

**HEME OXYGENASE-1: A PROTECTIVE GENE THAT REGULATES
INFLAMMATION AND IMMUNITY.**

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ABBREVIATIONS USED:

15d-PGJ2, 15-Deoxy- $\Delta^{12, 14}$ -prostaglandin J2; **ATP**, adenosine triphosphate; **ARE**, AU-rich elements; **cGMP**, cyclic 3':5'-guanosine monophosphate; **CO**, carbon monoxide; **COX**, cyclooxygenase; **EC**, endothelial cells; **ERK**, extracellular signal-regulated kinase; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **HMGB1**, high mobility group box 1; **HO-1**, heme oxygenase-1; **iNOS**, inducible nitric oxide synthase; **IL**, Interleukin; **ICAM-1**, intercellular adhesion molecule-1; **I κ B**, inhibitors of NF- κ B; **IKK**, I κ B kinases; **LPS**, lipopolysaccharide; **MCP-1**, monocyte chemoattractant protein-1; **M ϕ** , Monocyte/macrophages; **MHC**, major histocompatibility complex; **MAPK**, mitogen activated protein kinase; **MAPKAPK**, MAPK activated protein kinase, **MKK**, MAPK kinase; NAD(P)H-reduced nicotineamide adenine dinucleotide (phosphate); MIF-1 α , macrophage migration inhibitory factor-1 α ; **NF- κ B**, nuclear factor kappa B; **PRR**, pattern recognition receptors, **PTD**, protein transduction domain; **RAGE**, receptor for advanced glycation end-products; **TF**, tissue factor; **TNF- α** , tumor necrosis factor- α ; **VCAM-1**, vascular cell adhesion molecule-1.

1. SUMMARY

Heme oxygenase-1 (HO-1) is a stress responsive protein identified in 1968 as the rate-limiting enzyme in the catabolism of heme, yielding equimolar amounts of iron (Fe), biliverdin and the gas carbon monoxide (CO)(1). It was only in the last few years however, that the role of HO-1 in the regulation of inflammatory and immune responses became apparent. We shall address here accumulating evidence suggesting that HO-1 controls the extent of inflammatory and immune responses preventing tissue injury and disease. We will argue that CO mediates to a large extent the protective effects of HO-1.

2. INFLAMMATORY AND IMMUNE RESPONSES.

Inflammation, originally defined as *rubor, calor, tumor* and *dolor* (Aulus Cornelius Celsus; 30AD), is a beneficial host response to injury characterized by the migration of circulating leukocytes and soluble molecules from blood into tissues (2, 3). Inflammatory reactions are triggered via activation of endothelial cells (EC) and resident monocyte/macrophages (M ϕ) at sites of tissue injury. This occurs upon recognition of microbial pathogens and/or molecules released from injured cells by pattern recognition receptors (PRR) (4, 5). Inflammatory reactions are in most cases coupled to the activation of T and B cells that mediate a immune response directed specifically against molecules expressed by invading pathogens, i.e. antigens (4, 5). T cells can recognize antigens only when these are presented in the context of major histocompatibility complex (MHC) molecules expressed on the surface of antigen-presenting cells, i.e. dendritic cells (6). This requires that antigens must be “captured” at the sites of inflammation, coupled to MHC molecules, and transported into lymphoid organs where antigen presentation can occur (7, 8). Upon antigen recognition, T cells are activated, undergo profound phenotypic modifications and expand logarithmically to generate a large pool of antigen specific effector T cells. These can migrate into the site of inflammation and eliminate microbial pathogens.

3. PROGRESSION OF INFLAMMATORY AND IMMUNE RESPONSES: *ONSET, EFFECTOR AND RESOLUTION PHASES.*

Upon activation, EC up-regulate the expression of chemokines, cytokines and adhesion molecules that mediate the activation and recruitment of circulating leukocytes into sites of inflammation, i.e. the “onset” of inflammatory reactions. Activated leukocytes secrete high levels of pro-inflammatory cytokines, free radicals, enzymes and anti-microbial molecules that act together to clear microbial pathogens, i.e. the “effector” phase of inflammatory reactions. To avoid irreversible tissue injury from occurring the “resolution” phase of inflammatory reaction must be initiated as soon as microbial clearance has been achieved. This was thought to occur “spontaneously”, once pro-inflammatory stimuli are no longer present. An alternative explanation, however, is that the pro-inflammatory stimuli responsible for the onset and effector phases of inflammation also trigger the expression of anti-inflammatory genes responsible for its resolution. We will argue that this is the mechanism underlying the resolution of inflammatory reactions and will discuss data supporting the notion that expression of the anti-inflammatory gene HO-1 acts in such a manner (*reviewed in (9, 10)*).

4. INFLAMMATORY DISEASES

Inflammatory diseases designate an apparently disparate group of pathologic conditions that can be triggered by unfettered inflammatory responses (2, 3). This can occur in many instances, including when the expression of anti-inflammatory genes responsible for the resolution of inflammatory reactions is impaired. There is accumulating evidence to suggest that inadequate expression of one of such gene, i.e. HO-1, is a common event in the pathogenesis of inflammatory diseases such as endotoxic and septic shock (11) as well as atherosclerosis, coronary artery disease, abdominal aortic aneurysm, myocardial infarction, restenosis, chronic rejection of transplanted organs (12), idiopathic recurrent miscarriage or auto-immune diseases such as arthritis or multiple sclerosis (2).

5. CELLULAR MECHANISMS UNDERLYING THE REGULATION OF INFLAMMATORY AND IMMUNE RESPONSES.

There are a multitude of cell types, directly or indirectly, involved in the regulation of inflammatory and/or immune responses. We will focus here on EC and Mø and will address how these cells control inflammation in a manner that regulates the outcome of immune responses.

5.1. ENDOTHELIAL CELLS (EC).

EC are organized as a single cell monolayer that covers the entire lumen of blood vessels, i.e. the vascular endothelium (13, 14). Under non-inflammatory conditions EC promote vasodilatation and inhibit thrombosis as well as leukocyte adhesion (*reviewed in* (15)). However, when exposed to pro-inflammatory stimuli EC undergo a series of functional modifications such as to promote vasoconstriction, thrombosis and leukocyte adhesion, a phenomenon referred to as EC activation (16, 17). This results from the expression of a series of immediate early responsive genes encoding vasoconstrictors (e.g. endothelin-1), cytokines (e.g. interleukin 1beta (IL-1 β) and IL-6) chemokines (e.g. IL-8 and monocyte chemoattractant protein 1 (MCP-1)), adhesion molecules (e.g. E&P-selectins, intracellular adhesion molecule 1 (ICAM-1), vascular cellular adhesion molecule 1 (VCAM-1)) as well as pro-thrombotic molecules (e.g. tissue factor (TF)). Expression of these pro-inflammatory genes is responsible for the activation and recruitment of circulating leukocytes that occurs during the onset and effector phases of inflammatory reactions (*reviewed in* (15, 18, 19)). Mice genetically deficient in the expression of some of these genes, e.g. IL-1 β ^{-/-} (20), IL-6^{-/-} (21, 22) (23, 24), MCP-1^{-/-} (25-27), E&P-selectin^{-/-} (28-32), ICAM-1^{-/-} (33) or VCAM-1^{-/-} (33) mice, have impaired inflammatory responses but are less susceptible to develop inflammatory diseases such as autoimmune arthritis, encephalomyelitis (a rodent experimental model of multiple sclerosis) or atherosclerosis. This suggests that the expression of pro-inflammatory genes associated with EC activation must be tightly regulated so that the onset and effector phases of inflammatory responses can occur in a manner that does not lead to the development of inflammatory diseases.

5.2. MONOCYTE/MACROPHAGES (Mø)

Mø are a heterogeneous family of bone marrow derived mononuclear leukocytes that transmigrates continuously between blood and tissues. Because of their widespread

distribution Mø are likely to be exposed to invading microbes (34). These are recognized by Mø via PRR, an event that triggers the activation of Mø and the expression of a series of pro-inflammatory cytokines such as IL-1 β and tumor necrosis factor alpha (TNF- α), IL-6, macrophage migration inhibitory factor-1 α (MIF-1 α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (reviewed in (35)). These cytokines boost the pro-inflammatory phenotype associated with EC (see 5.1) and Mø activation up-regulating the expression of pro-inflammatory genes such as cyclooxygenase-2 (COX-2), NAD(P)H oxidase and inducible nitric oxide synthase-2 (iNOS). These produce high levels of prostaglandins, free radicals and nitric oxide (NO), respectively, which act in a concerted way to generate a potent oxidative stress response. In addition to these immediate early response there is a delayed pro-inflammatory response associated with Mø activation, characterized by the secretion of nuclear high mobility group box-1 (HMGB1) (36, 37)(see figure 1). HMGB1 is a non-histone DNA binding protein that when secreted from activated Mø is recognized by the receptor for advanced glycation end-products (RAGE) (38) as well as by other PRR (39). Signaling via this receptors sustains the expression of pro-inflammatory genes associated with EC (40) and Mø (41) activation. HMGB1 secretion has been recently shown to contribute in a critical manner to the deleterious effects of endotoxic (37) and septic shock (42) as well as to the pathogenesis of inflammatory diseases such as rheumatoid arthritis (43).

EC express high levels of nuclear HMGB1, which is not released upon PRR ligation by lipopolysaccharide (LPS) (see figure 2). This suggests that the pro-inflammatory effects of HMGB1 are mediated primarily via HMGB1 released from activated Mø. There are additional sources of HMGB1 that might influence the outcome of inflammatory and immune reactions. These include HMGB1 released from cells undergoing necrosis (44). As HMGB1 can activate dendritic cells, promoting T cell activation (45), this may explain how necrotic cells promote dendritic cell activation and therefore T cell activation and proliferation (46). We have recently obtained data indicating that HO-1 prevents nuclear translocation of HMGB1 in LPS activated Mø (*László Tokaji, unpublished observation*). Presumably this contributes in a critical manner to the ability of HO-1 to prevent the deleterious effects of HMGB1 release.

6. MOLECULAR MECHANISMS UNDERLYING THE REGULATION OF INFLAMMATORY AND IMMUNE RESPONSES.

Inflammatory reactions are regulated via the activation of signal transduction pathways that balance the expression of pro- and anti-inflammatory genes. Understanding the mechanisms underlying the activation of these signal transduction pathways may be critical in understanding how inflammatory and immune responses are regulated. We will focus on two signal transduction pathways that play a central role in inflammation and immunity, i.e. the p38 mitogen activated protein kinase (MAPK) and the nuclear factor kappa B (NF- κ B) signal transduction pathway.

6.2. THE P38 MAPK SIGNAL TRANSDUCTION PATHWAY

p38 MAPK regroup at least four distinct kinases, i.e. p38 α , p38 β , p38 γ and p38 δ (reviewed in (47)). Activation of these kinases involves phosphorylation of their canonical Thr-Gly-Tyr site (47). The p38 α isoform is the most thoroughly

characterized and has predominant expression among different cell types (47). Pro-inflammatory stimuli activate p38 MAPK via activation of upstream MAPK kinase (MAPKK), i.e. MKK3, MKK4, MKK6 and/or MKK7 (48-50). Among these, MKK3 and MKK6 play a predominant role in activating p38 α and p38 β with MKK-6 activating preferentially the p38 β MAPK isoform (48-51).

There is ample evidence showing that p38 MAPK activation controls the pro-inflammatory response of activated EC and M ϕ (*reviewed in* (47, 52, 53)). Activation of p38 MAPK up-regulates the expression of pro-inflammatory genes associated with M ϕ activation, including TNF- α and COX-2 (52, 54) and to a lesser extent IL-6 and IL-8 (55, 56). In addition, p38 MAPK activation also modulates the expression of other pro-inflammatory genes such as MCP-1 (57), IL-12p40 (58) as well as GM-CSF, vascular endothelial growth factor (VEGF), iNOS (*reviewed in* (59)) and metalloproteinases 1 and 3 (60).

Pyridinyl imidazoles that target the ATP binding site on p38 α and p38 β MAPK isoforms blocking their activation protect mice from developing inflammatory diseases such as endotoxic shock (61), arthritis (62), ischemic injury (63) and stroke (64). This indicates that p38 α and/or p38 β activation is a central event in the pathogenesis of these diseases. This conclusion should be cautioned by the demonstration that the inhibitory effect of pyridinyl imidazoles is not totally specific to the p38 MAPK signalling transduction pathway (65).

There is little evidence for biological effects attributable to activation of p38 α *versus* the p38 β MAPK isoforms. Mice genetically deficient in p38 α are embryonic lethal, which supports the notion that p38 α and p38 β are not functionally overlapping (66). Cardiomyocytes and fibroblasts derived from p38 α deficient mice are less susceptible to undergo apoptosis, as compared to wild type cells (67). Activation of p38 α is also pro-apoptotic in L929 fibroblasts (68), myocytes (69), HeLa cells (70) or Jurkat T cells (71) while activation of p38 β is anti-apoptotic in these cells (68-71). This strongly suggests that the p38 α and p38 β MAPK isoforms have antagonistic effects in controlling apoptosis, p38 α being pro-apoptotic and p38 β anti-apoptotic. This may be relevant for the control of inflammatory responses as anti-inflammatory genes such as HO-1 signal specifically via the p38 β MAPK isoform to exert their protective effects (see 7.2.3.).

Little is known about the specificity of the p38 α and p38 β MAPK isoforms in controlling the pro-inflammatory responses of activated M ϕ . Embryonic fibroblasts derived from p38 α deficient mice fail to express TNF- α , IL-1 β , IL-6 as well as type I interferon-dependent transcriptional response genes. This indicates that p38 α is required for the expression of these genes while p38 β is not (66). Whether p38 β opposes p38 α in regulating the expression of these genes remains to be established.

6.3. THE NF- κ B SIGNAL TRANSDUCTION PATHWAY

Most pro-inflammatory genes expressed during the onset of inflammatory responses, are regulated at the transcriptional level via activation of the nuclear factor kappa B (NF- κ B) family of transcription factors, i.e. p65/RelA, p50, p52, cRel and RelB (*reviewed in* (72, 73)). These form homo and heterodimers that under non-inflammatory conditions are retained in the cytoplasm by inhibitors of NF- κ B (I κ B) molecules (74), i.e. I κ B α , I κ B β , I κ B ϵ and I κ B γ (*reviewed in* (73)). Most pro-inflammatory stimuli leading to EC or M ϕ activation, trigger activate I κ B kinases (IKK), i.e. IKK α and IKK β (75, 76). This is a critical event in the process leading to

NF- κ B activation as I κ B phosphorylation (77) promotes its poly-ubiquitination and degradation by the 26s proteasome. This allows NF- κ B nuclear translocation (74) and regulation of gene transcription upon binding to κ B motifs (GGGRNNYYCC (where R is purine, Y is pyrimidine, and N is any base) within the promoter region of NF- κ B dependent genes, e.g. TNF- α , E&P-selectins, ICAM-1, VCAM-1, IL-1 β , IL-6, IL-8 as well as MCP-1 (*reviewed in* (72, 73)).

There are additional mechanisms that control NF- κ B activity independently of I κ B binding. These rely on the phosphorylation and/or acetylation of NF- κ B, e.g. p65/RelA at serine 276 (78), serine 529 (79) and/or serine 311 (80), an event strictly required for NF- κ B DNA binding and trans-activation activity (*reviewed in* (11)). P65/RelA serine 276 phosphorylation is mediated by protein kinase A (81) or MSK-1 (82) while serine 311 is phosphorylated directly by PKC ζ (80). As MSK-1 activation is controlled via p38 MAPK activation this may explain how p38 MAPK can in some instances regulate NF- κ B activation. Acetylation of p65/RelA at lysine 218, 221 and 310 also regulates DNA binding as well as trans-activation activity (*reviewed in* (83)). This is mediated by E1A-associated protein p300 and the cyclic AMP responsive element B binding protein (CBP), two acetyltransferases (84).

Based on the notion that NF- κ B activation plays a central stage in the expression of pro-inflammatory genes associated with EC and M ϕ activation, it was postulated that its activity must be regulated to insure the resolution of inflammatory responses. This was thought to rely on a negative feed back loop by which NF- κ B triggered the expression of I κ B molecules (85) that shuttle nuclear NF- κ B dimers into the cytoplasm suppressing their activity (86). However, inhibition of NF- κ B activity by over-expression of I κ B α sensitizes most cell types to undergo apoptosis (87-89). This suggests that NF- κ B activity cannot be controlled exclusively via I κ B molecules. It also suggests that NF- κ B activation has a dual effect in that it triggers not only the expression of pro-inflammatory genes responsible for the onset of inflammation but in addition is responsible for the expression of cytoprotective genes that prevent tissue injury and thus may have a critical role in the resolution of inflammatory reactions. We refer to these genes as protective genes.

7. HO-1: A PROTECTIVE GENE THAT MODULATES INFLAMMATORY AND IMMUNE RESPONSES.

Protective genes were originally defined according to their ability to suppress the expression of pro-inflammatory genes associated with EC activation and prevent EC apoptosis (9, 10). We will expand this definition here and refer to protective genes as those genes that when expressed during inflammatory reactions promote their resolution and suppress the development of inflammatory diseases. HO-1 is one of such genes.

Heme oxygenases (EC1.14.99.3) comprise three distinct genes, i.e. ho-1 (hmox1, P09601), ho-2 (hmox2, P30519) (*reviewed in* (90, 91)) and the poorly characterized ho-3 (hmox3, rat; O70453), a pseudo-gene that probably does not encode a functional protein (92). HO-1 and HO-2 are the rate-limiting enzymes in the catabolism of heme, a reaction that yields equimolar amounts of biliverdin, Fe and CO (1). Under non-inflammatory conditions HO-2 is constitutively expressed in most cell types while HO-1 expression is limited to liver Kupffer cells (93) and spleen red pulp M ϕ (94). However, during inflammatory reactions the expression of HO-2 remains unchanged while that of HO-1 is significantly up-regulated in most cell types (*reviewed in* (91, 95)). HO-1 acts as a protective gene by virtue of its ability to

suppress the expression of pro-inflammatory genes associated with EC and Mø activation (96-98) and to protect non-lymphoid cells from undergoing apoptosis (99).

The first demonstration that HO-1 contributed to the resolution of inflammatory responses was provided by the observation that inhibition of its enzymatic activity could exacerbate the deleterious effects of acute complement-dependent inflammatory responses (100). This suggested that HO-1 expression during inflammatory reactions had protective effects that were mediated via its enzymatic activity. The notion that HO-1 acted in a protective manner was subsequently confirmed by the observation that HO-1 genetic deficiency leads to an inflammatory syndrome that can be lethal in humans (101-104). There is a growing body of evidence suggesting that HO-1 expression can prevent the development of inflammatory diseases such as atherosclerosis. This is strongly supported by the observation that inhibition of HO-1 enzymatic activity (105, 106) or HO-1 genetic deletion are atherogenic (107, 108) in a variety of experimental conditions. In keeping with this notion there are also a growing number of reports showing that modulation of HO-1 expression can inhibit the development of inflammatory diseases, including atherosclerosis (*reviewed in* (109)).

7.1. CELLULAR BASIS OF THE PROTECTIVE EFFECTS OF HO-1.

Most pro-oxidant stimuli involved in the onset and effector phases of inflammatory responses can up-regulate the expression of HO-1 (110). We will consider here a simplified “model” in which heme acts as the main pro-oxidant responsible for HO-1 expression during the onset of inflammatory responses.

7.1.2. PROTECTIVE EFFECTS OF HO-1 EXPRESSION IN MØ.

Inflammatory reactions are associated with more or less severe hemolysis. In the presence of free radicals, hemoglobin released in this manner is readily oxidized into pro-inflammatory methemoglobin (111, 112) (*see* 7.1.3). The pro-inflammatory effects of methemoglobin are due to the conversion of Fe²⁺ into Fe³⁺ within the core of its heme groups. These can participate in the Fenton reaction amplifying free radical generation (113, 114). The pro-inflammatory effects of methemoglobin are controlled to great extent by haptoglobin, an acute phase response protein that binds to (met)hemoglobin (115). As a result of that (met)hemoglobin becomes recognizable by HbSr/CD163 (116), a hemoglobin scavenging receptor expressed by a subset of Mø present during the resolution of inflammatory lesions (117). Binding of (met)hemoglobin to HbSR results in its internalization. In addition there is also up-regulation of HO-1 and generation of CO that acts in an anti-inflammatory manner blocking TNF- α and inducing IL-10 secretion (97). IL-10 is a potent anti-inflammatory cytokine clearly involved in the resolution of inflammatory responses (*reviewed in* (118)). Both IL-10 and CO can inhibit the expression of pro-inflammatory genes associated with Mø activation, e.g. TNF- α , IL-1 β , and GM-CSF (97, 118). The anti-inflammatory effects of CO are independent of IL-10 (97). On the other hand IL-10 induces the expression HO-1 in activated Mø (119, 120) and its anti-inflammatory effects are dependent on the generation of CO (119). This would suggest that IL-10 acts in an anti-inflammatory manner, via a mechanism that relies on its ability to sustain the expression of HO-1 and the generation of CO. This is likely to occur in Mø that can internalize hemoglobin via the HbSR, providing the heme required for the generation of CO. This would also suggest that Mø that

expresses HbSR might act predominantly in an anti-inflammatory manner that would promote the resolution of inflammatory reactions (*see figure 2*).

There are other potent anti-inflammatory molecules that rely on the expression of HO-1 to exert their protective effects (*reviewed in (109)*). These include 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2), a cyclopentenone prostaglandin that displays potent anti-inflammatory effects. As for IL-10, the anti-inflammatory effect of 15d-PGJ2 in activated M ϕ relies on the expression of HO-1 and the generation of CO (121). This supports the notion that HO-1 acts a anti-inflammatory “sink” through which anti-inflammatory molecules such as IL-10 or 15d-PGJ2 exert their effects (*reviewed in (109)*).

7.1.3. ENDOTHELIAL CELLS

EC do not express HbSr and presumably for this reason are highly sensitive to the pro-inflammatory effects of methemoglobin (112, 122). When exposed to methemoglobin EC up-regulate the expression of pro-inflammatory genes that trigger the onset of inflammatory reactions. The pro-inflammatory effect of methemoglobin in EC is comparable to some of the most potent pro-inflammatory molecules, e.g. TNF- α or IL-1 β (122). EC also don't express the hemopexin receptor, a surface molecule that recognizes free heme bound to hemopexin (111). Presumably for this reason heme also acts in a pro-inflammatory manner in EC (123). However, its ability to promote the expression of pro-inflammatory genes associated with EC activation is probably negligible when compared to that of methemoglobin (122).

Both heme and methemoglobin sensitize EC to the cytotoxic effects of free radicals (111, 112, 124), leading to exposure of pro-thrombotic sub-endothelial matrix and promoting complement (125) as well as platelet (126) activation, thus leading to microvascular thrombosis. Both heme and methemoglobin induce the expression of HO-1 in EC (111, 112, 124), which prevents their deleterious effects (112, 127). The protective effects of HO-1 result not only from a inhibition of intra-cellular heme accumulation but also from the end products that it generates (*discussed in more detail in section 7.2.*).

Expression of HO-1 in EC has additional cytoprotective effects that are likely to contribute to its overall protective effect. One of these relies on the elimination of extra-cellular free heme, which inhibits the pro-inflammatory effects of activated neutrophils (128, 129).

7.1.4. DENDRITIC CELLS AND T CELLS

HO-1-deficient mice have enlarged spleen and lymph nodes as well as high numbers of activated peripheral CD4 T cells indicating that endogenous HO-1 expression may control the extent of CD4 T cell activation and proliferation(101, 102). Activation of human peripheral CD4 T cells by anti-CD3 plus anti-CD28 antibodies is associated with the up-regulation of HO-1 expression (130). Over-expression of HO-1 in CD4 T cells inhibits anti-CD3 plus anti-CD28 mediated IL-2 secretion and proliferation (130-132), an effect mimicked by CO (130, 133) as well as by biliverdin (131, 132). This would suggest that HO-1 acts as negative feed back loop to limit CD4 T cell activation/proliferation (*see figure 2*).

HO-1 inhibits pro-inflammatory gene expression, associated with the activation dendritic cells, e.g. TNF- α , IL-12, CD40 or MHC class II (*James McDaid et al. Unpublished observation*). As expression of these pro-inflammatory genes is strictly required for CD4 T cell activation and proliferation it is likely that this effect

of HO-1 would contribute in a significant manner to inhibit CD4 T cell activation and/or proliferation.

There are additional mechanisms by which HO-1 can modulate CD4 T cell activation. Induction of HO-1 expression *in vivo* induces CD4 T cells to undergo activated induced cell death, a regulatory mechanism that controls the extent of CD4 T cell responses (*McDaid et al. FAEB Journal, in press*). The recent observation that CO promotes Fas/CD95 mediated activated induced T cell death, suggests that CO mediates the pro-apoptotic effect of HO-1 in these cells (133) (*see figure 2*).

It is intriguing that HO-1 derived CO would act as an anti-apoptotic gene in non-lymphoid cells, e.g. EC, while promoting apoptosis of lymphoid cells, i.e. CD4 T cells. These apparently antagonistic effects may “work together” to resolve inflammatory and immune responses. By preventing EC apoptosis HO-1 would protect tissues from undergoing irreversible injury while by promoting CD4 T cell apoptosis it would limit these cells from causing further tissue injury. This may be a central mechanism by which HO-1 controls the extent of inflammatory and immune responses (*see figure 2*).

Expression of HO-1 regulates T cell activation in a manner that suppresses the rejection of transplanted organs (131, 134, 135). Transgenic expression of HO-1 in a transplant recipient affords significant prolongation of graft survival, in a mouse model of cardiac transplantation (136). This effect is associated with inhibition of alloreactive T cell activation as well as with a switch from a T helper type 1 to a T helper type 2, cytokine expression profile (136).

Given the critical role of self-reactive T cell activation in the development of autoimmune diseases we tested whether HO-1 expression would interfere with the pathogenesis of multiple sclerosis, an autoimmune disease of the central nervous system. Using an experimental model of multiple sclerosis in mice, i.e. experimental autoimmune encephalomyelitis (EAE), we found that this is the case. The protective effect of HO-1 is associated with a profound inhibition of auto-reactive T cell proliferation, via a mechanism that depends on the modulation of dendritic cell activation (*Angelo Chora, unpublished observation*). This is in keeping with similar data obtained by others in a model of EAE in rats (137, 138).

7.2. MOLECULAR BASIS OF THE PROTECTIVE EFFECTS OF HO-1.

Some of the biologic outcomes of HO-1 expression rely on its ability to limit heme availability. In addition, however, HO-1 can act in two other ways: i) via direct interaction with other proteins and/or ii) via the end products of heme degradation that it generates. Whilst there is evidence for direct protein/protein interaction involving HO-1 (139) the biological significance of this phenomenon is not fully established. On the other hand the contribution of the end products of heme degradation to the biologic effects of HO-1, is supported by the observation that more or less specific chemical inhibition of its enzymatic activity (140) can abolish these effects (141). The observation that exogenous Fe, CO or biliverdin can mimic some of the biologic effects of HO-1 strongly suggests that these end products of heme degradation contribute to these effects (119, 141).

7.2.1. DEPLETION OF CELLULAR HEME

Expression of HO-1 can decrease cellular heme content. This can interfere with biosynthesis of hemoproteins that regulate inflammatory reactions, i.e. NAD(P)H oxidase, COX-2 or iNOS. Such an effect has been recently illustrated for NAD(P)H

oxidase (142). HO-1 expression in Mø inhibits the maturation of gp91(phox), a hemeprotein subunit of NAD(P)H oxidase, strictly required to sustain its activity (142). By doing so, HO-1 can suppress O_2^- generation, an effect that is likely to modulate signal transduction pathways leading to the activation of NF- κ B (143, 144) or p38 MAPK in activated Mø (145, 146) (*see section 6*).

7.2.3. REGULATION OF CELLULAR Fe.

While reactive *per se*, cellular Fe released upon heme degradation up-regulates the expression of the Fe sequestering protein ferritin (147, 148) as well as that of an Fe pump. By that decreasing the cellular pool of labile Fe^{++} these proteins limit the availability of Fe^{++} to promote the generation of free radicals via the Fenton reaction. Presumably for this reason both ferritin (149, 150) and Fe pumps (151) can modulate signal transduction pathways activated by free radicals, e.g. p38 MAPK and NF- κ B (151, 152) (*see section 6*).

We have recently shown that HO-1 inhibits the activation of pro-inflammatory genes associated with EC activation (96). This effect is due to the decrease of intracellular free Fe^{++} that occurs when HO-1 is expressed in EC. This inhibits NF- κ B, i.e. p65/RelA transcription activity (96) (*Mark Seldon et al. unpublished observation*). The mechanism by which HO-1 targets p65/RelA is not clear but, presumably, occurs via modulation of p65/RelA phosphorylation and/or acetylation, two events that are strictly required to sustain p65/RelA transcriptional activity in EC (78, 83) (*see section 6*).

7.2.4. CARBON MONOXIDE (CO).

Cellular CO can bind almost exclusively to Fe atoms, such as, when these are contained within heme groups or iron sulphur clusters. Alternatively, CO diffuses through the cytoplasmic membrane, binding to Fe in the heme groups of hemoglobin, contained within circulating red blood cells. In this case CO is subsequently exhaled upon displacement by O_2 in the lungs.

Cellular CO can modulate the biological activity of proteins that contain within their structure a heme group or a Fe sulphur cluster. One of such proteins is the hemeprotein, guanylate cyclase, a ubiquitously expressed enzyme that generates cyclic 3':5'-guanosine monophosphate (cGMP). Upon binding to Fe in the heme group of guanylate cyclase, CO can stimulate the production cGMP. This effect is 100-65 times less potent than that of NO (153), which lead to the assumption that guanylate cyclase was not a physiologic target of CO (154). There is however, accumulating evidence to suggest that CO can exert physiologic effects via the activation of this signal transduction pathway. Generation of CO by EC (155) or smooth muscle cells (156) can block platelet aggregation (157), through a mechanism that is dependent on the expression of guanylate cyclase and the generation of cGMP in platelets (158, 159). The vasodilatory effect of CO (160, 161) is also dependent on the generation of cGMP, which acts in EC to inhibit the expression of vasoconstrictor molecules such as endothelin-1 (ET-1) and platelet-derived growth factor-B (PDGF-B) (162). These effects of CO are likely to contribute to the protective effect of HO-1, as inhibition of platelet aggregation and vasodilatation can prevent the occurrence of microvascular thrombosis. In addition from guanylate cyclase, CO targets the p38 MAPK signal transduction pathway to exert cytoprotective, anti-inflammatory and

anti-proliferative effects in EC, Mø and smooth muscle cells, respectively (*reviewed in* (109, 163)).

The anti-apoptotic effects of CO are exerted in a variety of cell types that include EC (99, 141, 164), fibroblasts (165), smooth muscle cells (166), β -cells of the pancreas (167), cardiac myocytes (168) and hepatocytes (169, 170). The molecular mechanisms mediating these effects are most probably cell type specific and only those involved in protecting EC from apoptosis will be discussed here (*reviewed in* (171)). CO protects EC from TNF- α , staurosporin, serum starvation (141) and hypoxia/reoxygenation mediated apoptosis (164). This effect is inhibited by pyridinyl imidazoles, a group of molecules that block p38 α and p38 β MAPK activity (172)(*see* 6.2). This suggests that CO acts via the p38 α and/or the p38 β MAPK isoforms to prevent EC from undergoing apoptosis. Based on the pro-apoptotic effects of p38 α and the anti-apoptotic effect of p38 β (*see* 6.2.) it is likely that the anti-apoptotic effect of CO would act via p38 β . We found that expression of HO-1 in EC specifically down regulates the expression of p38 α , while sparing that of p38 β (173)(*Gabriela Silva et al., unpublished observation*). This supports the notion that the anti-apoptotic effect of CO in EC acts via activation of the anti-apoptotic p38 β MAPK isoform. However, others have recently suggested that this effect is mediated via p38 α (164). This discrepancy may be related to the different methodology used to define specifically the involvement of p38 α (164).

While CO does not seem to modulate NF- κ B activity in EC (171, 174), NF- κ B activity is required to sustain the expression of anti-apoptotic genes, e.g. cIAP-2 and A1 that “interact functionally” with CO to prevent EC apoptosis (174). This effect is specific to these subset of NF- κ B dependent genes, as CO does not seem to interact functionally with other NF- κ B dependent anti-apoptotic genes such as A20 or MnSOD (174). Interaction of CO with A1 or c-IAP2 requires the activation of p38 MAPK (174). It is intriguing that HO-1 inhibits NF- κ B transcriptional activity (171) and at the same time requires NF- κ B transcriptional activity to sustain its anti-apoptotic effect (174). Our interpretation is that HO-1 has reached a “functional compromise” in that it decreases the levels of cellular Fe⁺⁺ to modulate NF- κ B mediated gene transcription, inhibiting the expression of a subset of pro-inflammatory genes (171) whilst allowing the expression of protective genes required to support the anti-apoptotic effects of CO (174).

The mechanism by which CO interacts with the pro-apoptotic signal transduction machinery of EC remains elusive. There is evidence, that in ischemia/reperfusion mediated EC apoptosis, CO can inhibit Fas/Fas-ligand expression, blocking caspase-3, -8, and -9 activation as well as mitochondrial cytochrome c release, all of which are involved in EC apoptosis (175). Activation of p38 MAPK has been shown to inhibit caspase-8 and caspase-3 activities in other cell types (176) but whether p38 MAPK acts in a similar manner in EC remains to be established.

In activated Mø the anti-inflammatory effect of CO is also mediated via activation of the p38 MAPK signal transduction pathway (97). This is suggested by the observation that the ability of CO to suppress TNF- α secretion is abolished in Mø derived from MKK3 deficient mice (97). Presumably, this is due to the inability of CO to modulate the p38 MAPK signal transduction pathway (*see* 6.2). The observation that CO does not interfere with TNF- α mRNA accumulation suggests that CO blocks TNF- α production by interfering with TNF- α translation (97), an effect mediated via p38 MAPK activation (*see* 6.2.). Whether the ability of CO to

inhibit TNF- α expression is regulated via the p38 α or p38 β MAPK isoforms remains to be established.

Expression of HO-1 also inhibits NF- κ B activation in activated M ϕ (98). This effect is likely to act in an anti-inflammatory manner, as NF- κ B is involved in the up-regulation of pro-inflammatory genes associated with M ϕ activation, e.g. TNF- α or GM-CSF (98). Contrary to EC, this effect of HO-1 seems to be mediated via CO, which blocks the phosphorylation and degradation of I κ B α , thus suppressing NF- κ B nuclear translocation and activity (98).

CO activates the p38 MAPK signal transduction pathway in smooth muscle cells, but contrary to EC or M ϕ this is mediated via guanylate cyclase activation and cGMP accumulation (177). Activation of p38 MAPK by CO leads to increases p21^{cip1/waf} expression (177), a well-established cell cycle inhibitor that modulates NF- κ B activation (178). Additionally, CO protects smooth muscle cell from undergoing apoptosis (179) and arrests their proliferation (177), an effect that is likely to contribute to the anti-atherogenic effects of HO-1.

CO can bind to the heme prosthetic group of neuronal PAS domain protein 2 (NPAS2), a mammalian transcription factor. Upon binding CO inhibits NPAS2 DNA binding activity and presumably the expression of its target genes. While these findings are probably not directly linked to the regulation of inflammation they demonstrate unequivocally that CO can regulate the activity of transcription factors that contain heme groups thus regulating gene expression directly (180).

8. HO-1 SUPPRESSES THE PATHOGENESIS OF INFLAMMATORY DISEASES: POTENTIAL TARGETS FOR THERAPEUTIC APPLICATIONS.

That, HO-1 acts as a protective gene in humans was first suggested by the association of a HO-1 genetic deficiency with the premature death of a six-year-old boy who succumbed to a complex inflammatory syndrome (103, 104). More subtle variations in the level of HO-1 expression are associated with different susceptibility to the development of inflammatory diseases. There is a guanine-thymine (GT)_n length polymorphism in the 5' regulatory region of the human HO-1 gene that dictates the extent of transcriptional inducibility. Long (GT)_n repeats are associated with low HO-1 expression and short (GT)_n repeats with high expression. Long (GT)_n repeats, have been linked to higher incidence of emphysema and decline of lung function in smokers as well as with increased severity of coronary artery disease, abdominal aortic aneurysm, myocardial infarction, Kawasaki disease, inflammation after balloon angioplasty, restenosis after coronary stenting or peripheral angioplasty, chronic rejection of renal transplants as well as idiopathic recurrent miscarriage (*reviewed in* (181)). There are two single nucleotide polymorphisms (SNP) in the regulatory region of HO-1, i.e. the G(-113)A and T(-413)A that have also been associated with higher incidence of hypertension and coronary artery disease (*reviewed in* (181)). This suggests that HO-1 suppresses the pathogenesis of these inflammatory diseases. Whether the protective effects of HO-1 can be used therapeutically to overcome the pathogenesis of inflammatory diseases remains to be established but is likely to be the case.

Modulation of HO-1 can be achieved in humans by the administration of protoporphyrins. This approach has been used to block HO-1 activity and revert moderate hyperbilirubinemia associated with type Crigler-Najjar type I syndrome (182). However, for those inflammatory diseases were induction of HO-1 expression would be desirable this approach may not wield the expected results, as individuals

with low level of HO-1 inducibility would probably not respond efficiently to this therapy. An alternative approach would be to use gene transfer based approaches, that have been shown to attenuate the deleterious effect of hypertension (183), cardiac ischemia reperfusion injury (184), atherosclerosis (185), acute arteriosclerotic lesions (108) as well as chronic rejection of transplanted organs (186, 187) in rodents.

There are specific pathological conditions, such restenosis that develop following coronary stenting, wherein the therapeutic effects may be accomplished by short-term expression of HO-1 (177). This could be achieved using protein transduction domains (PTDs) that enter cells “spontaneously” with very high efficiency (188). These can drive HO-1 protein transduction into cells of the vascular wall (189). As illustrated in figure 3 a chimeric PTD-HO-1 can be used to achieve high level of HO-1 expression in cells of the arterial wall. Whether this will protect vessels from restenosis remains to be established.

9. CONCLUDING REMARKS.

Expression of HO-1 should be recognized as a central event in the control of inflammation and immunity. Most of the mechanisms involved in the effects mediated by HO-1 remain to be established. In the process of unveiling these one will most probably gain better understanding on the control of inflammatory and immune reactions.

10. FIGURE LEGENDS.

FIGURE 1. HMGB1 TRANSLOCATION. (A) Peritoneal Mø harvested from untreated C57BL/6 mice were cultured in RPMI 1640, 10% fetal calf serum. Non-adherent cells were removed and adherent cells (Mø) were cultured for additional 24h in the absence or presence of bacterial LPS (10 ng/ml). (B). Human umbilical vein EC were cultured as described elsewhere (96) and exposed before reaching confluence to bacterial LPS (24h; 1000 ng/ml). Cells were washed in phosphate buffered saline fixed in 4% paraformaldehyde (15 minutes), permeabilized (Triton X-100 0.5%) and washed. DNA was stained with propidium iodide (DNA, red) and HMGB1 was stained with a specific rabbit polyclonal antibody directed against HMGB1 (Pharmingen, green channel) and revealed with a goat anti rabbit FITC labeled polyclonal antibody (Pierce). Cells were analyzed by confocal microscopy using 8µm-Z-position sectional scanning in a Leica SP2 confocal system. Notice the nuclear outwards translocation of HMGB1 in Mø exposed to LPS (A) versus EC where this does not occur (B).

FIGURE 2. CELLULAR EFFECTS OF HO-1 DERIVED CO. (A) HO-1 derived CO protects EC from undergoing apoptosis, an effect that presumably prevents irreversible tissue injury during inflammatory reactions (see 7.1.3). HO-1 also modulates the pro-inflammatory phenotype of activated Mø (see 7.1.2) it induces via CO the secretion of IL-10 a potent anti-inflammatory cytokine that induces the expression of HO-1 in Mø (see 7.1.2). This in turn will promote further production of CO and secretion of IL-10, maintaining the expression of HO-1. Expression of HbS_r probably allows for internalization of the heme required to sustain this positive feedback loop (see 7.1.2). (B) Expression of HO-1 induces CD4 T cells to undergo activated induced death via a mechanism that requires the expression of Fas/CD95 and the FasL/CD95L molecules. CO promotes activated induced cell death via this signal transduction pathway and presumably mediated this effect of HO-1 (see 7.1.4). By inducing apoptosis of activated CD4 T cells HO-1 may limit the extent of CD4 T cell activation in a manner that prevents further tissue injury. This may be a central mechanism by which HO-1 controls the extent of immune responses.

FIGURE 3. TRANSDUCTION OF HO-1 IN THE ARTERIAL WALL. The rat aorta was isolated, washed in phosphate buffered saline and exposed to a PTD-HO-1 in phosphate buffered saline (80 nM, 1 hour). Tissue was washed in phosphate buffered saline, included in Tissue-Tek (Sakura Finetek Europe B.V) and snap frozen in liquid nitrogen. Sections (8 µm) were fixed in acetone (10 minutes) and stained for PTD-HO-1 using a rabbit anti-human HO-1 polyclonal antibody (Stress Gene, Victoria, Canada). Primary antibody was detected using a Cy5-fluorophore anti rabbit IgG (Jackson Immuno Research laboratories inc.). Transduced (A and B) and non-transduced (C and D) sections were analyzed using 8µm-Z-position sectional scanning in a Leica SP2 confocal system.

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Figure 1

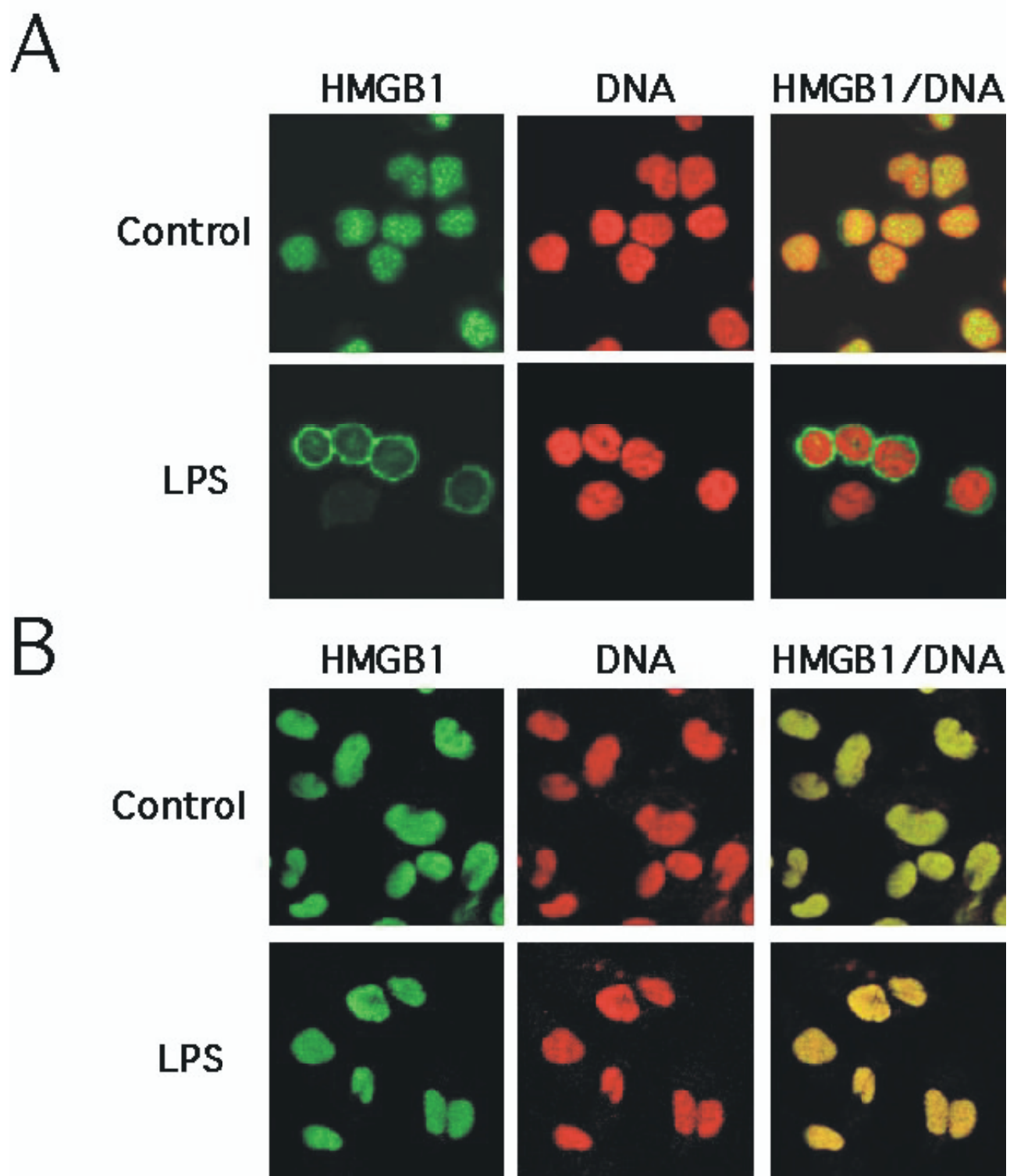
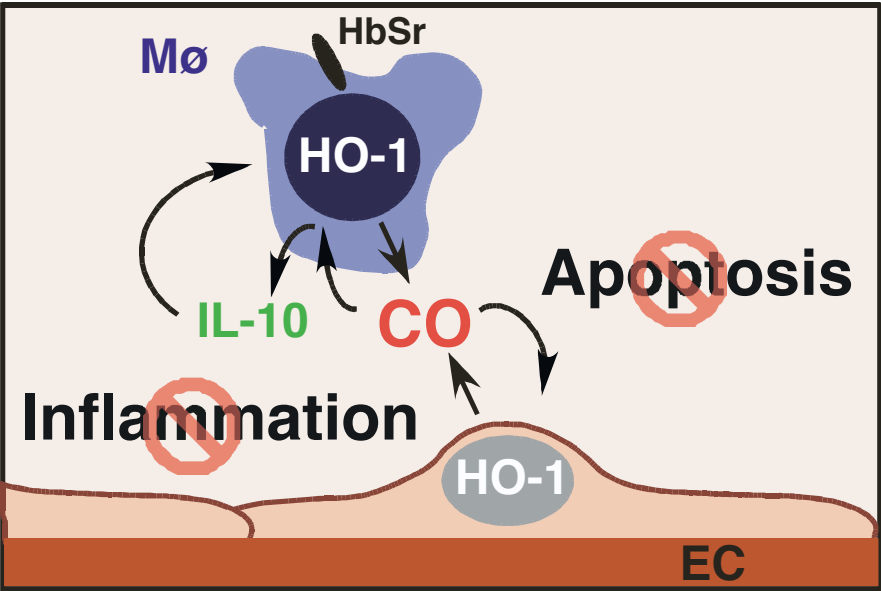


Figure 2

A



B

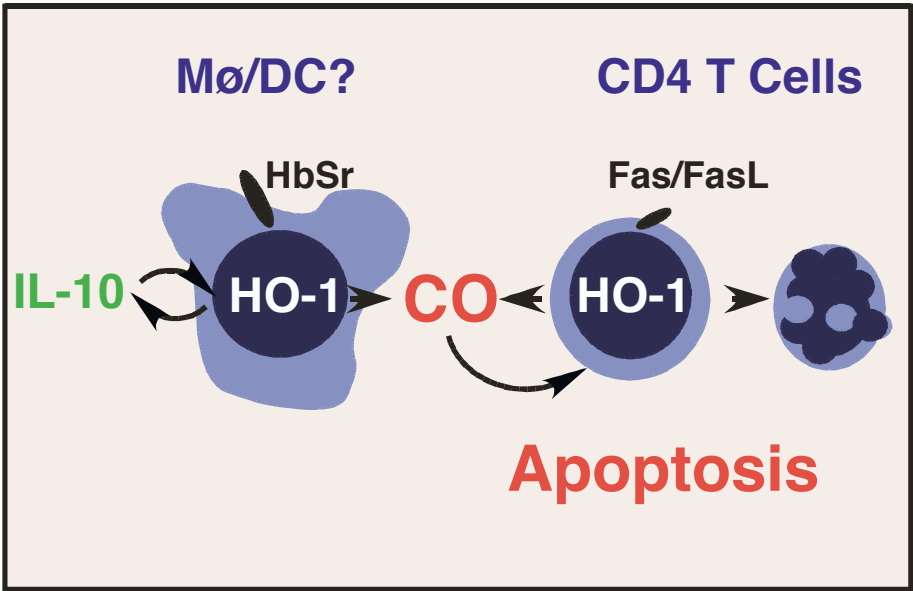


Figure 3

