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Article

Metabolic Adaptation to Tissue Iron Overload Confers Tolerance to Malaria

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SUMMARY

Disease tolerance is a defense strategy that limits the fitness costs of infection irrespectively of pathogen burden. While restricting iron (Fe) availability to pathogens is perceived as a host defense strategy, the resulting tissue Fe overload can be cytotoxic and promote tissue damage to exacerbate disease severity. Examining this interplay during malaria, the disease caused by *Plasmodium* infection, we find that expression of the Fe sequestering protein ferritin H chain (FtH) in mice, and ferritin in humans, is associated with reduced tissue damage irrespectively of pathogen burden. FtH protection relies on its ferroxidase activity, which prevents labile Fe from sustaining proapoptotic c-Jun N-terminal kinase (JNK) activation. FtH expression is inhibited by JNK activation, promoting tissue Fe overload, tissue damage, and malaria severity. Mimicking FtH's antioxidant effect or inhibiting JNK activation pharmacologically confers therapeutic tolerance to malaria in mice. Thus, FtH provides metabolic adaptation to tissue Fe overload, conferring tolerance to malaria.

INTRODUCTION

There are two evolutionarily conserved defense strategies against infection that limit host disease severity. One relies on the capacity of the host's innate and adaptive immune system to reduce pathogen burden, i.e., resistance to infection. The salutary effects of this defense strategy are illustrated by the protective effect of vaccination against a wide range of infectious diseases. There is, however, another host defense strategy that limits disease severity irrespectively of pathogen burden, i.e., disease tolerance (Ayres and Schneider, 2012; Medzhitov et al., 2012; Schneider and Ayres, 2008). Revealed originally in plants and thereafter in flies, disease tolerance also operates in mammals, as demonstrated for *Plasmodium* (Råberg et al., 2007; Seixas et al., 2009) and polymicrobial (Larsen et al., 2010) infection in mice.

Once infected, mammals restrict Fe availability to pathogens, reducing Fe acquisition via gut epithelial cells while inhibiting cellular Fe export and sequestering intracellular Fe within different tissues (Ganz, 2009; Weiss, 2002). A major "trade-off" of this host defense strategy is tissue Fe overload, which can act in a pro-oxidant and cytotoxic manner to promote tissue damage and exacerbate disease severity. Presumably, this pathologic outcome is countered by a series of mechanisms providing host metabolic adaptation to tissue Fe overload. We hypothesized that these mechanisms involve the expression of ferritin H chain (*Fth*), a stress-responsive gene (Torti et al., 1988) that regulates Fe metabolism (Harrison and Arosio, 1996; Hentze and Kühn, 1996; Vanoaica et al., 2010).

FtH is an evolutionarily conserved Fe sequestering protein that acts in a cytoprotective manner (Balla et al., 1992; Berberat et al., 2003; Cozzi et al., 2003; Pham et al., 2004). This cytoprotective effect relies on the ferroxidase activity of FtH, which converts Fe(II) into Fe(III), hence limiting the participation of Fe(II) in the production of free radicals via the Fenton chemistry (Pham et al., 2004). The antioxidant effect of FtH inhibits c-Jun N-terminal kinase (JNK) activation in vitro through a mechanism targeting redox-sensitive phosphates and conferring cytoprotection (Chang et al., 2006; Kamata et al., 2005; Pham et al., 2004). We now demonstrate that inhibition of JNK activation by FtH confers tolerance to malaria, the disease caused by *Plasmodium* infection.

RESULTS

FtH Confers Tolerance to Malaria in Mice

Plasmodium chabaudi chabaudi (*Pcc*) infection, i.e., administration of *Pcc*-infected red blood cells (RBCs), was associated with the induction of FtH expression in the liver of C57BL/6 mice (Figure 1A), namely in hepatocytes (Figures 1A and S1A). FtH protein expression (Figure S1B) was associated with concomitant, albeit less pronounced, *Fth* mRNA expression (Figure S1C). Ferritin L chain (FtL) protein (Figure S1B) and mRNA (data not shown) expression were not induced in the liver of *Pcc*-infected

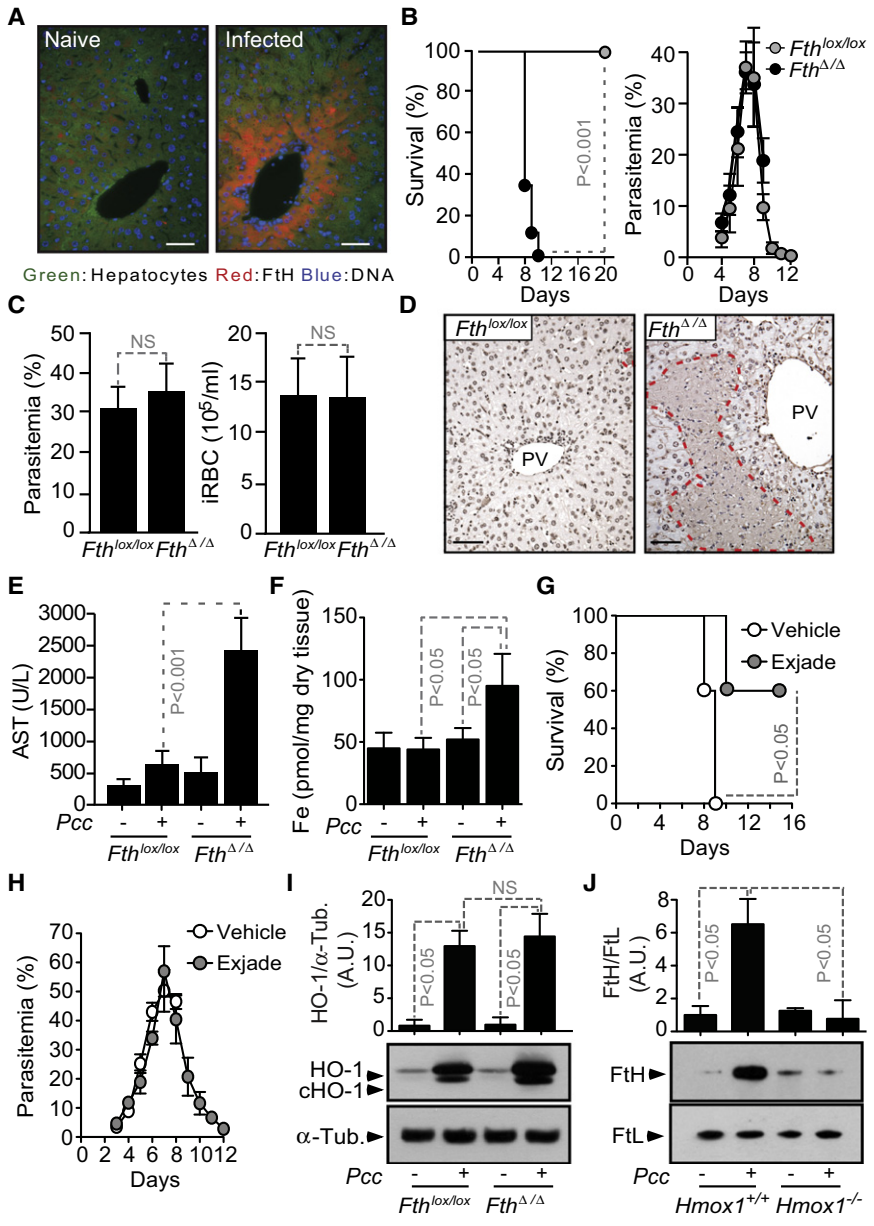

Figure 1. FtH Loss of Function Impairs Tolerance to *Plasmodium* Infection

(A) Expression of FtH detected by immunohistology in the liver of naive (not infected) or 7 days after *Pcc* infection in $Fth^{lox/lox}$ mice.

(B) Survival and mean parasitemia ± SD of *Pcc*-infected male $Fth^{lox/lox}$ (n = 6) and $Fth^{\Delta/\Delta}$ (n = 18) mice.

(C) Mean percentage (parasitemia) and number of infected RBC (iRBC) ± SD (n = 5 per group) in $Fth^{lox/lox}$ versus $Fth^{\Delta/\Delta}$ mice, 7 days post *Pcc* infection.

(D) PCD detected by TUNEL staining (brown) in the liver of mice, as in (C). PV: portal vein.

(E) Mean plasma AST concentration ± SD (n = 5/group) in mice, as in (C).

(F) Mean cellular labile Fe content per mg of dry tissue ± SD (n = 5 per group), in the liver of mice, as in (C).

(G and H) Survival (G) and mean parasitemia ± SD (H) of *Pcc*-infected $Fth^{\Delta/\Delta}$ mice receiving vehicle (PBS) (n = 5) or the Fe chelator Exjade (n = 5).

(I) HO-1 and α-tubulin (α-Tub.) expression in the liver of individual $Fth^{lox/lox}$ versus $Fth^{\Delta/\Delta}$ mice, detected by western blot before (–) or 7 days after (+) *Pcc* infection. Blot is representative of eight independent experiments, with similar trends quantified by densitometry, represented as mean arbitrary units (A.U.) of HO-1/α-tubulin ratio ± SD. cHO-1 indicates truncated, presumably nuclear HO-1 (Lin et al., 2007).

(J) FtH and FtL expression in the liver of individual C57BL/6 $Hmox\text{-}1^{+/+}$ and $Hmox\text{-}1^{-/-}$ mice, detected by western blot before (–) or 7 days after (+) *Pcc* infection. Blot is representative of four independent experiments, with similar trends quantified by densitometry and represented as mean arbitrary units (A.U.) of FtH/FtL ratio ± SD. See also Figure S1.

C57BL/6 mice. Moreover, there was no induction of FtH or FtL protein (Figure S1D) or mRNA (Figure S1E, data not shown) expression in heart, lung, kidney, or spleen of *Pcc*-infected C57BL/6 mice.

C57BL/6 $Fth^{lox/lox}$ mice, carrying a *Fth* allele flanked by *LoxP* sites and expressing physiologic levels of FtH (Figures S1F and S1G) (Darshan et al., 2009), cleared *Pcc* infection without overt lethality (Figure 1B). In contrast, C57BL/6 $Fth^{\Delta/\Delta}$ mice, in which the $Fth^{lox/lox}$ allele was deleted by the expression of Cre recombinase under the control of the *Mx1* promoter (Darshan et al., 2009; Kühn et al., 1995), did not express FtH in the liver (Figure S1F) and peripheral blood (Figure S1G), succumbing to *Pcc* infection (Figure 1B). Mortality of *Pcc*-infected $Fth^{\Delta/\Delta}$ mice was not associated with increased pathogen burden as compared to *Pcc*-infected $Fth^{lox/lox}$ mice (Figures 1B and 1C). *Pcc*-infected $Fth^{\Delta/\Delta}$ mice presented liver damage, revealed by widespread hepatocyte programmed cell death (PCD) (Figure 1D) and accumulation of aspartate aminotransferase (AST) in plasma (Figure 1E). This was associated with liver Fe overload (Figure 1F). Fe chelation by Deferasirox (Exjade) protected $Fth^{\Delta/\Delta}$ from infection (Figure 1G) without interfering with parasitemia (Figure 1H). This shows that FtH prevents the deleterious effects of tissue iron overload and confers tolerance to malaria in mice.

Expression of HO-1, a stress-responsive gene that confers tolerance to malaria in mice (Ferreira et al., 2008, 2011; Pamplona et al., 2007; Seixas et al., 2009), was similar in *Pcc*-infected $Fth^{lox/lox}$ versus $Fth^{\Delta/\Delta}$ mice, as assessed in the liver (Figure 1I). Expression of FtH, however, was not induced in the liver of *Pcc*-infected C57BL/6 *Hmox1*-deficient ($Hmox1^{-/-}$) mice (Figure 1J) that succumb to *Pcc* infection (Figure S1H) versus $Hmox1^{+/+}$ C57BL/6 mice that do not succumb to infection (Figure S1H). This suggests that HO-1 is required for FtH expression in response to *Pcc* infection and that the protective effect of HO-1 against *Pcc* infection (Seixas et al., 2009) acts via a mechanism involving FtH.

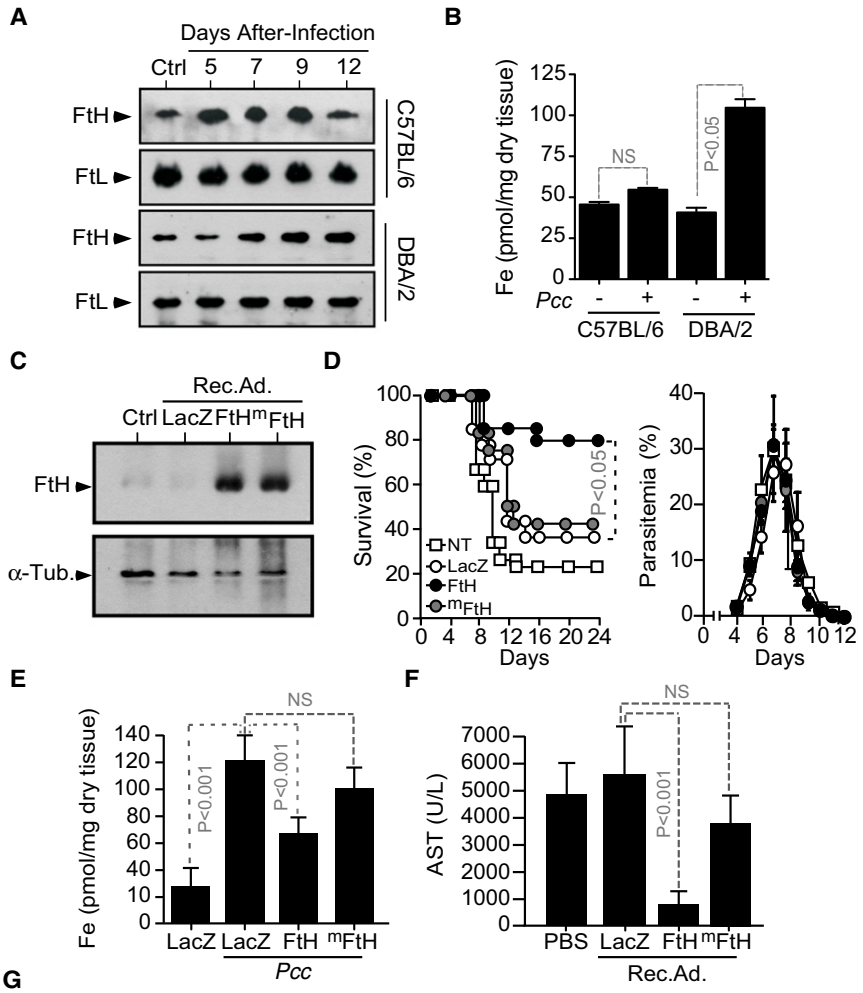

Figure 2. FtH Gain of Function Confers Tolerance to *Plasmodium* Infection

(A) FtH and FtL expression detected by western blot in the liver of individual DBA/2 versus C57BL/6 mice, not infected (Ctrl) or at the days indicated after *Pcc* infection. Blots are representative of five independent experiments with similar trend.

(B) Mean cellular labile Fe content per mg of dry tissue ± SD in the liver of C57BL/6 versus DBA/2 mice (n = 5 per group), noninfected (–), or *Pcc* infected (+).

(C) FtH and α-tubulin (α-Tub.) protein expression detected by western blot in the liver of individual DBA/2 mice 2 days after transduction or not (Ctrl) with LacZ, FtH, or ^{m}FtH Rec.Ad. Blots are representative of two independent experiments, with similar trends.

(D) Survival and mean parasitemia ± SD of *Pcc*-infected DBA/2 mice nontransduced (NT) (n = 12) or transduced with FtH (n = 20), ^{m}FtH (n = 12), or LacZ (n = 14) Rec.Ad.

(E) Mean cellular labile Fe content per mg of dry tissue ± SD in the liver of *Pcc*-infected DBA/2 mice, transduced with LacZ, FtH, or ^{m}FtH Rec.Ad. (n = 5 per group).

(F) Mean plasma AST concentration ± SD in mice, as in (E) (n = 5/group).

(G) PCD detected by TUNEL staining (brown) in liver of mice. Data in (E), (F), and (G) were pooled from three independent experiments, with similar results. Dotted red lines in (G) indicate areas of PCD. Images in (G) are 200× magnification, bar = 20 μm. Data in (B), (E), (F), and (G) are 7 days after *Pcc* infection. See also Figure S2.

Pcc infection is lethal in DBA/2 mice (Seixas et al., 2009), an outcome associated with delayed FtH mRNA (Figure S2A) and protein (Figure 2A) expression as well as with liver Fe overload (Figure 2B), as compared to C57BL/6 mice. There was no induction of FtL mRNA (Figure S2B) or protein (Figure 2A) expression in response to *Pcc* infection. Transduction of DBA/2 mice with a recombinant adenovirus (Rec.Ad.) encoding FtH (Figure 2C) was protective against *Pcc* infection (Figure 2D), irrespectively of pathogen load (Figure 2D and Figures S2C and S2D). Transduction with a Rec.Ad. encoding a mutated form of FtH (^{m}FtH) lacking ferroxidase activity (Broxmeyer et al., 1991; Lawson et al., 1989) failed to confer protection (Figures 2C and 2D). Transduction of DBA/2 mice with FtH but not ^{m}FtH Rec.Ad. prevented liver Fe overload (Figure 2E) as well as liver damage, revealed by decreased plasma AST concentration (Figure 2F) and hepatocyte PCD (Figure 2G). Transduction with FtH versus ^{m}FtH or LacZ Rec.Ad. was also protective against liver damage in *P. berghei* ANKA-infected DBA/2 (Figure S2E) or C57BL/6 (Figure S2F) mice (Adachi et al., 2001). This protective effect was not associated with modulation of parasitemia (Figures S2G and S2H), showing that the ferroxidase activity of FtH confers disease tolerance to malaria.

The Cytoprotective Effect of FtH Confers Tolerance to Malaria

Plasmodium infection is associated with accumulation of heme in plasma, as demonstrated in mice (Ferreira et al., 2011; Pamplona et al., 2007) as well as in humans (Andrade et al., 2010a). When released from hemoproteins, heme sensitizes hepatocytes to undergo PCD, causing liver damage and compromising host survival irrespectively of pathogen burden (Gozzelino et al., 2010; Larsen et al., 2010; Seixas et al., 2009). When transduced in vitro with FtH versus ^{m}FtH or LacZ Rec.Ad., primary mouse hepatocytes were protected against PCD induced by heme plus TNF (Figure 3A). This was associated with suppression of caspase-3 cleavage/activation in vitro (Figure 3B) as well as in vivo (Figure 3C), as assessed in the liver of

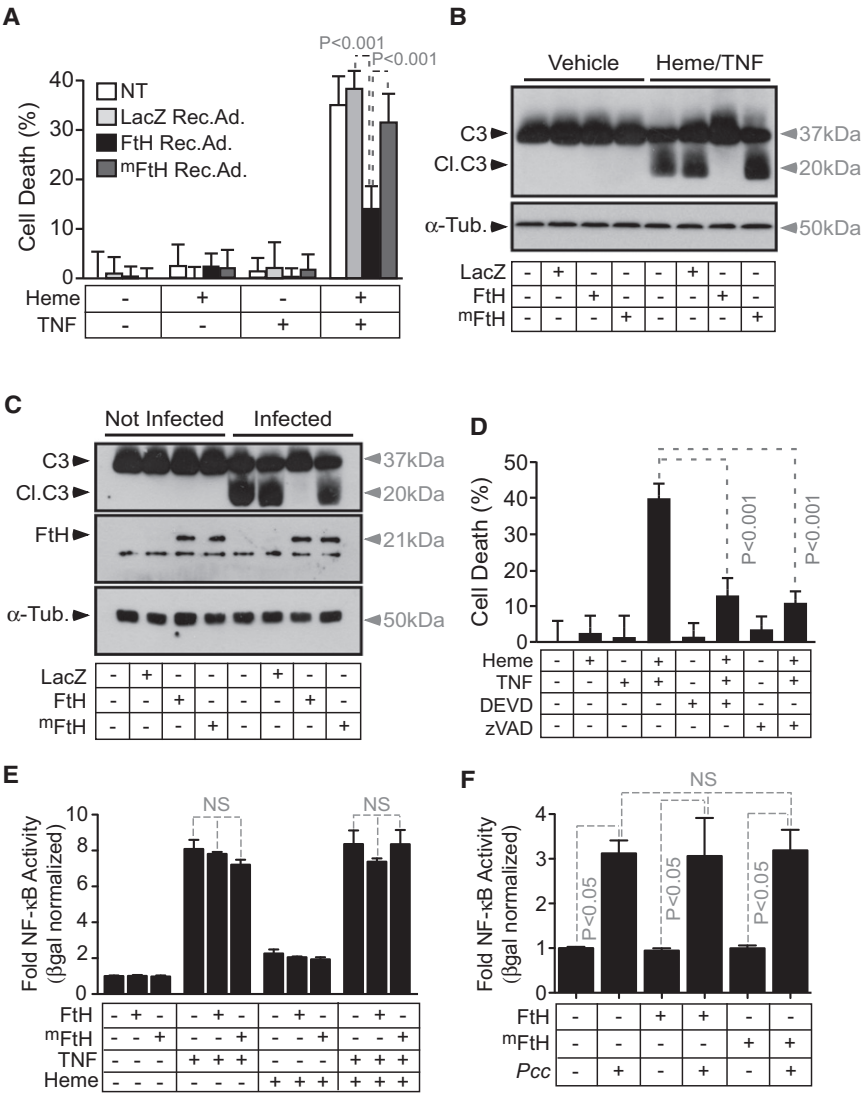

Figure 3. The Cytoprotective Effect of FtH against Free Heme Confers Tolerance to *Plasmodium* Infection

(A) Mean cytotoxicity ± SD in mouse primary hepatocytes transduced or not (NT) with FtH, ^{m}FtH, or LacZ Rec.Ad. and when indicated (+) exposed to heme (5 μM; 1 hr) plus TNF (5 ng/ml; 16 hr).

(B) Caspase-3 (C3), cleaved caspase-3 (Cl.C3), and α-tubulin (α-Tub.) detected by western blot in whole-cell extracts from Hepa 1-6 cells transduced as in (A) and when indicated (+) exposed to vehicle or heme (40 μM; 1 hr) plus TNF (50 ng/ml; 4 hr).

(C) Caspase-3 (C3), cleaved caspase-3 (Cl.C3), and α-tubulin (α-Tub.) detected by western blot in the liver of DBA/2 mice transduced or not (NT) with FtH, ^{m}FtH, or LacZ Rec.Ad., 7 days after *Pcc* infection.

(D) Mean cytotoxicity ± SD of primary hepatocytes treated as in (A) plus caspase-3 (DEVD) or pan-caspase (zVAD) inhibitor.

(E) Mean fold induction of NF-κB luciferase/β-galactosidase (β-gal) activity ± SD (n = 3) in primary hepatocytes from DBA/2 mice transduced with Rec.Ad. encoding NF-κB-luciferase and CMV-β-galactosidase reporters. When indicated, hepatocytes were cotransduced with FtH or ^{m}FtH Rec.Ad. and exposed to vehicle (–), heme (+), and/or TNF (+). Basal NF-κB luciferase/β-galactosidase activity (n = 1) was taken from untreated hepatocytes (–) not transduced with FtH or ^{m}FtH Rec.Ad (–).

(F) Mean fold induction of NF-κB luciferase/β-galactosidase activity ± SD in the liver of *Pcc*-infected DBA/2 mice (day 5; n = 5/group) transduced with Rec.Ad. encoding NF-κB-luciferase and CMV-β-galactosidase reporters. When indicated, mice were cotransduced with FtH or ^{m}FtH Rec.Ad. Basal NF-κB luciferase/β-galactosidase (β-gal) activity (n = 1) was taken from mice not infected (–) and not transduced with FtH or ^{m}FtH Rec.Ad (–). For each Rec.Ad. transduction, noninfected (NI) mice were considered at the basal level (n = 1). See also Figure S3.

DBA/2 mice transduced with FtH versus ^{m}FtH or LacZ Rec.Ad.. Pharmacologic caspase-3 (z-DEVD-fmk) or broad-caspase (z-VAD-fmk) inhibition protected primary hepatocytes from PCD in vitro (Figure 3D), showing that heme and TNF induce PCD via a mechanism involving caspase activation.

We asked whether the cytotoxic effect of heme acts via a mechanism involving nuclear factor kappa B (NF-κB), a transcription factor that regulates PCD (Beg and Baltimore, 1996). Heme did not modulate NF-κB activation in response to TNF, as assessed in vitro using a NF-κB-luciferase reporter assay in primary mouse hepatocytes (Figure 3E). Transduction with FtH versus ^{m}FtH or LacZ Rec.Ad. did not modulate NF-κB activation in response to heme and/or TNF (Figure 3E). The same was true for primary *Fth*$^{\Delta/\Delta}$ versus *Fth*$^{lox/lox}$ hepatocytes (Figure S3A). This shows that neither heme nor FtH modulate NF-κB activation in response to TNF in vitro.

Pcc infection was associated with NF-κB activation, as assessed in the liver of DBA/2 mice using an NF-κB-luciferase reporter assay (Figure 3F). NF-κB activity was induced to a similar extent in the liver of *Pcc*-infected DBA/2 versus C57BL/6 mice (Figure S3B). Transduction of DBA/2 mice with FtH Rec.Ad. failed to modulate NF-κB activation in response to *Pcc* infection, as compared to controls transduced with ^{m}FtH or LacZ Rec.Ad. (Figure 3F). This suggests that FtH does not modulate NF-κB activation during *Pcc* infection.

While transduction with a Rec.Ad. encoding the NF-κB inhibitor alpha (IκBα) (Figure S3C) sensitized hepatocytes to undergo PCD in response to TNF (Figure S3D) (Soares et al., 1998; Wang et al., 1996), as assessed at 24 hr (Figure S3D), it did not modulate the cytotoxic effect of heme and TNF, as assessed at 6 hr after heme and TNF exposure (Figure S3E). This suggests that heme and TNF induce PCD irrespectively of canonical NF-κB activation.

The Cytoprotective Effect of FtH Relies on Its Antioxidant Activity

The protective effect of FtH against *Pcc* infection was not associated with modulation of *Tnf* mRNA expression, as assessed

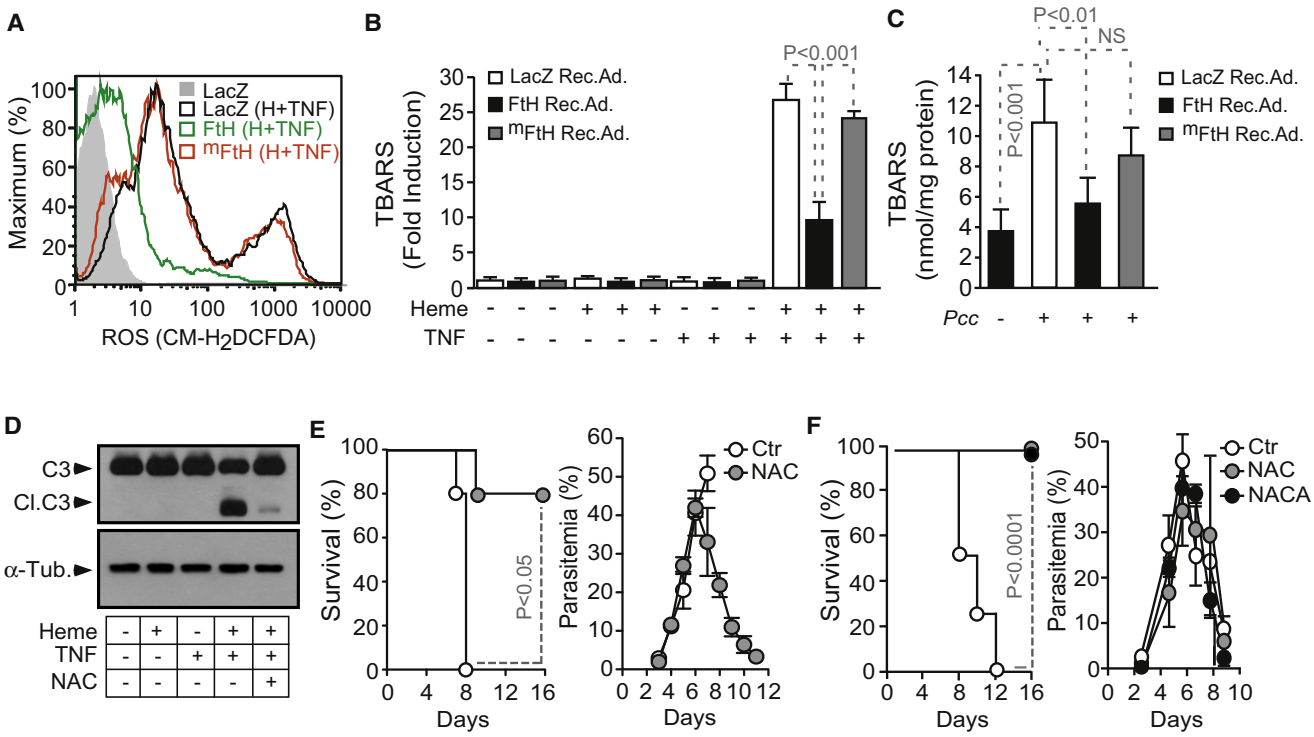

Figure 4. FtH Acts as an Antioxidant

(A) Intracellular free radicals were detected by flow cytometry using the broad-spectrum free radical probe CM-H_2DCFDA in Hepa 1-6 cells transduced with LacZ, FtH, or ^{m}FtH Rec.Ad. Cells were exposed to vehicle or heme (40 μM; 1 hr) plus TNF (50 ng/ml; 2 hr). Results are representative of two independent experiments.

(B) Mean induction of cellular concentration of thiobarbituric acid reactive substances (TBARS) ± SD (n = 5) in Hepa 1-6 cells transduced with LacZ, FtH, or ^{m}FtH Rec.Ad. Cells were exposed to heme (40 μM; 1 hr) and/or TNF (50 ng/ml; 4 hr). Basal TBARS levels were measured in Hepa 1-6 cells not exposed to heme (−) or TNF (−). Data from one out of two independent experiments are shown.

(C) Mean concentration of TBARS ± SD (n = 6) in the liver of DBA/2 mice transduced with LacZ, FtH, or ^{m}FtH Rec.Ad. and either not infected (−) or 7 days after *Pcc* infection. Data are pooled from three independent experiments with similar trend.

(D) Caspase-3 (C3), cleaved caspase-3 (Cl.C3), and α-tubulin (α-Tub.) detected by western blot in Hepa 1-6 cells exposed to heme and/or TNF as in (B) with or without N-acetylcysteine (NAC; 10 mM; 4 hr before heme and maintained thereafter). Blots are representative of two independent experiments.

(E) Survival and mean parasitemia ± SD of *Pcc*-infected *Fth*$^{\Delta/\Delta}$ mice receiving vehicle (PBS; n = 5) or NAC (n = 5).

(F) Survival and mean parasitemia ± SD of *Pcc*-infected DBA/2 mice receiving vehicle (PBS; n = 5), NAC (n = 5), or NACA (n = 5). NAC and NACA were administered at 15 mg/kg every 12 hr, i.p., from days 4 to 15 postinfection. See also Figure S4.

by qRT-PCR in the liver of *Fth*$^{\Delta/\Delta}$ versus *Fth*$^{lox/lox}$ mice (Figure S4A) or in the liver of DBA/2 mice transduced 2 days before *Pcc* infection, with FtH versus ^{m}FtH or LacZ Rec.Ad. (Figure S4B). Concentration of TNF (Figure S4C), total heme (Figure S4D), or heme not bound to hemoglobin (Figure S4E) was also similar in the plasma of *Pcc*-infected DBA/2 mice transduced with FtH versus ^{m}FtH or LacZ Rec.Ad. This suggests that it is the cytoprotective effect FtH that confers tolerance to malaria.

Transduction of hepatocytes in vitro with an FtH Rec.Ad. reduced the accumulation of free radicals (Figure 4A) and lipid peroxidation (Figure 4B) in response to heme plus TNF, as compared to control hepatocytes transduced with ^{m}FtH or LacZ Rec.Ad. This was also observed in vivo, in the liver of *Pcc*-infected DBA/2 mice transduced with FtH versus ^{m}FtH or LacZ Rec.Ad. (Figure 4C). The glutathione precursor N-acetylcysteine (NAC) suppressed caspase-3 activation in hepatocytes exposed in vitro to heme plus TNF (Figure 4D), in keeping with its cytoprotective effect (Seixas et al., 2009). When used at a dosage (15 mg/kg) 10× lower than previously described (Seixas et al., 2009), NAC protected *Fth*$^{\Delta/\Delta}$ mice from *Pcc* infection without interfering with parasitemia (Figure 4E). Moreover, NAC as well as N-acetylcysteine amide (NACA) protected DBA/2 mice from *Pcc* infection without interfering with parasitemia (Figure 4F). These observations link the protective effect of FtH with its antioxidant activity.

Ferritin Expression Is Associated with Reduced Liver Damage in Human Malaria

Similar to mice (Figures 1B, 1D, 1E, S2E, and S2F), individuals developing symptomatic forms of malaria in response to *Plasmodium vivax* infection presented varying levels of hepatic dysfunction/damage, as quantified according to AST, C reactive protein (CRP), and total bilirubin concentration in plasma (Figure 5; Table S1) (Andrade et al., 2010c) (see Supplemental Information). We have previously shown that these individuals have higher concentrations of non-hemoglobin-bound (free) heme and TNF in the plasma, as compared to noninfected individuals (Andrade et al., 2010a; Mendonça et al., 2012). Higher concentrations of free heme (Figure 5A) and TNF

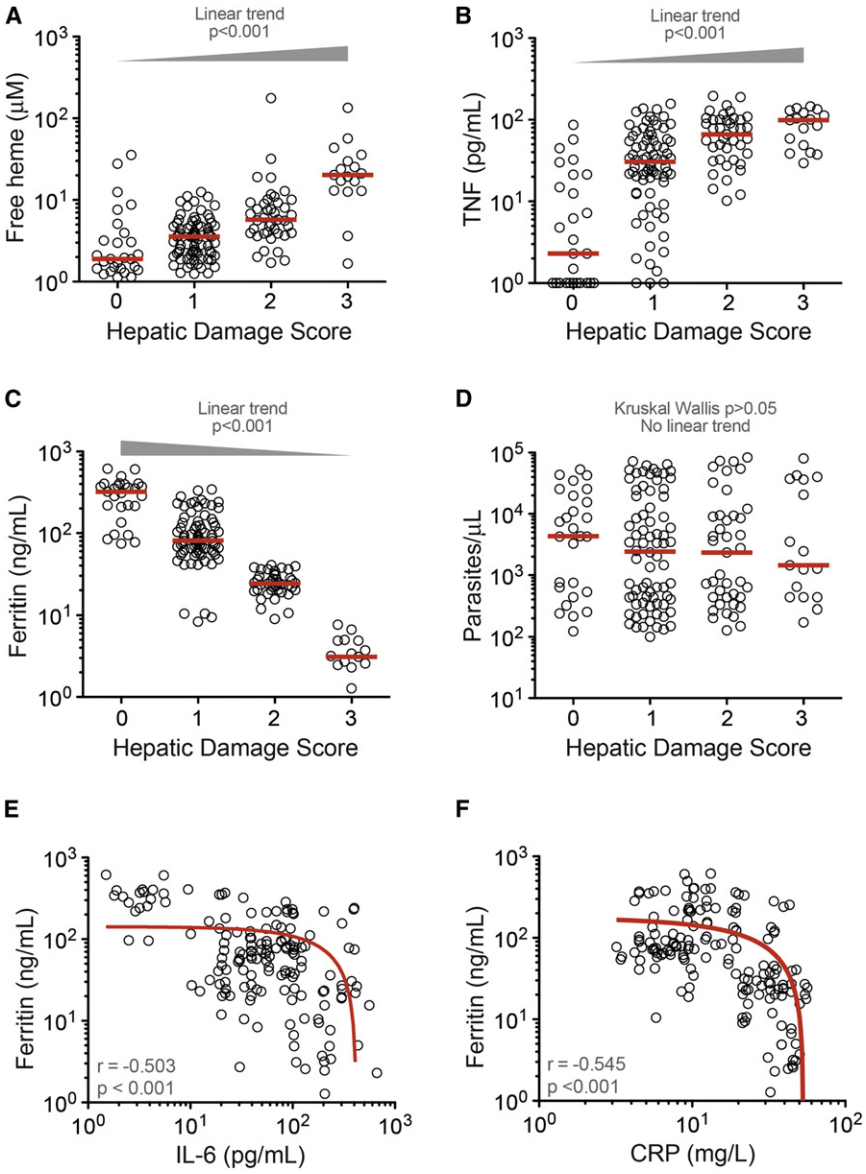

Figure 5. Tolerance to Human Malaria

(A–C) Concentration of non-hemoglobin-bound heme (free) (A), heme TNF (B), and ferritin (C) in plasma, plotted against hepatic damage score in individuals (N = 161) developing symptomatic forms of malaria in response to *Plasmodium vivax* infection.

(D–F) Number of parasites in the blood plotted against hepatic damage score in the same individuals as (A), (B), and (C). Ferritin concentration plotted against IL-6 (E) and CRP (F) concentrations in the same individuals as (A), (B), (C), and (D). Red lines in (E) and (F) represent curve fit for nonparametric linear regression. Circles represent individual values and red bars median values. See also Figure S5 and Table S1.

(Figure 5B) in the plasma of individuals developing symptomatic forms of malaria were associated with increased hepatic dysfunction/damage.

Ferritin concentration in plasma was higher in individuals infected with *Plasmodium vivax* versus noninfected individuals (median and interquartile range: malaria patients 64.77, 23.58–135 ng/ml, n = 161; noninfected donors 26.5, 16.5–40 ng/ml, n = 30, respectively; $p < 0.001$). However, among individuals developing symptomatic forms of malaria, higher ferritin concentrations in plasma were associated with reduced severity of liver dysfunction/damage (Figure 5C). There was no correlation between severity of liver dysfunction/damage and pathogen load, as determined by number of parasites in blood (Figure 5D). Higher concentrations of IL-6 and CRP in plasma were associated with lower ferritin concentrations (Figures 5E and 5F). This suggests that ferritin confers tolerance to malaria in humans and that unfettered inflammation, i.e., high IL-6 and CRP, is associated with decreased ferritin expression.

Transferrin saturation (TSAT; i.e., ratio of serum iron and total iron-binding capacity) was increased in individuals infected with *Plasmodium vivax*, as compared to noninfected individuals (median and interquartile range: malaria patients 33, 23%–50%, n = 161; noninfected donors 20.0, 18.0%–35.75%, n = 30, respectively; $p < 0.001$). In contrast to ferritin, higher TSAT in individuals developing symptomatic forms of malaria was associated with increased severity of liver dysfunction/damage (Figure S5A), irrespectively of pathogen burden (Figure S5B), which is consistent with previous studies (Das et al., 1997). Higher concentrations of IL-6 (Figure S5C), CRP (Figure S5D), and heme (Figure S5E) in plasma were associated with increased TSAT. Moreover, there was an inverse correlation between TSAT and ferritin concentration in plasma (Figure S5F), suggesting that unfettered inflammation dissociates ferritin expression from the iron status of individuals developing symptomatic forms of malaria.

FtH Inhibits JNK Activation during *Plasmodium* Infection

JNK was activated in a sustained manner in hepatocytes exposed in vitro to heme plus TNF (Figure 6A). A similar effect was observed in vivo, in the liver of *Pcc*-infected DBA/2 mice (Figure S6A). Inhibition of JNK1/2 expression using an shRNA Rec.Ad. protected primary mouse hepatocytes from undergoing PCD in vitro (Figure 6B), suppressing caspase-3 activation (Figure S6B). A similar effect was obtained when JNK1/2 activation was inhibited pharmacologically (data not shown). Transduction of hepatocytes in vitro with an FtH Rec.Ad. suppressed JNK1/2 activation in response to heme plus TNF, as compared to controls transduced with ^{m}FtH or LacZ Rec.Ad. (Figure 6C). A similar effect was observed in vivo, in the liver of *Pcc*-infected DBA/2 mice transduced with FtH versus ^{m}FtH or

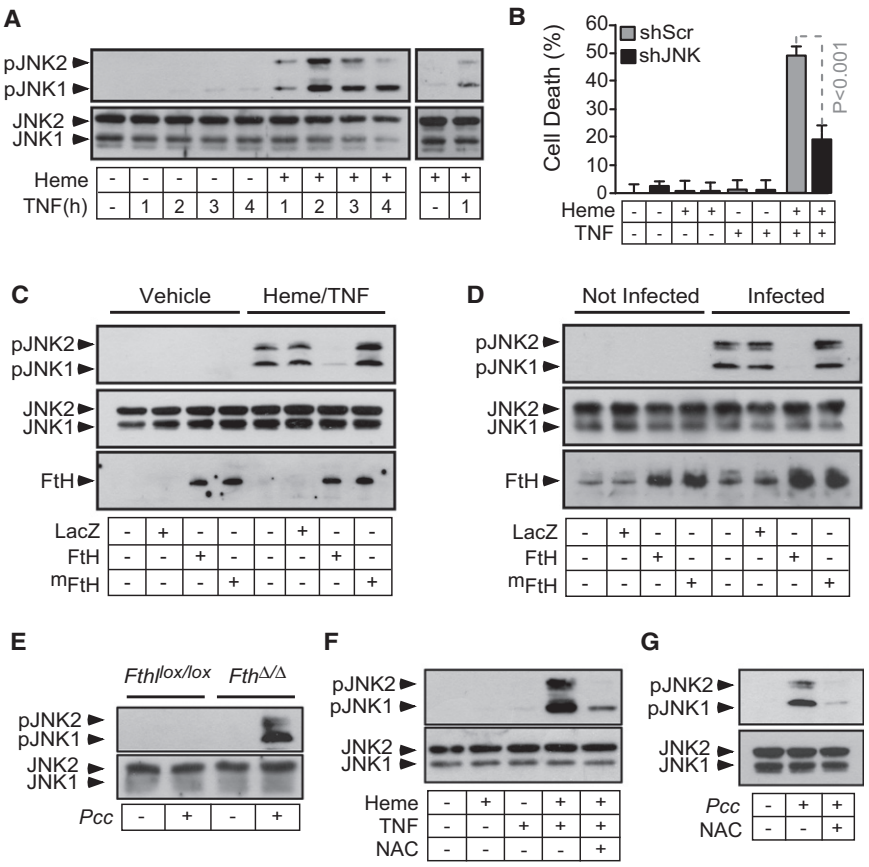

Figure 6. FtH Inhibits JNK Activation

(A) Phosphorylated (pJNK1/2) and total JNK1/2 detected by western blot in whole-cell extracts from Hepa 1-6 cells untreated (–) or exposed to heme (+) and/or TNF (+) for the indicated time (hr).

(B) Mean cytotoxicity ± SD in Hepa 1-6 cells transduced with Rec.Ad. and exposed to heme (+) or heme plus TNF (+) (n = 6). Data are from one out of two independent experiments, with similar trend.

(C) pJNK1/2, total JNK1/2, and FtH detected as in (A) in Hepa 1-6 cells transduced (+) or not (–) with FtH, ^{m}FtH, or LacZ Rec.Ad., treated as in (A).

(D and E) Same proteins detected as in (C) in liver extracts from (D) DBA/2 mice transduced (+) or not (–) with Rec.Ad. or (E) *Fth*$^{lox/lox}$ and *Fth*$^{\Delta/\Delta}$ mice not infected versus 7 days after *Pcc* infection.

(F) Proteins detected in Hepa 1-6 cells treated as in (A) with (+) or without (–) the antioxidant NAC.

(G) Proteins detected as in (A) in liver extracts from DBA/2 mice not infected (–) or 7 days after *Pcc* infection (+), treated (+) or not (–) with NAC. Blots are representative of at least two independent experiments, with similar trend. See also Figure S6.

LacZ Rec.Ad. (Figure 6D). Deletion of the *Fth* allele was also associated with increased JNK1/2 activation in the liver of *Pcc*-infected *Fth*$^{\Delta/\Delta}$ versus *Fth*$^{lox/lox}$ mice (Figure 6E). NAC inhibited JNK1/2 activation in hepatocytes in vitro (Figure 6F) as well as in vivo in the liver of *Pcc*-infected DBA/2 mice (Figure 6G). This suggests that the antioxidant effect of FtH inhibits JNK1/2 activation in vitro as well as in vivo during *Plasmodium* infection.

JNK and FtH Crossregulate Each Other to Control Tolerance to Malaria

In keeping with previous findings suggesting that JNK contributes to the pathogenesis of severe malaria in mice (Lu et al., 2006), we asked whether sustained JNK activation promotes liver damage associated with *Plasmodium* infection. JNK1/2 expression and activity were inhibited in the liver of DBA/2 mice by transduction, 3 days before *Pcc* infection, of shRNA Rec.Ad. targeting JNK1/2 (shJNK) versus a control scrambled (shScr) Rec.Ad. (Figures S6C and S6D). JNK inhibition was protective against *Pcc* infection, revealed by reduced lethality (Figure 7A). This was associated with inhibition of liver damage, revealed by reduced AST concentration in plasma (Figure 7B) as well as caspase-3 activation (Figure 7C) and PCD (Figure S6E) in the liver. This was not associated with modulation of pathogen burden (Figures 7A, S7A, and S7B) or modulation of TNF (Figure S7C) and heme (Figures S7D and S7E) concentration in plasma. This suggests that FtH confers tolerance to malaria via a mechanism involving JNK inhibition.

Pharmacologic inhibition of JNK activation using two distinct molecules, i.e., JNKi.SP (Figure 7D) or JNKi (data not shown), protected DBA/2 mice from *Pcc* infection without interfering with parasitemia (Figure 7D). This shows that JNK inhibition can act therapeutically to confer tolerance to malaria.

Transduction of hepatocytes in vitro with an shRNA Rec.Ad. targeting JNK1/2 was associated with induction of FtH and FtL protein (Figure S7F) as well as *Fth* mRNA (data not shown) expression in response to heme and TNF, as compared to control hepatocytes transduced with shScr Rec.Ad. A similar effect was observed for FtL protein (Figure S7F), but not for *Ftl* mRNA (data not shown) expression. This is consistent with the notion that JNK can inhibit ferritin expression in vitro (Antosiewicz et al., 2007).

Inhibition of JNK expression in vivo was associated with increased FtH and FtL protein (Figure 7E) as well as *Fth* and *Ftl* mRNA (data not shown) expression, as assessed in the liver of *Pcc*-infected DBA/2 mice, transduced 3 days before infection with shJNK versus shScr Rec.Ad. (Figure 7E, data not shown). Induction of FtH expression in DBA/2 mice transduced with shJNK versus shScr Rec.Ad. was associated with a reduction of liver Fe overload (Figure S7G). This suggests that JNK controls liver Fe overload during *Pcc* infection via a mechanism targeting the expression of FtH.

Pharmacologic JNK inhibition suppressed the accumulation of free radicals in hepatocytes exposed to heme plus TNF in vitro (Figure S7H), suggesting that JNK activation acts in pro-oxidant manner via downregulation of FtH expression (Figure S7F). Pharmacologic JNK inhibition failed to protect *Fth*$^{\Delta/\Delta}$ mice from *Pcc* infection (Figure 7F) or to protect primary *Fth*$^{\Delta/\Delta}$ hepatocytes from undergoing PCD in response to heme and

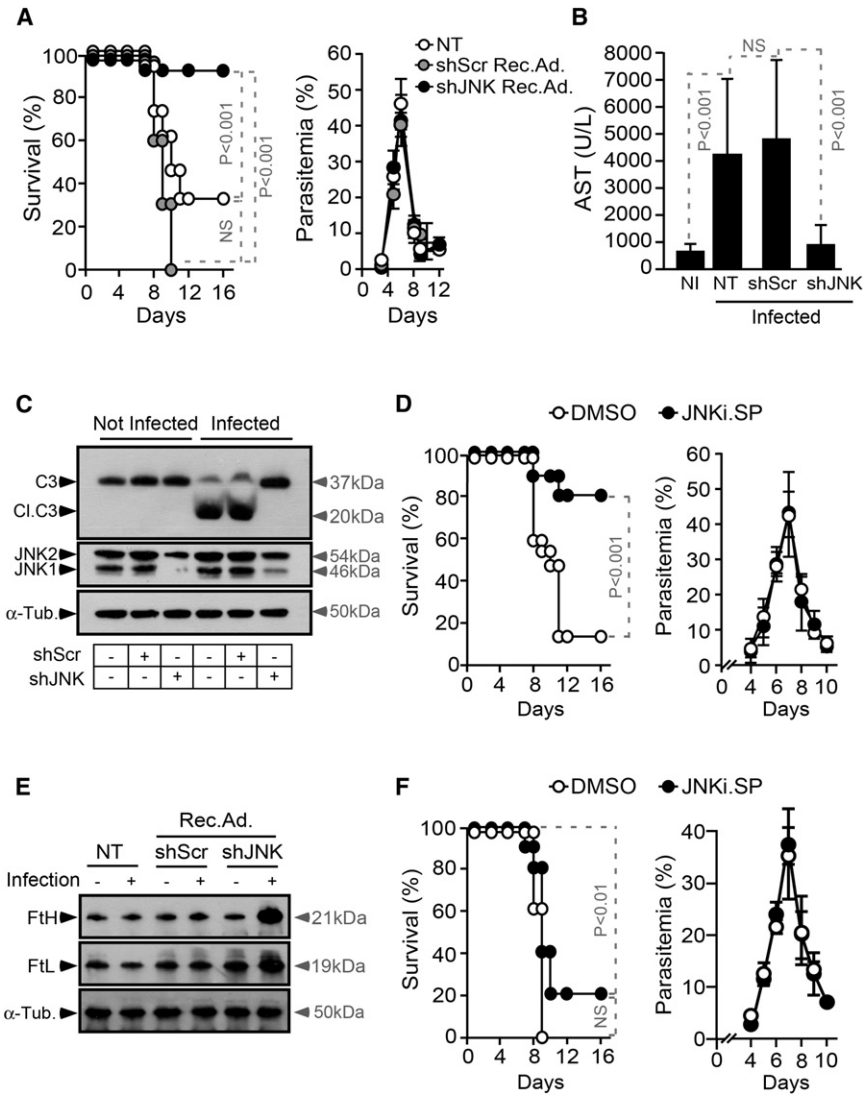

Figure 7. Functional Crosstalk between FtH and JNK Regulates Tolerance to Malaria

(A) Survival and mean parasitemia ± SD of *Pcc*-infected DBA/2 mice, not transduced (NT) (n = 15) or transduced with shJNK (n = 18) or control scrambled shRNA (shScr; n = 10) Rec.Ad.

(B) Mean plasma AST concentration ± SD in mice (n = 5/group) treated as in (A), 7 days after *Pcc* infection.

(C) Caspase-3 (C3), cleaved caspase-3 (Cl.C3), JNK1/2, and α-tubulin (α-Tub.), detected by western blot in the liver of mice treated as in (A).

(D) Survival and mean parasitemia ± SD of *Pcc*-infected DBA/2 mice receiving vehicle (DMSO in PBS) (n = 10) or JNK inhibitor SP600125 (JNKi.SP) (n = 10).

(E) FtH, FtL, and α-Tub. expression detected by western blot, 7 days after *Pcc* infection in the liver of mice treated as in (A).

(F) Survival and mean parasitemia ± SD of *Pcc*-infected *Fth*$^{\Delta/\Delta}$ C57BL/6 mice (n = 10 per group) treated as in (D). Data in (A), (B), (D), and (F) were pooled from three, four, two, and two independent experiments, respectively. Blots in (C) and (E) are representative of two independent experiments, with similar trend. See also Figure S7.

TNF in vitro (Figure S7I). This suggests that (1) the cytotoxic effect of JNK acts in vitro and in vivo via a mechanism involving the downregulation FtH expression and (2) the protective effect of JNK inhibition is dependent on the expression of FtH.

Given that NF-κB can regulate FtH expression (Kwak et al., 1995; Pham et al., 2004), we asked whether JNK regulates FtH expression via a mechanism involving NF-κB. Transduction of primary mouse hepatocytes with shJNK versus shScr Rec.Ad. did not modulate NF-κB activation in response to heme and/or TNF, as assessed in vitro using an NF-κB luciferase assay (Figure S7J). This suggests that JNK regulates FtH expression via a mechanism that does not involve NF-κB.

DISCUSSION

Resistance to infection, such as afforded by vaccination, confers protection against a broad range of infectious diseases. Given its overwhelming success, it has been difficult to understand why vaccination has so far failed to provide robust protection against specific infectious diseases such as malaria (Agnandji et al., 2011). This may be explained by the recognition that protection against malaria relies on an additional host defense strategy that does not target directly the pathogen, i.e., disease tolerance (Medzhitov et al., 2012; Råberg et al., 2007; Seixas et al., 2009).

Life-threatening complications of malaria are driven by multiorgan dysfunction/damage associated with unfettered inflammation (Miller et al., 2002). Heme accumulation in the plasma of individuals developing symptomatic forms of malaria (Andrade et al., 2010a) acts in a cytotoxic manner (Gozzelino et al., 2010; Seixas et al., 2009), contributing in a critical manner to the pathogenesis of severe forms of malaria in mice (Ferreira et al., 2008, 2011; Pamplona et al., 2007; Seixas et al., 2009). In keeping with this notion, expression of stress-responsive genes that counter the deleterious effects of heme, e.g., the heme catabolizing enzyme HO-1, provide tissue damage control and confer tolerance to malaria in mice (Ferreira et al., 2011; Pamplona et al., 2007; Seixas et al., 2009; Soares et al., 2009). The pathophysiologic relevance of this host protective response is supported by the finding that sickle trait, selected through human evolution based on its ability to confer protection against malaria, acts via activation of this stress-responsive pathway to confer disease tolerance to malaria (Ferreira et al., 2011; Rosenthal, 2011). Whether the protective effect of HO-1 impacts on the outcome of human malaria is not clear (Mendonça et al., 2012; Sambo et al., 2010; Walther et al., 2012).

As demonstrated hereby, the mechanism underlying tolerance to malaria involves another stress-responsive gene, namely

FtH, a Fe-sequestering protein that regulates Fe metabolism (Darshan et al., 2009; Harrison and Arosio, 1996; Hentze et al., 1987), limiting the cytotoxic effect of labile Fe (Balla et al., 1992; Berberat et al., 2003; Cozzi et al., 2003; Pham et al., 2004). Expression of FtH is induced in response to *Plasmodium* infection in mice (Figures 1A, 2A, and S1A–S1C) as well as in humans (Das et al., 1997). Presumably, the mechanism regulating the induction of FtH expression during malaria acts at a posttranscriptional level, driven by Fe generated through heme catabolism by HO-1 (Figure 1J) (Berberat et al., 2003; Eisenstein et al., 1991). Whether induction of FtH correlates with a threshold of parasitemia is not clear.

The salutary effect of FtH against *Plasmodium* infection acts irrespectively of pathogen load, conferring disease tolerance to malaria in mice (Figures 1 and 2) and presumably in humans (Figure 5). This suggests that individuals failing to upregulate the expression of FtH might be at higher risk of developing severe forms of malaria.

The cytoprotective effect of FtH relies on its ferroxidase activity (Figures 2 and 3) (Balla et al., 1992; Berberat et al., 2003; Cozzi et al., 2003; Pham et al., 2004), which limits the participation of labile Fe in the Fenton chemistry, thus inhibiting the production of free radicals (Figure 4). This in turn inhibits sustained JNK activation, as demonstrated in vitro (Figure 6C) as well as in vivo (Figures 6D and 6E). The salutary effects of FtH are mimicked by pharmacologic antioxidants such as NAC or NACA (Figures 4E and 4F). These inhibit JNK activation in vitro (Figure 6F) as well as in vivo (Figure 6G), conferring tolerance to malaria in mice (Figures 4E and 4F).

Sustained JNK activation, catalyzed by labile Fe produced during *Plasmodium* infection, plays a central role in the pathogenesis of severe malaria in mice (Figure 7). This notion is in keeping with the previous observation that deletion of the *Jnk2* allele confers protection against experimental cerebral malaria in mice (Lu et al., 2006), without interfering with pathogen load (Figures 7A, 7D, S7A, and S7B) (Lu et al., 2006). This suggests that targeting JNK activation might be used therapeutically to confer tolerance to malaria, which we found to be the case in mice (Figure 7D).

The mechanism via which JNK activation compromises tolerance to malaria involves the inhibition of FtH expression (Figures 7E, 7F, and S7F), leading to tissue Fe overload (Figure S7G), accumulation of free radicals (Figure S7H), cytotoxicity (Figures 6B, 7C, and S6B), tissue damage (Figures 7B and S6E), and ultimately host mortality (Figures 7A and 7D). We propose that induction of FtH expression during *Plasmodium* infection prevents pro-oxidant labile Fe from sustaining JNK activation, presumably via a mechanism involving the inhibition of redox-sensitive phosphatases regulating directly JNK activity (Chang et al., 2006; Kamata et al., 2005; Pham et al., 2004). Induction of FtH expression requires the expression of HO-1 (Figure 1J), thus suggesting that FtH contributes in a critical manner to the salutary effect of HO-1 against malaria (Figure S1H) (Seixas et al., 2009; Soares et al., 2009).

In conclusion, the pathogenesis of life-threatening complications of malaria is regulated by a functional crosstalk between FtH and JNK that controls host metabolic adaptation to tissue Fe overload during *Plasmodium* infection. FtH promotes tolerance to malaria, via inhibition of JNK activation, while JNK activation promotes the pathogenesis of life-threatening complications of malaria via inhibition of FtH expression. Whether FtH promotes tolerance to other types infection is likely to be the case, but this remains to be established.

EXPERIMENTAL PROCEDURES

Mice

Mice were bred and maintained under specific pathogen-free (SPF) conditions, according to protocols approved by local (Instituto Gulbenkian de Ciência) and national (Portuguese Official Veterinary Department; Direcção Geral de Veterinária) ethics committees. C57BL/6 $Fth^{lox/lox}$ mice (Darshan et al., 2009) were obtained originally from Lukas Kühn (Ecole Polytechnique Fédérale de Lausanne, Faculté des Sciences de la Vie, Institut Suisse de Recherche Expérimentale sur le Cancer).

Conditional Gene Deletion (Δ)

C57BL/6 $Fth^{lox/lox}$ mice were crossed with transgenic C57BL/6 Mx-Cre mice expressing the Cre recombinase under the control of the ubiquitous poly(I:C)-inducible Mx1 promoter (Darshan et al., 2009; Kühn et al., 1995).

Infections

Mice were infected intraperitoneally (i.p.) with 10^7 *Plasmodium chabaudi chabaudi* (*Pcc*) AS strain or 10^6 *Plasmodium berghei* ANKA-infected RBCs, referred to throughout the text as infection. Survival and parasitemia were monitored daily as described (Pamplona et al., 2007; Seixas et al., 2009).

Primary Mouse Hepatocytes and Cytotoxicity Assay

Primary mouse hepatocytes were isolated essentially as described (Gonçalves et al., 2007; Seixas et al., 2009), and cytotoxicity was assessed as described (Gozzelino et al., 2010; Larsen et al., 2010; Seixas et al., 2009).

Cell Culture and Reagents

Hepa 1-6 cells (C57BL/6 mouse liver hepatoma; ATCC, Barcelona, Spain) were cultured in DMEM (Invitrogen, Barcelona, Spain), 10% FCS, penicillin (20 U/ml), and streptomycin (20 U/ml) (Invitrogen) (37°C, 95% humidity, 5% CO_2), as described (Larsen et al., 2010; Seixas et al., 2009). Ammonium iron(II) sulfate hexahydrate $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and N-acetylcysteine (NAC) (Sigma, Sintra, Portugal), N-acetylcysteine amide (NACA) (provided by Dr. Glenn Goldstein, David Pharmaceuticals, New York), Exjade (Deferasirox, Novartis), mouse recombinant TNF (R&D Systems, Lisbon), heme (hemin, Frontier Scientific, Lancashire, UK), z-VAD-fmk and z-DEVD-fmk (MP Biomedicals, Porto, Portugal), SP600125 (LC Laboratories, Woburn, MA), JNK Inhibitor I (Alexis, Lausanne, Switzerland), and 5- (and 6-)chloromethyl-2′7′-dichlorodihy, drofluorescein diacetate acetyl ester (CM-H_2DCFDA) (Invitrogen) were used as indicated.

In Vivo Treatments

NAC and NACA were dissolved in PBS, adjusted to pH 7.4, and administered to DBA/2 mice (15 mg/kg i.p.) starting at day 4 postinfection and every 12 hr thereafter until day 15. The same dosage and schedule protocol was used in $Fth^{\Delta/\Delta}$ mice. JNKi.SP was dissolved in DMSO and administered to DBA/2 mice (2.5 mg/kg in DMSO 0.2% v/v, i.p., once a day) starting at day 4 postinfection until day 15. Exjade (Deferasirox, Novartis) was dissolved in PBS and administered to $Fth^{\Delta/\Delta}$ mice (10 mg/kg by gavage, once a day) starting at day 3 postinfection until day 15.

Recombinant Adenovirus

Generation of FtH and ^{m}FtH Rec.Ad. were previously described (Berberat et al., 2003). Rec.Ad. encoding a mutated form of IκBα (IκBα S32A/S36A) (Vector Biolabs, http://www.vectorbiolabs.com), β-galactosidase (LacZ) Rec.Ad. (a kind gift from Dr. R. Gerard, University of Texas Southwest Medical Center, Dallas, TX), and NF-κB luciferase reporter (*Photinus pyralis* luciferase gene under the control of a synthetic promoter containing five binding motifs for NF-κB [5′-GGGGACTTTCC-3′; NF-κB-Luc] [Vector Biolabs]) were previously described (Silva et al., 2009). Oligonucleotides used to construct a shRNA targeting JNK were previously described (Gururajan et al., 2005).

shJNK Rec.Ad and a Rec.Ad. encoding a control scrambled shRNA (shScr Rec.Ad.) were produced by Vector Biolabs.

Histopathology

Mice were perfused in toto with ice-cold PBS. Liver samples were harvested, fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and stained with hematoxylin and eosin as described (Pamplona et al., 2007). Detection of fragmented DNA in liver was carried out using the TUNEL method (In Situ Cell Death Detection Kit, Roche). FtH staining was performed as follows: Liver samples were collected in Tissue-Tek (Sakura), snap frozen in liquid nitrogen, and kept at −80°C until used. Liver samples were fixed in 1% PFA (15 min) and permeabilized with Triton X-100 (10 min), blocked with 5% goat serum and 1% BSA (60 min), and incubated with rabbit-anti-mouse FtH (1:100 dilution, overnight at 4°C) diluted in the same solution. Goat-anti-rabbit-Cy5 (1:300 dilution, 60 min) was used as a secondary antibody. DAPI reagent (15 min) was employed as nuclear stain. Cell autofluorescence (green; excitation λ = 480 nm, emission λ = 505) was used to detect hepatocyte morphology.

Human Samples

Plasma was collected between 2006 and 2007 from symptomatic individuals (N = 161) infected with *Plasmodium vivax* in Buritis, Rondônia, Brazil, as described before (Andrade et al., 2010a, 2010b, 2010c, 2011). Malaria diagnosis was performed using microscopic examination of thick smears and confirmed by qualitative nested PCR, as described (Andrade et al., 2009). Parasitemia was expressed as parasites/μl. Plasma samples from 30 age- and sex-matched healthy and noninfected individuals from the same malaria endemic area were used to compare ferritin and transferrin saturation levels with the cohort of individuals infected with *Plasmodium vivax*.

Hepatic Damage Score

Aspartate transaminase (AST), total bilirubin, and c-reactive protein (CRP) were measured in plasma samples obtained from individuals with *P. vivax* malaria in order to build a scoring system allowing us to assess in a quantitative manner hepatic damage severity (adapted from Andrade et al., 2010c). These parameters were measured at the clinical laboratory of Faculdade São Lucas and at the Pharmacy School of the Federal University of Bahia, Brazil.

Statistical Analysis

Data analysis was performed using unpaired Student's t test for data presenting Gaussian distributions, an assumption tested using the Kolmogorov-Smirnov method. When data did not follow Gaussian distribution, Mann-Whitney U test was used instead. Log-rank test was used when survival was assessed as an end point. Data from human samples were also tested for Gaussian distribution, and differences between the groups presented with diverse hepatic damage scores were analyzed using Kruskal-Wallis test with Dunn's multiple comparisons or nonparametric Kendal linear trend post test. The linear trend post test was used to evaluate the pattern of variation of free heme, TNF, ferritin, transferrin saturation, or parasite load correlating with the ascending degree of hepatic damage. The statistical analysis of the human data was performed using STATA10 software (StataCorp LP, College Station, TX). Correlations between free heme, TNF, CRP, IL-6, ferritin, transferrin saturation, parasitemia, hepatic damage score, and creatinine were analyzed by Spearman nonparametric test. Nonlinear curve fit models were used to illustrate the overall trend of data variation. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.10.011.

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