

Modulation of antigen processing by haem-oxygenase 1. Implications on inflammation and tolerance

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Summary

Haem-oxygenase-1 (HO-1) is an enzyme responsible for the degradation of haem that can suppress inflammation, through the production of carbon monoxide (CO). It has been shown in several experimental models that genetic and pharmacological induction of HO-1, as well as non-toxic administration of CO, can reduce inflammatory diseases, such as endotoxic shock, type 1 diabetes and graft rejection. Recently, it was shown that the HO-1/CO system can alter the function of antigen-presenting cells (APCs) and reduce T-cell priming, which can be beneficial during immune-driven inflammatory diseases. The molecular mechanisms by which the HO-1 and CO reduce both APC- and T-cell-driven immunity are just beginning to be elucidated. In this article we discuss recent findings related to the immune regulatory capacity of HO-1 and CO at the level of recognition of pathogen-associated molecular patterns and T-cell priming by APCs. Finally, we propose a possible regulatory role for HO-1 and CO over the recently described mitochondria-dependent immunity. These concepts could contribute to the design of new therapeutic tools for inflammation-based diseases.

Keywords: antigen presentation; carbon monoxide; cytokine; dendritic cells; haem-oxygenase 1.

Biological function of HO-1 activity

Several proteins, such as myoglobins, cytochromes and haemoglobins use the haem group as a cofactor.¹ As is

the case for most cellular components, these proteins are degraded after being damaged or aged.^{2,3} Because haem is a pro-oxidant molecule that can participate in the formation of oxidative radicals, leading to oxidative-toxic injury

that results in cell death, degradation of haem is required after the turnover of haem-containing protein.⁴ Therefore, animal cells contain a specific set of haem-degrading enzymes, known as haem-oxygenases (HOs).^{2,5-7} Depending on the tissue, three different HO isoforms can be expressed (HO-1, HO-2 and HO-3). HO-1 is mainly expressed in hepatic,⁸ endothelial,⁹ myeloid^{10,11} and respiratory epithelial cells.¹² HO-2 is expressed in testis, brain and vascular system.^{7,13-15} Although HO-3 is constitutively expressed, it has no catalytic activity, and genetic studies in rats have shown that the *Hmox3* gene is an HO-2-derived pseudogene.¹⁶

The HOs degrade the haem group into Fe³⁺, biliverdin and carbon monoxide (CO). Although Fe³⁺ and CO are conserved and employed as second physiological signals, biliverdin is rapidly converted into bilirubin by the biliverdin reductase system.^{2,7} Due to their functions, HOs are known as shock-stress-protecting enzymes.⁴

As a consequence of the high biological impact recently described for HO enzymatic activity, HO-1 function has been the most studied and characterized HO enzyme. An HO-1 deficiency leads to haem accumulation, causing several health burdens to the host.^{17,18} The first patient suffering from HO-1 deficiency was reported in 1999.¹⁹ In addition to all the metabolic, vascular and endothelial alterations, this patient suffered from an acute inflammatory state.^{18,19} Lymph node swelling and leukocytosis were observed in this patient, which was in agreement with an advanced health deterioration and subsequent death.¹⁹ Accordingly, HO-1 knockout (KO) mice display similar alterations, such as splenomegaly, lymph node swelling, altered CD4⁺ T-cell numbers and an enhanced T-cell activation state.^{18,20} Furthermore, HO-1 KO mice showed an unexpected increased susceptibility to lipopolysaccharide (LPS) -endotoxic shock.²¹ Splenocytes from HO-1 KO mice also showed an augmented secretion of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor- α . Consistent with these observations, monocytes from patients suffering from the autoimmune disease systemic lupus erythematosus, which manifests as exacerbated general inflammation, showed a reduced expression of HO-1.²² Similar results were seen in patients with multiple sclerosis, who displayed reduced levels of HO-1 in peripheral blood mononuclear cells during disease exacerbation.²³ These results suggest that HO-1, in addition to a pro-homeostatic function, can contribute to modulating the inflammatory response in the host.

Because antigen-presenting cells (APCs), such as dendritic cells (DCs), monocytes and macrophages express high levels of HO-1, the function and anti-inflammatory capacity of this molecule have been extensively studied.²⁴⁻²⁶ A better understanding of the biology and function for HO-1 within these APCs could contribute to the design of improved anti-inflammatory ther-

apies. In this article, we review recent findings for the role of HO-1 in the modulation of immunity.

Regulation of HO-1 gene expression

Regulation of HO-1 gene expression (*Hmox1*) is driven by pro-inflammatory and pro-oxidant molecules, such as pathogen-associated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs, e.g. haem group). These PAMPs and DAMPs activate signal transduction pathways that can modify intracellular equilibrium causing cell stress by activation of stress response genes.²⁷⁻²⁹ These pathways include mitogen-activated protein kinases (MAPK) and the c-Jun N-terminal kinases (JNK).^{30,31} HO-1 expression is regulated by the Keap1/Nrf2 and the Bach-1/Maf systems. During cellular stress or an inflammatory response,^{32,33} nuclear erythroid 2-related factor-2 (Nrf2) dissociates from kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), the molecule that retains Nrf2 at the cytoplasm (Fig. 1a). Activated kinases from the MAPK and JNK pathways catalyse the phosphorylation of Nrf2, allowing translocation to the nucleus and binding to antioxidant response elements (ARE) in the *Hmox1* promoter site³⁴ (Fig. 1b, c). In addition to pro-inflammatory stimuli, the anti-inflammatory cytokine IL-10 can also induce the transcription of the *Hmox1* gene through the p38-MAPK pathway to suppress the PAMP-mediated and pro-oxidant molecule-mediated inflammatory responses.^{35,36} Because HO-1 expression also induces IL-10 production, it is likely that a positive feedback loop takes place between IL-10 and HO-1 expression in the responding cells. On the other hand, the haem-binding protein Bach-1 has been shown to form a heterodimer with small Maf proteins and represses *Hmox1* transcription by competing with Nrf2 for the binding to AREs^{37,38} (Fig. 1). Only during stress responses, Bach-1 dissociates from V-maf musculoaponeurotic fibrosarcoma oncogene homologues (Mafs), allowing Nrf2 to heterodimerize with these molecules (Fig. 1b, c). Therefore, there is a constant competition between Nrf2 and Bach-1 for the binding to small Maf proteins at the ARE. Recently, it was shown that IL-10 and other anti-inflammatory molecules can regulate the Bach-1/Maf system by reducing the expression of miR-155, a Bach-1 repressor molecule.^{39,40} Hence, it is thought that micro RNAs can also contribute to the balance between cellular homeostasis and inflammatory response by modulating the access of Nrf2 to the *Hmox1* gene promoter. Consistently with this notion, Nrf2 KO mice develop several pathological manifestations including an enhanced proliferative response of CD4⁺ T cells and a lupus-like syndrome with the presence of antinuclear antibodies, intravascular deposition of immune complexes, glomerulonephritis and decreased survival rates, showing a similar phenotype to HO-1 KO mice.⁴¹

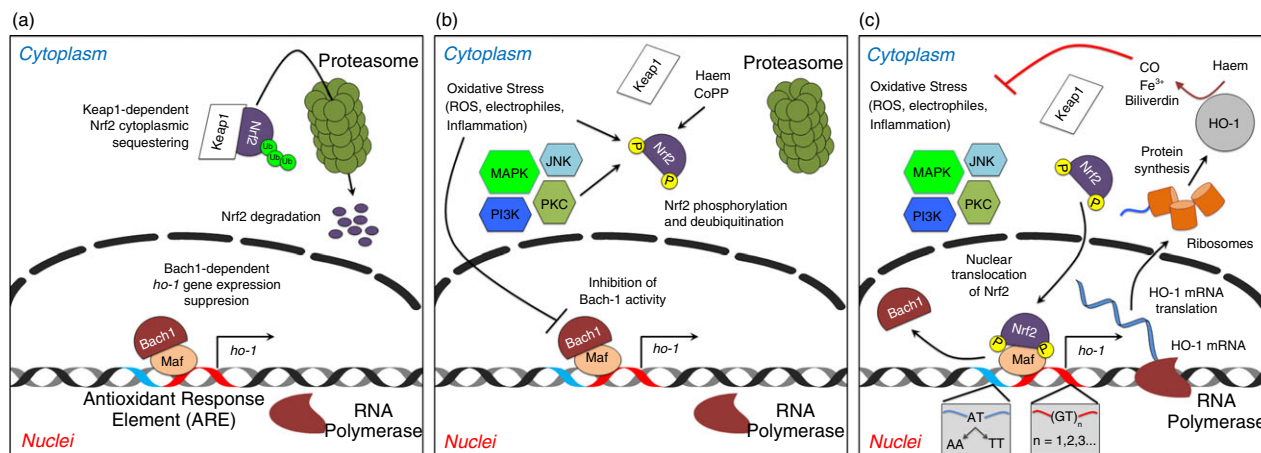


Figure 1. *Hmox1* gene expression and its regulation. (a) In resting state, Nrf2 remains as bound to Keap1 in the cytoplasm. Nrf2 is constantly being ubiquitinated and targeted for proteasomal degradation. During this process, Bach-1 heterodimerizes with small Maf proteins at Antioxidant Response Elements (ARE) in the *Hmox1* gene promoter site. (b) During cell stress caused either by pathogen-associated molecular pattern (PAMPs) and/or pro-oxidant molecules, Nrf2 is released from Keap1, increasing its stability and reducing its proteasome-dependent degradation. Different kinases access to phosphorylate Nrf2 and activate its translocation to the nucleus. The binding of Bach-1 to small Mafs is compromised by the same pro-inflammatory and pro-oxidants signals. (c) Phosphorylated Nrf2 migrates to the nucleus and displaces Bach-1. Heterodimers Nrf2/Maf induce the activation of the *Hmox1* gene promoter site and the recruitment of the RNA polymerase. The *Hmox1* gene transcription begins. Depending on the (GT)_n (*n* = number of repetitions) and the 413A > T (AT → AA; AT → TT) polymorphisms present in the promoter site, the amount of mRNA haem oxygenase 1 can vary.

Recent studies have shown that the expression of the *Hmox1* gene can be modulated by the presence of certain microsatellites located in the gene promoter (GT)_n.⁴² Individuals with short GT repetitions have been shown to have a reduced risk of suffering rheumatoid arthritis⁴³ or chronic pulmonary emphysema,⁴⁴ with a favourable outcome to sepsis,⁴⁵ as well as other diseases,⁴² due to an increased HO-1 promoter activity.²⁹ In addition, the single nucleotide polymorphism 413 A > T in the *Hmox1* gene promoter has been associated with increased transcription levels of HO-1 transcription.^{46–48} The presence of this polymorphism correlated with an augmented incidence of hypertension in women.⁴⁶ Interestingly, individuals harboring this genetic alteration manifested lower incidence of acute kidney injury⁴⁸ and ischaemic heart disease,⁴⁷ suggesting a differential protective capacity for the 413 A > T polymorphism. Hence, although the activities of Nrf2 and Bach-1 are essential to regulate the transcription of *Hmox1* gene and protein quantity, specific DNA modifications in the promoter region also contribute to regulating these processes.

Pharmacological modulation of HO-1 expression using metalloporphyrins is an interesting experimental approach to study the role of this enzyme in several biological processes.⁴⁹ Cobalt protoporphyrin IX (CoPP) is a haem group homologue that induces the up-regulation of *Hmox1* gene expression by promoting the degradation of Bach1 protein and decreasing degradation of Nrf2 protein.⁵⁰ Contrary to CoPP, Tin protoporphyrin IX (SnPP), a metalloporphyrin formed by a chelate of tin with the

porphyrin ring, is one of the most efficient inhibitors of HO-1 activity at the catalytic site.^{51,52} This molecule works as a competitive substrate for the haem group, although it enhances the synthesis of new enzyme without catalytic activity.⁵¹

Blockade of pro-inflammatory receptors by HO-1: the case of TLR4/MD2

As described above, HO-1 is up-regulated after PAMP recognition in APCs.^{29,32,33} Because PAMPs mainly signal through Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NODs), it has been suggested that these recognition pathways can play a central role in the induction of HO-1 expression.^{32,53} Therefore, the contribution of HO-1 activity to the function of immune cells expressing TLR and NOD receptors, such as DCs and macrophages,^{53–56} has been extensively studied. It has been shown that HO-1 over-expression inhibits the secretion of inflammatory cytokines after an LPS challenge in DCs.^{11,27,35,57,58} These studies underscored CO as the most important product responsible for the immune suppressive capacity of HO-1.^{27,59} CO can block the interferon regulatory factor 3/inducible protein 1 and the JNK/inducible protein 1 inflammatory pathways in DCs²⁷ and macrophages,¹⁰ respectively. Reduced signalling led to impaired *de novo* expression of different acute inflammatory cytokines, such as IL-6.⁶⁰ However, the molecular mechanisms used by CO to modify these pathways still remain unknown. Recent studies have

provided insight as to how this gas produced by HO-1 can block pro-inflammatory pathways.^{21,27,57,61} Two different groups have shown that CO directly interferes with the normal surface expression of the LPS-recognizing receptor in DCs,⁶² neutrophils⁶² and macrophages.⁶³ This receptor consists of a complex formed by the TLR4 and the myeloid differentiation factor 2 (hereafter TLR4/MD2). These studies suggested that CO modifies the native conformation of the TLR4/MD2 complex without altering the surface expression of either individual TLR4 or MD2. As a result, CO could impair a key step required for the proper conformational assembly of the TLR4/MD2 complex on the surface of APCs. This notion was further supported by the observation that CO exposure reduced the MD2-dependent glycosylation of TLR4 induced by LPS and inhibited both the transport and surface expression of these two molecules in hepatic cells.⁶⁴ Hence, a decrease in the expression of the TLR4/MD2 complex can reduce the sensitivity to LPS stimulation, reducing and dampening inflammation. Although DCs and macrophages did not up-regulate TLR4 after LPS stimulation, reduced TLR4 glycosylation can explain the capacity of CO to impair subsequent LPS stimulation in monocytes (Fig. 2). Hence, by reducing the MD2-dependent TLR4 glycosylation, CO would cause an absence of functionally assembled TLR4/MD2 on the surface of the monocyte without altering the total amount of individual TLR4 and MD2 over time. However, additional studies are required to demonstrate this hypothesis.

Due to the contribution of TLR4/MD2-dependent inflammation to LPS-mediated septic shock, a potential protective capacity for HO-1 and CO has been explored.⁶² CO-treated animals displayed reduced sensitivity to LPS-induced shock.^{35,62,64} Several explanations are possible, but most of them rely on the ability of CO to reduce the secretion of pro-inflammatory molecules and to increase the production of immune suppressive cytokines, such as IL-10.^{11,35} The role of IL-10 has been widely studied during inflammatory diseases because, in addition to inducing HO-1 expression as mentioned previously, this cytokine can efficiently suppress both innate and adaptive immunity.³⁵ Hence, the HO-1–CO system can reduce inflammation *in vivo* by, for instance, promoting the secretion of IL-10.^{35,65} CO-mediated protection has been observed in some inflammatory pathologies, such as acute pancreatitis,⁶³ haemorrhagic shock,⁶⁶ Alzheimer's amyloid- β 1-42-induced toxicity,⁶⁷ ischaemia/reperfusion,⁶⁸ autoimmunity²⁰ and graft rejection.⁶⁹ Because these pathologies are mainly mediated by innate or adaptive immune responses, it remains unknown whether CO can ameliorate disease progression by blocking the same or different inflammatory pathways in either innate or adaptive immune cells. Because the innate and adaptive immune responses can be linked by professional APCs that express HO-1 (DCs and macrophages), this enzyme's

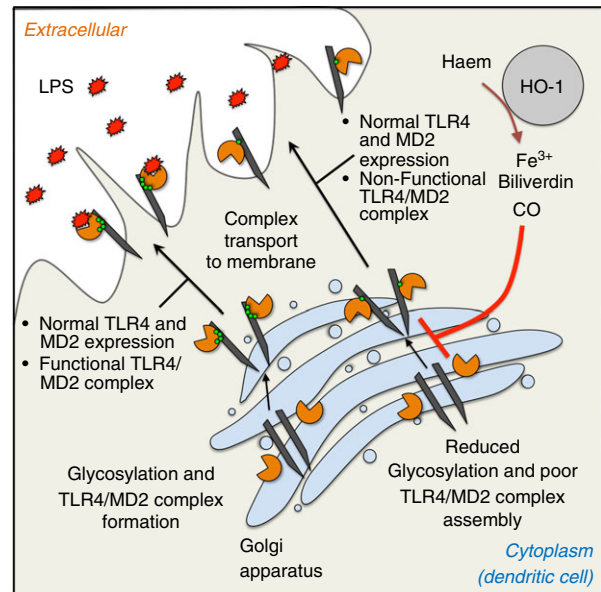


Figure 2. Carbon monoxide (CO) reduces the expression of the Toll like receptor 4/ myeloid differentiation factor 2 (TLR4/MD2) complex receptor in the surface of myeloid cells. TLR4 and MD2 form a glycosylation-dependent complex in the Golgi apparatus, which is then transported to the cell surface. Once in the surface, this complex can recognize lipopolysaccharide (LPS) and trigger an intracellular inflammatory cascade, which will lead to the activation of innate immune responses. After haem oxygenase 1 (HO-1) expression and CO production, a blockade in the glycosylation is produced in the Golgi apparatus and the standard generation and assembly of the TLR4/MD2 complex is compromised. Then, a non-well assembled complex is transported to the cell surface. Normal levels of TLR4 and MD2 are placed in the cell surface but its geometric association lack of effectiveness to recognize LPS. The innate immune response is compromised and reduced.

activity could be considered as a mechanism to down-modulate APC function and reduce detrimental innate and adaptive immune responses.

The HO-1–CO system as a modulator of antigen presentation by DCs

Dendritic cells are professional APCs that reside strategically in tissues that are normally exposed to foreign antigens and infectious agents.⁷⁰ PAMPs induce the maturation of DCs and, in conjunction with the capture of surrounding antigens, promote the migration of mature DCs to secondary lymphoid organs where they prime antigen-specific T cells (naive and memory).⁷¹ Because of the key role that DCs play during the innate and adaptive immune responses, a DC deficiency can cause significant immune suppression and an increased susceptibility to infections.^{72,73}

Haem-oxygenase 1 is constitutively expressed by DCs and can be up-regulated after PAMPs stimulation.^{11,74}

However, HO-1 over-expression is not observed at early times during maturation. Hence, it seems that this enzyme can contribute to the recovery of DCs after a long period of inflammatory stress. Recent studies have shown that HO-1 induction by a haem homologue, CoPP, reduces maturation in human and rat DCs, by decreasing the expression of surface maturation markers as well as the secretion of inflammatory cytokines.^{11,27} Furthermore, LPS-mediated reactive oxygen species (ROS) production also was blocked by HO-1 activity, which is consistent with an early defined role for this enzyme during oxidative stress. In agreement with these results, inhibition of the basal HO-1 activity by SnPP enhanced maturation of murine DCs after stimulation of the p38–MAPK, cAMP-responsive element binding protein and the activating transcription factor 1 pathways.⁷⁵ Reduced DC maturation due to CoPP-induced HO-1 expression abolished activation of allogeneic T cells.^{11,27,76} These observations were consistent with data showing an augmented priming of antigen-specific T cells by DCs in which HO-1 was inhibited by SnPP.⁷⁵ Similar results have been obtained with the HO-1 inhibitory molecule Tin mesoporphyrin, which increased the capacity of cytomegalovirus_{pp65}-peptide-pulsed peripheral blood mononuclear cells to prime virus-specific naive T cells.⁷⁷ Consistently with the ability of HO-1 to down-modulate the capacity of DCs to prime T cells, improved graft acceptance and reduced leucocyte infiltration were observed in an allogeneic aorta rat transplantation model after virus-mediated *Hmox1* gene transfer.⁵⁹ Notably, both reduced leucocyte recruitment and tissue acceptance were reproduced in CO-treated animals,^{59,78} suggesting that this gas was the molecule responsible for the HO-1-mediated inhibition of T-cell activation. The immune suppressive role proposed for CO has been corroborated by other studies showing that HO-1 expression and CO production reduce MHC-II expression in DCs. In addition, a deficiency of this enzyme increased the susceptibility to neuro-inflammation in a murine experimental autoimmune encephalomyelitis model by inducing both increased accumulation of effector T cells and central nervous system damage.⁷⁹

Either CoPP-treated or CO-treated human and murine DCs retained their capacity to secrete IL-10, despite losing their ability to produce IL-12p70,^{11,76} a finding that was reproduced in murine cells.^{27,62} However, although human DCs show reduced surface expression of maturation markers after CoPP/CO incubation, these molecules were not altered in murine DCs. These data suggest that the effect of HO-1 activity could vary among species.^{27,80} However, the precise explanation as to why the HO-1–CO system shows different pattern of responses between human/rat and mouse cells remains unknown.

Because DCs are professional APCs, the capacity of HO-1 to regulate antigen presentation has been an

intensive area of research.⁸⁰ In the murine system, it was shown that both the CoPP-mediated induction of HO-1 and CO were able to reduce the presentation of foreign antigens to naive CD4⁺ or CD8⁺ T cells (Fig. 3a). Furthermore, the effect of CO relied on a reduced capacity of both mature DCs and macrophages to target soluble extracellular antigens to intracellular lysosomal compartments. Conversely, HO-1 activity and CO treatment had no effect on the processing and presentation of larger sized antigens, as those contained in 3- μ m latex beads.⁸⁰

These data support a model in which the HO-1–CO system can discriminate between small and large antigens, by selectively inhibiting intracellular processing routes for the small soluble antigens.⁷⁶ Along these lines, it seems that size is a crucial parameter for defining the intracellular processing pathway for an antigen. This notion is supported by recent studies showing that when extracellular large-volume antigens make contact with cells, an endoplasmic reticulum (ER)-assisted phagocytosis occurs.^{81,82} As part of this process, the ER supports phagosome formation by providing membrane fragments that form a structure known as the ERgosome (ER + phagosome). This compartment does not seem to require to be transported to the perinuclear zone for antigen-processing because, at early times, it is enriched with lysosomal markers, proteasomal machinery and MHC molecules, all derived from cytoplasmic vesicles.^{83,84} Hence, presentation on MHC-II and cross-presentation on MHC-I of antigens attached to large bodies occurred regardless of CO treatment. In agreement with this observation is the fact that CO was unable to inhibit proteasome-dependent cross-presentation of intracellular soluble antigens, which suggested that only the endosome-to-lysosome route for extracellular soluble antigens can be targeted by CO.⁸⁰

Despite the HO-1–CO system only inhibiting the endosome-to-lysosome route, this pathway contributes to the onset of several antigen-specific T-cell-dependent pathologies. Hence, CO-mediated blockade of this pathway could be evaluated to interfere with the onset and progression of several inflammatory detrimental responses. Moreover, understanding how HO-1 controls antigen-dependent, as well as antigen-independent induction of immunity by DCs can contribute to designing new therapies to prevent and treat inflammatory diseases.

Regulation of mitochondrial function by CO: implications for immunity

Recent studies have shown that mitochondria play a key role in the initiation of the antiviral innate immune response.^{85,86} It has been observed that this organelle participates as a signalling platform for the activation of mitochondrial antiviral signalling protein, which ends mainly in the priming of the interferon response.^{86,87} Furthermore, mitochondria can contribute to the processing

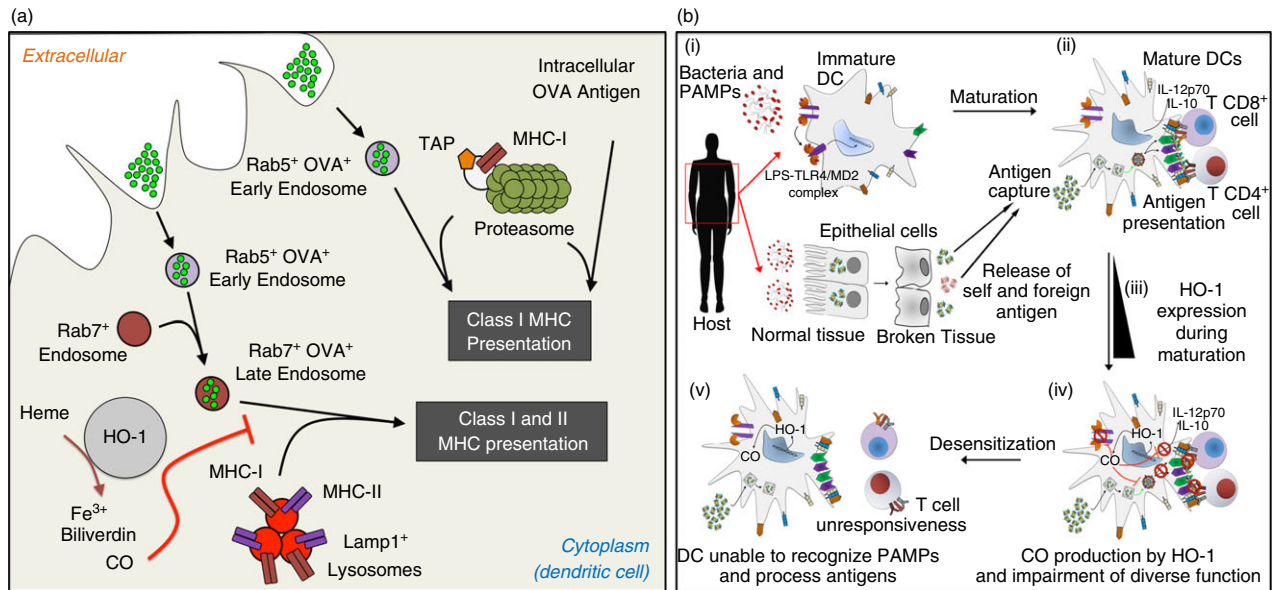


Figure 3. Carbon monoxide (CO) impairs the endosome-to-lysosome pathway to soluble antigens in myeloid cells. (a) After the extracellular antigen is captured, it fuses with Rab5⁺ early endosomes. After that, these vesicles can fuse with proteasome/MHC-I/TAP-containing endosomes, which drive cross-presentation. In parallel, antigen-containing Rab5⁺ vesicles can fuse with Rab7⁺ endosomes to form late endosomes and then, sequentially, they can fuse with lysosomes (Lamp1⁺). These lysosomes harbour a full repertoire of MHC molecules that receive and present the small peptides obtained after the antigen is processed by lysosomal proteases. Once haem oxygenase 1 (HO-1) is over-expressed and CO is produced, there is an interference in the fusion between antigen-containing late endosomes and lysosomes so compromising the correct antigen processing and antigen presentation to T cells. No effect of CO over cross-presentation has been observed. (b) (i) Under local presence of pathogen-associated molecular pattern (PAMPs); either by soluble molecules or presence of pathogens, dendritic cells (DCs) become activated. After binding the Toll like receptor 4/ myeloid differentiation factor 2 (TLR4/MD2) complex, LPS induces DC maturation by up-regulation of co-stimulatory molecules and secretion of cytokines. In addition, PAMPs cause local tissue damage and release of self- and non-self-antigens. (ii) Resident DCs capture soluble antigens presenting them to local T cells (something also observed in autoimmunity and graft rejection). Antigen-containing mature DCs can travel to secondary lymphoid organs and activate antigen-specific naive T cells. (iii) After PAMPs exposure [or treatment with cobalt protoporphyrin IX (CoPP), for example], DCs over-express HO-1, degrade haem-group and produce CO. This process will modulate the immunogenicity of DCs recovering their initial homeostasis. (iv) CO-producing mature DCs will lose their capacity to process antigens through the endosome-to-lysosome pathway. In addition, DCs reduce their secretion of cytokines. (v) Finally, mature DC-dependent innate and adaptive immune inflammation is suppressed. Tissue homeostasis is recovered and pathologies caused by PAMPs and either foreign or self-antigens are restricted.

of intracellular bacteria by enhancing ROS production after TLR signalling in macrophages.⁸⁸ Thus, innate immune cells, such as APCs, employ mitochondria to exert some of their immune functions.

Because APCs require internal regulators to control inflammatory pathways and to restore homeostasis, it is possible that mitochondria could decrease immunity. Traditionally, mitochondria have been considered as cellular organelles associated with energetic, metabolic and genetic roles, whose oxidative function is carried out mainly by cytochrome-containing complexes.^{89–91} Cytochromes are proteins that use Fe²⁺-to-Fe³⁺ porphyrinic groups as co-factors, which facilitate the transit of mobile electrons (high oxide-redox potential). Based on their chemical properties, the porphyrinic group and the Fe²⁺ also show high affinity for exposed electrons from diatomic gases, such as O₂, NO and CO.^{92,93} Hence, these gases in mitochondria can control both cytochrome-dependent

oxidative phosphorylation and ROS production. Experiments using soluble cell-free/purified mitochondria have shown that CO binds to complex I and III in the respiratory chain.^{94,95} CO affinity for these molecules prevents electron transport, reducing the mitochondrial membrane potential ($\Delta\Psi$), ATP and mitochondrial ROS generation (mROS, O₂⁻). Importantly, non-toxic doses of CO have been used in these *in vitro* experiments to avoid mitochondrial disorganization and destruction.⁹⁵ Because HOs are the only enzymes in mammal cells that produce CO at non-toxic levels, it is likely that they could play a relevant role at modulating both mitochondrial function and at controlling immune cells, such as APCs. However, this hypothesis remains to be evaluated.

Because the HO-1-CO system interferes with the initiation of the APC-dependent adaptive immune response, it is likely that a CO-dependent mitochondrial blockade could reduce T-cell priming.⁹⁶ Consistent with this

notion, it was shown that Kupffer cells require both a functional respiratory chain and high levels of mROS to prime antigen-specific CD4⁺ T cells.⁹⁷ These data agree with a recent study showing that mitochondrial stability is required to process and present soluble antigens by B cells to T cells in an ATP-dependent manner.⁹⁸ However, it remains unclear how and where mitochondria could be regulating antigen-dependent immunity and whether endogenously produced CO can regulate these processes. Recent data from our laboratory propose a role for mitochondria in the transport and processing of antigen-containing vesicles.⁹⁹ These observations suggest that the HO-1–CO system inhibits this pathway by dropping down mitochondrial ATP production without impairing the glycolysis-dependent DC maturation.⁹⁹ As a consequence, CO impairs mitochondrial function in DCs up to a point of down-modulating antigen-specific T-cell priming. Because DCs link the innate and the adaptive immune responses, their important function could be modulated by targeting the inflammatory activity of mitochondria by using molecules that regulate ROS and ATP production. It is likely that HO-1 activity and CO can act as natural regulators of the mitochondria-dependent inflammatory pathway in APCs by blocking the maturation of antigen-containing endosomes.

Regarding the interaction between mitochondria and HO-1, studies performed in human alveolar and bronchial epithelial cells have shown the translocation of HO-1 to the mitochondrial compartment after the exposure to cigarette smoke, LPS and haemin.¹⁰⁰ The localization of HO-1 at mitochondria *in vivo* has also been reported using the model of gastric mucosal tissue injury induced by non-steroidal anti-inflammatory drugs.¹⁰¹ This phenomenon resulted in the prevention of non-steroidal anti-inflammatory drug-induced mitochondrial dysfunction and oxidative stress, gastric mucosal cell apoptosis and gastric mucosal injury. The proposed mechanism is the stabilization of complex I-driven mitochondrial respiratory control and the transmembrane potential. These processes have been recently proposed as a novel cytoprotective effect of HO-1.¹⁰¹ Mitochondrial HO-1 translocation was also observed in macrophage RAW-264.7 cells after the treatment with CoCl₂ or exposure to hypoxia. However, in this case the in-organ localization of HO-1 caused mitochondrial dysfunction.¹⁰²

Taken together, these findings suggest that the anti-inflammatory effects of HO-1 could be in part explained by the suppression of the antigen-dependent immunity, which is directly associated with mitochondrial function. In addition, HO-1-mediated mitochondrial protection after the translocation of this enzyme to that organelle reduces cytotoxicity and massive cell death, reducing DAMPs release. These findings also uncover new therapeutic targets to control the HO-1–CO system as a manner to approach diseases caused by the adaptive immune response.

The HO-1–CO system reduces pathologies caused by the immune response

HO-1 reduces innate immunity-mediated inflammatory diseases

Several pathologies are associated with the effector function of the innate immune system.¹⁰³ Fast and acute inflammatory conditions, such as fever, organ swelling and septic shock are mainly mediated by the rapid recruitment of monocytes and neutrophils to the site of infection.¹⁰⁴ Cellular recruitment is associated with a gradient of inflammatory cytokines that are secreted either at the infection site or at damaged tissues (Fig. 3b(i)–(ii)).^{105–107} Hence, interference with the secretion of these pro-inflammatory cytokines is likely to reduce the pathology. Expression of HO-1 and the subsequent CO production can contribute to controlling several innate immunity-driven inflammatory pathologies.¹⁰⁸ For instance, CO can protect from the permeability induced by LPS in epithelial tissues by reducing inflammatory cytokine secretion and preventing the down-regulation of tight junction proteins, such as ZO-1 and occludin.¹⁰⁹ Furthermore, it has been recently shown that an IL-10-dependent HO-1 induction decreases both the recruitment of innate inflammatory cells and the secretion of inflammatory cytokines in a septic shock animal model.³⁵ Hence, blockade of myeloid cell-derived inflammatory cytokines by the HO-1–CO system can work as a mechanism to reduce the sensitivity to stimulation by PAMPs (Fig. 3b(iii)–(iv)).

The HO-1–CO system reduces adaptive immunity-dependent inflammation. Implications in tolerance during transplantation and pregnancy

Activated T cells can contribute to several immune-based pathologies. T cells become activated after recognizing antigens as peptide–MHC complexes (pMHC) on the surface of APCs. The pMHCs are generated by APCs as a result of the processing and presentation of internalized antigens, either foreign or self, as is the case of pathogen infections or autoimmune disorders, respectively.

Furthermore, allospecific T cells are primed by APCs expressing MHC molecules at variant from the host.^{110–112} Such an allogeneic recognition can lead to organ rejection in patients who have received a transplant to treat illnesses, such as kidney failure. During transplant rejection, DCs stimulate T cells through direct, indirect or semi-direct pathways of allorecognition.^{111,112} In the direct pathway, donor DCs can migrate out of the grafted tissue and present intact donor MHC/peptide complexes to allospecific T cells (which recognize non-self antigens).^{111,113} In the indirect pathway, recipient DCs process donor alloantigens (foreign/non-self antigens) and present

them to autologous reactive T cells.^{111,113} Finally, through the semi-direct pathway of allorecognition, recipient T cells recognize intact donor MHC/peptide complexes that have been transferred to the surface of recipient DCs by a process known as 'nibbling'.¹¹⁴ From these three pathways, the direct route is associated with early graft rejection and has been classified as the most powerful mechanism of rejection.¹¹⁵ However, this mechanism decreases with time because donor DCs mainly die by senescence. On the other hand, recipient DCs arise as the most potent factor able to activate host T cells during the time. Hence, the indirect pathway arises as the major cause of chronic graft rejection after presentation and recognition of alloantigens.^{112,113,115}

These mechanisms of allo-recognition pose an important challenge to improve graft acceptance, so new specific approaches are required to prevent T-cell activation by donor or recipient DCs after transplantation. Graft acceptance could be promoted either by suppressing *de novo* activation of T cells or inducing T-cell antigen-unresponsiveness. A promising approach to achieve these goals is the generation of antigen-specific regulatory T cells.^{116–118} This technique contributes to specific protection and graft survival. Although regulatory T cells show antigen-specificity, these cells are efficient at suppressing locally reactive effector T cells. Whether the regulatory T-cell approach can produce as a side effect local immune suppression leading to pathogen spread remains to be defined. A combination of different strategies, for example regulatory T cells together with DCs that induce T-cell unresponsiveness, might be a solution. However, these alternatives must be evaluated, both in animal models and in clinical studies.

It was recently shown that *in vivo Hmox1* gene transfer using adenoviral vectors improves long-term heart graft survival in a myeloid cell (DC) -dependent fashion.^{59,78} Also, in another model of graft survival, injection of pigs with a lentiviral vector encoding for *Hmox1* gene ameliorated the outcome of a Duchenne muscular dystrophy therapy with myogenic cell precursors.⁵⁸ Similar results were obtained in diabetes,^{119,120} renal transplantation^{121,122} and human cardiac stem cell transference¹²³ in which all the over-expression of HO-1 was induced with CoPP. This notion was further supported by the observation that regulatory T-cell-dependent suppression of activated T cells required the expression of HO-1 in APCs.¹²⁴ Similar observations were made in mouse models for inflammatory diseases, such as in lactobacillus-mediated infection where HO-1 activity was required to efficiently produce mesenteric Foxp3⁺ CD25⁺ CD4⁺ T cells.¹²⁵ Moreover, transfer of wild-type regulatory T cells into HO-1 heterozygous mice restored the ratios between regulatory and effector T cells and reduced inflammation in a model of necrotizing enterocolitis, supporting a possible direct role for HO-1 in the generation and function of

regulatory T cells.¹²⁶ Hence, the HO-1–CO system arises as a powerful candidate to restrict antigen presentation to T cells *in vivo* and also to impair the activation of lymphocytes in transplantation and autoimmunity. Furthermore, the generation of regulatory T cells during bacteria-dependent inflammation and graft acceptance seems to depend on the HO-1 activity.

Because these pathologies are mainly mediated by the presentation of extracellular antigens that have been processed by the endosome-to-lysosome pathway, the mechanism by which CO could be reducing the activation of T cells can be associated with a blockade of this route (Fig. 3b(v)). Moreover, as seen in rat and human models of organ transplantation, reduced DC maturation by HO-1 can impair the expression of both MHCs and co-stimulatory molecules. As a result, the priming of MHC mismatched T cells would be reduced. Hence, due to its impact on antigen presentation, modulation of the HO-1–CO system *in vitro* either by pharmacological or gene therapy could work as efficient strategies to induce antigen-specific tolerogenic DCs that are useful for cell transfer-based therapy during autoimmune diseases.

In addition, the HO-1–CO system has also been characterized as protective during pregnancy.^{127,128} During embryo development, new cells and antigens are produced. To protect the developing fetus from being attacked by the adaptive immune response of the mother, a tolerant immune state must be established in the fetal-maternal interface. Consistent with this notion, it has been shown that HO-1 expression can prevent natural abortion in a well-established mouse model.¹²⁹ HO-1 up-regulation induces BCL-associated athanogene-1 (Bag-1) and neuropilin-1, two markers associated with the development of regulatory T cells and the induction of tolerance.¹³⁰ Furthermore, recent studies showed that HO-1 regulates regulatory T-cell-mediated protection against abortion in mice because this enzyme reduced DC maturation and regulatory T-cell expansion.¹³¹ It is thought that reduced DC maturation avoided the priming of effector T cells in the mother, which protected embryo from the immune response. These mechanisms were supported by recent data suggesting that progesterone regulates both the expansion of regulatory CD8⁺ CD122⁺ T cells and the establishment of fetal tolerance after inducing expression of HO-1 in the placenta.¹³² In addition, the progesterone–HO-1 system reduced the expansion of cytotoxic CD8⁺ T cells that recognize non-self antigens expressed by the embryo.¹³² Consistently, a correlation was shown for pregnant women between current miscarriage and a reduced amount of regulatory T cells, despite having regular numbers of circulating DCs.¹³³ These data suggest that HO-1 regulates DC activity and the capacity to induce T-cell-mediated tolerance.

Because DCs are crucial for the establishment of peripheral tolerance, their contributions to tolerance dur-

ing pregnancy and to protecting the new embryo from immune recognition have been extensively studied. It has been shown that uterine DCs display an immature/IL-10-secreting phenotype during pregnancy and that maturation and IL-12 secretion are directly associated with abortion in mice.^{134,135} Indeed, absence of IL-10 during pregnancy leads to an altered phenotype for DCs, macrophages, effector and regulatory T cells, which contribute to LPS-mediated abortion.¹³⁶ These data suggest that production of IL-10 by APCs controls T-cell responses after recognition/presentation of embryo-derived antigens. In addition, uterine DCs might be crucial for embryo implantation because they accumulate before pregnancy during the oestrous cycle¹³⁷ and their remotion definitely affects the success of implantation.¹³⁸

Because mature uterine DCs release several inflammatory mediators, activation of these cells by PAMPs could promote inflammation in mother tissues and impair embryo development. Hence, it has been shown that bacterial infection is an important risk factor that can trigger abortion because, in addition to the inflammatory condition, pathogens down-regulate HO-1 expression in the placenta.^{139,140} Up-regulation of HO-1 by CoPP increased fetus development and augmented cell survival of the placental tissue.¹²⁸ This process is likely to be promoted by the capacity of HO-1/IL-10-expressing DCs to induce regulatory T-cell expansion after antigen presentation, which contributes to placental stability. It has also been shown that HO-1 regulates placental development.¹⁴¹ Consistently with this notion, the amount of apoptotic placental cells was reduced when HO-1 expression was promoted by transduction with an adenoviral *Hmox1* gene transfer system.¹²⁹ Another study showed decreased placenta size and weight, as well as impaired cell viability in mice heterozygous (*Hmox1*^{+/-}) for HO-1.^{141,142} Along these lines, it has been shown that both *Hmox1*^{+/-} and *Hmox1*^{-/-} mice show an impairment in uterine natural killer (uNK) -dependent maternal spiral arteries remodeling during implantation because of reduced numbers of uNK.^{143,144} Interestingly, treatment of *Hmox1*^{+/-} mice with low doses of CO improved both uNK proliferation, spiral arteries remodelling and also stabilized blood pressure, suggesting that this gas plays a key role during uNK-dependent intrauterine growth.^{143,144} Similar data were obtained in a trophoblastic stem cell line that resembles the development of trophoblasts into giant cells, which showed that HO-1 inhibition compromises cell viability and their ability to differentiate.¹⁴⁵ In agreement, trophoblastic tissue from women suffering spontaneous abortion displayed reduced HO-1 expression levels compared with healthy pregnancies.¹⁴² It is noteworthy that CO administration during implantation and the early placentation window improved cell survival and increased Bag-1 expression in the same manner as shown by the expression of HO-1, suggesting that the main

mediator for this enzyme in placenta stability is CO.¹⁴⁶ Hence, the HO-1–CO system not only plays an important role in the regulation and tolerance associated with the immune system, but also implies a direct function in the placental tissue development that will protect the embryo during the interaction with tissues of the mother.

Altogether, these results suggest that the HO-1–CO system can prevent and reduce the outcome of inflammatory pathologies related to innate and adaptive immunity (Fig. 3b). In the case of innate immunity, both CO-mediated cytokine secretion suppression and reduced cell recruitment to the site of PAMPs accumulation arise as the most important regulatory mechanism used by HO-1. In the case of adaptive immunity, CO-mediated suppression of both fusion between antigen-containing endosomes and lysosomes, antigen surface presentation and impairment of DC maturation is associated with reduced T-cell priming, lower tissue destruction and an establishment of tolerance.

Concluding remarks

HO-1 is expressed in APCs, such as DCs and macrophages. The production of CO by this enzyme mediates the suppression of different inflammatory pathways. HO-1 and CO reduce the capacity of APCs to recognize PAMPs and suppress both pro-inflammatory cytokine secretion and antigen presentation. These modulatory processes uncouple the whole immune network by blocking the activation of either antigen-specific or allogeneic T cells. HO-1–CO-mediated reduction of immunity controls the onset and progression of several diseases, such as sepsis, organ rejection and autoimmunity. The exploitation of the molecular targets of the HO-1–CO system, such as mitochondria, arises as a promising alternative to create anti-inflammatory therapies. More research is needed to evaluate whether by inhibiting mitochondria until non-toxic levels, as CO does it, can lead to improving life quality in patients suffering from inflammatory ailments.

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Disclosures

The authors have declared that no conflict of interest exists.

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