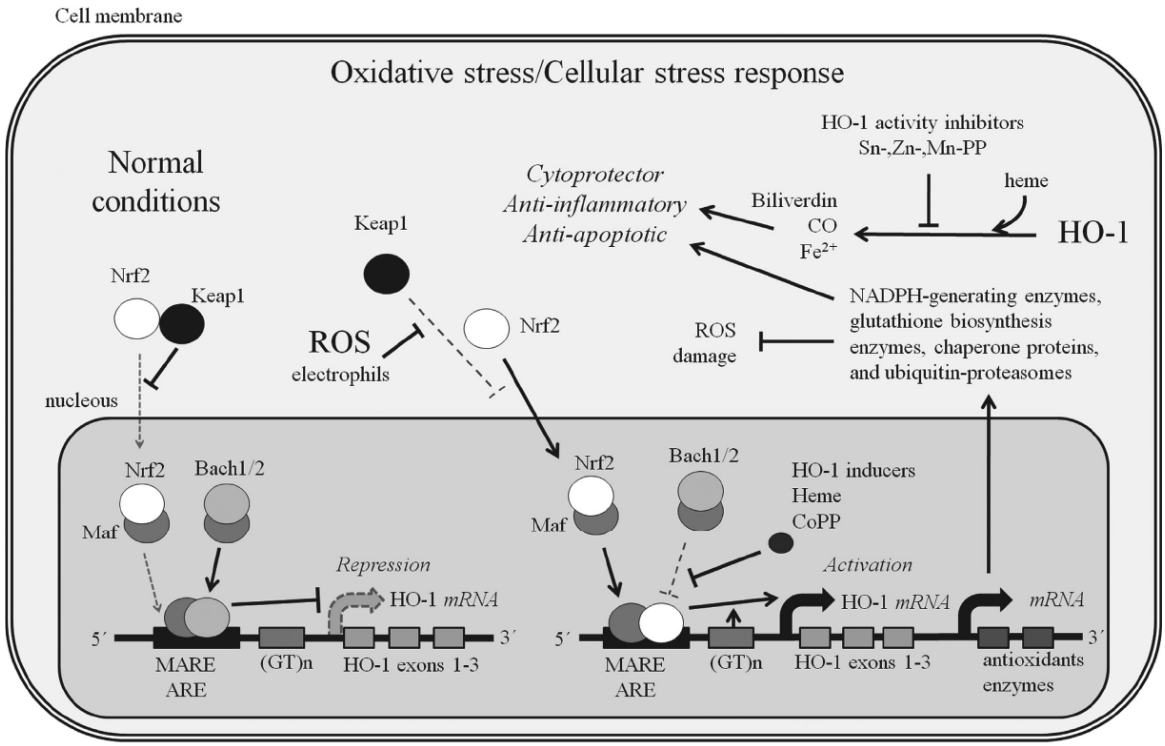

Fig. (1). ***Bach1 and Nrf2 transcription factors modulate HO-1 mRNA transcription.*** Under normal conditions, Keap1 sequesters Nrf2 in the cytoplasm and Bach1/2 with small Maf proteins bind to Maf response elements (MARE), including antioxidant response elements (ARE), repressing HO-1 gene transcription and allowing HO-1 transcription when heme-Bach1 interaction occurs. In human, a dinucleotide guanine-thymidine repeat $(GT)n_{15\text{-}40}$ polymorphism at HO-1 promoter region may impact in determining the level of the transcription induction. In stress conditions, such as oxidative stress, ROS may react to Keap1 releasing Nrf2 to translocate into the nucleus. Nrf2 forms heterodimers with small Maf proteins and binds to ARE to increase the expression of antioxidant enzymes such as HO-1 and glutathione biosynthesis enzymes. These antioxidant enzymes remove ROS derived from environmental and intracellular sources, limiting further oxidative damaged. Heme (and classical HO-1 inducer such as CoPP) binds to Bach1, thus allowing Nrf2 to induce HO-1 transcription. HO-1 protein degrades heme group producing CO, biliverdin and Fe^{2+}leading to cytoprotective, antiapoptotic and antiinflammatory effects. Mn-, Sn- and Zn-PP inhibit HO-1 activity by competitive blocker of heme substrate.

anti-apoptotic effect mediated by HO-1 improves transplant survival. In a recent study, it has been shown that HO-1 induction by a retroviral vector suppresses TNF-α-induced cell death [59]. Thus, HO-1 arises as a cell sentinel mediating anti-apoptotic effects associated with inflammatory stimuli that, if sustained are likely to cause release of intracellular components that can support a positive feedback for inflammation. HO-1 overexpression in the mitochondria or in the vicinity of mitochondria membranes is associated with a change in redox status of cells, which would prevent Cytochrome C release and the activation of caspases, which would contribute to prevent cell death [60, 61].

Cytoprotective Effects of HO-1 and its by-products

HO-1 may also have anti-proliferative properties that are mediated by CO and biliverdin. While proliferation of vascular smooth muscle cells contributes to atherosclerosis pathogenesis, it has been shown that CO and biliverdin can reduce this process and induce cell cycle arrest by suppressing the expression of cdk2 activators and cyclin D1 [62-64].

CO, in conjunction with bilirubin, may exert different functions, including the modulation of adiponectin levels, which prevents adipogenesis, inflammation and myocardial ischemia [65], the regulation of vascular function and stimulation of ion channels [66] leading to vasorelaxant effects [67]. Furthermore, CO inhibits cytochrome P450 activity, leading to vasoconstriction inhibition [68]. In addition, it has been suggested that CO could by itself contribute to cellular signaling, mainly as an activator of guanylatecyclase [69].

4. PHARMACOLOGICAL AND GENETIC TOOLS TO MODULATE HO-1 ACTIVITY

Pharmacologic Induction of HO-1 Activity

Given that HO-1 has cytoprotective properties, this enzyme has been postulated as a potential therapeutic target for immune-mediated diseases where both, cell integrity and balance, can be drastically compromised. Thus, up-regulation of HO-1 activity could be of clinical interest for these ailments. In experimental animal models, it has been demonstrated that HO-1 activity is increased in whole tissues after treatment with its natural substrate heme, as well as various metals, xenobiotics, endocrine factors, synthetic metalloporphyrins and stress factors [22]. Cobalt protoporphyrin IX (CoPP) has been reported as a classical inducer of the HO-1 expression in different tissues and capable of increasing significantly this enzyme activity (Fig. **1**) [22]. However, it has been shown that CoPP could also act as a competitive inhibitor of the activity of the HO system in *in*

vitro assays [70, 71]. In addition, hemin has also been shown to be a potent HO-1 inductor *in vivo* [22]. Interestingly, aspirin, one of the most common anti-inflammatory drugs, also induces HO-1 expression [72]. Moreover, there are some less common molecules that act as HO-1 inducers, such as AG1067, a probucol analog [73], probucol [22], paclitaxel [22], rapamycin [22] and curcumin [74].

Biliverdin and bilirubin can be protective by preventing oxidation-mediated cell death and contribute to endothelial cell integrity by their antioxidant potential [75]. However, due to the potentially detrimental effects that accumulation of these molecules could cause in some tissues, their use in the clinic remains uncertain. For instance, high levels bilirubin are harmful to the central nervous system [76]. However, regulated biliverdin administration can be beneficial in several experimental models of disease, as this molecule can protect against lung injury due to lipopolysaccharide-induced shock [77], it can prevent liver damage caused by ischemia and reperfusion [78] and it can increase islet allograft due to its immune-suppressive and antioxidant properties [79, 80]. Furthermore, weekly administration of non-toxic amounts of CoPP in a mouse model for type 1 diabetes increased adiponectin levels, which promotes resistance to oxidative stress and reduces pathogenesis [81]. Moreover, it has been demonstrated that hemin causes a strong induction of HO-1 expression in leukocytes, after administration in a single intravenous dose, in a phase I clinical trial [82].

Importantly and together with HO-1 induction, continuous administration of high amounts of CoPP as an HO-1 inducer may be toxic, mainly by consuming heme and therefore decreasing hemoproteins, such as cytochrome P450 [83-85]. Thus, excessive or sustained administration of molecules that induce HO-1 could be deleterious for the patient.

Although CO therapy may be beneficial in a wide spectrum of disorders such as multiple sclerosis, Type 1 Diabetes, SLE and nephrotoxicity, exposure to high levels of CO may be harmful [13, 17, 58, 86, 87]. In fact, CO binds hemeproteins and blocks mitochondrial electron transport, resulting in a release of detrimental superoxide anions, elevated titers of carboxyhemoglobin and reduction of oxygen transport to different tissues [87, 88]. Different molecules that can supply CO in solution and prolong its effects have been developed, such as CO-releasing molecules (CORMs) [89], which are effective *in vivo* and *in vitro* [90-92].

Thus, rational administration of HO-1 inducers could increase the activity of enzyme with a sustained delivery of their products, such as CO and biliverdin/bilirubin that can be of potential use for clinical application in several diseases, such as hypertension, diabetes, cardiovascular disease and immune-mediated diseases.

While most therapeutic approaches have focused on augmenting HO-1 activity, some pathologic conditions could be tackled by inhibiting HO-1 activity. In 1990s, HO-1 blockade using Sn-mesoporphyrin resolved the problem of progressive jaundice in newborns at risk of brain damage due to uncontrolled hyperbilirubinemia [22, 75, 76]. Blocking HO-1 activity in neonates was one of the first clues of HO-1 benefits in a clinical setting. It has also been described that treatment with zinc-protoporphyrin (ZnPP) improves mouse survival in a tumor-induced model mainly by making tumor cells more sensible to reactive oxygen species derived from the antitumor response [93]. Besides ZnPP, metalloporphyrins such as Sn- and Mn- protoporphyrin are potent competitive inhibitors of the HO-1 enzymatic activity (Fig. **1**) [22]. Although ZnPP is a potent HO-1 inhibitor, it has also been reported *in vitro* that ZnPP, at low concentrations, can produce a synergism of HO-1 induction by heme [94]. In contrast to HO-1 induction, there is no gene therapy related to HO-1 inhibition.

HO-1 Transcriptional Regulation by a Dinucleotide Guanosine-thymine Repeat Polymorphism

Transcriptional activity of HO-1 coding gene (HMOX1) can be regulated by a dinucleotide guanosine-thymine repeat (GT)n polymorphism at the promoter region, which determines the expression level of this enzyme [95]. Polymorphisms with short (GT)n repeats (< 25) promote higher levels of HO-1 expression as compared to long (GT)n repeats (n > 25) [95]. It has been suggested that (GT)n microsatellite length may influence the susceptibility to different diseases, including inflammatory ailments [95]. Previous studies have shown that short HMOX1 (GT)n polymorphisms associate with susceptibility to childhood-onset SLE but not with Juvenile Rheumatoid arthritis (JRA) [96]. Furthermore, RA patients carrying the short polymorphism showed a better clinical prognosis than did those with the long polymorphism [95]. Healthy individuals carrying the short polymorphism showed a higher percentage of monocytes expressing HO-1 than did individuals with the long polymorphism [97]. Thus, the short polymorphism displayed a significant protective effect in RA susceptibility [97]. Furthermore, these short and long polymorphisms have been associated with other pathologic conditions. For example, type 2 diabetes patients carrying the long polymorphism showed higher lipid peroxidation derived molecules in serum and higher frequencies of coronary artery disease than did patients with the short polymorphism [98, 99] (Fig. **1**). These observations suggest that HMOX1 polymorphism may impact on the HO-1 activity modulating pathologic conditions, such as RA, coronary artery disease and diabetes and may provide a novel target for immune mediated diseases treatment.

Gene Therapy to Modulate HO-1 Expression

Genetic tools have recently been designed to promote HO-1 induction in different tissues to promote cytoprotection and cell survival. Viral vectors carrying HO-1 gene have been used as treatment for different pathologic conditions [10, 59, 100-103].

While much work has been done to evaluate HO-1 gene therapy for transplant rejection and vascular diseases, there are scarce reports testing the effectiveness of HO-1 gene delivery in inflammatory diseases. In a muscular dystrophy model, it was shown that HO-1 lentiviral infection of porcine myogenic precursor cells can protect from staurosporine-induced apoptosis *in vitro* [104]. In addition, when these HO-1-transduced myogenic precursors were transplanted in pig skeletal muscle, a fivefold increase in cell survival was reported five days after transplantation [104]. Further, microinjection of adenovirus vector carrying human HO-1 gene (Ad-hHO-1) into the eye produced a local increase in the enzymatic activity, which was undetectable in peripheral tissues. This approach could be considered as a potential therapy for oxidative stress-mediated

ocular disease [105]. In contrast, when Ad-hHO-1 was delivered intravenously it resulted in expression of human HO-1 mRNA in several tissues including liver, kidney, heart, lung, and brain, which may be considered as a potential therapy for different conditions, such as tissue toxicity and hypertension [106]. Further, transduction of bone marrow-derived macrophages with an adenovirus vector containing HO-1 gene results in overexpression of this enzyme with a consequent reduction of nitric oxide (NO) and TNFα release, augmenting IL10 production after LPS stimulation [107]. In a model for renal ischemia/reperfusion injury (IRI), Ad-hHO-1-transduced macrophages injected intravenously localized preferentially and rapidly at the injured kidney prevented local platelet and fibrin aggregation and improved renal function [107]. Similar results were obtained in a model of liver transplantation when Ad-hHO-1-transduced macrophages transferred simultaneously with transplanted hepatocytes led to an extension in animal survival due to a reduction in the expression of TLR-4, as well as in the production of proinflammatory cytokines and liver infiltration [108]. Intracardiac delivery of retroviral vectors carrying HO-1 gene reduces the production of the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE) and ameliorates hypertension in spontaneously hypertensive rats [109]. In addition, it has been reported that the transfer of adenoviral vectors encoding for HO-1 to pregnant mice undergoing abortion improved pregnancy outcome [110]. Authors showed that HO-1 gene therapy reduced the pro-inflammatory cytokine IFNγ and increased IL4 production in both spleen and decidual lymphocytes, which is important in placental tolerance [110, 111]. In a model of chronic allogeneic aorta rejection in rats, it was shown that Ad-hHO-1-transduced aorta overexpressed HO-1 after ten days of allogeneic transplantation, leading to a reduction of intima and adventitia layer infiltration by $CD45^+$ cells, macrophages and $CD4^+$ T cells, as well as reduced expression of co-stimulatory molecules, which results in a favorable graft outcome [102]. Further, it was shown that Ad-hHO-1 transduction of pancreatic islets improves allogenic transplantation survival by reducing leukocyte infiltration and apoptosis [112].

Additionally, transgenic animals overexpressing HO-1 (tHO-1) have been generated showing similar results as obtained by pharmacologic induction or by the transduction of viral vectors encoding for HO-1 [113, 114]. tHO-1 mice expressing HO-1 under the control of the neuron-specific enolase promoter showed reduced stroke volumes and limited ischemic cerebral edema [114]. In a model of cardiac allograft transplantation, tHO-1 heart graft donor into WT recipient or WT heart graft donor into tHO-1 recipient improved allograft survival. Moreover, WT hearts transplanted into tHO-1 recipients displayed a decrease in $CD4^+$ T cell infiltration and activation measured by CD25 expression, prolonging graft survival [115]. Hearts transplanted into HO-1 Tg recipients showed decreased $CD4^+$ lymphocyte infiltration and diminished immune activation, as determined by CD25 expression [115]. All these results suggest that HO-1 gene therapy could be considered as a potential strategy to improve therapies based on cell transfer or transplantation.

HO-1 Deficiency

The first reported patient with HO-1 deficiency occurred in 1999 which showed growth retardation, hemolytic anemia, abnormal coagulation function, premature death, endothelial damage and atherosclerotic changes in the vasculature, linking HO-1 system with atherosclerosis [116]. Also, this patient showed major alterations in the immune system such as leukocytosis and lymph node swelling.

Similarly to human HO-1 deficiency, HO-1 KO mice showed splenomegaly and lymph node swelling, leukocytosis, altered $CD4^+$ T cells ratio, and an elevated amount of activated T cells [117]. Also, HO-1 KO mice showed increased vulnerability and a decreased survival in response to LPS-mediated challenge, a phenomenon that is thought to be associated to the modulation of mitochondrial function by HO-1 [117]. Furthermore, HO-1 KO mice showed an increase in cytokine responses, such as IL-1, IL-6 and TNF-α in LPS-stimulated splenocytes [118]. Similarly, when HO-1 KO splenocytes were stimulated with CD3/CD28 antibodies, the production of IL-2, IFN-γ and other cytokines was increased [118]. The exacerbated immune responses shown by HO-1 KO mice suggested that HO-1 activity may be closely related to a regulatory role in the activation of myeloid and lymphoid cells.

5. THE ROLE OF HO-1 IN IMMUNE CELLS

The Role of HO-1 in the Regulation of Dendritic Cells Immunogenicity

Several inflammatory diseases, such as autoimmune diseases, are caused by alteration in the function of T cells [119-122]. The onset of this exacerbated response is triggered when antigen presenting cells (APCs) capture self-antigens, degrade and present them to self-reactive naïve T cells. The most important APCs with the capacity to prime resting T cell are the DCs [123]. These cells mainly reside in peripheral tissues where they constantly sense antigens and danger signals [124-128], to transfer these to lymphoid organs and present these antigens to naïve and memory T cells [123, 126], thus eliciting an antigen-specific adaptive immune response. In response to danger-inflammatory signals, DCs undergo a process known as maturation [126-128]. It has been reported that in the absence of pro-maturation stimuli, DCs remain unable to successfully process and present antigens to naïve T cells [129, 130]. DC maturation is important because it enhances the communication with T cells during antigen presentation, which involves TCR binding to MHC-peptide complexes, the expression of co-stimulatory molecules and the release of pro-inflammatory cytokines, such as IL-12 and IL-6 [123, 131-134]. In addition, DCs can also secrete anti-inflammatory cytokines such as IL-10, which regulate T cell priming by counteracting the inflammatory environment [135].

Recently, it has been reported that HO-1 induction by CoPP in humans and rats impairs DC maturation during LPS-mediated stimulus [38] (Fig. **2A**). Interestingly, HO-1 induction was not only associated with the inhibition of maturation markers, but also with the expression and secretion of anti-inflammatory molecules. This was the case for IL-10, an anti-inflammatory cytokine whose secretion by DCs was not altered by the induction of HO-1 expression by CoPP, supporting the tolerogenic profile promoted by the activity of this enzyme (Fig. **2A**). Moreover, HO-1 induction can impair the intracellular LPS-induced generation of ROS, which is a typi-

cal process of the innate immune that contributes to antigen processing [38, 136]. In this context, we have shown that maturation of murine and human DCs can also be inhibited by CO [92]. Thus, inhibition of DC maturation by HO-1 is attributable to CO with a reduced activation of IRF3 pathway, leading to a subsequent decrease in IL-12 levels [92] (Fig. **2A**). However, Mashreghi *et al.* reported that CoPP inhibition of DCs maturation by LPS stimulation is independent of HO-1 and dependent on STAT3 [137]. Authors showed that CoPP prevented maturation of DCs from both HO-1 deficient and wild type mice while STAT3 inhibition reversed the anti-inflammatory effects of CoPP on DCs [137].

As the main roles of DCs are the capture and presentation of antigens (including autoantigens) to T cells, HO-1 induction could promote the tolerogenic capacity of DCs and reduce the activation of autoreactive T cells. It has been demonstrated that plasmacytoid DCs from SLE patients release large amounts of IFN-alpha (IFN-α), which induces the maturation of conventional DCs, that in turn activates autoreactive T and B cells [138, 139]. In this context, it has been shown that treating HO-1 induction in DCs or treatment with CO reduces antigen presentation in both MHC-I and –II [140]. Interestingly, only the presentation of small soluble antigens (proteins), but not the presentation of antigens associated to large particles, such as 3 μm latex beads in DCs was impaired by CO treatment. Thus, it seems that antigen size is important for antigen presentation inhibition by HO-1 activity. Along these lines, it has been described that small antigens, such as ovalbumin (OVA), can enter DCs by the mannose receptor (MR) or the pinocytic route [141, 142]. Once intracellular, MR-driven capture of OVA leads antigens to $Rab5^+$ compartments where proteasomal degradation takes place. Then, class I MHC presentation is rapidly directed toward cells surface. OVA captured by pinocytosis follows the vesicular pathway, leading antigens to a sequential fusion of endosomal compartments that finally converge in the fusion of late endosome-containing OVA with lysosomes [129, 141, 143, 144]. Thus, interference with both MR and pinocytic routes can lead DCs to decrease their capacity to target soluble antigens to degradative compartments and present them on MHC-I and –II molecules. Consistent with this notion, we have recently shown that either HO-1 induction or treatment of murine DCs with CO at low doses impairs presentation of soluble antigens to T cells [140]. We observed that CO impairs the targeting of soluble OVA to lysosomes by stalling this antigen in late endosomes. Despite OVA was fully degraded in late endosomal compartment, fusion of these vesicles with lysosomes did not take place, preventing the loading of peptides on MHC-I and -II molecules. No alterations were observed for the proteasomal/degradative route, because both targeting of soluble antigens to early endosomes ($Rab5^+$) and presentation of OVA as an endogenous antigen were not impaired by CO. Thus, CO produced by HO-1 decreased the capacity of DCs to present soluble antigens by reducing the destination of these molecules to lysosomal compartments and thus reducing the generation of pMHC complexes. Interestingly, we did not observe any alteration in the capacity of DCs to present bead-bound OVA. These large particles were captured by DCs trough phagocytosis. It has been recently proposed that large particles are captured by phagocytic cells by using, in part, the endoplasmic reticulum (ER) membrane [145]. The fusion of early phagosomes with ER produces a large vesicle known as the Ergosome [145-147]. It has been shown that this compartment is able to produce pMHC-I complexes even before fusing with lysosomes [147]. Thus, despite CO impairs endosomal LPS-driven soluble OVA processing, the mechanism by which large phagosomes are processed within DCs renders them resistant to HO-1 activity, because it seems that they do not require fusion with lysosomes. However, recent findings have proposed that large particles, which are captured by ER-mediated phagocytosis, acquired both MHC-I and -II in conjunction with other lysosomal and ERs proteins [148]. Thus, it is proposed that large cargo rapidly fuses with compartments that carry MHC-I and -II molecules and, after antigen degradation, emerging peptides can be loaded on MHCs. These peptides are derived from the phagosome-to-cytosol process with subsequent proteasomal degradation and re-internalization of fragments into the vesicle using TAPs proteins [146-148]. Also, antigens can be processed by phagoluminal lysosomal proteases such as Cathepsin D [146-148]. Interestingly, despite it still remains controversial, phagosome maturation inside APCs has been proposed as LPS-independent [149, 150]. Thus, despite HO-1 activity, by means of CO, is able to reduce LPS-dependent endosomal pathways by causing a stalling in the soluble antigen trafficking in DCs (Fig. **2B**), CO is unable to block Ergosome maturation and antigen presentation for large particles.

Capture of external soluble antigens is a conserved mechanism by which DCs sense foreign and self-antigens to promote either activation or tolerance by T cells [151]. During graft rejection, DCs play an important role in directing the inflammatory response that finally leads to the destruction of the engrafted tissue [152, 153]. Disruption of new grafts has been associated with the activation of T cells that secrete elevated titers of pro-inflammatory cytokines that impair cell-to-cell junctions together with activated cytotoxic T $CD8^+$ cells. Thus, reducing T cell activation is a promising alternative to decrease graft rejection. However, as described above, T cells become activated after receiving stimulus from innate immune cells designed to present extracellular antigens, such as DCs. In this context, it has been recently proposed that targeting the function of these cells is a promising therapy to generate antigen-specific tolerance [153]. As inflammation associated to graft rejection is similar in various aspects to that observed for autoimmune diseases, it is possible that we could learn about how to handle self-inflammatory conditions by studying graft rejection models and the tools used in this field by conferring tolerance to certain antigens. It has been shown that transference of tolerogenic DCs significantly reduces graft rejection [152-154] and that induction of HO-1 *in vivo* protects against graft rejection [101, 102, 155-157]. CO has been postulated as the candidate molecule that mediates the tolerogenic profile that supports graft acceptance in these studies [102]. Moreover, CO exposure or HO-1 induction in donor organs reduced the immunogenicity of DCs by decreasing MHCII and co-stimulatory molecules thus improving graft survival [158]. Also, Schumacher *et al.* state that HO-1 expression on DCs contributes to maintain pregnancy mainly by inducing an immature phenotype and avoiding presentation of paternal antigens to maternal T cells [159]. Consistent with this notion is the observation that the transfer of hemagglutinin

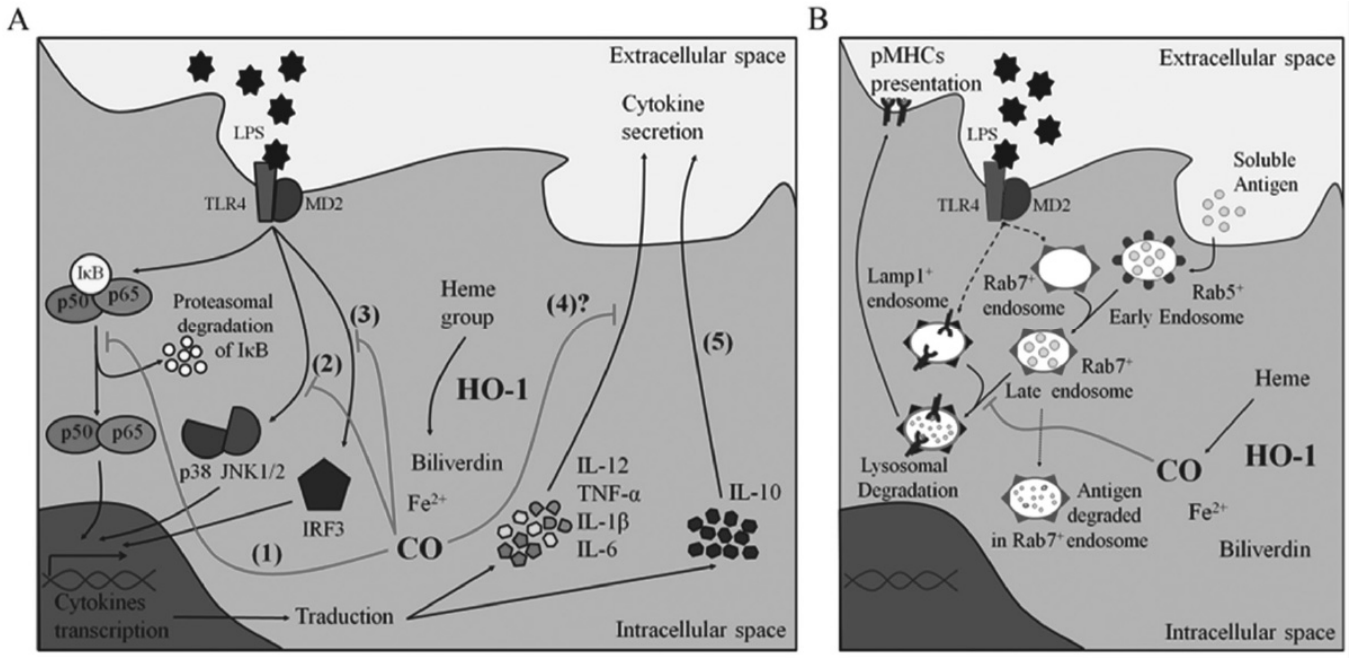

Fig. (2). ***HO-1 produces CO and impairs both the production of Fig. (3) inflammatory cytokines and the presentation of soluble antigens in myeloid cells***. **A**) After TLR4/MD2 engagement by LPS, three independent pathways are triggered (NFκB, p38/JNK1/2 and IRF3). Altogether, these pathways promote the production of either inflammatory or anti-inflammatory cytokines. After HO-1 induction, heme-group is degraded and CO, Fe^{2+} and biliverdin are produced. CO is able to inhibit the activation of (1) NFκB, (2) p38/JNK1/2 and (3) IRF3 signaling pathways. This leads to a decrease in the amount of inflammatory cytokines produced. Still remains unknown whether CO can block the traffic of inflammatory cytokines toward the extracellular milieu (4). In addition, CO has been reported as unable to affect IL-10 production and secretion (5). Inhibitory pathways are represented as gray lines. **B**) Dendritic cells capture soluble extracellular antigens and target these molecules to early endosomes ($Rab5^+$). Then, after TLR4/MD2-mediated stimulation of $Rab7^+$-vesicles (interlinear arrow), these compartments fuse with early endosomes forming late endosomes ($Rab7^+$). Next, $Lamp1^+$ lysosomes fuse with $Rab7^+$-late endosomes in a TLR4/MD2-dependent manner (interlinear arrow) to produce antigen degradation and loading of peptides over class I and II MHCs (pMHCs). pMHCs are transported to the surface of DCs to further prime T cells. After heme degradation and CO production by HO-1, CO impairs the fusion of $Rab7^+$-late endosomes with $Lamp1^+$-lysosomes, thus reducing the target of the soluble antigen-derived peptides to MHCs. As a consequence, antigens are stalled and degraded in $Rab7^+$-vesicles but without chances to be loaded on MHCs molecules (dotted arrow). Class I and II MHCs presentations are finally impaired by CO.

(HA)-specific $CD8^+$ T cells primed by CO-treated DCs loaded with an HA-derived peptide to Tg mice expressing HA in the pancreas did not induce diabetes. Instead diabetes was developed when these mice were transferred with HA-specific $CD8^+$ T cells primed by untreated DCs loaded with HA. These observations support CO treatment as a promising new tool to induce tolerance in immune-mediated diseases [15]. HA-specific $CD8^+$ T cells primed by CO-treated DCs showed reduced β1 integrin and reduced ability to lyse isolated pancreatic islets [15]. The generation of tolerogenic DCs by pulsing them with CO gas, protected mice against an immune system-mediated disease, such as type1 diabetes.

Although much advance has been made in understanding the effects of CO over DCs, the mechanism by which CO causes tolerance and reduces T cell activation by DCs remains unknown. As in autoimmune diseases, DCs may take up self-antigens and present them to self-reactive T cells, CO treatment could reduce intracellular antigen trafficking and modulate DC maturation to reduce T cell activation, as discussed above [140].

Suppression of Macrophage Function by HO-1 and CO

Similar to DCs, HO-1 induction in macrophages might revert inflammatory processes. It has been shown that macrophages respond to TLR ligands, such as LPS, via the TLR4/MD2 complex [160, 161]. After LPS recognition, these cells secrete elevated amounts of inflammatory cytokines, including TNF-α, IL-6 and IL-12 [160, 161]. Also in acute phases of infection by Gram-negative bacteria, where LPS reaches abnormal levels in tissues, macrophages, supported by DCs activity, release inflammatory cytokines in an uncontrolled manner [160-162] leading even to death [163]. Thus, down regulation of the inflammatory capacity of macrophages is a promising approach to address bacteria-driven sepsis.

It has been reported that in macrophages the response to LPS challenge can be suppressed by CO upon HO-1 induction [164]. Interestingly, the authors of this work described that CO has the capacity to decrease the secretion of TNF-α, IL-1β and (MIP)-1β by macrophages after LPS challenge (Fig. **2A**). In contrast, CO administration increased the secretion of the anti-inflammatory cytokine IL-10 by macrophages, which is similar to what was observed for DCs. Indeed, authors reported that the mechanism underlying this effect of CO involves the MAPK signaling pathway, because mice lacking these kinases were unable to respond to CO treatment [164]. Consistent with this notion is the observation that CO requires the activation of the JNK signaling

pathway to reduce inflammation in a mouse model for sepsis in mice [37]. Contrary to wild-type, mice lacking either *Jnk1* or *Jnk2* genes were unable to prevent inflammatory cytokine production in response to LPS after CO administration. Wild type animals drastically decreased the secretion of both IL-6 and IL-1β in the presence of CO. However, *Jnk* knock-out animals failed to respond to CO and cytokine secretion remained unaltered. *In vitro*, this correlated with reduced secretion of these cytokines by LPS-pulsed CO-treated macrophages. Thus, CO exerts its immune-modulatory mechanism by interfering with the JNK signaling pathway [37].

It has been shown that CO induces IL-10 secretion and that IL-10 has the capacity to induce HO-1 expression in macrophages by activating the p38 signaling pathway [165], which could be beneficial during inflammatory responses. Indeed, *in vivo* administration of IL-10 protects mice from LPS-mediated sepsis by inducing HO-1 expression. Although there is no direct experimental evidence showing that macrophages are the immune cells mediating this protective effect during sepsis, it is likely that innate cells, such as macrophages and DCs, could play an important role in these events. In fact, although it has been shown that DCs are more effective at secreting inflammatory mediators upon pathogen-associated molecular patterns (PAMPs) stimulation, the role of macrophages during this process cannot be ruled out [162, 166].

The mechanisms responsible for the capacity of HO-1 and CO to reduce macrophage inflammatory response to LPS remain obscure. It has been recently shown that macrophages from mice with cystic fibrosis produce exacerbated amount of inflammatory cytokines when challenged with LPS [167]. In normal macrophages, LPS challenge induces a sustained activation of NF-κB/MAPK signaling pathway, which produces pro-inflammatory cytokines and ROS. This leads to the stress-induced expression of HO-1 and to the recruitment of HO-1 to cell surface with caveolin-1, suppressing TLR4-mediated signaling by CO production [167]. Interestingly, macrophages from cystic fibrosis patients showed a decreased expression of caveolin-1, which recruited less HO-1 to the cell surface leading to sustained NF-κB/MAPK signaling and production of ROS, altering macrophage homeostasis [167]. These data support the importance of HO-1 and CO as protective agents against inflammation and suggest that the proper cellular localization of HO-1 is required to ensure an effective anti-inflammatory response [167].

Interestingly, in models that use inflammation mediated by both macrophages and pathogens in intestinal diseases, HO-1 induction or CO administration enhanced the killing of Gram negative bacteria, suggesting a different role for this enzyme [168]. Other studies have reported that CO-treated macrophages engulf elevated numbers of Gram-negative bacteria [169] and that CO administration enhances formation of the phagolysosomes [170]. Thus, it is likely that in macrophages, the capture, processing and presentation of antigens could be different from DCs when exposed to CO [140]. Further studies are required to clarify these apparent discrepancies. Studies about the role of HO-1 and CO as anti-inflammatory molecules need to be analyzed in depth for DCs and macrophages, because these mechanisms can be exploited to restrict inflammatory condition during pathogen-driven illness or autoimmune diseases.

Suppression of T Cell Function by HO-1 and CO

It has been reported that both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells express HO-1 and activation by CD3/CD28 antibodies induces the expression of this enzyme, suggesting a possible direct role for HO-1 in T cell function [171]. It has been shown that HO-1 induction as well as CO exposure could abolish IL-2 production and T cell proliferation after CD3/CD28 stimulation [172]. In activated T cells, CO blocks upstream signaling cascades of MEK1, thereby resulting in ERK inactivation and abolishing IL-2 secretion [172]. Thus, HO-1 activity could be associated with reduced T cell activation. Also, pharmacological overexpression of HO-1 in Jurkat T cells prevented Fas-mediated apoptosis triggered by anti-Fas antibodies [173]. Interestingly, it has been shown that HO-1 expression was dramatically increased in Foxp3-transfected Jurkat cells and pharmacological blockade of HO-1 activity abolished the suppressive function of $CD4^+CD25^+$ regulatory T cells, playing a critical role in maintaining peripheral tolerance and modulating immune responses [174].

HO-1 would be clinically important as a focal point in the development of strategies to reverse the detrimental effects of T cell-mediated diseases. Also, it has been recently demonstrated that a repressor of HO-1, Bach2, is highly expressed in T cells. Bach2 is up-regulated during T cell development as naive T cells express high Bach2 levels [175]. The endogenous HO-1 substrate heme, promotes Bach2 degradation in B cells in conjunction with effector differentiation to plasma cells [176]. Consistent with this notion, naive murine $Bach2^{-/-}$ (CD62LhiCD44lo) T cells in spleen and lymph node were decreased [175], suggesting that Bach2 is required for the homeostasis of peripheral T cells and might modulate effector memory-related genes and function in naive and effector-memory T cells. Most importantly, $Bach2^{-/-}$ mice showed anti-nuclear and anti-double stranded DNA autoantibodies, extensive perivascular and alveolar infiltration by lymphocytes and macrophages in lungs and mild infiltration in gut and stomach [177]. Also, $Bach2^{-/-}$ T cells evidenced a bias to Th2 related cytokines, such as IL-4 and IL-10 as well as Blimp-1 and Gata3 [175]. Other studies have shown that Bach2 cDNA transfection into effector memory T cells suppressed effector-memory T cell related genes, suggesting that Bach2 expression contributes to the maintenance of naive T cells. Also, previous work confirmed that Bach2 binds to Hmox1 (HO-1 encoding element) [175] and in $Bach^{-/-}$ T cells, HO-1 mRNA was increased. Further, $Bach2^{-/-}$ mice showed to be defective in immune responses *in vivo* [175]. In addition, it has been reported that naive T cells from $Bach2^{-/-}$ mice showed reduced numbers of $FOXP3^+$ cells and an impaired ability to form $FOXP3^+$ induced-Treg cells upon stimulation in the presence of TGF-β [177]. These studies suggest that Bach2 represses genes associated with effector cell differentiation. Bach2, which could be modulated by heme groups, may limit immune activation, playing a critical role in the maintenance of regulated immunity.

Suppression of B Cell Function by HO-1 and CO

Although there are few reports showing that HO-1 induction may modulate humoral immunity, the role of Bach2 in B/plasma cell modulation has been studied in more detail.

HO-1 KO mice show higher serum IgM levels as compared to wild type mice, indicating that HO-1 ant its by- products, or CO/heme levels, would be involved in modulating antibody production [118]. The transcription factor B lymphocyte-induced maturation protein 1 (Blimp-1), a master regulator of plasma cells [178], was induced by heme stimulation in B cell cultures, then promoting plasma cell differentiation by inactivating Bach2, a repressor of Blimp-1 [176]. Also, authors showed that the administration of heme concomitantly with antigen, reduced the production of antigen-specific IgM *in vivo*, suggesting that heme also modulates the early phase of B-cell responses to antigen [176]. Furthermore, it has been shown that Bach2 is required for class switch recombination/somatic hypermutation of immunoglobulin genes [179]. Heme binds to Bach2, inducing its degradation and Bach1/Bach2 redundantly represses the expression of HO-1 in B cells [176], supporting the notion that HO-1/heme/CO are key modulators in humoral immunity. $Bach2^{-/-}$ mice also have reduced numbers of naive T and B cells, which show a rapid differentiation to effector cells, suggesting a major role of this transcription factor in naive cells homeostasis [175].

6. INVOLVEMENT OF HO-1 IN AUTOIMMUNE DISEASES

Systemic Lupus Erythematous (SLE)

Deregulated inflammation is a common feature of chronic autoimmune disease as SLE. The autoimmune response in SLE is mediated by both cells and humoral components of the immune system [180]. SLE is characterized by the presence of autoantibodies against dsDNA, nucleosomes, ribonucleoproteins and other nuclear components [181]. Systemic damage present in SLE is mainly due to immune complexes deposition in blood vessels, followed by granulocyte and complement activation, leading to a wide spread inflammation and tissue injury [119, 120]. Lupus symptoms are accompanied by alterations in the immune system such as the presence of pathogenic B- and T- cells that lead to autorreactive responses to nuclear self-antigens, as well as an increase in inflammatory cytokines producing a major inflammatory status [119, 120, 180]. Despite much progress has been made in developing novel immunotherapies, this has not been the case for the treatment of lupus, most probably due to its complexity, continuing with systemic immunosuppressant agents such as corticosteroids, azathioprine, mycophenolatemofetil and cyclophosphamide [182, 183].

Recently, our group has reported that SLE patients showed a decreased HO-1 expression in peripheral blood monocytes [18]. Furthermore, mice lacking the FcγRIIb receptor, a spontaneous lupus murine model, also showed decreased levels of HO-1 in DCs, T $CD4^+$ cells and $CD11b^+$ cells [13]. In this model, animals suffering lupus displayed elevated numbers of monocytes/granulocyte population in spleen. Here, when lupus prone mice were chronically treated with gaseous CO, monocyte/granulocyte expansion was limited [13]. Interestingly, $FcγRIIb^{-/-}$ mice in spleen contained less numbers of regulatory $TCD4^+$ $Foxp3^+$ cells than wild type mice, which was restored when animals received CO as an inhalator gas. In addition, histone specific autoantibodies were also decreased after CO-treatment. CO treatment, as well as HO-1 induction, were able to ameliorate the development of proteinuria in this model [13] (Fig. **3A** and **B**). Thus, the inflammatory environment mediated by elevated number of monocytes, neutrophils and self-antigen-specific T cells can be controlled by the expansion of regulatory/anti-inflammatory T cells after administration of CO. In addition, it has been shown that HO-1 induction by hemin administration in other lupus prone mice, MRL/lpr, was able to decrease autoantibodies (IgG anti DNA) production, ameliorate renal glomerulonephritis by decreasing immune complex deposition, and decrease the levels of the proinflammatory cytokine IFN-γ in serum [184].

Rheumatoid Arthritis

The role of HO-1 in rheumatic diseases could be further corroborated in the collagen induced arthritis model. In this experimental model of rheumatoid arthritis, the administration of CO decreased serum anti-collagen antibodies, ameliorated disease activity, and showed lower inflammation and cartilage erosion [185]. In other approach, when the role of endogenous HO-1 level was evaluated in arthritis development using HO-1 deficient mice, $HO\text{-}1^{-/-}$ and $HO\text{-}1^{+/-}$ mice developed accelerated clinical symptoms of arthritis compared with $HO\text{-}1^{+/+}$ animals after intraperitoneal injection of arthritic K/BxN mice serum (Fig. **3C**) [186]. In this work, the authors showed that $HO\text{-}1^{+/-}$ mice evidenced more synovial infiltration and accumulation of inflammatory cells in joint spaces and proteoglycan depletion than WT mice. Also, IL-6 and matrix metalloproteinase-3 (MMP-3) were increased in ankles of $HO\text{-}1^{+/-}$ and $HO\text{-}1^{-/-}$ arthritic mice compared to $HO\text{-}1^{+/+}$ group [186]. Complementary, in mice treated with arthritogenic anti-type II collagen antibody plus LPS mixture, the CO exposure ameliorates clinical outcome of arthritis in footpads with a decrease in IL1-β and MCP-1 mRNA expression in the hind paws (Fig. **3D**) [187]. All this data in HO-1 deficient mice highlights HO-1 as a potential target for autoimmune disease therapy as well as a biological disease marker.

In the human disease, serum HO-1 levels were increased in systemic juvenile idiopathic arthritis during the active and inactive phase of the disease and being higher than levels observed in polyarticular juvenile idiopathic arthritis (p-JIA), Kawasaki disease, SLE or mixed connective tissue disease, promoting serum HO-1 levels as a useful marker for the differential diagnosis of systemic juvenile idiopathic arthritis [188, 189]. These data suggest that HO-1 modulation as well as CO exposure would be a potential strategy for SLE treatment.

Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) and is characterized by local inflammation, demyelization, and axonal loss. MS has remittent course leading to long-term disability. Although MS has been extensively studied, its etiology remains unknown and it is believed that immunological, environmental, genetic and infectious factors contribute to disease development [190, 191]. Most of current knowledge of immunomodulatory effects of HO-1 in MS was obtained from studies over

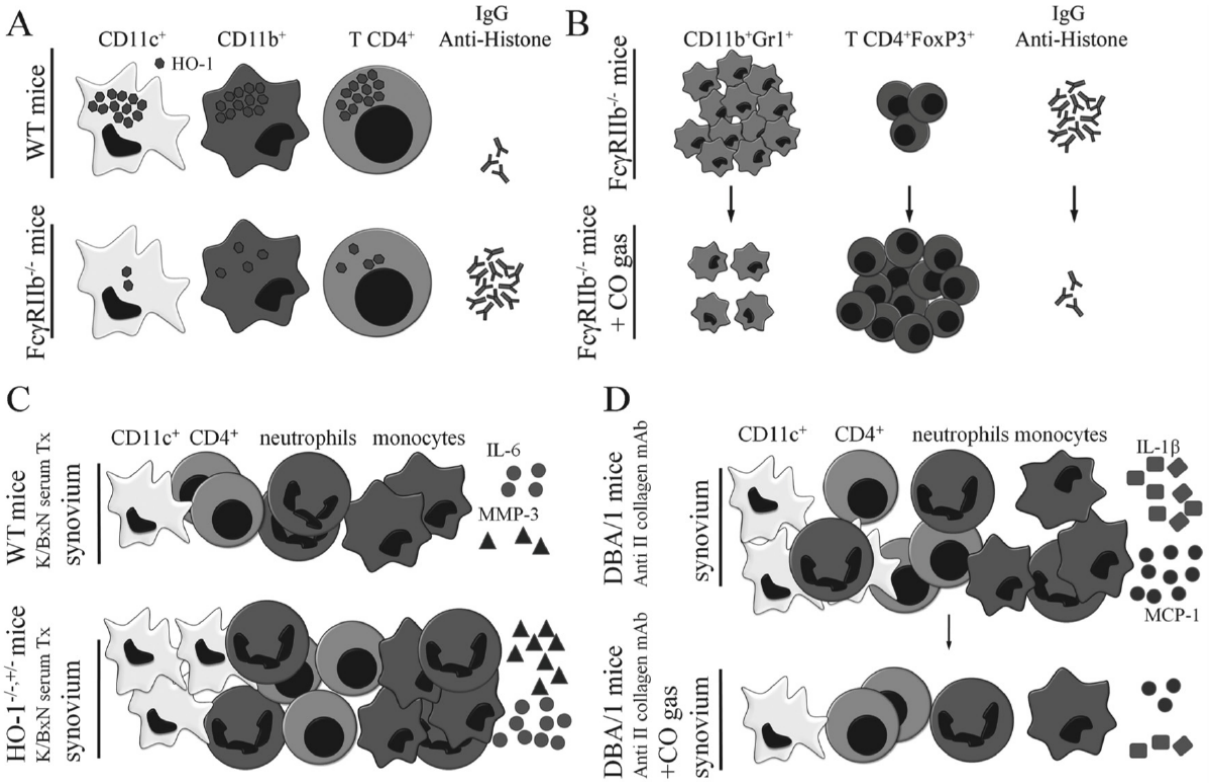

Fig. (3). ***HO-1/CO treatment reduces immune disorders in systemic autoimmune diseases.*** *Systemic Lupus Erythematosus*, **A**) Spleen $CD11c^+$, $CD11b^+$ and $CD4^+$ T cells express HO-1 in wild-type (WT) mice. Anti-histone IgG levels in serum are low. In the spleen of $Fc\gamma RIIb^{-/-}$ mice (suffering lupus, positive for anti-nuclear antibodies) there are decreased levels of HO-1 in $CD11c^+$, $CD11b^+$ and T $CD4^+$ cells. In addition, serum levels of anti-histone IgG are increased. **B**) Lupic $Fc\gamma RIIb^{-/-}$ mice display in spleen an inflammatory syndrome associated to increased amount of $Gr1^+CD11b^+$ inflammatory monocytes, reduced amounts of regulatory T $CD4^+Foxp3^+$ cells and increased levels of anti-histone IgG in serum. After chronic gaseous CO treatment, spleen levels of $Gr1^+CD11b^+$are reduced, regulatory $CD4^+Foxp3^+$T cells are increased and the levels of anti-histone IgG in serum are re-established to normal values. *Rheumatoid arthritis*, **C**) Role of HO-1 level in arthritis development using HO-1 deficient mice in the model of arthritic K/BxN mice serum transfer. $HO\text{-}1^{-/-}$and $HO\text{-}1^{+/-}$mice developed accelerated clinical symptoms such as synovial inflammation and proinflammatory cytokines production in compared with $HO\text{-}1^{+/+}$ animals after K/BxN mice serum transfer. **D**) Arthritic mice induced by the administration of anti-type II collagen antibody plus LPS mixture improve clinical outcome after CO exposure ameliorates inflammatory infiltrate in footpads with a decreased in IL1-β and MCP-1 expression.

experimental autoimmune encephalomyelitis (EAE) mice model. Both, MS and EAE are characterized by the presence of oxidative stress [192] and lipid oxidation [193]. During acute phases of EAE, HO-1 expression was markedly increased in microglia/macrophages and astrocytes. In CNS lesions from MS patients, HO-1 was mainly expressed in oligodendrocytes [194]. In contrast, it has been reported that peripheral blood mononuclear cells (PBMCs) from MS patients showed a reduced expression of HO-1during disease exacerbation, while corticosteroid treatment led to an increase in HO-1 expression [195] (Fig. **4A**). Interestingly, HO-1 induction by hemin was able to limit EAE development, while HO-1 inhibition by SnMP worsened the disease [196]. The protective effect of HO-1 in EAE was associated with inhibition of MHC class II expression by antigen presenting cells and inhibition of Th1 IFN-γ secretion, increasing Treg, decreasing IL-2, and $CD8^+$ T cell accumulation, proliferation, and function within the CNS (Fig. **4B**). CO acts on APC to inhibit the expression of MHC-II thus limiting reactivation of pathogenic Th cells within the CNS. Exogenous CO mimicked these effects, suggesting that CO contributes to the protective action of HO-1 [16]. Confirming these results, pre-treatment with CORM-A1 also protects from EAE development, showing amelioration of clinical score, disease incidence and reduction in the infiltration of polymorphonuclear cells [17, 197].

In addition, HO-1 may have a partial role in maintaining the integrity of the blood–spinal cord barrier, ameliorating damage due to proinflammatory leukocytes infiltration [198]. Also, HO-1/CO treatment reduces neuroinflammation in murine cerebral malaria, a disease that showed immune mechanism similar to EAE, by maintaining blood brain barrier integrity thus limiting T cell infiltration [199]. Moreover, our group has shown that mice gestated under hypothyroidism, and that in the adulthood have been induced with EAE have higher EAE score, increased demyelination and greater $CD4^+$ and $CD8^+$ infiltrating cells in the spinal cord compared to mice gestated in euthyroidism [1]. Interestingly, the expression of HO-1 is reduced in these mice compared to mice gestated in euthyroidism (data not published). Although the mechanism of HO-1 protection from EAE remains to be unveiled, immunomodulation as well as cytoprotective properties on CNS may have a major role in preventing neuroinflammation disease. These data pose endogenous HO-1 as a major protective factor in EAE, and that HO-1 modulation could become a novel therapy for MS.

Type 1 Diabetes

In type 1 diabetes, β cell damage and vascular injury are partially caused by ROS generation, leading to apoptosis [200-203]. Most of the knowledge about HO-1 protective effects in diabetes is related to vascular and endothelial cell function attributed to heme group degradation and the concomitant biliverdin and CO production. The protective effect of HO-1 may be associated with a decreased infiltration of dendritic cells in the pancreas [202]. HO-1 has a protective effect in pancreatic cells and β-cells by increasing anti-apoptotic proteins such as Akt and Bcl-xL, by reducing the pro-oxidant injury, and preventing the development of type 1 diabetes [81]. Hu *et al.* (2007) showed that HO-1 transduction ameliorated destructive insulitis and the incidence of diabetes in mice [204]. This would be associated with a lower T-helper cell (Th1)–mediated immune response, in which IL-2 and IFN-γ producing cells from spleen are decreased, as well as serum levels of these cytokines. Also, HO-1 transduction decreased the $CD11c^{+}$ $MHC\text{-}II^{+}$ cell population in spleen, suggesting that T cell priming is affected [204] (Fig. **4C**). Interestingly, Nikolic *et al.* showed that CORM-A1 treatment delayed the onset and reduced diabetes symptoms in NOD mice while reestablished insulin levels [86]. Also, this study showed that CORM-A1 treatment reduced both the expression of IFN-γ and the proliferative capacity of lymph node cells. Most importantly, CORM-A1 treatment increased the proportion of Treg cells, reduced the numbers of IL-17 and IFN-γ producing cells while increased IL-10 cytokine production [86]. In addition to the anti-inflammatory functions, CORM-A1 prevented islet cells apoptosis by downregulation of cytochrome *c* expression and suppression of caspase 3 activity [86].

Interestingly, HO-1 would be involved in different immuno-supressive signaling pathways. It has been reported that HO-1 expression would be regulated by PPARs agonists, which are known to improved diabetes in obese diabetic mice [205], consistent with the observed adiponectin increase [65]. Thus, HO-1 could be induced by other molecules that, in conjunction, can work together to decrease inflammation and produce a positive outcome for diabetic mice. The therapeutic utility of inducing HO-1 or CO exposure has yet to be tested in human diabetes.

Experimental Autoimmune Uveitis

Experimental models of autoimmune uveitis (EAU) have been used to evaluate the therapeutic potential of HO-1 relevant to immune mediated inflammatory diseases of the eye such as Behcet's disease, sympathetic ophthalmia, ocular sarcoidosis and Vogt-Koyanagi-Harada (VKH) disease [206-208]. Jang *et al.* demonstrated that HO-1 induction by hemin administration to rats immunized with interphotoreceptor retinoid-binding protein (IRBP) showed a lower grade of clinical inflammation than did the IRBP-control group examine with a biomicroscope [208]. Further, authors showed that HO-1 inhibition by the administration of Sn-PP to EAU rats accelerated the onset of clinical symptoms as well as worsening them [208]. Similarly, in an acute model of ocular inflammation, it was reported that hemin administration to LPS-treated rats significantly decreased the number of infiltrating polymorphonuclear cells in the anterior chamber of the eye [209]. In addition, HO-1 induction by hemin administration reduced iNOS and IL-6 mRNA expression in the iris-ciliary body after LPS injection. Most importantly, authors demonstrated that hemin pretreatment reduced protein, NO, IL-6 and TNF-α levels in Aqueous humor from the anterior chamber after LPS injection [209]. Similarly, Rossi *et al.* showed that hemin administration reduced the amounts of IL-1β and CXCL8 in ciliary bodies in the endotoxin-induced uveitis model under both normal and hyperglycemia conditions highlighting the potential role of HO-1 induction in the treatment of ocular diseases secondary to diabetes [210].

Clinically important is the fact that PBMCs from patients with active Behcet´s Disease (BD) expressed lower HO-1 mRNA levels than PBMCs from healthy controls [211]. TLR4 is involved in immune cells activation after ligation with specific DAMPS associated with Behcet´s Disease such as HSP60 [212]. Interestingly, Kirino *et al.* reported that PBMCs from BD patients showed an increase in TLR4 mRNA levels as compared to those from healthy controls [211]. Furthermore, the expression of TLR4 mRNA was inversely correlated with HO-1 mRNA levels in PBMCs from active BD patients [211].

7. INVOLVEMENT OF HO-1 IN INFLAMMATORY DISEASES

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is characterized by recurrent and severe gastrointestinal inflammation. As observed in EAE and type 1 diabetes models, CO-exposure improved the clinical score of mice with IBD. It has been demonstrated that CO administration or HO-1 induction expression by heme/CoPP ameliorates tissue destruction and inflammatory infiltrate in intestine of $IL10^{-/-}$ spontaneous IBD mice model compared with air-exposed mice in model [213]. Furthermore, authors showed that CO administration and HO-1 induction in $IL\text{-}10^{-/-}$ mice showed less weight loss and a reduction in IL12p40 and TNF secretion by explants. Complementary to this studies, the same group demonstrated that both CO treatment and HO-1 induction in IBD $TCRa^{-/-}$ mice reduced colitis, increased IL-10 and decreased IL-4 and IL-17 secretion by colonic cells compared with air-exposed $TCRa^{-/-}$ mice [214]. Similar results with CO/HO-1 were observed in dextran sulfate sodium-induced (DSS) colitis mice [215, 216]. Authors demonstrated that hemin significantly attenuated DSS induced colitis as evidenced by reducing inflammatory cell infiltration, IL-6 and IL-17 production, epithelia apoptosis and tissue destruction. In addition, HO-1 inhibition by SnPP pretreatment enhanced DSS-induced colitis inflammation, IL-6 production, mucosal bleeding and weight loss [215]. Also, hemin markedly increased $Foxp3^{+}$ Treg cells population in mesenteric lymph node of DSS-induced colitis mice. Ulcerative colitis patients with active disease showed augmented HO-1 levels than patients with quiescent disease or healthy controls by immunohistochemical staining [217]. In contrast, Crohn´s disease patients showed no difference in HO-1 levels compared to healthy controls or patients during inactive phase. All these data promote CO/HO-1 axis as a potential tool for IBD treatment.

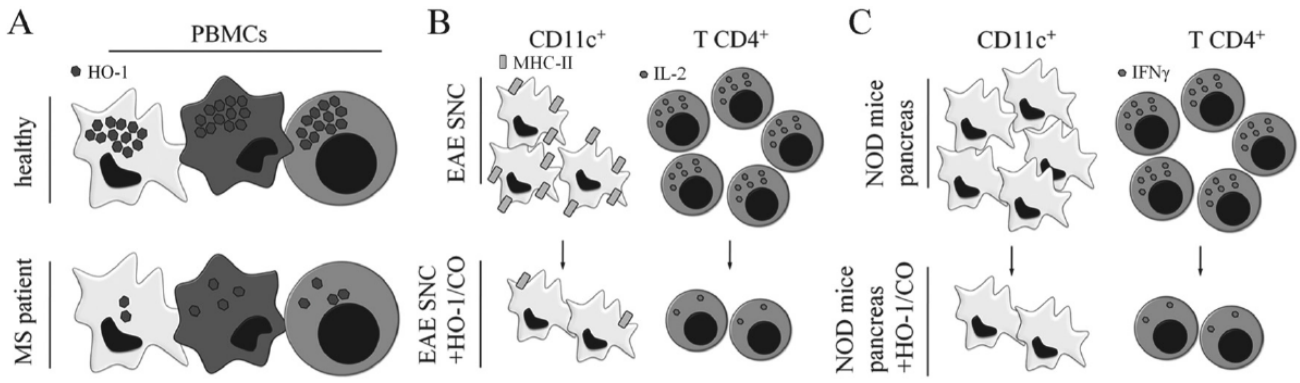

Fig. (4). ***HO-1/CO treatment reduces immune disorders in organ-specific autoimmune diseases.*** *Multiple Sclerosis*, **A**) MS patients showed a decreased HO-1 expression in PBMCs when disease is active. **B**) HO-1 induction as well as CO treatment in EAE mice ameliorates disease by decreasing MHC-II expression on $CD11c^+$cells and modulating cytokine production by T cells such as IL-2. *Type 1 Diabetes,* **C**) HO-1/CO treatment in NOD Type 1 diabetes mice block disease development by decreasing pro-inflammatory cytokines by T cells such as IFNγ and IL-2, insulitis and decreasing $CD11c^+$cells infiltration on β-islet areas.

Asthma and Allergy

Asthma is a chronic disease characterized by airflow obstruction airways inflammation, hyper-reactivity and remodeling. As most of immune mediated diseases, the etiology of asthma is multifactorial, in which Th2 related cytokines play a predominant role. In OVA-sensitized/challenged mice, an allergic airway inflammation model, pretreatment with hemin reduced inflammatory infiltration, including eosinophil infiltration, in bronchoalveolar lavage fluid and decreased levels of serum OVA-specific IgE, compared to OVA mice [218]. Also, authors showed that the administration of hemin increased Foxp3 mRNA expression in the lung and increased the percentage of $CD4^+$ CD25 high regulatory T cells in blood compared with OVA mice, suggesting that regulatory T cells play a role in hemin protection of allergic airway inflammation [218, 219]. The protection of hemin in the OVA allergic airway inflammation model was specifically prevented by the administration of HO-1 enzymatic inhibitor SnPP [219]. Also, in the neutrophilic lung inflammation model, transgenic mice for TCR specific for MHC-II restricted OVA peptide exposed to OVA aerosols, hemin administration reduced the total number of neutrophils and lymphocytes with much less tissue injury [220]. In addition, hemin reduced numbers of Th17 cells in spleen, IL-17 levels in bronchoalveolar lavage fluid and Th17-related transcription factor retinoic acid-related orphan receptor γt (RORγt) mRNA expression in lung tissues, mediators that were enhanced by OVA challenge. In contrast to the effect of hemin over Th17 profile, Th1 and Th2 profiles were not affected by HO-1 induction [220].

A small study in nonsmoking adolescents with moderate to severe asthma showed that HO-1 nasal epithelial mRNA expression was inversely associated with upper and lower airway clinical outcomes promoting HO-1 as a potential disease marker of airway severity [221].

Hepatitis and Liver Injury

A therapeutic potential for HO-1 induction in immune-mediated liver injury models has also been suggested. Odaka *et al.* showed that hemin treatment prevented the infiltration of inflammatory cells around the liver central vein and hepatocyte necrosis in halothane–hypoxia model rats [222]. In addition, it has been reported that HO-1 inhibition by Sn-MP further increases the proinflammatory cytokine TNF-α and NF-κB activation in carbon tetrachloride-liver injury model rats [223]. Similar cytoprotective effects of HO-1 induction were observed in the acetaminophen-induced hepatotoxicity model [224]. Sass *et al.* showed that HO-1 induction by CoPP reduced hepatocyte apoptosis in various models for liver injury, such as anti-CD95 antibody treatment and LPS or TNF-α administration in D-galactosamine sensitized mice, mainly by inhibiting caspase-3 activity [225]. Further, overexpression of HO-1 by adenoviral gene transfer (injected intravenously) also reduced apoptosis and liver damage after anti-CD95 antibody treatment [225]. In addition, CO exposure prevented liver apoptosis induced by anti-CD95 antibody treatment [225] and CoPP administration reduced the expression of the proinflammatory cytokines TNF-α and IFN-γ in LPS/D-galactosamine treated mice [226]. Although several reports have evaluated the therapeutic potential of HO-1 induction during hepatic injury, the contribution of HO-1/CO for autoimmune hepatitis remains unknown [222-225].

On the other hand, HO-1 inhibition has been shown to be beneficial during excessive iron accumulation after bile duct ligation in rats preventing fibrosis by decreasing TGF-β1, TIMP-1 and MMP expression while HO-1 induction increased liver fibrosis [227].

Most importantly, it has been shown that patients with chronic liver disease due to non-alcoholic steatohepatitis, chronic hepatitis C, and liver cirrhosis showed an increased HO-1 mRNA expression than healthy controls [228, 229]. Further, HO-1 expression correlated with lipid peroxidation end-product levels suggesting that HO-1 induction may be part of the response against the oxidative damage resulting critical in disease progression [228]. Thus, HO-1 induction could be a novel therapeutic option for chronic liver disease treatment.

8. ONGOING CLINICAL TRIALS

Heme has been proved for the treatment of human porphyria [230] and hemin has been approved as a therapeutic agent by the U.S. Food and Drug Administration (FDA) for

the treatment of acute porphyrias. Currently, some clinical trials and preclinical toxicology tests suggest that doses of CO gas or CORM taken from experimental mice model therapies, surrounding 20% of COHb levels, showed no side effects in healthy volunteers (ClinicalTrials.gov identifier: NCT00094406). Some clinical trials using CO exposure therapy are initiating and are mainly focused on preventing lung inflammation (ClinicalTrials.gov identifier: NCT000 94406; ClinicalTrials.gov Identifier: NCT00122694) and transplants rejection (ClinicalTrials.gov Identifier: NCT00 531856). In the next years, results of these studies will demonstrate if the CO therapy is a feasible tool for use in routine medical practice and more precisely in autoimmune disease treatment.

9. CONCLUDING REMARKS

The multiple effects of HO-1 modulation, HO-1 gene therapy as well as CO gas/CORM exposure occur at different points of cellular and molecular biology such as cell activation, proliferation, apoptosis, cytokine response with a major role in redox balance. The efficiency of HO-1/CO axis in modulate proinflammatory response has been proven in different pathological conditions. Although further research must be done to evaluate the effectiveness of HO-1 gene therapy in immune mediated diseases, this strategy may have major clinical impact. The use of CORMs has significantly increased actual knowledge of the anti-inflammatory properties of HO-1 activity, neglecting side effects of hypoxia secondary to inhaled CO gas. Although, HO-1/CO is able to abrogate immune mediated disease in different experimental mice models, translating its efficacy in human autoimmune disease treatment such as SLE, MS and type 1 diabetes is a worthy challenge.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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