



“Towards a Molecular Basis of Host Tolerance to Plasmodium Infection” (Seguindo a base molecular da tolerância do hospedeiros à infecção por Plasmodium)

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Heme (iron protoporphyrin IX) acts as a prosthetic group in a variety of (hemo)proteins that are essential to support aerobic life (1). One of the largest pools of hemoproteins in our body is most probably hemoglobin (Hb) expressed in red blood cells (RBC). Hb is a tetrameric ($\alpha_2\beta_2$) hemoprotein that accounts for 97% of the total RBC dry content. When confined inside RBC, $\alpha_2\beta_2$ Hb tetramers are maintained in a reduced (Fe^{++}) state (2). However, if released from RBC, the Hb tetramers dissociate into $\alpha\beta$ dimers, which react avidly with reactive oxygen/nitrogen species (ROS/RNS). This results in Hb oxidation, from ferrous (Fe^{++}) to ferric (Fe^{+++}) Hb (3, 4) that releases its heme prosthetic groups (1, 5).

We have recently demonstrated that free heme can act as a potent cytotoxic agonist, sensitizing several cell types to undergo programmed cell death in response to pro-inflammatory agonists (1, 6). These include TNF, a cytokine that plays a central role in the regulation of inflammatory responses. While the pro-inflammatory effect of TNF can be observed under a variety of experimental conditions, its cytotoxic effect is more elusive. The reason for this being that most cell types can express a series of immediate-early TNF-responsive genes that afford cytoprotection against TNF. The expression of these cytoprotective genes is regulated at the transcriptional by the transcription factor nuclear factor kappa B (NF- κ B)(7). We found that in the presence of free heme this cytoprotective mechanism fails to suppress TNF-mediated programmed cell death (6).

The mechanism underlying the cytotoxic effect of protein-free heme does not act via modulation of NF- κ B-responsive genes, since i) pharmacologic inhibition of mRNA synthesis or ii) protein synthesis as well as iii) inhibition of NF- κ B activation using an I κ B α dominant negative mutant, all fail to interfere with the ability of free heme to catalyze TNF-mediated apoptosis (6). Instead, free heme promotes the unfettered production of ROS/RNS in response to TNF, via a reaction catalyzed by the Fe atom contained within its protoporphyrin IX ring (6). This pro-oxidant effect produces high levels of lipid peroxidation, leading to sustained activation of the c-jun-N-terminal kinase (JNK) signal transduction pathway in response to TNF (*R. Gozzelino, unpublished observation*). This leads to the activation of caspase- 8 and -3 and ultimately to apoptosis (8, 9). The cytotoxic effect of free heme is inhibited by water-soluble (N-acetylcysteine) or lipid-soluble (butylated hydroxyanisole) anti-oxidants (10), revealing that ROS/RNS and lipid peroxidation are required to support heme-driven programmed cell death (6). The same anti-oxidants suppress heme-driven JNK activation, another key component of the signal transduction

pathway via which free heme exerts its cytotoxic effects (*R. Gozzelino, unpublished observation*).

To the best of our knowledge, there are no other molecules produced under pathophysiologic conditions that can override the cytoprotective effect of NF- κ B-responsive genes and sensitize cells to undergo TNF-mediated apoptosis. Our finding that free heme acts in such a manner should have important implications for the understanding of the mechanisms underlying the involvement of TNF in the pathogenesis of a variety of immune mediated inflammatory conditions. This appears to be particularly relevant for inflammatory conditions associated with hemolysis, such as malaria, the disease caused by *Plasmodium* infection (5).

Malaria remains one of the main causes of morbidity/mortality worldwide (11). Epidemiologically however, less than 1-2% of *Plasmodium*-infected individuals succumb to severe forms of malaria (11). This suggests that *Plasmodium* has co-evolved with its human host to reach an evolutionary “trade-off” in which infection “rarely” compromises host’s viability. This trade-off is thought to rely almost exclusively on the ability of the host’s immune system to control parasite burden (12), a defense strategy referred to as resistance to infection (13-15). 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 (13-15). The mechanisms underlying host tolerance to *Plasmodium* infection remain poorly understood (16).

We have recently demonstrated that heme released from oxidized Hb, such as it occurs during the blood stage of *Plasmodium* infection, plays a central role in the pathogenesis of severe forms of malaria in mice (5, 6, 17, 18). We have also shown that the deleterious effects of free heme can be prevented, if the infected host expresses adequate levels of heme oxygenase-1 (HO-1; encoded by the *Hmox1* gene) (5, 6, 17), a stress-responsive enzyme that catabolizes heme into equimolar amounts of biliverdin, iron (Fe) and the gasotransmitter carbon monoxide (CO)(19). Presumably for this reason, induction of HO-1 expression is strictly required to insure host survival in response to *Plasmodium* infection (5, 6, 17). The protective mechanism of HO-1 against *Plasmodium* infection relies on its ability to afford cytoprotection against free heme (6, 16), limiting tissue injury and thus disease severity. This host defense strategy, which limits disease severity irrespectively of parasite burden is defined as tolerance to infection (13-15). Our finding that HO-1 affords tolerance to *Plasmodium* infection is, to the best of our knowledge, the very first demonstration of such a molecular mechanism in the context of malaria (16).

We reasoned that if host tolerance to *Plasmodium* infection should operate in humans, then this defense strategy should have been selected through evolution as to afford protection in human populations living in endemic areas of *Plasmodium* infection. We found that this is most probably the case for sickle cell trait, an hemoglobinopathy (20) that has been selected through evolution based on its potent protective effect against malaria (21).

Sickle cell disease is a molecular disease (20) caused by a single replacement of a glutamic acid by a valine at amino-acid residue six of the β -chain of Hb occurring in either homozygous or compound heterozygous form (22). While asymptomatic *per se*, the heterozygous sickle cell trait confers a survival advantage against malaria (23-26). Despite the widespread recognition of this protective effect for several decades, understanding of its mechanism of action has remained elusive. The prevailing view is that hemoglobinopathies, such as sickle cell disease, should afford resistance to *Plasmodium* infection, thus limiting disease severity based on their ability to decrease pathogen load (23, 24, 27). However, this does not explain why hemoglobinopathies such as sickle cell trait, afford protection against cerebral malaria, a lethal and prevalent form of severe malaria that occurs under relatively low parasite load (25, 28). In the manuscript submitted for publication and enclosed hereby for appreciation for the “**Prémios de investigação Básica, Pfizer, 2009**” ([see attached manuscript by A. Ferreira et. al.](#)) we demonstrate that sickle cell trait affords tolerance to *Plasmodium* infection in mice, limiting disease severity without interfering with parasite load. Briefly, we found that transgenic Hb^{SAD} mice that develop an hemoglobinopathy resembling in many aspects the human sickle cell trait (29) have increased levels of HO-1. When infected by *Plasmodium*, Hb^{SAD} mice are protected against experimental cerebral malaria (ECM), a lethal neuroinflammatory syndrome that recapitulates the basic pathologic features of human cerebral malaria (28). HO-1 expression and activity are strictly required to suppress the onset of ECM in Hb^{SAD} mice, as demonstrated by functional deletion of the *Hmox1* locus as well as by pharmacologic inhibition of its enzymatic activity. The protective effect of HO-1 against *Plasmodium* infection in Hb^{SAD} mice is mediated by CO, an end-product of heme catabolism that inhibits further accumulation of free heme in plasma (17) and consequently prevents free heme from eliciting the pathogenesis of ECM (17). Neither HO-1 nor CO modulate pathogen load revealing that hemoglobinopathies and in particular the sickle cell trait can afford host tolerance to *Plasmodium* infection. The evolutionary conserved nature of the HO-1/CO system (30) as well as its induction during sickle cell disease in humans (31-34) argues strongly that a similar mechanism might underlie the protection afforded by sickle cell trait against *Plasmodium* infection in humans.

Given that free heme can be produced under a variety of pathophysiologic conditions, our findings should have important implications not only to understand the pathogenesis of severe malaria but also for that of other immune mediated inflammatory diseases. This notion is strongly supported by our recent finding that free heme plays a critical role in the pathogenesis of severe sepsis driven by polymicrobial infection in mice ([see attached manuscript by R. Larsen et. al.](#)). We have also obtained conclusive evidence that HO-1 limits the cytotoxic effects of free heme produced during polymicrobial infection, thus suppressing the onset of severe sepsis irrespectively of pathogen load. This observation supports the notion that HO-1 affords host tolerance to polymicrobial infection.

In conclusion we have unveiled what we believe to be a central mechanism of host defense (tolerance) against infection. The potential therapeutic implications of these findings might benefit millions of lives.

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