Sickle Hemoglobin Confers Tolerance to *Plasmodium* Infection

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SUMMARY

Sickle human hemoglobin (Hb) confers a survival advantage to individuals living in endemic areas of malaria, the disease caused by *Plasmodium* infection. As demonstrated hereby, mice expressing sickle Hb do not succumb to experimental cerebral malaria (ECM). This protective effect is exerted irrespectively of parasite load, revealing that sickle Hb confers host tolerance to *Plasmodium* infection. Sickle Hb induces the expression of heme oxygenase-1 (HO-1) in hematopoietic cells, via a mechanism involving the transcription factor NF-E2-related factor 2 (Nrf2). Carbon monoxide (CO), a byproduct of heme catabolism by HO-1, prevents further accumulation of circulating free heme after *Plasmodium* infection, suppressing the pathogenesis of ECM. Moreover, sickle Hb inhibits activation and/or expansion of pathogenic CD8$^+$ T cells recognizing antigens expressed by *Plasmodium*, an immunoregulatory effect that does not involve Nrf2 and/or HO-1. Our findings provide insight into molecular mechanisms via which sickle Hb confers host tolerance to severe forms of malaria.

INTRODUCTION

Several point mutations in the β-chain of human Hb, e.g. HbS ($^\beta$6Glu->Val) (Jallow et al., 2009; Williams et al., 2005b), HbC ($^\beta$6Glu->Lys)(Modiano et al., 2001) and HbE ($^\beta$26Glu->Lys)(Hutagalung et al., 1999), can confer a survival advantage to *Plasmodium* (*P.*) infection (Williams, 2006). When present in the homozygous form, some of these mutations, e.g. HbS, become pathologic causing hemolytic anemia, leading to accumulation of high levels of cell-free Hb and heme in plasma (Reiter et al., 2002). Individuals carrying the HbS mutation in the heterozygous form, i.e. the A/S sickle cell trait, also accumulate low (nonpathologic) levels of heme in plasma (Muller-Eberhard et al., 1968) and are protected against malaria (Allison, 1954; Beet, 1947; Jallow et al., 2009; Williams, 2006).

Once released from Hb, a phenomenon favored in the case of HbS (Hebbel et al., 1988), free heme becomes cytotoxic (Balla et al., 1992; Seixas et al., 2009; Gozzelino et al., 2010). This deleterious effect is countered by the expression of heme oxygenase-1 (HO-1, encoded by the *Hmox1* gene)(Balla et al., 1992; Ferreira et al., 2008; Gozzelino et al., 2010), a stress-responsive enzyme that catabolizes free heme into biliverdin, iron and carbon monoxide (CO)(Tenhunen et al., 1968). HO-1 expression is induced by free heme, through a mechanism that involves the ubiquitination-degradation of Kelch-like ECH-associated protein 1 (Keap1) (Kensler et al., 2007), a cytoplasmic repressor of the transcription factor NF-E2-related factor-2 (Nrf2)(Alam et al., 1999). Upon nuclear translocation, Nrf2 binds to the stress-responsive elements in the *Hmox1* promoter (Ogawa et al., 2001), a regulatory mechanism that plays a central role in the control of *Hmox1* expression in response to heme (Kensler et al., 2007).

HO-1 is protective against a wide variety of immune mediated inflammatory diseases (Nath et al., 1992; Soares and Bach, 2009) including experimental cerebral malaria (ECM)(Pamplona et al., 2007), a lethal neuroinflammatory syndrome that develops in *P. berghei* ANKA infected C57BL/6 mice and that mimics some of the pathologic features of human cerebral malaria (CM)(Mishra and Newton, 2009). This protective effect is mediated by CO, which binds cell-free Hb and inhibits its oxidation, thus preventing heme release from oxidized Hb (Hebbel et al., 1988) and hence the pathogenesis of ECM (Pamplona et al., 2007). Given that expression of HO-1 (Belcher et al., 2006; Jison et al., 2004) and production of CO (Sears et al., 2001) are induced in individuals carrying the HbS mutation we hypothesized that this might explain why individuals carrying the A/S sickle cell trait have a survival advantage against malaria (Allison, 1954; May et al., 2007; Williams et al., 2005b). We provide evidence demonstrating that this is the case.

RESULTS

Sickle Hb Confers a Survival Advantage against Malaria in Mice

Inoculation of C57BL/6 mice (*Hb^{wt}*) with *P. berghei* ANKA infected red blood cells (RBC) led within 6 to 12 days to the
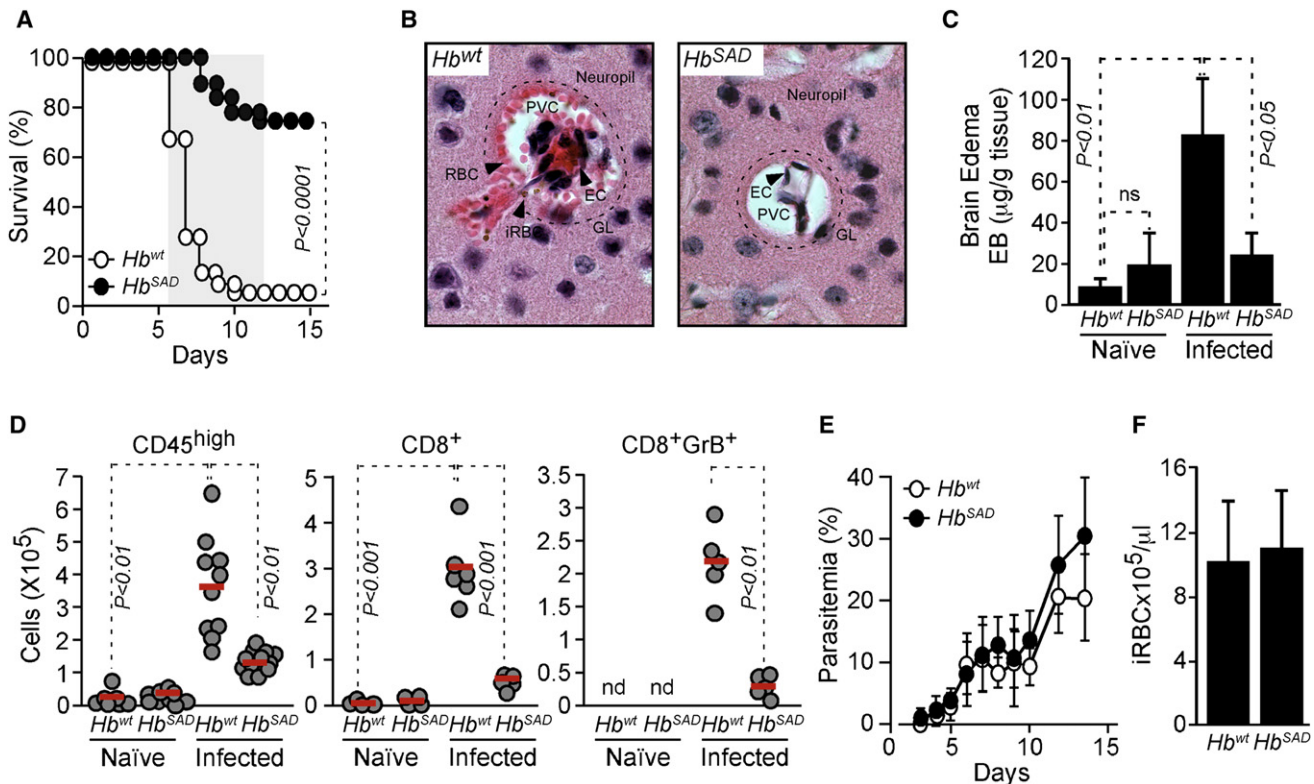
Figure 1. Hb^{SAD} Prevents ECM Onset

(A) Survival of *P. berghei* ANKA infected *Hb^{wt}* (n = 91) and *Hb^{SAD}* (n = 76) mice (10 independent experiments with survival advantage p < 0.05). Grey shading: expected time of ECM.

(B) Representative H&E stained microvessel in the BBB of infected *Hb^{wt}* and *Hb^{SAD}* mice, at ECM onset in *Hb^{wt}* mice (n = 3/group). EC: endothelial cell; PVC: perivascular compartment; GL: glia limitans (dotted line); RBC: red blood cells; iRBC: infected RBC. Magnification: 100x.

(C) Mean brain edema in naïve versus infected *Hb^{wt}* and *Hb^{SAD}* mice ± standard deviation (n=4/group), at the time of ECM onset in *Hb^{wt}* mice. ns: not significant.

(D) Brain leukocyte sequestration in naïve versus infected *Hb^{wt}* and *Hb^{SAD}* mice, at the time of ECM onset in *Hb^{wt}* mice. Dots represent single mice (n = 4 -14/group). Red lines represent mean values. nd: not determined.

(E) Mean percentage of infected RBC in *Hb^{wt}* and *Hb^{SAD}* mice ± standard deviation. Same mice as in (A).

(F) Mean number of infected RBC in *Hb^{wt}* (n=7) and *Hb^{SAD}* (n=9) mice ± standard deviation at the time of ECM onset in *Hb^{wt}* mice.

See also Figure S1 and Figure S2.

development of clinical signs of ECM (Figure 1A). Incidence of ECM was significantly reduced in hemizygous C57BL/6 *Hb^{SAD}* mice (Figure 1A) expressing a β-chain of human Hb carrying the $^{\beta}$6Glu->Val (Hb**S**) mutation as well as two additional mutations, $^{\beta}$23Ala->Ile (**A**ntilles^{23I}) and $^{\beta}$121Asp->Gln (D-Punjab^{121Q}), known to enhance HbS polymerization in humans and mice (Trudel et al., 1991). Naïve *Hb^{SAD}* mice present a very mild sickle cell syndrome, which does not lead to anemia (Table S1A available online; Trudel et al., 1994), similar to the asymptomatic human A/S sickle cell trait that affords protection against malaria (Allison, 1954; Beet, 1947; Jallow et al., 2009). *Hb^{SAD}* mice that did not develop ECM, succumbed 20-25 days postinfection from hyperparasitemia-induced anemia (data not shown), a condition unrelated to ECM (Schofield and Grau, 2005).

When infected with *P. berghei* ANKA, C57BL/6.Sv/129 *Hb^{A/a}* mice expressing normal human Hb as well as endogenous mouse Hb (Wu et al., 2006) developed clinical signs of ECM and succumbed 6 to 12 days after infection (Figure S1A). Littermate control C57BL/6.Sv/129 *Hb^{a/a}* mice, expressing only the endogenous mouse Hb developed ECM but with lower incidence, as compared to *Hb^{A/a}* mice (Figure S1A). This suggests that normal human Hb might promote, rather than prevent, the pathogenesis of ECM in C57BL/6.Sv/129 mice. While the reason for this is not clear, it is possible that human Hb alters mouse RBC physiology in a manner that would promote the development of ECM. However, this effect becomes negligible as C57BL/6.Sv/129 mice are backcrossed into the C57BL/6 genetic background (Figure S1D), in which ECM incidence is higher than 95% (Figure 1A). Given that *Hb^{SAD}* suppresses the pathogenesis of ECM in C57BL/6 mice and that the deleterious effect of normal human Hb becomes negligible under this genetic background, it is reasonable to infer that the protective effect of *Hb^{SAD}* is attributable to the mutations in the human β-globin chain rather than to human Hb *per se*.

Hb^{SAD} mice that did not succumb within 6–12 days postinfection also did not develop the pathologic hallmarks of ECM, including blood brain barrier (BBB) disruption (Figures 1B), perivascular RBC accumulation in brain (Figure 1B) and brain edema

(Figure 1C). These pathologic features were present in Hb^{wt} (Figures 1B and 1C) and $Hb^{A/a}$ mice, i.e. brain edema (Figure S1B).

Given that BBB disruption and brain edema occur in *P. berghei* ANKA infected C57BL/6 mice via a $CD8^+$ T cell-dependent mechanism (Belnoue et al., 2002; Schofield and Grau, 2005), we asked whether the protective effect of Hb^{SAD} against ECM was associated with inhibition of $CD8^+$ T cell sequestration in the brain. The number of $CD45^{high}$ leukocytes and $CD8^+$ T cells, including granzyme B-positive (GrB^+) $CD8^+$ T cells, was reduced in *P. berghei* ANKA infected Hb^{SAD}, as compared to Hb^{wt} mice at ECM onset (Figure 1D).

When infected with *P. berghei* ANKA, Hb^{wt} mice also developed severe lung injury and a mild form of liver injury (Figure S2) with no apparent injury to the kidneys or to the heart (*data not shown*). The extent of lung and liver injury was reduced in *P. berghei* ANKA infected Hb^{SAD} versus Hb^{wt} mice (Figure S2).

Sickle Hb Confers Tolerance to *Plasmodium* Infection in Mice

Protection of Hb^{SAD} mice against ECM was not associated with reduction of pathogen load, as assessed by the percentage of infected RBC, i.e., parasitemia (Figure 1E) as well as by the number of circulating infected RBC (Figure 1F) versus control Hb^{wt} (Figures 1E and 1F) or $Hb^{A/a}$ mice (Figures S1C and S1E). While the protective effect of the human sickle cell trait against malaria has been associated with decreased pathogen load (Allison, 1954; May et al., 2007; Williams et al., 2005b), there are several instances where this does not appear to be the case (Crompton et al., 2008; Livincstone, 1971; Motulsky et al., 1966), which is in keeping with the observation that Hb^{SAD} confers protection against ECM without interfering with pathogen load. These observations suggest that mutations in the β-chain of human Hb, such as those in Hb^{SAD} can afford tolerance to *Plasmodium* infection, a host defense strategy that limits disease severity by preventing tissue damage, without targeting the pathogen. This contrasts to resistance to infection, the well-recognized host defense strategy that limits disease severity by decreasing pathogen load (Raberg et al., 2007; Schneider and Ayres, 2008).

Parasite sequestration was similar in Hb^{wt}, $Hb^{SAD}Hmox1^{+/+}$, and $Hb^{SAD}Hmox1^{+/-}$ mice, as assessed using a transgenic luciferase-*P. berghei* ANKA strain (Figure 4S). This supports further the notion that induction of HO-1 by sickle Hb confers host tolerance to *Plasmodium* infection.

Sickle Hb Induces the Expression of HO-1 that Confers Tolerance to *Plasmodium* Infection

Humans and rodents carrying the HbS mutation express high levels of HO-1 in the hematopoietic compartment (Belcher et al., 2006; Jison et al., 2004). Consistent with this, naïve Hb^{SAD} mice express high levels of *Hmox1* mRNA in bone marrow and peripheral blood cells, as compared to naïve Hb^{wt} mice (Figure 2A). Naïve Hb^{SAD} mice also expressed higher levels of *Hmox1* mRNA in the kidneys (Figure S3A), which is consistent with the chronic development of kidney injury in these mice, revealed clinically upon aging (Sabaa et al., 2008). Hb^{SAD} mice expressed similar levels of *Hmox1* mRNA in the liver, heart, lung and spleen (Figure S3A), as compared to Hb^{wt} mice. $Hb^{A/a}$ mice expressed similar levels of *Hmox1* mRNA in the

bone marrow and peripheral blood versus littermate control $Hb^{a/a}$ mice (*Suppl.* Figure 3B), demonstrating that expression of a $β^S$ related variant but not a normal β-globin chain is required to induce *Hmox1* expression.

Given that HO-1 is protective against severe forms of malaria in mice (Pamplona et al., 2007; Seixas et al., 2009), we asked whether its induction in Hb^{SAD} mice (Figure 2A) is required to suppress the development of ECM (Figure 1A). Deletion of one *Hmox1* allele ($Hmox1^{+/-}$) reduced *Hmox1* mRNA expression in bone marrow and whole blood leukocytes of Hb^{SAD} mice (Figure S3C), without causing overt postnatal lethality (Table S2A). When challenged by *P. berghei* ANKA infection, $Hb^{SAD}Hmox1^{+/-}$ mice succumbed to ECM (Figure 2B), with concomitant development of BBB disruption (Figure 2C), brain edema (Figure 2D) and sequestration of $CD45^{high}$ leukocytes (*data not shown*), $CD8^+$ T cells and activated GrB^+CD8^+ T cells in the brain (Figure 2E) but without noticeable hematological changes (Table S1B).

The protective effect of Hb^{SAD} against lung and liver injury, associated with *P. berghei* ANKA infection, was lost in $Hb^{SAD}Hmox1^{+/-}$ mice (Figure S2). This was not associated with increased parasite load (Figure 2F).

Pharmacologic inhibition of HO activity by zinc protoporphyrin IX (ZnPPIX) (Figures S5A and S5B), increased ECM incidence in Hb^{SAD} mice versus vehicle-treated controls (Figure S5C). This effect was not associated with modulation of parasitemia (Figure S5D), suggesting that heme catabolism by HO-1 confers tolerance to *Plasmodium* infection in Hb^{SAD} mice.

Induction of HO-1 by Sickle Hb Inhibits the Production of Chemokines Involved in the Pathogenesis of ECM

Several chemokines can contribute to the pathogenesis of ECM and presumably to that of human CM (Campanella et al., 2008; Schofield and Grau, 2005; Mishra and Newton, 2009). Expression of mRNA encoding *Ccl2* (*Mcp-1*), *Ccl3* (*MIP1α*), *Ccl5* (*Rantes*), and *Cxcl10* (*Ip-10*) were decreased in the brain of Hb^{SAD} mice that did not develop ECM versus Hb^{wt} mice that succumbed to ECM (Figure 3A). This inhibitory effect involved HO-1, since expression of mRNA encoding these chemokines was increased in the brain of infected $Hb^{SAD}Hmox1^{+/-}$ versus $Hb^{SAD}Hmox1^{+/+}$ (Figure 3A). The involvement of CXCL10/IP-10 in the pathogenesis of ECM (Campanella et al., 2008) suggests that its inhibition might contribute functionally the protective effect of Hb^{SAD} against ECM. Expression of mRNAs encoding other chemokines, such as *Cxcl11* (*Ip-9*) or the chemokine receptors *Ccr2* and *Cxcr3* was also inhibited by Hb^{SAD} but in a manner that was not impaired in $Hb^{SAD}Hmox1^{+/-}$ versus $Hb^{SAD}Hmox1^{+/+}$ mice (Figure 3B). This suggests that the inhibitory effect of Hb^{SAD} over the expression of these genes, probably does not involve HO-1. Expression of mRNA encoding the chemokine *Ccl19* (*MIP-3β*) and the chemokine receptor *Ccr7* was not modulated by Hb^{SAD} and/or did not involve HO-1 (Figure 3C). This was also the case for several other genes previously involved or not in the pathogenesis of ECM (Figure S6 and Figure S7).

Sickle Hb Confers Tolerance to *Plasmodium* Infection via HO-1 Expression in Hematopoietic Cells

We performed syngenic bone marrow transplants from $Hb^{SAD}Hmox1^{+/+}$ or $Hb^{SAD}Hmox1^{+/-}$ mice into lethally irradiated
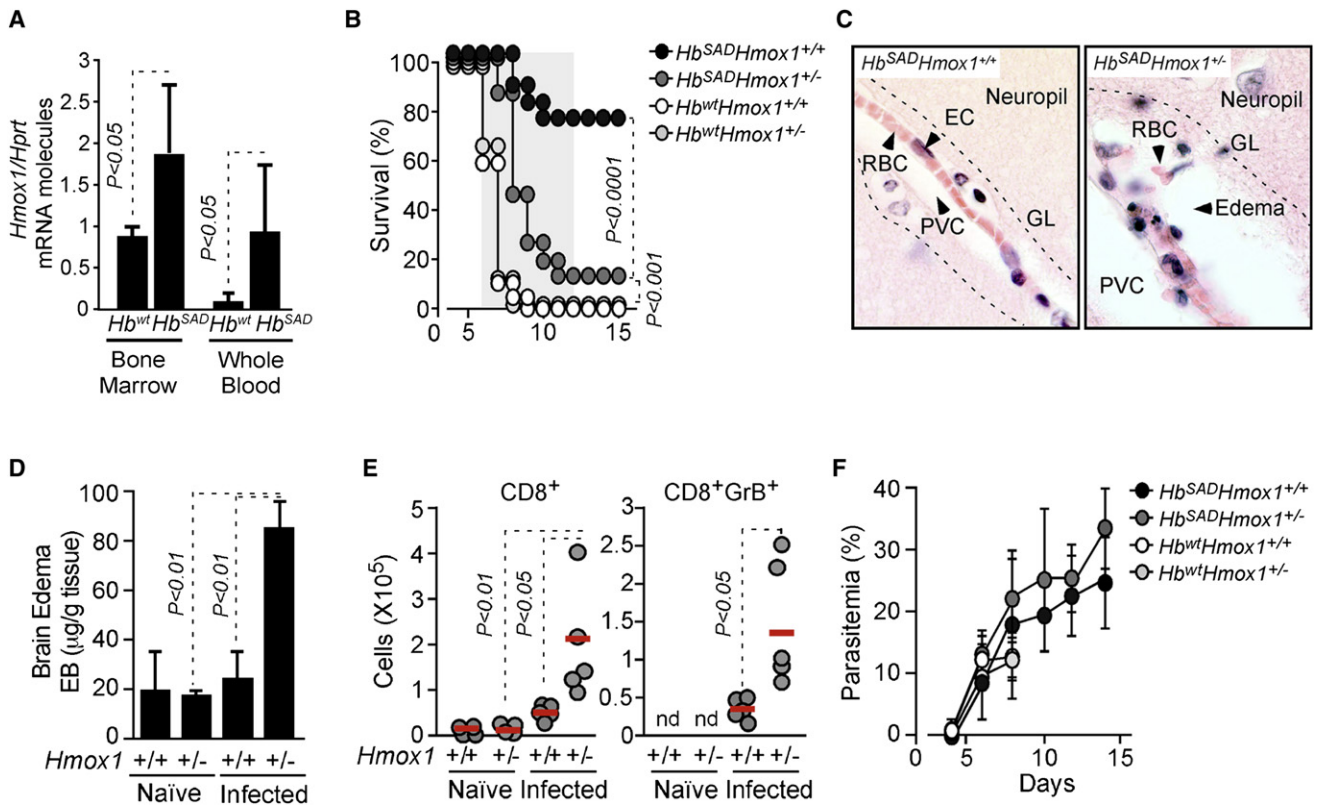
Figure 2. HO-1 Mediates the Protective Effect of Hb^{SAD} against ECM

(A) Mean ratio of *Hmox1* versus hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) mRNA molecules in naïve *Hb^{wt}* and *Hb^{SAD}* mice ± standard deviation (n=4/group).

(B) Survival of *P. berghei* ANKA infected *Hb^{wt}Hmox1$^{+/+}$* (n=19), *Hb^{SAD}Hmox1$^{+/+}$* (n = 13), *Hb^{wt}Hmox1$^{+/-}$* (n =15) and *Hb^{SAD}Hmox1$^{+/-}$* (n = 15) mice. (three independent experiments; survival advantage p < 0.05). Grey shading: expected time of ECM.

(C) Representative H&E stained micro-vessel in the brain of infected *Hb^{SAD}Hmox1$^{+/+}$* and *Hb^{SAD}Hmox1$^{+/-}$* mice, at ECM onset in *Hb^{SAD}Hmox1$^{+/-}$* mice (n=3/group). EC: endothelial cells; PVC: perivascular compartment; GL: glia limitans (dotted line); RBC: red blood cells. Magnification: 100×.

(D) Mean brain edema in naïve versus infected *Hb^{SAD}Hmox1$^{+/+}$* and *Hb^{SAD}Hmox1$^{+/-}$* mice, at ECM onset in *Hb^{SAD}Hmox1$^{+/-}$* mice ± standard deviation (n = 3-4/group).

(E) CD8$^+$ T cells and activated GrB$^+$CD8$^+$ T cells in brains of naïve versus infected *Hb^{SAD}Hmox1$^{+/+}$* and *Hb^{SAD}Hmox1$^{+/-}$* mice, at the time of ECM onset in *Hb^{SAD}Hmox1$^{+/-}$* mice. Dots represent single mice (n = 4–5/group). Red lines represent mean values. nd: not determined.

(F) Mean percentage of infected RBC (parasitemia) in the same mice as in (B). See also Figure S2, Figure S3, Figure S4, and Figure S5.

Hb^{wt}Hmox1$^{+/+}$ or *Hb^{wt}Hmox1$^{+/-}$* mice to generate chimeric *Hb^{SAD}* mice in which one *Hmox1* allele is deleted in the hematopoietic (*Hb^{SAD}Hmox1$^{+/-}$* → *Hb^{wt}Hmox1$^{+/+}$*) or nonhematopoietic (*Hb^{SAD}Hmox1$^{+/+}$* → *Hb^{wt}Hmox1$^{+/-}$*) compartment. Chimeric *Hb^{SAD}* mice carrying two functional *Hmox1* alleles in the hematopoietic and in the nonhematopoietic compartments (*Hb^{SAD}Hmox1$^{+/+}$* → *Hb^{wt}Hmox1$^{+/+}$*) did not succumb to ECM (Figure 4A) or develop brain edema (Figure 4B) in response to *P. berghei* ANKA infection. Control chimeric mice in which the bone marrow of *Hb^{wt}Hmox1$^{+/+}$* mice was transferred into lethally irradiated *Hb^{SAD}Hmox1$^{+/+}$* mice, develop ECM (Figure 4A) and brain edema (Figure 4B), confirming that cells derived from the hematopoietic compartment confer the protective effect of *Hb^{SAD}*.

Chimeric *Hb^{SAD}* mice carrying a single functional *Hmox1* allele in hematopoietic cells (*Hb^{SAD}Hmox1$^{+/-}$* → *Hb^{wt}Hmox1$^{+/+}$*) succumbed to ECM (Figure 4A), developing brain edema (Figure 4B). The reverse was not true in that deletion of a single *Hmox1* allele in

nonhematopoietic cells (*Hb^{SAD}Hmox1$^{+/+}$* → *Hb^{wt}Hmox1$^{+/-}$*) did not impair the protective effect of *Hb^{SAD}* against ECM (Figure 4A), confirmed by lack of brain edema (Figure 4B). Similar results were obtained when transferring bone marrows from *Hb^{SAD}Hmox1$^{+/+}$* or *Hb^{SAD}Hmox1$^{+/-}$* mice into *Hb^{SAD}Hmox1$^{+/+}$* or *Hb^{SAD}Hmox1$^{+/-}$* mice Figure S8). Lethality after day 12 postinfection (Figure 4A) was most probably due to the development of a "composite disease" in which high levels of parasitemia (>20%) synergize with sickle human Hb to cause death, without overt clinical or pathological signs of ECM. These observations reveal that the protective effect of *Hb^{SAD}* requires the induction of HO-1 expression in hematopoietic cells, consistent with the observed induction of HO-1 expression in blood and bone marrow cells of naïve *Hb^{SAD}* mice (Figure 2A). The protective effect of HO-1 expression in the hematopoietic compartment was not associated with modulation of pathogen load (Figure 4C and Figure S8C), confirming that HO-1 affords tolerance to *Plasmodium* infection.
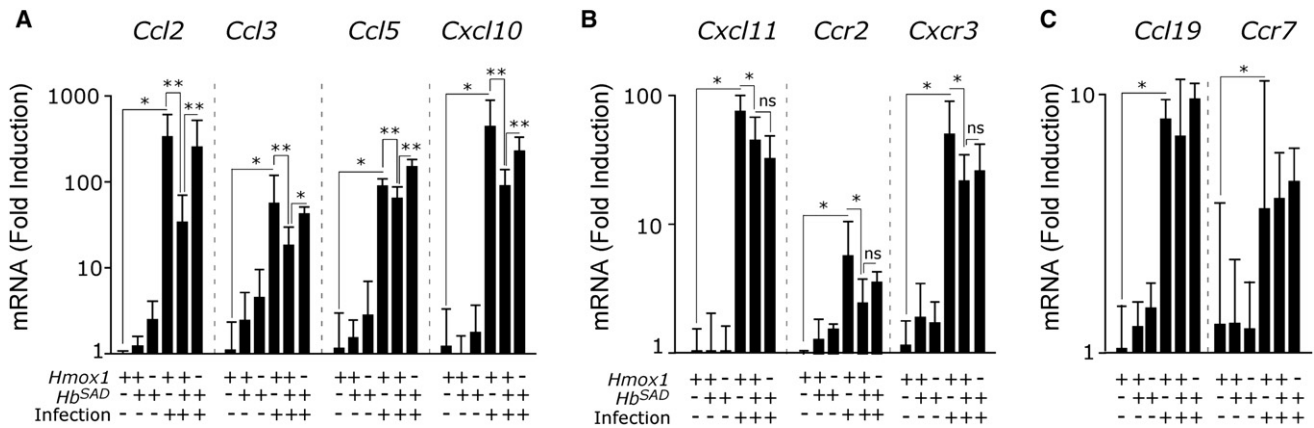
Figure 3. Induction of HO-1 by Hb^{SAD} Inhibits Chemokine Production in the Brain

Quantification of mRNA encoding chemokines and chemokine receptors in the brains of naïve (-) and *P. berghei* ANKA infected (+) mice carrying one (-) or two (+) functional *Hmox1* alleles and expressing (+) Hb^{SAD} or not (-). Results are shown as mean fold induction over naïve *Hb^{wt}Hmox1$^{+/+}$* mice ± standard deviation (n = 4–8/group), analyzed at ECM onset in *Hb^{wt}* or *Hb^{SAD}Hmox1$^{+/-}$* control groups.

(A) Genes inhibited by Hb^{SAD} under the control of HO-1.

(B) Genes inhibited by Hb^{SAD}, presumably not under the control of HO-1.

(C) Genes not regulated by Hb^{SAD}. *p < 0.05; **p < 0.01; ns P > 0.05. See also Figure S6 and Figure S7.

Sickle Hb Inhibits the Activation/Expansion of CD8+ T Cells Recognizing Antigens Expressed by *Plasmodium*

The number of splenic CD8+ T cells recognizing specifically a MHC I-restricted epitope derived from glycoprotein B (gB$_{498-505}$) of herpes simplex virus-1 expressed by transgenic *P. berghei* ANKA (Lundie et al., 2008) was reduced in *Hb^{SAD}* versus *Hb^{wt}* mice, as assessed five days after infection (Figure 4A *and* Figure S9A). The number of splenic GrB+CD8+ T cells was also reduced in *Hb^{SAD}* versus *Hb^{wt}* mice five days after infection (Figure 4E and Figure S9B). This reveals that *Hb^{SAD}* prevents overt expansion of pathogenic CD8+ T cells, an effect that should contribute to the protective effect of *Hb^{SAD}* against ECM (Belnoue et al., 2002; Lundie et al., 2008). We then asked whether this immunoregulatory effect of *Hb^{SAD}* involved the expression of HO-1. The number of gB$_{498-505}$-specific CD8+ T cells and GrB+CD8+ T cells was not different in the spleen of *Hb^{SAD}Hmox1$^{+/-}$* versus *Hb^{SAD}Hmox1$^{+/+}$* mice five days after infection (Figures 4D and 4E and Figures S9A and S9B). This suggests that *Hb^{SAD}* controls the activation and/or expansion of splenic CD8+ T cells, via a mechanism that probably does not involve HO-1.

Sickle Hb Induces HO-1 Expression via a Mechanism Involving Nrf2

Given that Nrf2 plays a central role in the transcriptional regulation of HO-1 expression (Alam et al., 1999) we asked whether induction of HO-1 expression in whole blood leukocytes of naïve *Hb^{SAD}* mice (Figure 2A) involved this transcription factor. Deletion of one *Nrf2* allele in *Hb^{SAD}* mice (*Hb^{SAD}Nrf2$^{+/-}$*) was sufficient to reduce the level of *Hmox1* mRNA expression in whole blood leukocytes, to those of naïve *Hb^{wt}Nrf2$^{+/+}$* mice (Figure 5A). This suggests that sickle Hb induces *Hmox1* transcription and expression via a mechanism involving Nrf2. Incidence of ECM increased significantly in *P. berghei* ANKA infected *Hb^{SAD}Nrf2$^{+/-}$*

versus *Hb^{SAD}Nrf2$^{+/+}$* mice (Figure 5B), confirmed by the development of brain edema (Figure 5C). A similar effect was observed in a limited number of *Hb^{SAD}* mice in which both *Nrf2* alleles were functionally deleted, i.e. *Hb^{SAD}Nrf2$^{-/-}$* mice (n = 5; 20% survival). It should be noted that deletion of both *Nrf2* alleles in *Hb^{SAD}* mice lead to overt postnatal lethality (Table S2B). Loss of protection against ECM in *Hb^{SAD}Nrf2$^{+/-}$* versus *Hb^{SAD}Nrf2$^{+/+}$* mice was not associated with a regain of CD8+ T cell activation and/or expansion in the spleen, as assessed five days after infection (Figure S10). This suggests that the immunoregulatory of *Hb^{SAD}* probably does not involve Nrf2, which is consistent with the observation that this effect also does not seem to involve HO-1, a gene regulated by *Hb^{SAD}* via Nrf2 (Figure 5A).

The protective effect of *Hb^{SAD}* against lung and liver injury associated to *P. berghei* ANKA infection was lost in *Hb^{SAD}Nrf2$^{+/-}$* versus *Hb^{SAD}Nrf2$^{+/+}$* mice (Figure S2). This was not associated with increased parasite load in *Hb^{SAD}Nrf2$^{+/-}$* versus *Hb^{SAD}Nrf2$^{+/+}$* mice (Figure 5D), which is consistent with the notion that induction of HO-1 expression by Nrf2 confers tolerance to *Plasmodium* infection.

Sickle Hb Confers Tolerance to *Plasmodium* Infection via a Mechanism Involving CO Produced through Heme Catabolism by HO-1

Consistent with similar observations in individuals carrying the HbS mutation in the homozygous (Reiter et al., 2002) or heterozygous (Muller-Eberhard et al., 1968) form, naïve *Hb^{SAD}* mice had higher concentration of free heme in plasma, as compared to age-matched control naïve *Hb^{wt}* mice (Figure 6A). When pre-exposed in vitro to low levels of free heme cells are protected against a subsequent heme challenge (Balla et al., 1992). We asked whether free heme would exert a similar protective effect in vivo. Administration of free heme to *Hb^{wt}* mice prior to
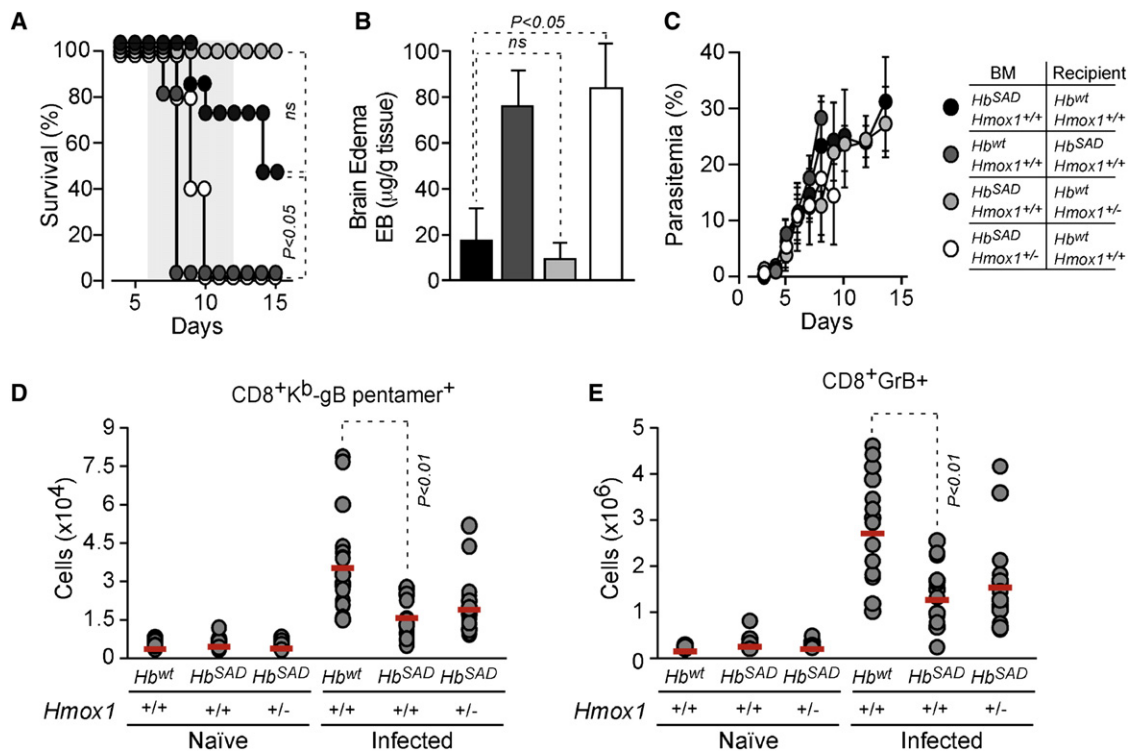
Figure 4. The Protective But Not The Immunoregulatory Effect of Sickle Hb against ECM Involves the Expression of HO-1 in Hematopoietic Cells

(A) Survival of *P. berghei* ANKA infected chimeric mice resulting from the adoptive transfer of bone marrow from $Hb^{SAD}Hmox1^{+/+}$ mice into $Hb^{wt}Hmox1^{+/+}$ recipients (n = 6); from $Hb^{wt}Hmox1^{+/+}$ mice into $Hb^{SAD}Hmox1^{+/+}$ recipients (n = 5); from $Hb^{SAD}Hmox1^{+/+}$ mice into $Hb^{wt}Hmox1^{+/-}$ recipients (n = 8) and from $Hb^{SAD}Hmox1^{+/-}$ into $Hb^{wt}Hmox1^{+/+}$ recipients (n = 7). Recipients were lethally irradiated before the adoptive transfer. Grey shading indicates expected time of ECM. Pooled from four independent experiments.

(B) Mean brain edema ± standard deviation (n = 3/group) in chimeric mice, produced as in (A).

(C) Mean percentage of infected RBC in chimeric mice ± standard deviation, same mice as in (A).

(D) Number of splenic CD8$^+$ T cells specific for gB$_{498-505}$ in $Hb^{wt}Hmox1^{+/+}$, $Hb^{SAD}Hmox1^{+/+}$ and $Hb^{SAD}Hmox1^{+/-}$ mice not infected (naïve) or 5 days after *P. berghei* ANKA infection.

(E) Number of splenic CD8$^+$GrB$^+$ T in the same mice as in (d). Circles in (d) and (e) represent single mice and red lines mean values (n = 10 and n = 16 in noninfected and infected groups, respectively), pooled from 4 independent experiments with similar results.

See also Figure S8 and Figure S9.

P. berghei ANKA infection suppressed ECM incidence, as compared to vehicle-treated Hb^{wt} mice (Figure 6B).

The protective effect of heme was dose-dependent, with higher dosage leading to (1) increased HO-1 expression in whole blood cells (Figure S1A) and spleen (Figure S1B) and to a lesser extent in the bone marrow (Figure S11C) and (2) suppression of ECM (Figure 11D). This protective effect was not associated with modulation of parasitemia (Figure S11D), suggesting that low concentration of free heme in the plasma of naïve Hb^{SAD} mice (Figure 6a) can confer tolerance to *Plasmodium* infection.

We asked whether accumulation of low levels of free heme in Hb^{SAD} contributes to the immunoregulatory effect exerted by Hb^{SAD} on CD8$^+$ T cells (Figures 4D and 4E). Administration of free heme to Hb^{wt} mice, prior to infection with transgenic *P. berghei* ANKA expressing gB$_{498-505}$, reduced the number of splenic gB$_{498-505}$-specific CD8$^+$ T cells (Figures S12A and S12B) as well as GrB$^+$CD8$^+$ T cells, as compared to vehicle treated Hb^{wt} mice five days after infection (Figures S12C and

S12D). This supports further the notion that the protective effect of Hb^{SAD} against ECM is mediated, to a large extent, via the accumulation of low levels of circulating free heme.

Plasma free heme concentration increased significantly following *P. berghei* ANKA infection in Hb^{wt} mice (Figure 6A), an effect we have previously shown to contribute in a critical manner to the pathogenesis of ECM (Ferreira et al., 2008; Pamplona et al., 2007). Albeit less pronounced this increase was also observed in Hb^{SAD} mice (Figure 6A). When challenged with free heme after infection, Hb^{SAD} succumbed to ECM (Figure 6C), confirmed by the occurrence of brain edema (Figure 6d). This reveals that free heme has a dual effect in the control of ECM onset, being protective when present at slightly above normal concentration before infection (Figure 6B) while highly pathogenic when present at higher levels after infection (Figure 6C). Free heme did not interfere with pathogen load (Figures S11E and S11F), revealing that when present at slightly above normal concentration before infection free heme promotes tolerance to malaria, while impairing tolerance to malaria when present at
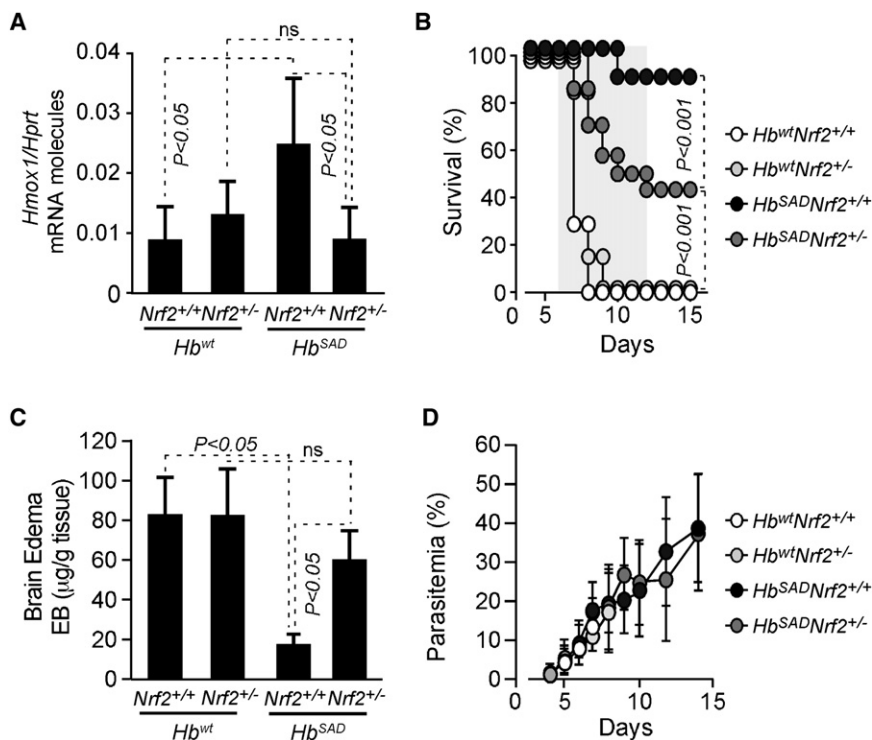
Figure 5. Sickle Human Hb Prevents the Onset of ECM via the Induction of HO-1 expression by Nrf2

(A) Mean ratio of *Hmox1* versus hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) mRNA molecules in peripheral blood mononuclear cells of naïve $Hb^{wt}Nrf2^{+/+}$, $Hb^{wt}Nrf2^{+/-}$, $Hb^{SAD}Nrf2^{+/+}$ and $Hb^{SAD}Nrf2^{+/-}$ mice ± standard deviation (n = 6–8/group).

(B) Survival of *P. berghei* ANKA-infected $Hb^{wt}Nrf2^{+/+}$ (n = 6), $Hb^{wt}Nrf2^{+/-}$ (n = 13), $Hb^{SAD}Nrf2^{+/+}$ (n = 10) and $Hb^{SAD}Nrf2^{+/-}$ (n = 14) mice, three independent experiments. Grey shading indicates expected time of ECM.

(C) Brain edema was measured by Evans blue (EB) accumulation in brains of infected $Hb^{wt}Nrf2^{+/+}$, $Hb^{wt}Nrf2^{+/-}$, $Hb^{SAD}Nrf2^{+/+}$ and $Hb^{SAD}Nrf2^{+/-}$ mice, at the time of ECM onset. Mean ± standard deviation (n=4-5/group).

(D) Mean percentage of infected RBC (parasitemia) ± standard deviation, same mice as in (B). See also Figure S10.

higher concentrations after infection. Heme administration at the same dosage and schedule to naïve Hb^{wt} or Hb^{SAD} mice did not result in lethality (data not shown).

When applied via inhalation to wild type mice, CO suppresses the pathogenesis of ECM via a mechanism that relies on the inhibition of heme release from Hb (Pamplona et al., 2007). We asked whether the protective effect of Hb^{SAD} against ECM was mediated via this mechanism. Inhaled CO suppressed the incidence of ECM in $Hb^{SAD}Hmox1^{+/-}$ mice (Figure 6E), confirmed by the lack of brain edema (Figure 6F). A similar protective effect was observed when CO was applied to *P. berghei* infected Hb^{SAD} mice treated with the enzymatic HO inhibitor ZnPPIX (Figures S5E and S5F). CO did not modulate parasitemia (Figure 1G and Figures S11G and S11H). Instead, its protective effect was associated with reduction of plasma free heme concentration, below that of naïve $Hb^{SAD}Hmox1^{+/-}$ mice (Figure 6G). Administration of free heme to infected $Hb^{SAD}Hmox1^{+/-}$ mice abrogated the protective effect of CO, restoring ECM incidence (Figure 6H), confirmed by brain edema (Figure 6I). Heme was not toxic when administered at the same dosage and schedule to naïve $Hb^{SAD}Hmox1^{+/-}$ mice receiving CO, i.e. 0% mortality. These observations demonstrate that sickle Hb suppresses the onset of ECM via the induction of HO-1 and the production of CO, which inhibits the accumulation of free heme thus affording tolerance to *Plasmodium* infection (Figure 7).

DISCUSSION

The protective effect of sickle human Hb against malaria is thought to rely on the reduction of parasite load (Allison, 1954;

May et al., 2007; Williams et al., 2005b), implying that sickle human Hb affords resistance (Raberg et al., 2007; Schneider and Ayres, 2008) to *Plasmodium* infection. Consistent with this notion, sickle human Hb decreases RBC permissiveness to *Plasmodium* invasion and growth (Friedman, 1978; Pasvol et al., 1978) while increasing phagocytosis of infected RBC, as assessed ex vivo (Ayi et al., 2004). Whether these effects account for the protective effect of sickle human Hb in vivo remained to be established. We used a well-established mouse model of malaria allowing for genetic manipulation of the host, to test in vivo the relative contribution of host specific genes to the protective effect of sickle Hb against *Plasmodium* infection. For technical and ethical reasons these studies can only be performed in rodent models of malaria.

As demonstrated hereby, sickle Hb affords protection against *Plasmodium* infection in mice (Figure 1A and Figure 2), a finding consistent with previous reports using different mouse and *Plasmodium* strains (Hood et al., 1996; Shear et al., 1993). This survival advantage occurs irrespectively of parasite load (Figures 1E and 1F) and is not associated with modulation of parasite sequestering in different organs (Figure S4), revealing that sickle human Hb confers tolerance (Raberg et al., 2007; Schneider and Ayres, 2008) to *Plasmodium* infection. This is consistent with our recent observation that heme catabolism by HO-1 also suppresses development of multiple organ dysfunction associated with the pathogenesis of severe sepsis in mice (Larsen et al., 2010), a lethal outcome of polymicrobial infection that resembles, in some aspects, severe malaria (Clark et al., 2004). Since the survival advantage conferred by HbS against malaria in human populations can occur without overt decrease of parasite load (Crompton et al., 2008; Livincstone, 1971; Motulsky et al., 1966), tolerance to *Plasmodium* infection might also operate in individuals expressing sickle Hb.
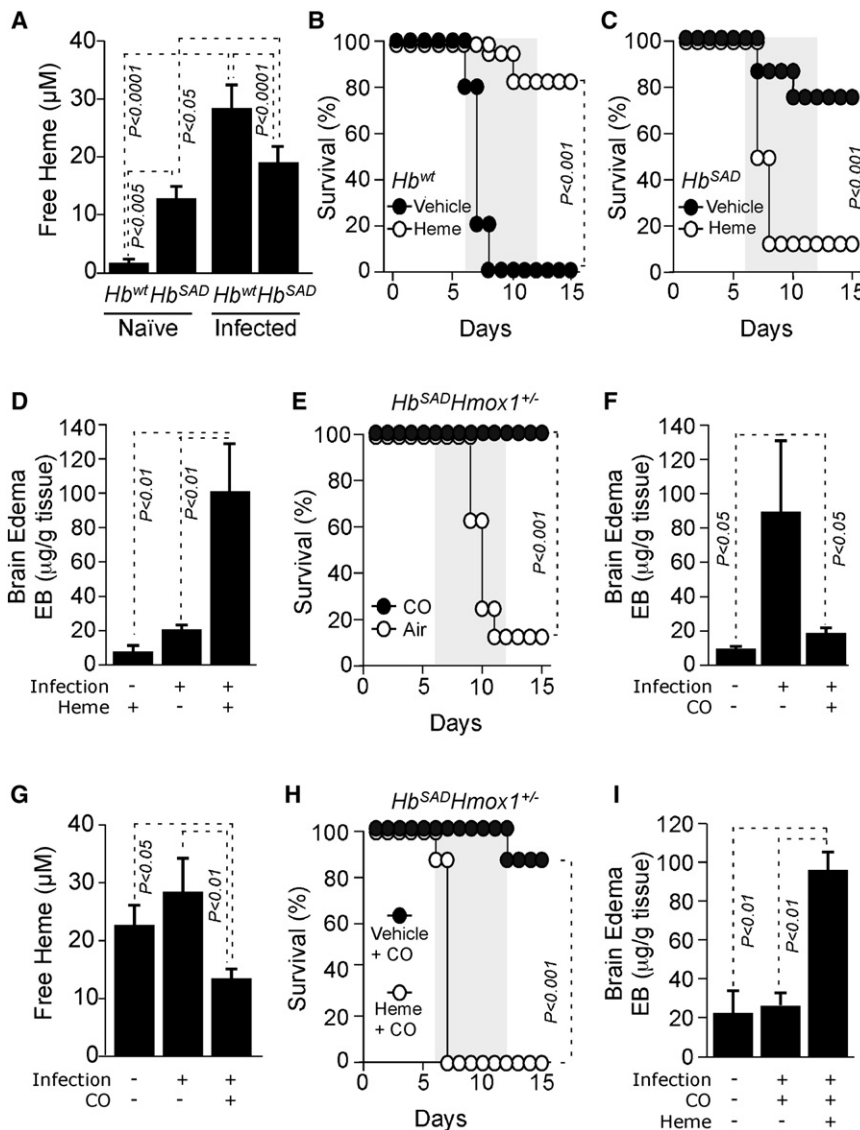
Figure 6. Hb^SAD Inhibits Free Heme Accumulation via the Production of CO

(A) Mean plasma free heme concentration in naïve versus *P. berghei* ANKA infected Hb^{wt} and Hb^{SAD} mice at ECM onset in Hb^{wt} mice ± standard deviation (n = 4–15/group).

(B) Survival of infected Hb^{wt} mice receiving vehicle (n = 25) or heme (35–40 mg/kg, every 48 hr, day 2 preinfection to 4 postinfection) (n = 17). Pooled from four independent experiments, with similar results.

(C) Survival of infected Hb^{SAD} mice receiving vehicle (n = 8) or heme (20 mg/kg, every 12 hr, day 4–7 postinfection) (n = 8). Pooled from two independent experiments, with similar results.

(D) Mean brain edema in Hb^{SAD} mice treated as in (c) at ECM onset in heme-treated Hb^{SAD} mice ± standard deviation (n = 4/group).

(E) Survival of infected $Hb^{SAD}Hmox1^{+/-}$ mice exposed to air (n = 8) or CO (250 ppm, days 4-7 post infection) (n = 12). Pooled from three independent experiments, with similar results.

(F) Mean brain edema in $Hb^{SAD}Hmox1^{+/-}$ mice treated as in (E), at ECM onset in air-treated mice ± standard deviation (n =3–4/group).

(G) Mean free heme in plasma of $Hb^{SAD}Hmox1^{+/-}$ mice treated as in (E) ± standard deviation (n = 4–6/group).

(H) Survival of infected $Hb^{SAD}Hmox1^{+/-}$ mice exposed to CO (250ppm; days 4–7 postinfection) and receiving vehicle (n = 8) or heme (20 mg/kg, every 12 hr, days 4–7 postinfection) (n = 8). Pooled from two independent experiments, with similar results.

(I) Mean brain edema in $Hb^{SAD}Hmox1^{+/-}$ mice treated as in (H), at ECM onset in heme-treated mice ± standard deviation (n = 3–4/group). Grey shading in (B, C, E, and H) indicates expected time of ECM. See also Figure S11 and Figure S12.

prevents the cytotoxic effects of free heme (Balla et al., 1992; Gozzelino et al., 2010), hence limiting the pathological outcome of sickle cell anemia in mice (Belcher et al., 2006).

We provide evidence for the existence of a specific molecular mechanism via which sickle human Hb confers tolerance to *Plasmodium* infection. When expressed at nonpathological levels in mice, sickle Hb leads to the accumulation of low concentrations of free heme in plasma (Figure 6A). The same is true for individuals carrying the sickle cell trait (Muller-Eberhard et al., 1968), which affords protection against malaria (Allison, 1954; Beet, 1947; Jallow et al., 2009; Williams, 2006). Presumably, this is due to the higher rate of heme release from sickle versus normal human Hb (Hebbel et al., 1988). In the absence of overt inflammation, free heme induces HO-1 expression without causing cytotoxicity (Balla et al., 1992; Gozzelino et al., 2010). Presumably, this explains how sickle human Hb induces the expression of HO-1 in human (Jison et al., 2004) and mouse (Figure 2A) peripheral blood mononuclear cells as well as in human endothelial cells (Bains et al., 2010; Nath et al., 2001). Expression of HO-1

The mechanism via which sickle Hb induces the expression of HO-1 in vivo involves Nrf2 (Figure 5A), a transcription factor previously shown to regulate *Hmox1* expression (Alam et al., 1999; Kensler et al., 2007). Induction of HO-1 via Nrf2 affords protection against malaria in Hb^{SAD} mice expressing sickle Hb (Figure 2B and Figure 5B). This protective effect occurs irrespectively of parasite load (Figure 2F and Figure 5D) or parasite sequestration in different organs (Figure S4), revealing that sickle human Hb affords tolerance to *Plasmodium* infection via the Nrf2/HO-1 system.

The protective effect of HO-1 against sickle cell anemia (Belcher et al., 2006) and against malaria is mediated by the same end-product of heme catabolism, namely CO (Figures 6E and 6F). This gasotransmitter inhibits Hb oxidation and subsequently heme release from Hb (Hebbel et al., 1988) (Figure 6G), thus preventing free heme from participating in the pathogenesis
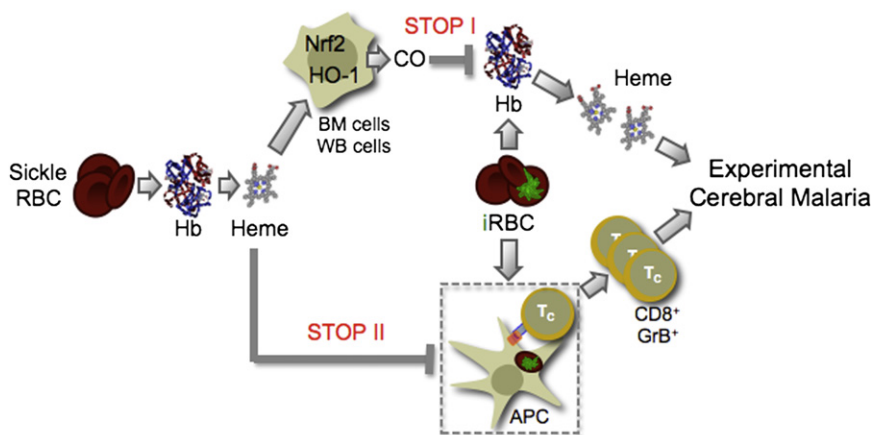
Figure 7. Protective Effect of Sickle Hb against ECM

Release of Hb from sickle RBC leads to chronic accumulation of cell-free Hb and release of its heme prosthetic groups. Free heme induces HO-1 expression in bone marrow (BM) and white blood (WB) cells, via a mechanism involving Nrf2. Heme catabolism by HO-1 produces CO that prevents (STOP I) further heme release from the cell-free Hb after *Plasmodium* infection, suppressing the pathogenesis of ECM. Moreover, sickle human Hb exerts an immunoregulatory effect that appears to act independently of Nrf2 and/or HO-1 and that inhibits (STOP II) cytotoxic GrB$^+$CD8$^+$ T cells (T$_C$) activation and expansion. APC: Antigen presenting cell.

of ECM (Figures 6H and 6I)(Pamplona et al., 2007) (Figure 7). However, CO might have additional protective effects that contribute to prevent the lethal outcome of *Plasmodium* infection (Ferreira et al., 2008).

Other end-products of heme catabolism by HO-1, such as labile iron, might also contribute to the protective effect conferred by sickle Hb against malaria. While cytotoxic *per se*, labile iron induces the expression of ferritin H chain (FtH)(Berberat et al., 2003; Eisenstein et al., 1991), which confers cytoprotection against free heme in vitro (Balla et al., 1992). We have obtained preliminary evidence suggesting that the cytoprotective effect of FtH is required to support the salutary effects of HO-1 (Seixas et al., 2009) against the onset of noncerebral forms of severe malaria in mice (Raffaella Gozzelino, personal communication.

The pathogenesis of ECM relies on a "multiple hit" system in which free heme synergizes with other cytotoxic agonists, e.g. pathogenic CD8$^+$ T cells, to trigger disease onset (Ferreira et al., 2008; Pamplona et al., 2007). While sickle Hb suppresses both, accumulation of pathogenic free heme (Figure 6A) and activation and/or expansion of pathogenic CD8$^+$ T cells (Figure 4D and e), it appears to do so via different mechanisms. Namely, it inhibits the accumulation of free heme after infection, via the HO-1/CO system (Figures 6A and G) while restraining the activation and/or expansion of pathogenic CD8$^+$ T cells (Figure 4D), via a mechanism that does not seem to involve HO-1 (Figure 4D and e, Figure S9) or Nrf2 (Figure S10). This latter effect is in keeping with the likely immunoregulatory basis of the protective effect of sickle cell trait against severe malaria in human populations (Williams et al., 2005a). While the immunoregulatory effect of sickle Hb appears to be driven by free heme (Figure S12) its molecular mechanism acts via a signal transduction pathway that remains to be established and that might target antigen presenting cells, e.g. dendritic cells, and/or CD8$^+$ T cells.

Its possible that chronic hemolysis, associated with oxidation of cell-free Hb and production of circulating free heme, acts as a general protective mechanism against severe forms of malaria in human populations. This would contribute to explain the survival advantage conferred by a variety of RBC mutations against *P. falciparum* infection. In keeping with this notion, many of these RBC mutations can induce hemolysis (spontaneously or upon oxidative challenge) associated with the accumulation of circulating free heme (Muller-Eberhard et al., 1968). Some of these also afford protection against CM as illustrated for HbC (May et al., 2007; Modiano et al., 2001), glucose 6 phosphate dihydrogenase (G6PD) deficiency in males (Guindo et al., 2007), β- or α-thalassemia that confer protection mainly against severe anemia caused by *P. falciparum* infection (May et al., 2007) as well as other RBC cytoskeleton or membrane protein defects (Williams, 2006). The notion that chronic hemolysis might be protective *per se* against severe forms of malaria is strongly supported by the observation that heme administration to naïve mice, is sufficient *per se* to elicit a protective response (Figure 6D and Figure S12), relying, presumably on the induction of the Nrf2/HO-1 system. This is however, difficult to prove because the same Nrf2/HO-1 system provides protection against the pathological outcome of some of these RBC mutations, as demonstrated for sickle cell disease (Belcher et al., 2006).

In conclusion, we suggest that induction of the Nrf2/HO-1 system associated with sickle cell trait and probably with other often clinically silent genetic RBC defects might provide a general protective mechanism against *Plasmodium* infection in human populations. We propose that modulation of the Nrf2/HO-1 system might be used therapeutically to treat severe forms of malaria and in particular CM.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 *Hmox1*$^{+/-}$ mice were provided by Shaw-Fang Yet (Brigham and Women's Hospital, Boston)(Yet et al., 1999). C57BL/6 *Nrf2*$^{-/-}$ mice were provided by the RIKEN BioResource Center (Koyadai, Tsukuba, Ibaraki, Japan)(Itoh et al., 1997). C57BL/6 *Hb*^{SAD} mice were provided by Annie Henri (INSERM U733 IUH Hôpital Saint-Louis, Paris)(Trudel et al., 1991). C57BL/6.Sv129 *Hb*$^{A/A}$ mice were provided by Tim M. Townes and Tom M. Ryan (University of Alabama at Birmingham, USA)(Wu et al., 2006). *Hb*$^{A/a}$ mice expressing one copy of the human Hb chains (*Hb*^{A}) and one copy of the endogenous mouse Hb chains (*Hb*^{a}) were produced from C57BL/6.Sv129 *Hb*$^{A/A}$ x C57BL/6 *Hb*^{wt} mice (or C57BL/6 *Hb*$^{a/a}$) breeding. Mice were genotyped by PCR (*Hmox1* and *Nrf2*) and isoelectric focusing (*Hb*), as described elsewhere (Pamplona et al., 2007; Trudel et al., 1991). Experimental protocols were approved by the "Instituto Gulbenkian de Ciência animal care committee"

and by the "Direcção Geral de Veterenária (DGV)" of the Portuguese Ministry of Agriculture, Rural Development and Fisheries (License 018831-2010-09-03).

Bone Marrow Chimeras
Bone marrow chimeras were generated in *Hmox1*^{+/+}, *Hmox1*^{+/-} mice expressing or not the *Hb*[SAD] allele (8–10 weeks) as described (Seixas et al., 2009).

Parasites, Infection, and Disease assessment
Mice were infected with (GFP)-*P. berghei* ANKA (Pamplona et al., 2007), GFP-Luciferase *P. berghei* ANKA (MR4-866) or a (GFP)-*P. berghei* transgenic parasite expressing different MHC II and MHC I restricted epitopes including the MHC I-restricted epitope derived from glycoprotein B of herpes simplex virus-1 (gB[498-505])(Lundie et al., 2008), provided by William R. Heath (Walter and Eliza Hall, Melbourne, Victoria, Australia). Parasitemias were determined by flow cytometry (Pamplona et al., 2007). Infected mice were monitored twice daily for clinical symptoms of ECM.

Visualization and Quantification of Luciferase Activity
***P. berghei* ANKA Infected Mice**
Luciferase activity was visualized by imaging of dissected tissues using an electron multiplying-charge-coupled device (EM-CCD) photon-counting camera (ImagEM, Hamamatsu).

Protoporphyrins
Iron-protoporphyrin IX (FePPIX; heme) and zinc-protoporphyrin IX (ZnPPIX) were dissolved in 0.2 M NaOH, neutralized (pH 7.4) with 0.2 M HCl and administered (i.p.), as described (Pamplona et al., 2007).

CO Treatment
Mice were placed in a gastight 60 L capacity chamber and exposed continuously between days 4–7 postinfection to CO at a flow rate of ~12 L/min (final concentration of 250 parts per million; ppm), as described (Pamplona et al., 2007; Sato et al., 2001). CO concentration was monitored using a CO analyzer (Interscan Corporation, Chatsworth).

Histology
Brains were harvested, when clinical signs of ECM were noticed in control mice. Tissue was fixed in buffered 4% (vol/vol) paraformaldehyde and histological analysis was performed on perfusion-fixed tissues.

BBB Permeability
Mice were injected intravenously (i.v.) with 0.1 ml of 2% Evans Blue (Sigma) when clinical symptoms of ECM were noticed in control mice (Pamplona et al., 2007).

Analyzes of Splenic CD8^{+} T Cell Activation
Intracellular granzyme B staining were performed as described elsewhere (Lundie et al., 2008). Analyzes of CD8^{+} T cells recognizing the MHC I-restricted epitope gB[498-505] (SSIEFARL) from glycoprotein B of herpes simplex virus-1, was performed as described elsewhere (Lundie et al., 2008).

Leukocyte Brain Infiltration
Leukocytes were isolated from the brain of *P. berghei* ANKA infected mice when clinical symptoms of ECM were detectable in control groups. Brain leukocyte infiltration was quantified by flow cytometry (Pamplona et al., 2007).

Quantitative Real-Time Reverse Transcription PCR
Mice were sacrificed at day ECM onset in *Hb*[wt] mice. *Hmox1* mRNA was quantified by Quantitative real-time reverse transcription PCR (qRT-PCR) (Roche System)(Pamplona et al., 2007). TaqMan® Gene Signature Mouse Immune Array (Applied Biosystems) was used to quantify all other mRNAs (7900HT ABI system), according to manufactures recommendations.

Serum Biochemistry
Hematograms were measured by focused flow technology (Hemavet Multispecies Hematology System, HV950FS, Drew Scientific Inc., Centro Diagnóstivo Veterinário, Lisboa, Portugal). Plasma Hb was determined by spectroscopy at λ=577. Total plasma heme was measured using the 3,3′, 5,5′ tetramethylbenzidine (TMB) peroxidase assay (BD Biosciences), at λ=655 nm.

Statistical Analysis
Nonparametric Mann-Whitney *U* test was used to assess statistical significance between averages in different samples in which $n<5$. In samples with $n \geq 5$ the unpaired Student's *t*-test for unequal variances was used. Normal distributions were confirmed using the Kolmogorov-Smirnov test. Significant differences in survival were evaluated by the generation of Kaplan-Meier plots and by performing log-rank analysis for all experiments in which survival was assessed as an end-point. Statistical analysis for the progeny-expected ratios was performed using Pearson's chi-squared tests. *$P<0.05$ or **$P<0.01$ were considered statistically significant.

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