

Heme oxygenase-1 affords protection against noncerebral forms of severe malaria

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Infection by *Plasmodium*, the causative agent of malaria, is associated with hemolysis and therefore with release of hemoglobin from RBC. Under inflammatory conditions, cell-free hemoglobin can be oxidized, releasing its heme prosthetic groups and producing deleterious free heme. Here we demonstrate that survival of a *Plasmodium*-infected host relies strictly on its ability to prevent the cytotoxic effects of free heme via the expression of the heme-catabolizing enzyme heme oxygenase-1 (HO-1; encoded by the *Hmox1* gene). When infected with *Plasmodium chabaudi chabaudi* (*Pcc*), wild-type (*Hmox1*^{+/+}) BALB/c mice resolved infection and restored homeostasis thereafter (0% lethality). In contrast, HO-1 deficient (*Hmox1*^{-/-}) BALB/c mice developed a lethal form of hepatic failure (100% lethality), similar to the one occurring in *Pcc*-infected DBA/2 mice (75% lethality). Expression of HO-1 suppresses the pro-oxidant effects of free heme, preventing it from sensitizing hepatocytes to undergo TNF-mediated programmed cell death by apoptosis. This cytoprotective effect, which inhibits the development of hepatic failure in *Pcc*-infected mice without interfering with pathogen burden, is mimicked by pharmacological antioxidants such as N-acetylcysteine (NAC). When administered therapeutically, i.e., after *Pcc* infection, NAC suppressed the development of hepatic failure in *Pcc*-infected DBA/2 mice (0% lethality), without interfering with pathogen burden. In conclusion, we describe a mechanism of host defense against *Plasmodium* infection, based on tissue cytoprotection against free heme and limiting disease severity irrespectively of parasite burden.

heme | heme-oxygenase-1 | cell death | infection

Malaria, the disease caused by *Plasmodium* infection, remains one of the main causes of morbidity/mortality worldwide (1). Epidemiologically however, less than 1–2% of infected individuals succumb to severe forms of malaria (1). This suggests that *Plasmodium* has co-evolved with its human host to reach an evolutionary “tradeoff” in which infection “rarely” compromises host viability. This tradeoff is thought to rely almost exclusively on the ability of the host’s immune system to control parasite burden (2), a defense strategy referred to as resistance to infection (3, 4). However, there is an additional host defense strategy that operates during *Plasmodium* infection and that limits disease severity irrespectively of parasite burden, i.e., tolerance to infection (3, 4). The mechanisms underlying host tolerance to *Plasmodium* infection remain poorly understood.

Plasmodium replication inside RBC leads to hemolysis and therefore to the production of cell-free hemoglobin (5, 6). When exposed to reactive oxygen species (ROS), cell-free hemoglobin is oxidized and releases its heme prosthetic groups (6). Circulating free heme is deleterious (6, 7), an effect countered by several host defense strategies (5), including the up-regulation of the heme catabolizing enzyme heme oxygenase-1 (HO-1, encoded by the *Hmox1* gene) (6).

HO-1 is a stress-responsive enzyme that converts the protoporphyrin IX ring of heme into biliverdin, releasing iron (Fe) and producing carbon monoxide (CO) (8). The heme/HO-1 system controls the pathogenesis of experimental cerebral malaria in mice (5, 6), a neuroinflammatory syndrome that resembles cerebral

malaria in children infected by *Plasmodium falciparum* (9). We provide hereby a mechanistic basis for a broader involvement of the heme/HO-1 system in the pathogenesis of cerebral as well as noncerebral forms of severe malaria, such as those associated with hemolytic anemia and hepatic damage leading eventually to multi-organ failure. Moreover we demonstrate that the cytoprotective effect of HO-1 against free heme affords host tolerance against *Plasmodium* infection.

Results

Expression of HO-1 Sustains the Survival of *Plasmodium chabaudi chabaudi* (*Pcc*)-Infected Mice. Contrary to wild-type (*Hmox1*^{+/+}) BALB/c mice that survived *Pcc* infection (0% mortality) (Fig. 1A), *Pcc* infection was lethal in HO-1 deficient (*Hmox1*^{-/-}) BALB/c mice (100% mortality) (Fig. 1A). Higher virulence of *Pcc* infection in *Hmox1*^{-/-} mice was not attributable to higher parasitemia (Fig. 1A), suggesting that the protective effect of HO-1 acts irrespectively of pathogen burden, a host defense strategy referred to as tolerance to infection (3, 4).

We asked whether the protective effect of HO-1 could be dissociated from the host immune response against *Pcc* infection. We used syngenic bone marrow transplants from *Hmox1*^{+/+} or *Hmox1*^{-/-} donors into lethally irradiated *Hmox1*^{+/+} or *Hmox1*^{-/-} recipients to generate chimeric mice in which *Hmox1* was deleted specifically in nonhematopoietic (*Hmox1*^{+/+} → *Hmox1*^{-/-}) vs. hematopoietic (*Hmox1*^{-/-} → *Hmox1*^{+/+}) tissues. The assumption being that host immunity against *Pcc* infection is confined to the hematopoietic compartment. Mortality associated with *Pcc* infection increased from 0% in control chimeric mice (*Hmox1*^{+/+} → *Hmox1*^{+/+}) to 100% in chimeric mice in which the *Hmox1* locus was deleted in nonhematopoietic tissues (*Hmox1*^{+/+} → *Hmox1*^{-/-}) (Fig. 1B). This effect was associated with a slight, but significant increase in parasitemia (Fig. 1B). Chimeric mice in which the *Hmox1* locus was deleted in hematopoietic tissues (*Hmox1*^{-/-} → *Hmox1*^{+/+}) were resistant to *Pcc* infection, i.e., 20% lethality (Fig. 1B). There was no modulation of parasitemia in these chimeric mice (Fig. 1B), suggesting that expression of HO-1 outside the host’s adaptive immune system affords tolerance to *Pcc* infection.

We tested whether HO-1 would afford protection to *Pcc* infection in the absence of host adaptive immunity. SCID BALB/c mice, lacking T and B cells, did not clear *Pcc*, succumbing 11–15 days postinfection (100% mortality), from hyperparasitemia, i.e., >60%

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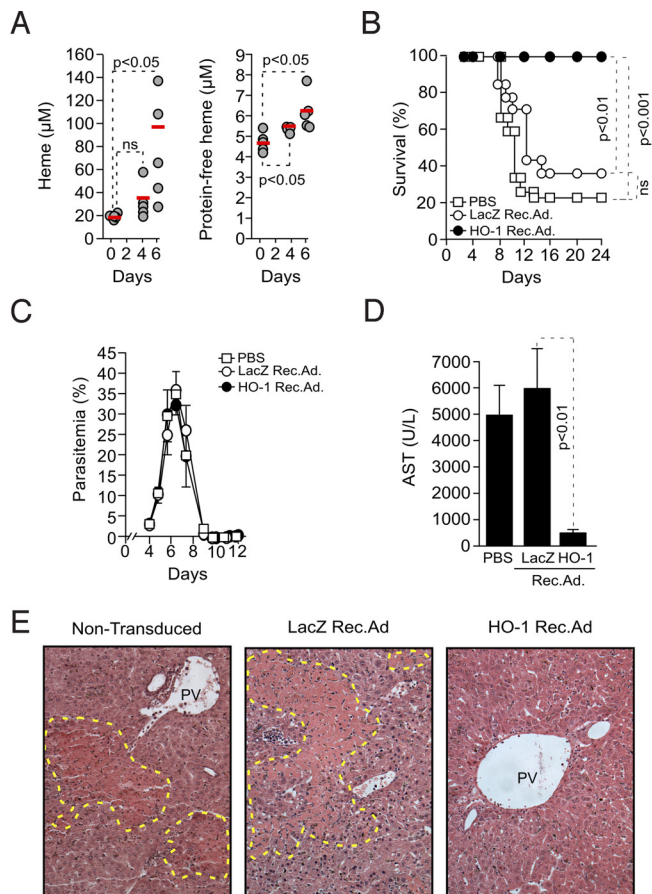


Fig. 3. Expression of HO-1 in the liver suppresses liver failure in *Pcc*-infected mice. (A) Total and protein-free heme in plasma of *Pcc*-infected DBA/2 mice. Each circle represents an individual mouse. (B) Survival and (C) mean parasitemia \pm SD in *Pcc*-infected DBA/2 mice receiving vehicle (PBS) ($n = 12$), transduced with HO-1 ($n = 12$) or LacZ ($n = 8$) Rec.Ad. (D) Mean plasma AST concentration \pm SD, 8 days post-*Pcc* infection ($n = 4$ mice/group). (E) Hematoxylin & eosin-stained livers from DBA/2 mice, 7 days post-*Pcc* infection (200 \times ; representative of eight mice). PV, portal vein. Yellow dotted line surrounds necrosis/apoptosis area.

effect of HO-1 was not due to modulation of parasitemia (Fig. 3C), suggesting that expression of HO-1 in the liver affords tolerance to *Plasmodium* infection.

Survival of *Pcc*-infected DBA/2 mice transduced with the HO-1 Rec.Ad. was due to suppression of hepatic failure as revealed by an average 10-fold reduction of aspartate aminotransferase (AST) plasma concentration (Fig. 3D) and liver portal and centrilobular vein necrosis (Fig. 3E), as compared to nontransduced or LacZ Rec.Ad.-transduced controls. This suggests that expression of HO-1 in the liver prevents the onset of severe malaria in *Pcc*-infected DBA/2 mice via a mechanism associated with preservation of liver function.

TNF Triggers Hepatic Failure in *Pcc*-Infected DBA/2 Mice. DBA/2 mice produced high levels of TNF in response to *Pcc* infection, reaching peak concentrations in plasma 8 days postinfection (3–4 ng/mL) and decreasing thereafter to basal levels in surviving mice (i.e., 25% of *Pcc*-infected mice) (Fig. 4A) (12). Administration of an anti-TNF-neutralizing Ab from day 5 to 9 postinfection reduced lethality to 25% vs. 75% and 66.7% in vehicle (PBS) or isotype-matched Ab-treated controls, respectively (Fig. 4B). There was no change in parasitemia in mice receiving the anti-TNF-neutralizing Ab, vs. control Ab (Fig. 4C). The protective effect of the anti-TNF-

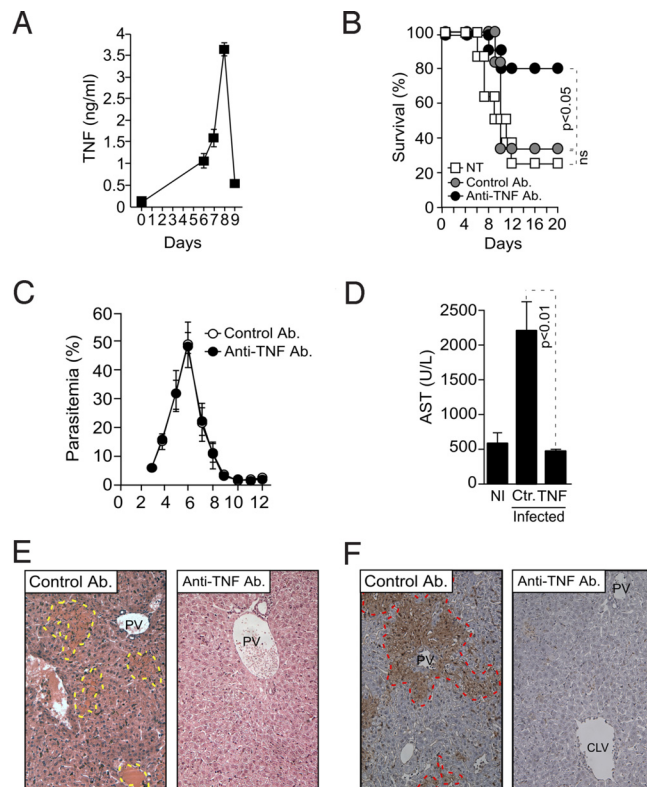


Fig. 4. *Pcc*-infected DBA/2 mice succumb to TNF-mediated hepatic failure. (A) Mean plasma TNF concentration \pm SD in *Pcc*-infected DBA/2 mice ($n = 5$). (B) Percent survival of *Pcc*-infected DBA/2 mice treated with anti-TNF, isotype-matched control antibody, or not treated (NT) ($n = 12$ mice per group). (C) Mean parasitemia \pm SD in *Pcc*-infected DBA/2 mice treated with an anti-TNF neutralizing antibody or an isotype-matched control antibody ($n = 12$ per group). (D) Mean plasma AST concentration \pm SD in noninfected (NI; $n = 4$) and *Pcc*-infected DBA/2 mice treated with anti-TNF ($n = 4$) or isotype-matched control antibody (Ab; Ctr; $n = 4$). (E) Hematoxylin & eosin-stained liver sections, 8 days post-*Pcc* infection, in DBA/2 mice treated with neutralizing anti-TNF or isotype-matched control antibody (Ab). Images (200 \times) are representative of eight mice. Dotted yellow line surrounds areas of necrosis/apoptosis. (F) Liver section from DBA/2 mice treated as in panel E stained by TUNEL. Dotted red line surrounds areas of necrosis/apoptosis. PV, portal vein; CLV, centrilobular vein. Images (200 \times) are representative of eight mice.

neutralizing Ab was associated with (i) decreased AST plasma concentration (a marker of hepatic failure) (Fig. 4D); (ii) decreased liver portal and central lobular vein necrosis (Fig. 4E); and (iii) inhibition of hepatocyte programmed cell death (Fig. 4F). These observations suggest TNF is critically involved in the pathogenesis of hepatic failure associated to *Pcc*-infection in DBA/2 mice.

Free Heme Sensitizes Hepatocytes to Undergo TNF-Mediated Apoptosis. We asked whether free heme interacts functionally with TNF to trigger hepatic failure following *Pcc* infection. At concentrations in the range of those detected in vivo, neither free heme (5 μ M) nor TNF (5 ng/mL) were cytotoxic per se to primary mouse hepatocytes in vitro (Fig. 5A). However, combination of free heme with TNF was cytotoxic, i.e., >25% cell death (Fig. 5A), suggesting that free heme interacts functionally with TNF to trigger hepatocytes to undergo programmed cell death, presumably leading to hepatic failure in *Pcc*-infected DBA/2 mice.

When exposed in vitro to free heme plus TNF, hepatocytes undergo of programmed cell death by apoptosis, as revealed by chromatin condensation, nucleus shrinking (Fig. 5B) and pro-caspase-3 processing (Fig. 5C), all of which are hallmarks of apoptosis. However, we cannot exclude that heme might also elicit

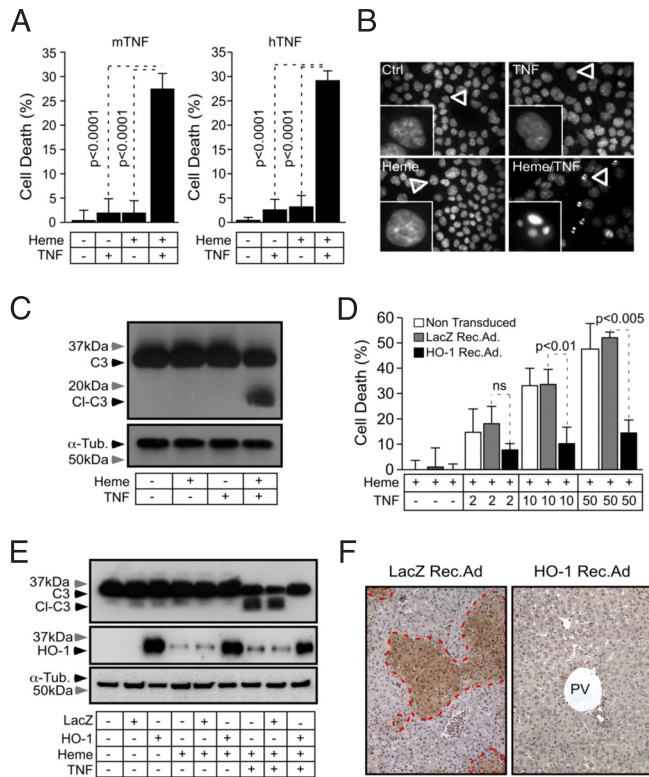


Fig. 5. Free heme sensitizes hepatocytes to TNF-mediated apoptosis. (A) Mean cytotoxicity of primary mouse hepatocytes exposed to heme (5 μ M for 1 h) plus mouse (m) or human (h) recombinant TNF (5 ng/mL for 16 h) \pm SD ($n = 6$) in one out of three independent experiments. Cytotoxicity was assessed by crystal violet staining. (B) DNA staining (Hoechst 33342) of untreated (Ctrl), heme (40 μ M for 1 h), and/or TNF (10 ng/mL for 6 h) treated Hepa1-6 cells (400 \times). Results are representative of three independent experiments. (C) Caspase-3 (C3), cleaved caspase-3 (Cl-C3), and α -tubulin (α -Tub) were detected by western blot in primary hepatocytes treated as in panel B. Results are representative of two independent experiments. (D) Hepa1-6 cells were transduced or not with HO-1 or LacZ Rec.Ad. and exposed to heme (40 μ M for 1 h) alone or heme (40 μ M for 1 h) plus TNF (ng/mL for 6 h). Results are expressed as mean \pm SD from triplicates in one out of two independent experiments. TNF is expressed in ng/mL. (E) Hepa1-6 cells were treated as in panel D. Caspase-3 (C3), cleaved caspase-3 (Cl-C3), and α -tubulin (α -Tub) were detected by western blot. Results shown are representative of two independent experiments. (F) Liver sections were stained by TUNEL, 8 days post-*Pcc* infection in DBA/2 mice transduced with HO-1 or control LacZ Rec.Ad. Dotted red lines surround areas of necrosis/apoptosis. PV, portal vein. Images (200 \times) are representative of eight mice.

other forms of programmed cell death such as necroptosis, as observed in other cell types (13). Transduction of hepatocytes in vitro with a HO-1 Rec.Ad. afforded cytoprotection against heme plus TNF-mediated apoptosis, i.e., average 73% reduction in cytotoxicity at maximal heme and TNF concentrations vs. nontransduced or LacZ Rec.Ad.-transduced controls (Fig. 5D). The cytoprotective effect of HO-1 was associated with inhibition of caspase-3 cleavage/activation (Fig. 5E). When transduced in vivo HO-1 suppressed hepatocytes apoptosis in *Pcc*-infected DBA/2 mice, as compared to nontransduced or LacZ Rec.Ad.-transduced control mice (Fig. 5F). Similar results were obtained using pO₂ concentrations of 2%, which are closer to the physiologic steady state conditions in the mouse liver. Overall, these observations suggest that HO-1 prevents the free heme produced during *Plasmodium* infection from sensitizing host hepatocytes to undergo TNF-mediated apoptosis. This cytoprotective effect prevents the development of a lethal form of hepatic failure in *Pcc*-infected DBA/2 mice.

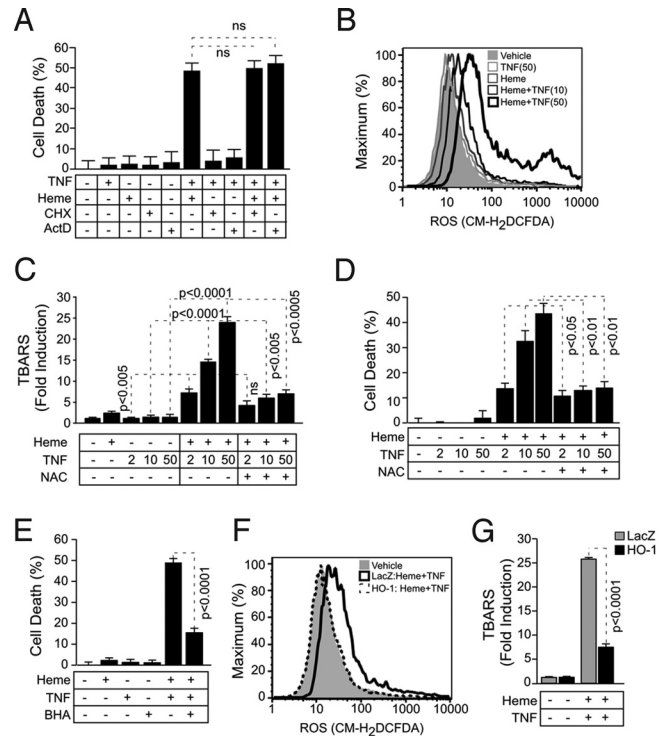


Fig. 6. The antioxidant effect of HO-1 suppresses the cytotoxic effect of free heme plus TNF in hepatocytes. (A) Hepa1-6 cells were exposed (+) to heme (40 μ M for 1 h) and/or (+) TNF (50 ng/mL for 6 h). When indicated, transcription was inhibited by ActD (5 μ g/mL) and translation by CHX (5 μ g/mL). Cytotoxicity was assessed by crystal violet staining. Results are expressed as mean \pm SD from one out of two independent experiments, each in triplicate. Notice that ActD or CHX did not interfere with the pro-apoptotic effect of heme plus TNF. (B) Free radicals were measured by flow cytometry in Hepa1-6 cells exposed to vehicle, TNF (for 6 h), heme (40 μ M for 1 h), or heme (40 μ M for 1 h) plus TNF (for 6 h). (C) TBARS in Hepa1-6 cells treated as in panel A are expressed as mean \pm SEM obtained from the mean values of three independent experiments, each in duplicate. NAC (10 mM for 4 h before heme and continuously thereafter). (D) Cytotoxicity in Hepa1-6 cells treated with heme plus TNF with or without NAC as in panel C. Results are expressed as mean \pm SD from triplicates in one representative experiment out of two independent experiments, each in sextuplicate. (E) Cytotoxicity in Hepa1-6 cells treated with heme (40 μ M for 1 h) plus TNF (50 ng/mL for 6 h) with or without BHA (100 μ M for 24 h before heme and continuously thereafter). (F) Free radicals were measured by flow cytometry in Hepa1-6 cells nontransduced (vehicle) or transduced with LacZ or HO-1 Rec.Ad. exposed to heme (40 μ M for 1 h) plus TNF (50 ng/mL for 6 h). (G) TBARS in Hepa1-6 cells transduced with LacZ or HO-1 Rec.Ad., treated with heme plus TNF as in panel A, are expressed as mean \pm SEM obtained from the mean values of three independent experiments, each in duplicate. TNF is expressed in ng/mL (panels C and D).

Free Heme and TNF Synergize to Trigger Oxidative Stress in Hepatocytes. We tested whether the cytotoxic effect of free heme requires newly gene expression. Inhibition of mRNA synthesis by actinomycin D (ActD) or inhibition of protein synthesis by cycloheximide (CHX), did not interfere with the pro-apoptotic effect of heme plus TNF in hepatocytes, i.e., >45% cell death (Fig. 6A). Timing of TNF exposure in these assays was limited to 6 h, to avoid ActD or CHX from sensitizing hepatocytes to undergo TNF-mediated apoptosis (Fig. 6A). However, when exposure to TNF was prolonged to 12–24 h, both ActD and CHX sensitized hepatocytes to undergo TNF-mediated apoptosis (Fig. S2), confirming that these reagents blocked RNA and protein synthesis, respectively.

Accumulation of intracellular free radicals was negligible in untreated hepatocytes as well as in hepatocytes exposed in vitro to heme or TNF alone, as assessed by flow cytometry, using a broad range cell-permeable free radical probe (CM-H₂DCFDA) (Fig. 6B). However, when exposed to heme and TNF, hepatocytes

produced high levels of intracellular free radicals, a dose-response effect that increased with higher concentrations of TNF (Fig. 6B).

Neither TNF nor heme per se triggered the production of thiobarbituric acid reactive substances (TBARS), a lipid peroxidation derivative, in hepatocytes (Fig. 6C). However, exposure to heme plus TNF triggered an average 20-fold increase in TBARS, as compared to untreated control hepatocytes (Fig. 6C). This effect was dose-dependent in that higher TNF concentrations increased TBARS (Fig. 6C). That accumulation of TBARS is due to free radical production was confirmed by the observation that N-acetylcysteine (NAC), a glutathione precursor that acts as a broad antioxidant, reduced by an average 2.5- to 3.5-fold the levels of TBARS in hepatocytes exposed in vitro to heme plus TNF, as compared to control hepatocytes not treated with NAC (Fig. 6C). Similar results were obtained using pO₂ concentrations of 2%, which as argued above are closer to the physiologic steady state conditions in the mouse liver. This data reveals that free heme acts as a pro-oxidant catalyst in hepatocytes, promoting oxidative stress, i.e., lipid peroxidation, in response to TNF.

HO-1 Negates the Pro-Oxidant Effect of Free Heme and Affords Protection Against Apoptosis. When exposed in vitro to the antioxidant NAC, hepatocytes became resistant to heme plus TNF-mediated apoptosis, i.e., 4-fold decrease in cytotoxicity, as compared to control hepatocytes (Fig. 6D). A similar effect was observed using the lipid phase antioxidant butylated hydroxyanisole (BHA) (Fig. 6E), suggesting that lipid peroxidation is functionally involved in the pro-apoptotic effect of heme plus TNF. Transduction of hepatocytes in vitro with a HO-1 Rec.Ad. suppressed the accumulation of both intracellular free radicals (Fig. 6F) and TBARS (Fig. 6G) in response to heme plus TNF, as compared to control hepatocytes transduced with LacZ Rec.Ad. This suggests that HO-1 acts as an antioxidant to prevent hepatocyte apoptosis. Similar results were obtained using pO₂ concentrations of 2%.

HO-1 Prevents the Onset of Severe Malaria Through Its Antioxidant Activity. *Pcc*-infected *Hmxo1*^{-/-} BALB/c mice that developed hepatic failure (Figs. 1 and 2) accumulated higher levels of TBARS in the liver, as compared to *Pcc*-infected *Hmxo1*^{+/+} BALB/c mice that did not develop hepatic failure (Fig. 7A). When treated with the antioxidant NAC, starting 4 days after *Pcc* infection, DBA/2 were protected against hepatic failure, i.e., 0% vs. 80% mortality in NAC vs. vehicle-treated *Pcc*-infected mice, respectively (Fig. 7B). The protective effect of NAC was associated with a 7-fold decrease in AST plasma concentration (Fig. 7C), demonstrating that this antioxidant prevents the development of hepatic failure in *Pcc*-infected DBA/2 mice. The protective effect of NAC was not due to modulation of parasitemia (Fig. 7D), suggesting that NAC affords tolerance to *Pcc* infection.

Discussion

Survival of a *Plasmodium*-infected host is thought to rely almost exclusively on its ability to generate an effector immune response capable of limiting pathogen burden, i.e., a host defense strategy referred to as resistance to infection (3, 4). However, this immune response can contribute to the development of lethal forms of severe malaria (2). This suggests that resistance to *Plasmodium* infection can compromise the host's viability, a phenomenon observed in other types of infection, including polymicrobial infections leading to severe sepsis (14). Therefore, viability of a *Plasmodium*-infected host must involve additional defense strategies that do not aim at limiting pathogen burden, but instead aim at limiting the deleterious effects associated with the immune response targeting *Plasmodium*, i.e., a host defense strategy referred to as tolerance to infection (3, 4). The molecular mechanism(s) underlying tolerance to *Plasmodium* infection remain to be identified. As demonstrated hereby, expression of HO-1, outside the

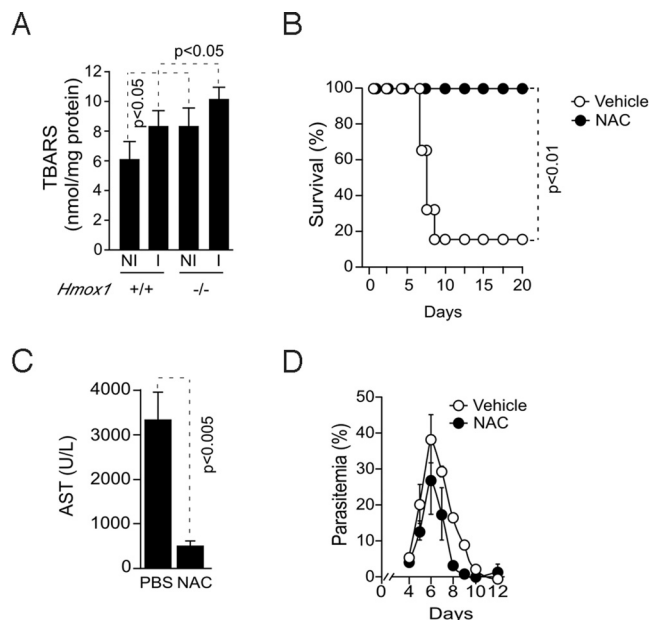


Fig. 7. The antioxidant effect of HO-1 suppresses the onset of severe malaria. (A) TBARS in the liver of noninfected (NI) or *Pcc*-infected (I) wild-type (*Hmxo1*^{+/+}) or HO-1 deficient (*Hmxo1*^{-/-}) BALB/c mice, 5 days post-*Pcc* infection. Results are expressed as mean \pm SD ($n = 3$ mice/group). (B) Survival of *Pcc*-infected DBA/2 mice receiving NAC (150 mg/kg) or vehicle (PBS) as control ($n = 5$ –6 mice/group). (C) Mean plasma AST concentration \pm SD in *Pcc*-infected DBA/2 mice receiving NAC ($n = 4$) or vehicle (PBS) ($n = 4$). (D) Mean parasitemia \pm SD of *Pcc*-infected DBA/2 mice receiving NAC ($n = 5$) or vehicle (PBS) ($n = 5$).

hematopoietic (immune) compartment, plays a central role in determining host tolerance to *Plasmodium* infection in mice (Fig. 1).

Production of protein-free heme is a pathological event inherent to the blood stage of *Plasmodium* infection, as illustrated hereby for *Pcc* infection (Fig. 3A) and as previously shown for infection with other *Plasmodium* strains in mice (5, 6). Perhaps more important is the notion that free heme plays a central role in the pathogenesis of experimental cerebral malaria in mice (5, 6). Our present data suggests that free heme also plays an important role in the pathogenesis of noncerebral forms of severe malaria in mice. This pathological effect is due to a unique biologic property of free heme, namely its ability to sensitize nonhematopoietic cells, i.e., hepatocytes, to undergo TNF-mediated apoptosis (Fig. 5). As shown hereby this cytotoxic effect plays a critical role in the development of tissue/organ injury, i.e., hepatic failure (Fig. 2), ultimately leading to death of *Pcc*-infected mice (Fig. 1).

The cytotoxic effects of TNF are revealed only when the expression of immediate-early TNF-responsive cytoprotective genes is artificially repressed (15, 16). To the best of our knowledge, there are no molecules that have been shown to act under pathophysiological conditions to override this protective mechanism and promote TNF cytotoxicity. Therefore, the finding that free heme acts in such a manner (Fig. 5) is unique, in that it provides not only evidence for the existence of a functional interaction between free heme and TNF in the pathogenesis of severe malaria, but suggests that the same pathophysiological mechanism might apply to other inflammatory conditions.

The ability of free heme to sensitize hepatocytes to undergo TNF-mediated apoptosis is not suppressed when mRNA or protein synthesis are inhibited (Fig. 6A), suggesting that the cytotoxic effect of free heme acts independently of newly gene transcription and/or protein synthesis. Instead free heme acts as a potent pro-oxidant catalyst that fosters the production of free radicals in response to TNF, presumably via the participation of its Fe atom in the Fenton chemistry. In keeping with this notion,

hepatocytes accumulate high levels of intracellular free radicals in response to heme plus TNF (Fig. 6B), leading to an unfettered oxidative stress response, i.e., lipid peroxidation (Fig. 6C). Antioxidants such as NAC (Fig. 6D) or BHA (Fig. 6E) suppress the pro-apoptotic effect of free heme plus TNF.

The finding that free heme acts as a pro-oxidant catalyst (Fig. 6B) to promote TNF-mediated apoptosis in hepatocytes (Fig. 5), led to the hypothesis that pharmacologic antioxidants might be used therapeutically (i.e., after *Plasmodium* infection) to suppress the pathogenesis of severe malaria. When administered to DBA/2 mice 4 days after *Pcc* infection, the antioxidant NAC prevents the development of hepatic failure and insures host survival (Fig. 7B and C), a finding in keeping with previous reports suggesting that NAC might exert salutary effects in *P. falciparum*-infected individuals (17, 18).

In conclusion, we provide evidence for an unsuspected pathologic mechanism leading to the development of noncerebral forms of severe malaria. Namely, free heme produced during the blood stage of *Plasmodium* infection sensitizes nonhematopoietic cells, i.e., hepatocytes, to undergo TNF-mediated apoptosis. Presumably, this sequence of events is critically involved in the pathogenesis of noncerebral forms of severe malaria associated with the development of multi-organ injury (19), including hepatic failure (20, 21). The cytotoxic effect of free heme is suppressed when the heme-catabolizing enzyme HO-1 is expressed in hepatocytes, limiting the severity of hepatic disease irrespective of parasite burden and presumably therefore independently of the host immune response against *Plasmodium*. Identification of this host defense strategy against *Plasmodium* infection should help in the development of therapeutic approaches aimed at limiting the severity of malaria without the need to target *Plasmodium*.

Materials and Methods

Mouse Hepatocytes and Reagents. Hepa1–6 cells were cultured as described in the *SI Text*. Primary mouse hepatocytes were isolated as described (22).

Cytotoxicity Assay. Hepatocytes were seeded (96-well plates, 10×10^3 cells/well for Hepa1–6 cells, and 25×10^3 cells/well for primary hepatocytes; for 48 h), washed with PBS, and exposed for 1 h to heme in HBSS (Invitrogen). Control hepatocytes were washed with PBS and exposed to HBSS without heme. Hepatocytes were then washed in PBS and exposed to human (h) or mouse (m) recombinant TNF in DMEM, 10% FCS (Hepa1–6 cells), or 4% FCS (primary hepatocytes; Invitrogen). Viability was assessed by a crystal violet assay, as described (23). Immunofluorescence was performed as in *SI Text*. Flow cytometry was performed as described in *SI Text*.

Lipid Peroxidation. Cellular thiobarbituric acid reactive substances were measured according to the manufacturer instructions (OXI-TEC; Alexis).

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Mice and Parasites. BALB/c, BALB/c.SCID, DBA/2, and BALB/c.*Hmx1*^{-/-}.SCID mice were maintained under specific pathogen-free conditions, according to the Animal Care Committee of the “Instituto Gulbenkian de Ciência.” BALB/c *Hmx1*^{-/-} mice were generated originally by Shaw-Fang Yet (24). Mice (6–8 weeks) were genotyped as described (6) and infected i.p. with 10^6 *Pcc* (AS)-infected RBC. Course of infection was monitored daily as described (25).

Bone Marrow Chimeras. *Hmx1*^{+/+} and *Hmx1*^{-/-} BALB/c mice (8–10 weeks) were lethally irradiated (900 rad, 2.35 min, ¹³⁷Cs source) (Gammacell 2000; Mølsgaard Medical) and reconstituted 4 h thereafter with 5×10^6 total bone marrow cells from *Hmx1*^{+/+} and *Hmx1*^{-/-} BALB/c mice (6 weeks). Chimerism was assessed 8–10 weeks thereafter by PCR as described (6, 26).

CO Exposure. Mice were exposed to CO as described (6, 26).

Plasma Cytokines. Plasma TNF was measured by ELISA (R&D Systems). BBB permeability was assessed as described (6).

Heme. Protein-bound and protein-free heme were measured in plasma, as described (6). Liver homogenates were centrifuged (for 3 min at 4 °C at $16,000 \times g$), proteins were precipitated (10% trichloroacetic; vol/vol, for 1 h on ice), centrifuged (for 3 min at 4 °C at $14,000 \times g$), and free heme was measured in the protein-free supernatant as described (6).

Histopathology. Liver samples were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (6). Detection of fragmented DNA was carried out using the In Situ Cell Death Detection kit from Roche (TUNEL method). HO-1 staining was performed as described (27). In vivo treatments using anti-TNF mAb was performed as described in *SI Text*. Serum biochemistry was performed as described in *SI Text*. Quantitative real-time reverse transcription PCR (qRT-PCR). Expression of *Hmx1* mRNA was assessed as described (26).

Recombinant Adenoviruses. The LacZ, HO-1 Rec.Ad. were previously described (23). Mice were transduced with Rec.Ad. (i.p.; 4×10^8 pfu/mouse) 40 h before challenge with *Pcc*. Hepa1–6 cells were transduced with 10^3 pfu/cell, 48 h before treatment. Protein extraction and immunoblotting were performed as described in *SI Text*. Statistical analysis was performed as described in *SI Text*.

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