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REVIEW

A central role for free heme in the pathogenesis of severe malaria: the missing link?

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Abstract Malaria, the disease caused by *Plasmodium* infection, is endemic to poverty in so-called underdeveloped countries. *Plasmodium falciparum*, the main infectious *Plasmodium* species in sub-Saharan countries, can trigger the development of severe malaria, including cerebral malaria, a neurological syndrome that claims the lives of more than one million children (<5 years old) per year. Attempts to eradicate *Plasmodium* infection, and in particular its lethal outcomes, have so far been unsuccessful. Using well-established rodent models of malaria infection, we found that survival of a *Plasmodium*-infected host is strictly dependent on the host's ability to up-regulate the expression of heme oxygenase-1 (HO-1 encoded by the gene *Hmox1*). HO-1 is a stress-responsive enzyme that catabolizes free heme into biliverdin, via a reaction that releases Fe and generates the gas carbon monoxide (CO). Generation of CO through heme catabolism by HO-1 prevents the onset of cerebral malaria. The protective effect of CO is mediated via its binding to cell-free hemoglobin (Hb) released from infected red blood cells during the blood stage of *Plasmodium* infection. Binding of CO to cell-free Hb prevents heme release and thus generation of free heme, which we found to play a central role in the pathogenesis of cerebral malaria. We will address hereby how defense mechanisms that prevent the deleterious effects of free heme, including the expression of HO-1, impact on the pathologic outcome of *Plasmodium* infection and how these may be used therapeutically to suppress its lethal outcomes.

Keywords *Plasmodium* · Malaria · Heme · Heme oxygenase-1 · Carbon monoxide · Inflammation

Abbreviations

BBB	blood brain barrier
CO	carbon monoxide
COHb	carboxyHb
Hb	hemoglobin
Hpx	hemopexin
FtH	ferritin heavy chain
HO-1	heme oxygenase-1
Hp	haptoglobin
Fe	iron
ICAM-1	intracellular adhesion molecule-1
LT	lymphotoxin
LDL	low-density lipoprotein
MAPK	mitogen activated protein kinases
Mø	monocyte/macrophages
MetHb	methemoglobin
NO	nitric oxide
PAMP	pathogen-associated molecular patterns
PRR	pattern recognition receptors
PMN	polymorphonuclear cells
ppm	parts per million
RBC	red blood cell
ROS	reactive oxygen species
RNS	reactive nitrogen species
TLR	toll-like receptor family
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1

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Severe malaria

Malaria affects an estimated 300–500 million new individuals every year [1]. The life cycle of *Plasmodium*, the causative agent of malaria, involves initially a clinically silent "liver stage" (reviewed in [2, 3]) and thereafter a "blood stage" (reviewed in [3, 4]) associated with the appearance of overt clinical signs including prostration, respiratory distress, pulmonary edema, convulsions, circulatory collapse, abnormal bleeding, jaundice (reflective of hepatic failure), hemoglobinuria, severe anemia, and/or impaired consciousness [1] (reviewed in [5, 6]). Severe malaria is defined by the occurrence of one or more of these clinical signs in individuals with no apparent cause of disease other than *Plasmodium* infection. Cerebral malaria, probably the most lethal form of severe malaria, is defined clinically by the development of coma, occurring at least 1h after termination of a seizure or correction of hypoglycemia, in individuals where asexual forms of *Plasmodium* are detected and in which other causes of encephalopathy have been excluded (reviewed in [5]). Lethal forms of severe malaria, including cerebral malaria, are in most cases associated with impaired consciousness, respiratory distress and severe anemia (reviewed in [5, 7]), accounting for the death of more than one million children (<5 years old) per year mainly in sub-Saharan Africa [1].

Pathogenesis of severe malaria

Epidemiologically, lethality associated with *Plasmodium* infection is a rare event, i.e., less than 1–2% of afflicted individuals succumb to infection [1]. This suggests that *Plasmodium* co-evolved with humans to reach an evolutionary "trade-off" in which its life cycle does not compromise that of its host. The cellular and molecular mechanism(s) insuring host viability are thought to rely almost exclusively on its ability to mount an effective immune response against *Plasmodium*. Over the years, however, it has become apparent that this immune response can contribute in a critical manner to the pathogenesis of severe malaria. Based on its resemblance to the human disease, *Plasmodium* infection in mice has been widely used to identify cellular and molecular mechanisms underlying the pathogenesis of severe and/or cerebral malaria [8, 9] (reviewed in [3]). The validity of this approach is supported by the finding that some of the key genes involved in the pathogenesis of severe malaria in mice, e.g., tumor necrosis factor (Tnf), lymphotoxin (Lt), intracellular adhesion molecule-1 (Icam-1), and nitric oxide synthases (Nos), are also involved in the pathogenesis of severe malaria in humans (reviewed in [3]).

Plasmodium infection elicits in its host an innate immune response that is critically involved in the pathogenesis of severe malaria. As for other infectious agents, *Plasmodium* expresses pathogen-associated molecular patterns (PAMP) that can be recognized by host germline-encoded pattern recognition receptors (PRR; reviewed in [10]). These signal in innate immune cells, such as monocyte/macrophages (Mø), to elicit a potent pro-inflammatory response. Glycosylphosphatidylinositol, a glycolipid expressed at the surface of *Plasmodium*, is recognized via PRR belonging to the Toll-like receptor (TLR) family, i.e., TLR2/TLR1 or TLR2/TLR6 complexes and to a lesser extent TLR4 [11]. Hemozoin, a crystal derived from heme and generated by *Plasmodium* (see "The HO system"), can also be recognized by TLR9 [12, 13].

The contribution of these PRR to the pathogenesis of severe malaria is suggested by the following set of observations. Polymorphisms in human TLR4 (Asp299Gly/Thr399Ile) and TLR9 (T-1486C) are associated with susceptibility to severe malaria in children [14] and with the outcome of *Plasmodium* infection during pregnancy [15], respectively. There is also a polymorphism (Ser180Leu) in Mal/TIRAP (an adaptor molecule required for TLR2 and TLR4 signaling) associated with susceptibility to severe malaria [16]. The involvement of these PRR in the pathogenesis of severe malaria in rodents remains controversial, with some reports suggesting an active role [13] while others not [17, 18].

The contribution of host adaptive immunity to the pathogenesis of severe malaria is well illustrated in rodent models of *Plasmodium* infection (reviewed in [3, 19]). Once primed by dendritic cells, presumably in the spleen, (T_H) cells foster the co-activation of $CD8^+$ T helper cells. It has been suggested that activated $CD8^+$ T cells can target brain microvascular endothelial cells, triggering endothelial cell activation and ultimately granzyme- and perforin-dependent programmed cell death, promoting the onset of cerebral malaria (reviewed in [3, 19]).

The evolutionary "trade-off" allowing *Plasmodium* and its host to coexist might require an additional mechanism of host defense that does not necessarily involve the host immune response targeting *Plasmodium* [20]. We reasoned that such a mechanism might operate in a manner that limits the deleterious effects of red blood cell (RBC) lysis, a phenomenon inherent to *Plasmodium* infection. Hemolysis is associated with release of an estimated 20% to 40% of the initial pool of RBC hemoglobin (Hb) into the circulation (reviewed in [21]). Cell-free Hb can release its heme prosthetic groups, which, as discussed in the next section, is a highly deleterious event that can promote the onset of severe malaria (Fig. 1).

Deleterious effects of free heme

Erythrocytic forms of *Plasmodium* are in intimate contact with Hb, degrading approximately 60% to 80% of the total RBC Hb content to use it as a vital source of amino acids (reviewed in [21]). Hb degradation, however, generates free heme and reactive oxygen species (ROS), the combination of which is highly deleterious to erythrocytic schizonts [22] as well as to the infected host.

At concentrations below 5 mM, such as outside RBC, cell-free Hb tetramers dissociate spontaneously into dimers (reviewed in [23]; Fig. 1). In the presence of ROS or reactive nitrogen species (RNS), cell-free Hb dimers are readily oxidized into methemoglobin (metHb), releasing its heme prosthetic groups [24–26] (reviewed in [23, 27]; Fig. 1). Free heme can be highly cytotoxic to endothelial cells [24–26], a pathologic event that exposes the pro-thrombotic sub-endothelial matrix to the coagulation cas-
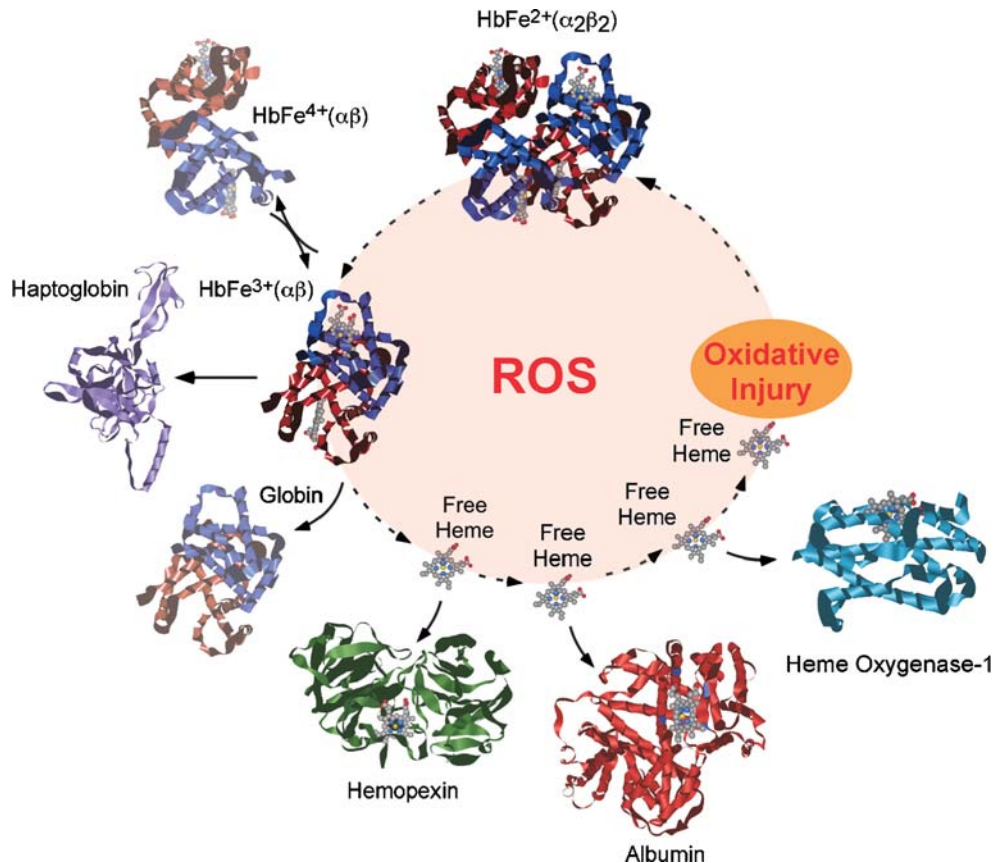

Fig. 1 Defense mechanisms against the free heme generated via Hb oxidation. When released from infected RBC, ferrous HbFe^{2+} ($\alpha_2\beta_2$) tetramers disaggregate spontaneously into Hb ($\alpha\beta$) dimers. In the presence of ROS (or RNS), cell-free Hb ($\alpha\beta$) dimers are readily oxidized into ferric HbFe^{3+} ($\alpha\beta$), i.e., metHb. In this process, ferryl HbFe^{4+} ($\alpha\beta$), a short-lived intermediate is also generated [93], which can result in a potent pro-inflammatory response (Gabriela Silva, unpublished observations). Under homeostatic conditions, ferric HbFe^{3+} ($\alpha\beta$) is scavenged by haptoglobin (*Hp*). The very high affinity of Hp towards Hb (>10^{10} mol^{-1} in humans) is sustained by two Hp-binding sites in the Hb β chain, i.e., β^{11-25} and $\beta^{131-146}$ and one in the α chain, i.e., $\alpha^{121-127}$ [94]. Binding of Hp to Hb also stabilizes heme, inhibiting its release [95]. During *Plasmodium* infection, plasma Hp is depleted, allowing ferric HbFe^{3+} ($\alpha\beta$) to release heme. Plasma free heme can be scavenged by hemopexin (*Hpx*), a plasma protein folded into two homologous β-propeller domains joined by a linker and binding a single heme molecule via His^{213} and His^{266} with the highest affinity (K_d<10^{-12} M in humans) of any other protein described so far [96]. Additional non-covalent interactions between hydrophobic residues of Hpx and the porphyrin ring and propionate groups of heme act as electrostatic anchors that contribute to heme binding and to coordinate its Fe^{3+} as well. Once the scavenging capacity of Hpx is exhausted, plasma free heme can be scavenged by albumin, which has two tyrosine residues, i.e., Tyr^{138} and Tyr^{161} that provide a D-shaped cavity limited by p–p stacking interaction with the porphyrin ring of heme with an affinity that is however, 10^4 times lower to that of Hpx (K_d=10^{-8} M in humans) [97]. In addition, this 3D structure supplies a donor oxygen (from Tyr^{161}) to the Fe atom of heme, maintaining it in its reduced state, i.e., Fe^{2+}. The interaction of heme with human albumin is further stabilized by two salt bridges, provided by the albumin Arg^{114} and Lys^{190} residues (reviewed in [98]). When the scavenging capacity of Hp, Hpx and albumin are exhausted, there is yet an ultimate defense mechanism to avoid free heme accumulation in plasma, i.e., heme catabolism by HO-1. If this is still not sufficient to clear all the free heme generated from Hb oxidation, a pathologic positive-forward feedback loop is formed in which free heme promotes the generation of ROS that sustain the oxidation of cell-free Hb, with further generation and accumulation of free heme in plasma, ultimately leading to irreversible oxidative injury

cade, leading to formation of more or less extensive microvascular thrombi with concomitant vaso-occlusion and tissue ischemia.

The underlying cause of free heme cytotoxicity involves most probably its highly hydrophobic nature, which allows the Fe contained within its protoporphyrin IX ring to enter and cross cell membranes as well as other lipid structures [25]. The Fe contained within the protoporphyrin IX ring can act as a potent pro-oxidant, donating electrons that foster the generation of ROS and/or RNS via the Fenton chemistry. This can also occur when Fe is released from the protoporphyrin IX ring of heme, either through non-enzymatic oxidative degradation or enzymatic cleavage, catalyzed by heme oxygenases (HO) [28] (see "Expression of HO-1 modulates the onset of severe malaria"). There is also the possibility that as for other hydrophobic molecules [29], endothelial cells might express "heme receptors" that could mediate its cytotoxic effects [30].

Additional mechanisms via which free heme can be deleterious to endothelial cells include its ability to promote oxidative modification of low-density lipoproteins (LDL) [24, 25]. Heme-assisted LDL oxidation, involving spontaneous insertion of heme into LDL particles coupled with oxidative interactions between LDL and heme, can lead to oxidative modifications in LDL that are highly cytotoxic to endothelial cells [24].

Free heme can act as a polymorphonuclear (PMN) cell chemoatractant [31], sustaining PMN cell activation [32] and survival [33], thus fostering the generation of ROS that can synergize with free heme to promote further endothelial cell cytotoxicity. As activation of PMN leukocytes by free heme is blocked by pertussis toxin, these cells might recognize free heme specifically via G-protein-coupled receptors [31].

In addition to targeting endothelial cells, free heme can also exert deleterious effects in other cell types. We have recently found that free heme sensitizes non-hematopoietic cells to undergo TNF-mediated programmed cell death (Seixas et al., manuscript submitted for publication). Probably explaining the critical involvement of TNF to the onset of severe malaria [34].

Defense mechanisms against the deleterious effects of free heme

Plasmodium evolved a series of defense mechanisms against the deleterious effects of free heme, such as its polymerization into hemozoin, an inert crystal in which the Fe atoms in the protoporphyrin IX ring of heme can no longer act as pro-oxidant catalysts (reviewed in [21]). The importance of this defense mechanism is revealed by the potent therapeutic effects of anti-malarial drugs that inhibit

hemozoin formation [35]. While this strategy, i.e., hemozoin formation, allows erythrocytic forms of *Plasmodium* to replicate efficiently within the confined space of a RBC, it does generate a major caveat, namely that *Plasmodium* replication will unavoidably lead to hemolysis and Hb release into the circulation (reviewed in [21]).

Given the highly deleterious effects of free heme to the infected host, there might be a reason why accumulation of cell-free Hb has been maintained throughout evolution. One possible explanation is that cell-free Hb can act as a host innate defense mechanism [36]. This notion is supported by the recent observation that cell-free Hb can recognize molecular patterns expressed specifically by microbial organisms, an effect that when coupled to heme-assisted ROS production, can lead to the clearance of blood-borne microbial pathogens [36]. Whether this innate defense mechanism can target *Plasmodium* remains to be tested experimentally.

Despite its putative beneficial effects, several defense mechanisms have evolved to cope with the cytotoxicity of cell-free Hb. Under homeostatic conditions, cell-free Hb can be readily scavenged by haptoglobin (Hp), a tetrameric ($\alpha_2\beta_2$) plasma protein (human; P00738). Hb/Hp complexes are recognized and internalized via the Hp receptor (CD163) expressed by monocyte/macrophages (Mø) in the red pulp of the spleen [37]. This allows for heme degradation via the HO system ("Defense mechanisms against the deleterious effects of free heme"; Figs. 2 and 3). Contrary to the β chain of Hp (β, ~40 kDa) its α chain has two allelic variants, i.e., *Hp1* (α_1, ~8.86 kDa) and *Hp2* (α_2, ~17.3 kDa) that give rise to three possible Hp phenotypes, i.e., *Hp1.1*, *Hp1.2*, and *Hp2.2* [38]. These have graded affinities for cell-free Hb, i.e., *Hp1.1>Hp1.2>Hp2.2*, as well as for CD163, i.e., *Hp2.2>Hp1.2>Hp1.1* [37]. Individuals expressing the *Hp2.2* phenotype are less likely to develop severe malaria [39], suggesting the existence of a stringent process of natural selection targeting this Hp allele in endemic areas of *Plasmodium* infection.

Under hemolytic conditions, such as during *Plasmodium* infection, circulating Hp can be depleted, leading to accumulation of cell-free Hb in plasma. In keeping with this notion, 20–40% of children infected by *Plasmodium falciparum* in sub-Saharan countries present undetectable levels of plasma Hp [40]. Presumably, for this reason, cell-free metHb does accumulate in the plasma of individuals suffering from severe malaria [41, 42], which should lead to the prompt release of its heme prosthetic groups (Fig. 1). When this occurs, there are additional mechanisms that can prevent the accumulation of free heme in plasma. One of these relies on hemopexin (Hpx), a monomeric plasma protein (~63 kDa; human; P02790; Figs. 1 and 2). Under homeostasis, Hpx can scavenge most of the free heme that accumulates in plasma, forming Hpx/heme complexes.

Fig. 2 Defense mechanisms against free heme are coupled to heme degradation by HO-1. When cell-free ferric $HbFe^{3+}$ ($\alpha\beta$), i.e., metHb, accumulates in plasma, it can be scavenged by circulating haptoglobin (*Hp*) to be delivered to the Hp receptor (CD163) expressed by Mø in the red pulp of the spleen. This allows for sheltering of the heme prosthetic groups of $HbFe^{3+}$ ($\alpha\beta$) as well as for catabolism of the heme groups by HO-1, constitutively expressed by $CD163^+$ Mø. If released from ferric $HbFe^{3+}$ ($\alpha\beta$), free heme can be scavenged by very high affinity binding to hemopexin (*Hpx*) and delivered to liver hepatocytes and Mø expressing the Hpx receptor (CD91). Expression of HO-1 in liver hepatocytes and Mø ensures heme catabolism. During the onset of severe malaria, the pool of plasma Hp and Hpx is exhausted, and free heme accumulates in plasma. Free heme can cross cell membranes, e.g., in endothelial cells, where it synergizes with oxidants to induce the expression of HO-1 by which it is catabolyzed. Pathology will only develop when all these defense systems are overwhelmed
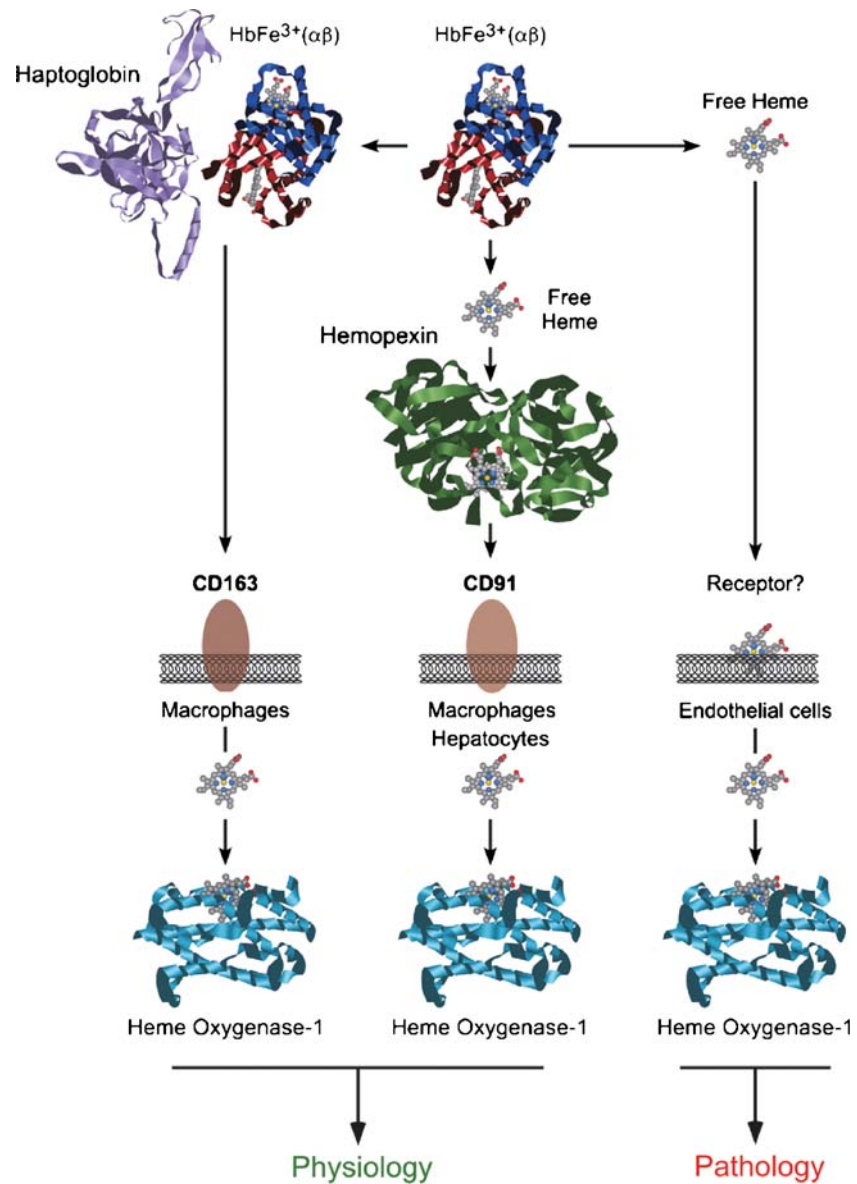
These are recognized by the Hpx receptor (CD91) expressed in hepatocytes, Mø, fibroblasts, adipocytes, neurons, and trophoblasts [43] (Fig. 2). Internalization of these complexes via CD91-assisted endocytosis allows for heme degradation via the HO system [43] (Figs. 2 and 3). Once the scavenging capacity of plasma Hpx is exhausted, free heme can still be scavenged by plasma albumin (~66 kDa; human; P02768; Fig. 1). Whether heme/albumin complexes are recognized by specific receptors allowing for heme degradation via the HO system has not been established.

We have recently described that the onset of severe malaria in mice is associated with the accumulation of high concentrations of free heme in plasma [8], suggesting that the capacity of plasma Hp, Hpx, and albumin to prevent free heme accumulation is exhausted during the onset of severe malaria (reviewed in [23, 27]). However, there is yet an ultimate defense mechanism to avoid the deleterious effects of free heme, namely its catabolism by the cellular HO system (Fig. 3).

The HO system

Heme oxygenases (EC1.14.99.3) are encoded by three distinct genes, i.e., *HMOX1* (human; P09601), *HMOX2* (human; P30519), and *Hmox3* (rat; O70453). *HMOX1* and *HMOX2* encode two distinct proteins, i.e., HO-1 (32 kDa) and HO-2 (37 kDa), respectively, while *Hmox3* is most probably a pseudogene. Under oxidative stress, HO-1

Fig. 3 The heme oxygenase system. HO-1 catabolizes free heme into biliverdin. Initially, free heme intercalates in the heme-binding pocket of apo HO-1, which cleaves the protoporphyrin IX ring using one O_2 molecule and electrons donated by NAD(P)H/CytP450 reductase via a reaction releasing Fe^{2+} (*yellow circle*) from its inner core and generating carbon monoxide (*CO*; *gray* and *red circle*). Biliverdin, the protoporphyrin structure released from the heme pocket of HO-1, can be converted into bilirubin by biliverdin reductase (*BVR*), which uses NAD(P)H as electron donors. All three end products of this reaction, i.e., Fe, CO, and bilirubin, can dampen the deleterious effects of inflammatory reactions
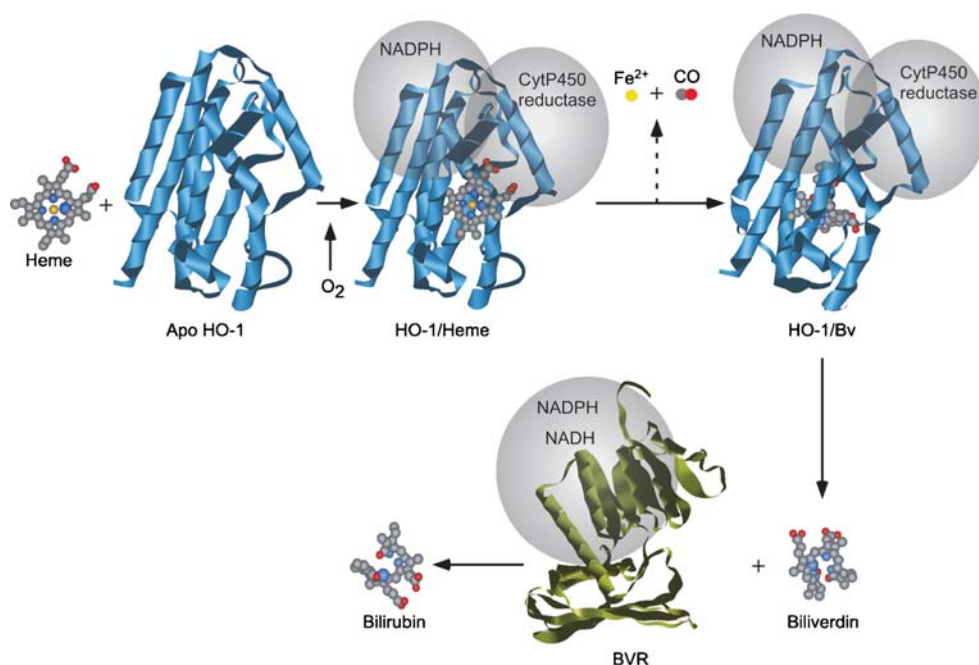
expression is induced ubiquitously, while the expression of HO-2 remains unchanged [8]. Both HO isoforms can cut the protoporphyrin IX ring of free heme, releasing Fe from its inner core and generating carbon monoxide (CO; Fig. 3). The remaining protoporphyrin structure gives rise to biliverdin, which can be converted by biliverdin reductase into bilirubin, a potent antioxidant [44] (Fig. 3). Biliverdin reductase can also trigger signal transduction and probably mediate in this manner some of the biologic effects attributed to biliverdin (reviewed in [45]).

Heme catabolism by the HO system rids cells of a membrane-permeant Fe, i.e., heme. However, the Fe released via heme catabolism represents a potential hazard, unless sequestered by ferritin [46], which acts as a vital antioxidant in various experimental models [46–48]. Ferritin is a multimeric (24-subunit) protein (H chain, 21 kDa, L chain, 20.7 kDa) with high Fe storing capacity (4,500 mol of Fe per mole of ferritin). The proportion of H and L subunits in the ferritin shell depends on the Fe status of the cell (reviewed in [49]). The protective effects of ferritin are attributable in large measure to the ferroxidase activity of the H chain subunit [50], which catalyzes the oxidation of (ferrous) Fe^{2+} formed by superoxide or endogenous reductants into (ferric) Fe^{3+}. This process, i.e., Fe nucleation, facilitates Fe insertion into multimeric ferritin structures, allowing for the functional storage and inactivation of labile (ferrous) Fe^{2+} that is otherwise capable of fueling the production of toxic hydroxyl radicals ($OH^{\cdot}$) via the Fenton chemistry.

It is now well established that induction of HO-1 expression plays a pivotal role in the resolution of inflammatory conditions (reviewed in [51]). Malaria seems to provide yet another situation where the potent protective effects of HO-1 are exerted [8].

Expression of HO-1 modulates the onset of severe malaria

Plasmodium infection, in both mice and humans, is associated with the induction of high levels of HO-1 expression that is neither restricted to a specific cell type nor to a specific organ [8, 52–54]. We found that mouse strains that express high levels of HO-1 in response to *Plasmodium* infection do not succumb to severe and/or cerebral malaria, while those that express low levels of HO-1 do so [8] (Seixas et al., manuscript submitted for publication). Perhaps more importantly, deletion of the *Hmox1* locus by homologous recombination is sufficient per se to promote the onset of severe and/or cerebral malaria [8] (Seixas et al., manuscript submitted for publication). These observations reveal that induction of HO-1 expression is part of a protective mechanism that suppresses the onset of severe and/or cerebral malaria in mice.

Contrary to HO-1, the expression of HO-2 is not induced in response to *Plasmodium* infection in mice [8], which would suggest that HO-2 is not critically involved in modulating the pathogenesis of severe and/or cerebral malaria. However, as the outcome of *Plasmodium* infection in *Hmox2*-deficient mice has not been reported, we cannot at this point exclude the possibility that HO-2 might

modulate the pathogenesis of severe and/or cerebral malaria.

Probably critical to our understanding of the mechanism of action of HO-1 is the observation that its protective effects do not correlate with modulation of "parasite burden", i.e., percentage of infected RBC [8]. This suggests that HO-1 can prevent the lethal outcome of *Plasmodium* infection without interfering with the host immune response controlling parasite burden [8], a general host defense mechanism against infection referred to as "tolerance" [20]. Based on the extremely well-conserved nature of this enzymatic system among mice and humans, it is possible that HO-1 expression might also be protective against the onset of severe and/or cerebral malaria in *Plasmodium*-infected children.

If one assumes that HO-1 might play a central role in modulating the pathogenesis of severe malaria in humans, then the prediction would be that spontaneous mutations selected through evolution based on their protective effect against severe malaria might act via HO-1. Sickle cell trait, caused by a single point mutation in one of the Hb β-globin chain *locus*, is probably the best-characterized of such mutations. Sickle cell trait can be associated with the occurrence of mild hemolysis and heme release into the circulation. When exposed chronically to low levels of free heme, cells up-regulate the expression of HO-1 [25, 26, 46], probably explaining why individuals carrying this mutation can have high level of HO-1 expression [55]. It is therefore tempting to speculate that up-regulation of HO-1 might contribute to the protective effect of this hematological condition against the onset of severe malaria. It is also possible that a similar protective mechanism, involving HO-1, could contribute to explain how other hemolytic disorders such as α- and β-thalassemia or glucose 6-phosphate dehydrogenase deficiency also afford protection against severe malaria. However, this remains to be tested experimentally.

Spontaneous disruption of the *HMOX1* locus in humans is lethal [56], excluding the possibility of establishing a linear functional link between HO-1 expression and the outcome of *Plasmodium* infection. However, there is a (GT) n microsatellite polymorphism in the human *HMOX1* promoter that regulates in a quantitative manner its response to many stimuli (reviewed in [57]). Individuals with fewer (GT)n repeats have a high HO-1 inducibility, while those with higher (GT)n repeats have a lower response (reviewed in [57]). There are now multiple studies demonstrating that individuals responding more strongly, i.e., fewer (GT)n repeats, are also less likely to develop a series of pathologies (reviewed in [57]). However, in the case of *Plasmodium* infection, this relationship does not seem to be straightforward. In a recent study, children that succumbed to cerebral malaria were shown to carry more frequently the homozygous short (GT)n repeats ($n < 28$; high HO-1 inducibility) than those that do not develop this syndrome [58]. As a cautionary note, however, this study was performed in a very limited sample size, and no correlation between the (GT)n polymorphism and HO-1 expression was assessed in the context of *Plasmodium* infection [58]. Nevertheless, assuming that in *Plasmodium*-infected children the homozygous short (GT)n repeats afford high level of HO-1 expression but no protection against the onset of cerebral malaria, this might be explained by the following set of observations in mice. Expression of HO-1 is significantly induced during the hepatic stages of *Plasmodium* infection [59]. More importantly, when *Hmox1* is deleted by homologous recombination, generation of liver merozoites is reduced by ~70–80% [59]. This would suggest that a viable interaction of *Plasmodium* with its host is strictly dependent on the induction of HO-1 expression during both liver and blood stages of infection. Namely, HO-1 expression might favor initially the generation of liver merozoites and their progression into the blood, while expression of HO-1 thereafter might prevent the onset of severe malaria. While this interpretation does not take away the critical role of HO-1 in suppressing the pathogenesis of severe malaria, it suggests that *Plasmodium* interaction with its human host might have evolved in a way that subverts the HO-1 system to promote both *Plasmodium* and host survival.

CO and expression of heavy chain ferritin (FtH) prevent the onset of severe malaria

Congruous with previous observations in other experimental models of disease [60–63], we found that CO can prevent the development of cerebral malaria in mice [8]. Briefly, under experimental conditions in which more then 95% of *Plasmodium*-infected mice develop cerebral malaria, CO inhalation (250 ppm) at different times after infection reduces the incidence of cerebral malaria to less than 5% [8]. As for HO-1, CO does not modulate parasite burden, suggesting that it affords host tolerance against *Plasmodium* infection [20]. That is, CO limits disease severity, i.e., death, associated with a given parasite burden.

The finding that CO can prevent the onset of cerebral malaria in mice could have important implications for the treatment of this disease in humans, as the protective effect of CO is achieved when applied as late as 4–5 days post-infection at a dosage as low as 250 ppm [8]. This suggests that CO might be used therapeutically, after infection, to prevent the onset of cerebral malaria in humans.

We have also tested whether other end products of heme catabolism by HO-1 exert protective effects. Biliverdin, which we have shown to elicit potent protective effects in

other experimental models of disease [64], failed to prevent the development of cerebral malaria in mice [8]. This suggests that biliverdin/bilirubin is probably not the main effector mechanism via which HO-1 affords protection against severe and/or cerebral malaria. However, as a cautionary note, failure of biliverdin to exert protective effects may be due to its inability to cross the blood brain barrier (BBB).

Finally, we have recently found that expression of FtH in non-hematopoietic cells suppresses the cytotoxic effects of free heme and prevents the lethal outcome of severe malaria in mice (Seixas et al., manuscript submitted for publication). This salutary effect, which requires FtH ferroxidase activity, acts independently of host immunity against *Plasmodium*, suggesting that in a similar manner to CO, expression of FtH can limit disease severity and prevent the lethal outcome of severe malaria under a given parasite burden [20].

Are the protective effects of CO mediated via signal transduction?

Understanding further the mechanism(s) of action of CO might improve the possibility of using this gaseous molecule for therapeutic purposes in humans. There are now several studies suggesting that CO exerts anti-inflammatory [61], cytoprotective [65], and anti-proliferative effects [62] that act in a concerted way to prevent the deleterious effects associated with the development of many pathologic conditions, such as hyperoxic lung injury, rejection of transplanted organs, ischemia and reperfusion injury, severe sepsis or arteriosclerosis, among others (reviewed in [51]). These studies also suggested that CO acts as a signal transduction molecule to down-modulate inflammation and afford cytoprotection as well as to prevent unfettered cell proliferation (reviewed in [51]). These effects should contribute to the ability of CO to prevent the onset of severe and/or cerebral malaria.

CO can react with reduced divalent metals and in particular with Fe^{2+} contained within the heme groups of hemoproteins (reviewed in [66]). The relative affinity of CO for different heme prosthetic groups is dictated not only by their Fe "redox status" (Fe^{2+} versus Fe^{3+}) but also by the amino acid sequence in the "heme-binding motifs" of these hemoproteins. This was demonstrated originally for Hb and later for guanylate cyclase, an enzyme that generates the signal transduction molecule cyclic GMP (cGMP) [67] (reviewed for Hb in [68]). In the case of guanylate cyclase, the amino acid sequence of its heme-binding motifs impose that CO binds to heme in a manner that is distinct from that of other biologically active gases such as nitric oxide (NO)

[69]. The net result being that the relative ability of CO to induce cGMP production is significantly lower than that of NO [70]. This led to the notion that guanylate cyclase is a physiologic target of NO, but probably not of CO, despite experimental evidence suggesting that CO can exert biologic effects via cGMP (reviewed in [71]). Whether the guanylate cyclase/cGMP pathway is involved in the protective effect of CO against severe malaria remains to be established.

CO can bind a variety of other hemoproteins, including cytochrome C oxidase, the terminal electron acceptor in the mitochondrial electron transport chain [72]. As for guanylate cyclase, cytochrome C oxidase is also a physiologic target of NO (reviewed in [73]). Binding of CO to cytochrome C oxidase can trigger a small burst of mitochondria-derived ROS [74–76] that activates several signal transduction pathways. Among these is the p38 family of mitogen-activated protein kinases (MAPK) [74, 75], a common molecular denominator via which the anti-inflammatory [61], cytoprotective [65], and anti-proliferative effects [62] of CO are exerted in several cell types.

The p38 family of MAPK is composed of four isoforms encoded by different genes, i.e., *P38α* (38 kDa), *P38β* (39 kDa), *P38γ* (43 kDa), and *P38δ* (40 kDa; reviewed in [77]). These share sequence homologies ranging from 74% (*P38α* versus *P38β*) to 98% (*P38β* versus *P38β2*) and a canonical dual phosphorylation site (Thr–Gly–Tyr; reviewed in [77]). The kinase activity of the p38α and p38β isoforms can be inhibited by pyridinyl imidazoles, a class of chemical compounds that does not target the p38γ or p38δ isoforms. The observation that pyridinyl imidazoles can abrogate the anti-inflammatory [61], cytoprotective [65], and anti-proliferative [62] effects of CO led to the hypothesis that these effects might act via the p38α and/or the p38β isoforms. In keeping with this notion, suppression of p38β expression impairs the cytoprotective [65, 78, 79] and anti-proliferative [80] effects of CO, suggesting that CO acts specifically via this p38 MAPK isoform. Moreover, we found that the mechanism via which CO signals via p38β also involves the specific targeting of the p38α isoform for degradation by the 26S proteasome pathway [78]. Presumably, CO alters the ratio of p38α versus p38β expression, allowing signaling via the cytoprotective p38β to predominate over that of cytotoxic p38α [78]. Whether (1) mitochondria-derived ROS, (2) p38α degradation, and/or (3) signaling via p38β contribute to the protective effect of CO against severe malaria remains to be established.

There are probably many other signal transduction pathways via which the protective effects of CO can be exerted (reviewed in [71]). Exposure of naïve Mø to CO in vitro can induce the expression of peroxisome proliferator-activated receptor-gamma (PPARγ) [75], a nuclear hormone receptor that down-regulates a subset of pro-

inflammatory genes associated with Mø activation [81]. Blocking mitochondria-driven ROS and/or deleting the expression of PPARγ abrogates the anti-inflammatory effects of CO, suggesting that this effect acts via up-regulation of PPARγ, presumably via mitochondria-driven ROS [75]. Yet, another signal transduction pathway triggered by CO in Mø involves the transcription factor hypoxia-inducible factor 1 alpha (HIF-1α) [76]. Activation and stabilization of HIF1α by ROS arising from mito-chondria induces the transcription of TGF-β [76], a potent anti-inflammatory and cytoprotective cytokine that could contribute to the salutary effects of CO in many inflam-matory conditions, including in severe malaria. Again, the contribution of these signal transduction pathways in the control of the pathogenesis of severe malaria is not defined.

In most studies addressing the mechanisms of action of CO, the assumption has been that this gas targets one or several "upstream" signal transduction pathways that modulate the activity of transcription factors regulating gene expression. However, there are instances where CO can target transcription factors directly. This is the case for the neuronal Per-ARNT-Sim (PAS) domain protein 2 (NPAS2), a transcription factor implicated in the regulation of circadian rhythms [82]. Binding of CO to the heme prosthetic group of NPAS2 controls NPAS2 heterodimeri-zation with the brain and muscle arnt-like protein-1 (BMAL1), a transcription factor that regulates NPAS2 DNA binding and transcriptional activity [82]. Whether NPAS2 regulates Plasmodium interaction with its human host remains to be established, but is likely to be the case. Cycles of RBC schizont infection (hemolysis, invasion, replication) are highly synchronous [83] and therefore could be controlled by host transcription factors that regulate circadian rhythms, such as NPAS2.

Most, if not all, of the biological functions attributed to CO are thought to rely on its binding to Fe^{2+} in the heme prosthetic groups, e.g., guanylate cyclase, cytochrome C oxidase, NAD(P)H oxidase (reviewed in [71]). The postulate has been that CO can alter the 3D structure of these hemoproteins and modulate in this manner their biologic activity, i.e., signal transduction (reviewed in [71]). While this notion has provided a useful framework to explain the protective effects of CO, our recent finding of an alternative mechanism via which CO can suppress the onset of severe malaria might challenge this view.

## Mechanism underlying the protective effects of CO: inhibition of heme release from Hb

We found that one of the mechanisms via which CO prevents the onset of cerebral malaria relies on its ability to bind cell-free Hb and form carboxyHb (COHb; Fig. 3). When bound to cell-free Hb, CO slows down its rate of oxidation and, subsequently, the rate of heme release from Hb [8] (Fig. 3). The critical involvement of free heme in the pathogenesis of cerebral malaria is supported by the following set of observations: (1) the protective effect of CO is associated with a profound decrease (~95%) in the accumulation of free heme in plasma of Plasmodium-infected mice and (2) administration of heme precipitates the onset of cerebral malaria, abrogating the protective effect of CO [8].

There is further evidence that free heme can dictate susceptibility to cerebral malaria in mice. We found that a given Plasmodium strain that triggers cerebral malaria in mice also leads to higher levels of free heme accumulation in plasma, as compared to another related Plasmodium strain that does not trigger cerebral malaria (Fig. 4a). More-over, administration of free heme is sufficient per se to precipitate the onset of cerebral malaria in mice infected with the Plasmodium strain that does not trigger cerebral malaria naturally (Fig. 4b). In a similar manner, a Plasmodium-infected mouse strain that does not develop cerebral malaria naturally (is resistant) has lower levels of plasma free heme, as compared to a mouse strain that develops cerebral malaria naturally (is susceptible; Fig. 4c). Again, heme administration is sufficient per se to precipitate the onset of cerebral malaria in the naturally resistant mouse strain (Fig. 4d) [8]. Taken together, these observations suggest that accumulation of free heme in plasma can explain at least to some extent why some Plasmodium strains trigger cerebral malaria while others do not, as well as why some mouse strains are resistant to cerebral malaria while others are susceptible.

While free heme seems to be critically involved in the pathogenesis of cerebral malaria in mice, there is yet no direct evidence linking functionally free heme to the pathogenesis of severe and/or cerebral malaria in humans. There is indirect evidence, however, to suggest that this might be the case. Individuals developing severe malaria have high levels of plasma metHb [41, 42]. As metHb releases free heme, the prediction is that free heme should accumulate in the plasma of these individuals, a hypothesis we are currently testing. Moreover, individuals that suc-cumb to cerebral malaria express high levels of HO-1 in the brain [52, 53] and present high levels of plasma COHb, a "biological fingerprint" of CO production by the HO system [84]. Taken together, these observations suggest that some of the key components involved in the pathogenesis of severe malaria in mice, i.e., metHb, HO-1 and CO are also present during the pathogenesis of severe malaria in humans. Whether the heme/HO-1 system should also play a pivotal role in determining the onset of severe malaria in humans remains to be established.

Fig. 4 Free heme dictates the onset of cerebral malaria in mice. C57BL/6 or BALB/c mice were inoculated (i.p.) with *P. berghei* ANKA or *P. berghei* NK65-infected RBC (10⁵), i.e., infection [8]. **a** Survival and heme concentration in protein-free plasma of C57BL/6 mice infected with either *P. berghei* ANKA (*black circles*) or NK65 (*white circles*) at days 0 (*n*=7), 4 (*n*=4 for *P. berghei ANKA*), 6 (*n*=30 and *n*=16 for *P. berghei ANKA* and NK65, respectively), 10 (*n*=10), and 15 (*n*=10) as it relates to day of infection, i.e., day 0. Results are shown as mean±standard deviation. (*unpaired *t* test between *P. berghei ANKA* and *P. berghei* NK65-infected mice at day 6 post-infection, *p*<0.01). **b** Survival of C57BL/6 mice infected with either *P. berghei* ANKA or *P. berghei* NK65 receiving vehicle (*white circles*; *n*=10), 60 μmol/kg (*n*=5; *gray circles*) or 90 μmol/kg (*n*=12; *black circle*) of free heme (i.p.; starting at day 6 post-infection and every 12 h thereafter). **c** Survival and heme concentration in protein-free plasma of C57BL/6 mice (*black circle*) or BALB/c mice (*white circle*) infected with *P. berghei* ANKA at days 0 (*n*=7 and *n*=6 for *P. berghei* ANKA-infected C57BL/6 mice and BALB/c mice, respectively), 4 (*n*=4 for *P. berghei ANKA*-infected C57BL/6 mice), 6 (*n*=30 and *n*=15, for *P. berghei* ANKA-infected C57BL/6 mice and BALB/c mice, respectively), 10 (*n*=10) and 15 (*n*=12) as it relates to day of infection, i.e., day 0. Results are shown as mean±standard deviation (*unpaired *t* test between *P. berghei ANKA*-infected C57BL/6 and BALB/C mice at day 6 post-infection, *p*<0.01). **d** Survival of *P. berghei* ANKA-infected BALB/c mice receiving vehicle (*white circle*; *n*=10), 60 μmol/kg (*n*=9; *grey circles*), or 90 μmol/kg (*n*=6; *black circle*) free heme (i.p; starting at day 6 post-infection and every 12 h thereafter)
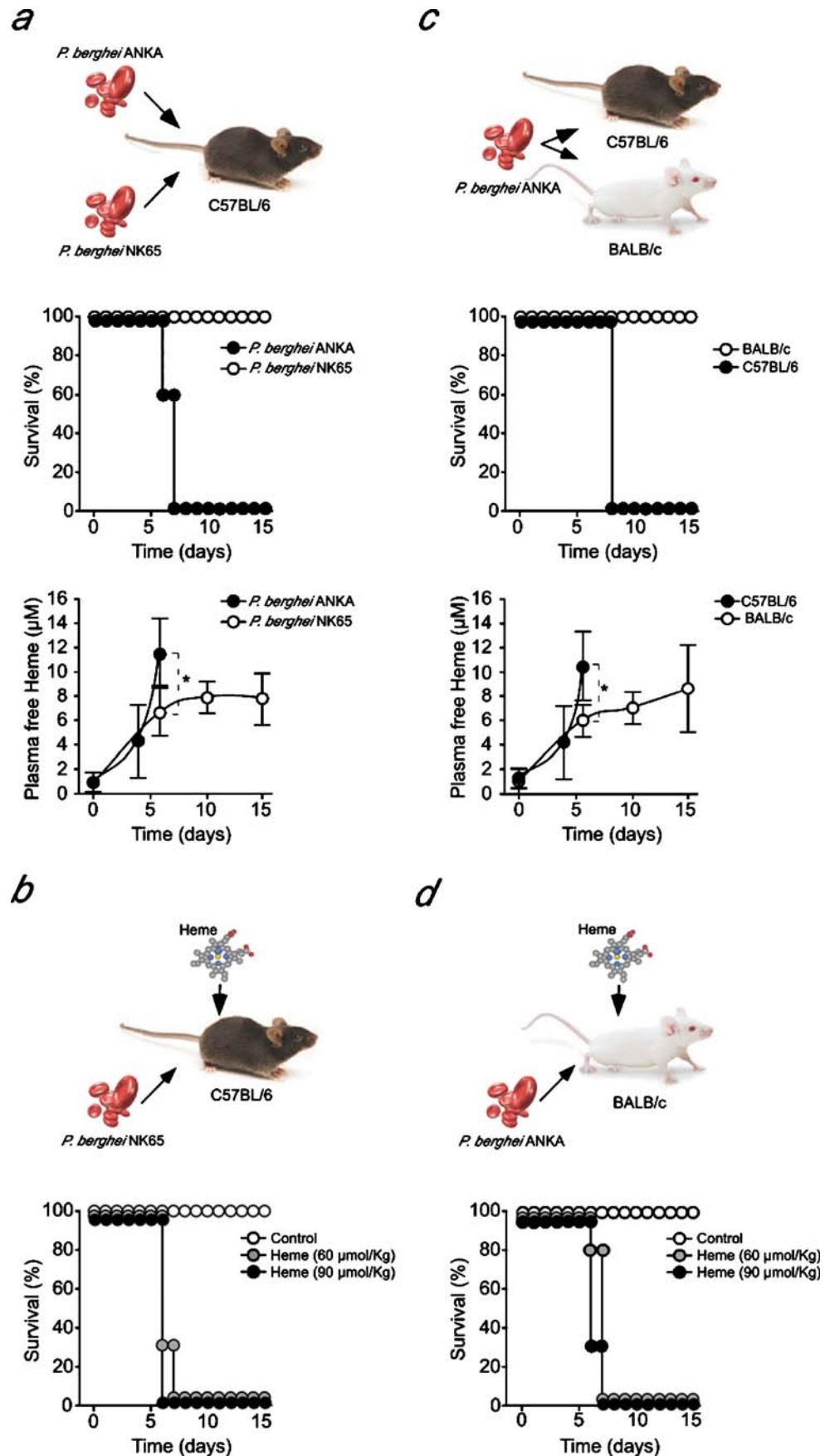
Effector mechanism(s) via which free heme triggers the onset of cerebral malaria

The mechanism(s) via which free heme precipitates the pathogenesis of severe malaria, and in particular that of cerebral malaria, are not fully elucidated. Cerebral malaria is associated with the development of BBB disruption and brain edema in both mice [8] and humans [85] (reviewed in [5]). However, the molecular mechanism(s) leading to BBB disruption are not fully understood. Postmortem studies in humans diagnosed with cerebral malaria suggest that BBB leakage is associated with adhesion of infected RBCs to brain microvascular endothelial cells, a phenomenon referred to as RBC sequestration (reviewed in [3, 4]). A functional link between these two events, i.e., BBB leakage and RBC sequestration, is supported by a series of observations suggesting that adhesion of infected RBCs to endothelial cells in vitro can induce the disruption of tight junctions, a multiprotein structure that maintains BBB integrity (reviewed in [3, 4]). However, there are probably additional mechanisms that can operate, independently of RBC sequestration, to trigger BBB disruption.

Using an in vitro assay in which brain endothelial cells form tight junctions, free heme was found to promote the disruption tight junction function [8]. This effect requires the presence of ROS, which would explain why free heme triggers BBB disruption only in *Plasmodium*-infected but not in naïve mice [8]. This observation also suggests that CO suppresses BBB disruption by preventing the accumulation of free heme in the brain microvasculature [8]. Given the above, we propose that CO might be used to suppress the onset of cerebral malaria for as long as plasma free heme concentration is kept below a certain threshold level at which BBB disruption occurs and CO would lose its protective effect [8]. Whether a similar therapeutic effect would be attained in children diagnosed as having or at risk of developing cerebral malaria remains to be established. Finally, and assuming that free heme is involved in the pathogenesis of severe and/or cerebral malaria in humans, one could consider measuring plasma free heme concentration to diagnose this pathologic condition.

There are several unsolved issues raised by these findings including the identification of the sources of ROS that oxidize cell-free Hb, leading to the generation of free heme and to the onset of cerebral malaria. Based on the observation that NAD(P)H gp91[phox]-deficient mice can develop cerebral malaria, it has been suggested that ROS are probably not involved in its pathogenesis [86]. Our interpretation is somehow different. We propose that while ROS are most probably involved in the pathogenesis of cerebral malaria, gp91[phox] is probably not the main source of ROS leading to the onset of this disease. By extending this line of thought, one could consider many other putative

sources of superoxide generating systems. These include the gp91[phox] homologue Nox genes, e.g., Nox4 and Nox1, both of which are major components of vascular NAD(P)H oxidase-like complexes operating in the brain microvasculature. In addition, the brain vasculature also expresses relatively high levels of xanthine oxidase and cyclooxygenase-1, both of which are potent superoxide anion producers. Another possibility is that the reducing potential of the brain microvasculature might be altered during *Plasmodium* infection in a way that would promote the accumulation of ROS. This could be a direct consequence of decreased expression of antioxidant genes, e.g., FtH, superoxide dismutase, catalase, or glutathione peroxidase. While the participation of these host genes in the pathogenesis of severe malaria and/or cerebral malaria remains to be tested, it becomes apparent that many possible sources of ROS or their regulators may be involved in its pathogenesis (Fig. 5).
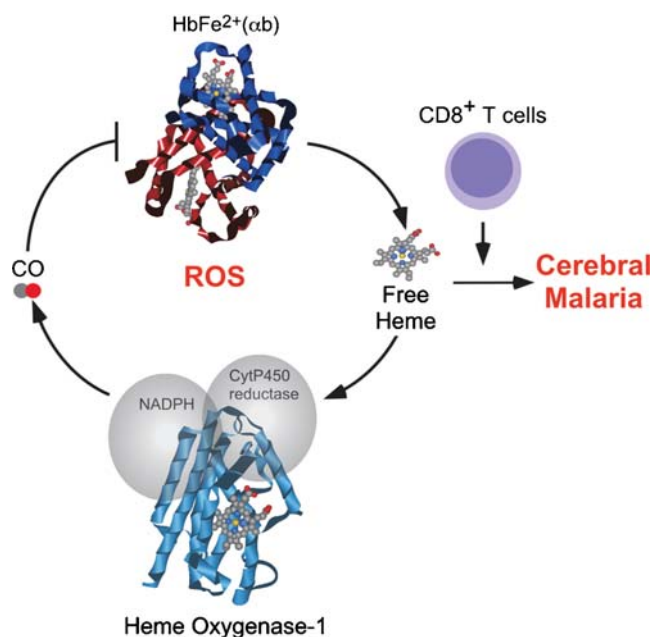

Fig. 5 CO prevents the onset of cerebral malaria via inhibition of heme release from cell-free Hb. When exposed to ROS generated in the brain vasculature by enzymes such as NAD(P)H oxidase, xanthine oxidase, cyclooxygenase, lipoxygenases, and/or riboflavin, dimeric cell-free ferrous HbFe^{2+} ($\alpha\beta$) is oxidized into ferric HbFe^{3+} ($\alpha\beta$), i.e., metHb (not shown), which releases heme promptly. Accumulation of free heme in plasma of *Plasmodium*-infected mice allows the Fe contained within the protoporhyrin IX ring of heme to participate in further generation of ROS via the Fenton chemistry. ROS synergize with activated CD8$^+$ T cells to trigger the development of cerebral malaria. When the expression of HO-1 is induced, free heme availability is reduced via two pathways: (1) heme degradation and (2) generation of CO, which binds dimeric cell-free ferrous HbFe^{2+} ($\alpha\beta$) and suppresses further heme release from Hb. The combined effect of HO-1 and its end product CO suppresses the onset of cerebral malaria in mice [8]

Integrating the heme/HO-1/CO system with other mechanisms contributing to the pathogenesis of cerebral malaria

Unfettered expression of pro-inflammatory genes is most probably a central component in the pathogenesis of severe malaria and/or cerebral malaria [87]. Given the above, the anti-inflammatory effects of CO should contribute to prevent the onset of these pathologic conditions [8, 61] (reviewed in [66]). In support of this notion, CO reduces the expression of key pro-inflammatory genes involved in the pathogenesis of cerebral malaria in mice, e.g., ICAM-1/CD54, vascular cell adhesion molecule-1 (VCAM-1/CD106), TNF, LT, and interferon-gamma (IFN-γ) [8].

As discussed under "Deleterious effects of free heme", recognition of *Plasmodium*-derived PAMP by host PRR might be involved in the pathogenesis of severe malaria in humans [10]. When bound to the heme prosthetic group of the gp91^{phox} subunit of the superoxide anion-generating enzyme NAD(P)H oxidase, CO inhibits ROS production in Mø [88], thus effect inhibits TLR2, 4, 5, and 9 signaling [88]. It is possible therefore that inhibition of NAD(P)H oxidase by CO or other gp91^{phox}-like superoxide-generating enzymes should contribute to prevent the involvement of these PRR in the onset of severe malaria.

While immunocompetent mice require the expression of HO-1 to prevent the onset of cerebral malaria, this is no longer the case when mice lack T and B cells, i.e., severe combined immunodeficiency or when CD8$^+$ T cells are depleted from immunocompetent mice [8]. This would suggest that HO-1 down-modulates the activation or effector function of CD8$^+$ T cells and/or that it acts in the brain microvasculature to prevent the cytotoxic effects of CD8$^+$ T cells. The observation that pharmacologic induction of HO-1 or exposure to CO results in a profound inhibition of CD8$^+$ T cell sequestration in the brain of *Plasmodium*-infected mice [8] suggests that CO suppresses CD8$^+$ T cells activation and/or effector function. As free heme can act as a potent T cell mitogen [89, 90], it is possible that inhibition of heme release from cell-free Hb would also down modulate the activation and/or effector function of CD8$^+$ T cells.

There is yet another mechanism via which CO might exert protective effects that could prevent the pathogenesis of cerebral malaria. *Plasmodium* infection is associated with a profound reduction of NO bioavailability both in mice [9] and in humans [91], an effect that precipitates the onset of cerebral malaria in mice [9]. Reduction of NO bioavailability occurs via several pathways, including the release of arginase by infected RBCs, an enzyme that degrades the rate-limiting substrate for NO synthesis, i.e., arginine (reviewed in [23]). In addition, cell-free Hb also acts as a potent NO scavenger, an effect that reduces NO

bioavailability [9, 92]. It has been recently shown that in a similar way to CO, NO inhalation or administration of pharmacologic compounds that release NO suppresses the onset of severe malaria in mice [9]. As NO and CO target the same heme prosthetic groups in cell-free Hb, it is possible that these two gases may interact functionally. For example, it is conceivable that once bound to cell-free Hb, CO could reduce its NO scavenging capacity, thus increasing NO bioavailability and suppressing in this manner the onset of cerebral malaria [9]. This might occur even if CO binds to the heme groups of cell-free Hb with an affinity that is ~1,500 lower to that of NO, as contrary to CO, NO is very labile, reacting avidly with ROS to generate peroxynitrite. This hypothesis is currently being tested in our laboratory.

Concluding remarks

The data we have obtained in rodent models of malaria suggest that survival of a *Plasmodium*-infected host is strictly dependent on its ability to induce the expression of the heme-catalyzing enzyme HO-1. CO, an end product of heme catabolism by HO-1, supresses the development of severe malaria, i.e., cerebral malaria. The protective effect of CO is mediated not only via its cytoprotective and anti-inflammatory effects but also via inhibition of heme release from cell-free Hb generated during the blood stage of *Plasmodium* infection. One of the key notions that arise from these findings is that at least in mice, free heme seems to play a critical role in the pathogenesis of cerebral malaria. If this also proves to be the case in humans, then one might be able to use plasma free heme concentration to diagnose the onset of severe and/or cerebral malaria and eventually use therapeutic approaches that target free heme, including CO administration, to overcome the onset of these pathologic conditions.

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