The triumph of good over evil: protection by the sickle gene against malaria

Short title: Sickle trait protection against malaria

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Abstract

The mechanisms underlying *P. falciparum* resistance in individuals with sickle trait have been under active investigation for over a half century. This Perspective reviews progress in solving this challenging problem including recent studies that have exploited the genomics and proteomics of the parasite. The formation of Hb S polymer in the parasitized AS red cell leads to impaired parasite growth and development along with enhanced clearance from the circulation and reduced deposition in deep post-capillary vascular beds. Enhanced generation of reactive oxygen species in sickled AS red cells is a pathogenetic feature shared by parasitized thalassemic and G6PD deficient red cells, triggering abnormal topology of the red cell plasma membrane with decreased and disordered display of PfEMP-1, a *P. falciparum* adhesion protein critical for endothelial adherence. A mouse model of Hb S confers host tolerance to *P. berghei*, through inhibition of pathogenic CD8+T cells and induction of heme oxygenase -1. An additional and apparently independent mode of protection is provided by the selective expression in AS red cells of two species of microRNA that integrate into *P. falciparum* mRNAs and inhibit translation and parasite growth.
Mankind’s most commonly encountered genetic disorders lie snugly within the confines of the red blood cell. Hemoglobin mutants S, C and E, as well as α- and β-thalassemias, glucose-6-phosphate dehydrogenase deficiency and Southeast Asian ovalocytosis were initially identified because of their association with abnormal red cell morphology and/or anemia along with less common life-threatening clinical phenotypes. These genes have all arisen in areas in which falciparum malaria is endemic, and their rise to high levels of prevalence is believed to be due to their conferring significant degrees of protection against this dreaded pathogen.* Thus malaria has imposed extreme selective pressure on the human genome, far more than any other infectious disease, and the red cell has been the prime target for evolutionary adaptation. These mutations are prime examples of Haldane’s1,2 notion of “balanced polymorphism” in which genes are fixed at a high frequency in susceptible populations because the enhanced fitness enjoyed by heterozygotes more than outweighs the morbidity and mortality suffered by homozygotes or compound heterozygotes.

The βS globin mutation has arisen at least four times in Africa and once in Arabia or India. Supportive epidemiologic evidence for the protection of Hb S heterozygotes was initially provided by Allison.3,4 He5 and Smith6 estimated that under conditions of selection for fitness against malaria, about 45 generation or 1,000 years were required for the frequency of the sickle gene to reach a stable equilibrium. It is likely that selection for sickle trait and other red cell defects occurred 3,000 to 5,000 years ago, with the emergence of agriculture which converted large tracts of tropical rain forest into breeding grounds for Anopheles gambiae.7

The enhanced resistance of sickle trait individuals to falciparum malaria is substantial. Infected AS children have lower parasite densities than AA children and are 50-90% less likely to progress to a severe form of malaria or to die from the disease.8-11 The degree of enhanced

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*A meta analysis of the hemoglobinopathies mentioned above has failed to confirm that Hb E and beta thalassemia provide significant protection against P. falciparum.12*
fitness varies considerably with geographic locale.\textsuperscript{8,13} A recent meta analysis encompassing 62 studies confirmed the robust protection from AS hemoglobin from severe disease but found little or no protection against uncomplicated malaria or asymptomatic parasitemia.\textsuperscript{12} This result is fully in keeping with a recent genome-wide association study involving 1,325 cases of severe anemia or cerebral malaria in Ghana, compared to 828 unaffected controls, that revealed the highest protection scores with SNPs at the \(\beta\)-globin locus on chromosome 11.\textsuperscript{14}

This brief overview will focus on the mechanisms by which individuals with sickle trait have enhanced resistance to falciparum malaria. This topic is particularly timely in view of advances in genomics and proteomics that have greatly enhanced the scope and depth of malaria research. Moreover, three important papers published recently in high profile journals present new insights into this process.\textsuperscript{15-17}

**Earlier Studies**

The presence of \(\beta^S\) globin in AS individuals might confer resistance against malaria by interfering with a number of steps in the complex interaction between parasite and host red cells. During the last half century a number of plausible mechanisms have been proposed. As discussed below they are not mutually exclusive.

**Sickling of circulating infected red cells** Luzzatto and his colleagues in Nigeria\textsuperscript{18} incubated red cells of AS children infected with \textit{P. falciparum} and found that the formation of sickle shapes under low oxygen tension occurred much more readily in cells that contained parasites than in those that were uninfected. Subsequently, Roth et al\textsuperscript{19} showed that the enhanced sickling was restricted to AS red cells containing small plasmodium (ring) forms. Extensive sickle polymer was observed in red cells containing trophozoites and schizonts, but these large inclusions probably precluded formation of sickled shapes. The enhanced Hb S polymerization in parasitized AS red cells is likely due to the increased oxygen consumption that accompanies the robust metabolic activity of the intracellular parasite. However parasitization
could also enhance polymer formation by lowering intracellular pH or by increasing intracellular hemoglobin concentration. These simple but elegant experiments suggested that rapid clearance and destruction of circulating parasitized red cells protects AS individuals from severe infestation.

**Impaired parasite growth and oxidant damage** The development of a continuous culture system for human red cells infected with *P. falciparum* allowed more prolonged *in vitro* studies of the impact of sickle trait. Under normoxic conditions the invasion, growth and multiplication of *P. falciparum* in AS cells was the same as that of AA red cells. In contrast, under hypoxic exposure there was a reduction in fraction of parasites in AS cells along with a block in the maturation of ring forms to trophozoites and schizonts (Figure 1A). This result could be due to the formation of Hb S polymers, even in the absence of morphologic sickling. Parasites appear to be fragile and may be disrupted and killed by polymers. Alternatively the impairment in parasite growth could be due to polymer-induced red cell dehydration. Even more compelling is the notion that enhanced oxidative damage to RBC membrane threatens parasite viability. Infection of normal AA red cells with *P. falciparum* generates reactive oxygen species that elevate biochemical markers of oxidant damage to the red cell membrane. Red cells containing Hb S are likely to be even more vulnerable. The polymerization of Hb S leads to deposition of hemichrome and iron in the membrane of SS red cells making them highly sensitive to oxidant damage. It is plausible that similar changes occur in parasitized AS red cells. This added oxidative stress may cause considerable impairment of parasite growth and development and thus contribute to the protection conferred by sickle trait against *P. falciparum* infection.

Ayi et al reported that AS (as well as β-thalassemia and G6PD deficient) red cells containing ring forms (but not trophozoites) were more prone to uptake by macrophages than normal or alpha thalassemia red cells (Figure 1A). This enhanced phagocytosis appears to be
triggered by a combination of hemichrome formation, aggregates of Band 3 and deposition of autologous IgG immunoglobulin and complement (C3) on red cells.\textsuperscript{32}

There is experimental evidence that oxidant damage is a common mechanism shared by AS red cells, β- and α-thalassemias as well as G6PD deficiency in mediating resistance to malaria.\textsuperscript{33,34} It is likely that when these red cells are parasitized they are subjected to additional oxidative stress due to enhanced generation of reactive oxygen species by both the parasite and the mutant red cell. However it is uncertain whether the enhanced oxidant stress of these mutant red cells impacts more on impairing parasite growth or enhancing uptake by phagocytes.

Recent Advances

**Impaired endothelial adhesion** A major and clinically dominant feature of falciparum malaria is the sequestration of ring-containing red cells by endothelial adherence deep within post-capillary beds. Low oxygen tension in these sites is likely to induce sickle polymer formation in AS red cells thereby stunting parasite growth and development, similar to what has been observed in the \textit{in vitro} culture experiments cited above.

Impressive advances in the genomics and proteomics of \textit{P. falciparum} has enabled a better understanding of the mechanism underlying vascular cytoadherence. Among the more than 5,300 genes within the \textit{P falciparum} genome, over half encode proteins of unknown function.\textsuperscript{35-38} Many have no homology to mammalian proteins. During its 48-hour intra-erythrocytic life cycle the parasite exports a large number of these proteins into host red cells. Considerable progress has been made recently in identifying specific interactions between a number of novel parasite proteins with host proteins either in the erythrocyte cytoskeleton or on the plasma membrane. These interactions lead to dramatic changes in red cell morphology, deformability and, importantly, to endothelial cell adherence.

One of these proteins, \textit{P. falciparum} erythrocyte membrane protein-1 (PfEMP-1) is of particular interest.\textsuperscript{39,40} It is expressed on knob-like protrusions on the surface of parasitized red
cells and serves as the parasite’s primary ligand for mediating endothelial adherence, binding primarily to CD36, the main cytoadherence receptor on the surface of host endothelial cells and monocytes. In the brain, PfEMP-1 binds to ICAM-1. PfEMP-1 also binds to complement receptor 1 on the surface of non-infected red cells, leading to the formation of rosettes that impair cerebral blood flow. Cholera et al found that AS red cells parasitized with \textit{P. falciparum} trophozoites bound to human microvascular endothelial cells and blood monocytes about half as effectively as did comparably infected AA red cells. Moreover AS red cells had slightly reduced surface expression of PfEMP-1 along with more uneven distribution of expression on their surface (Figure 1B). Similar results have been obtained with both AC and alpha thalassemia red cells. Thus these benign and highly prevalent hemoglobin polymorphisms alter the red cell topography of the knob projections that are essential for adherence in post-capillary venules, perhaps by the oxidant dependent perturbations mentioned above. Impaired cytoadherence may explain why AS children are less likely to have severe disease than AA children with comparable parasite density.

Recently Cyrklaff et al have utilized electron tomography to study the dramatic structural perturbations that occur during the development of \textit{P. falciparum} in human red cells. At the trophozoite stage, 20-26 hours after invasion, the parasite develops a trafficking system to direct the export of some of its endogenous proteins including PfEMP-1 to the plasma membrane of the host red cell. In normal uninfected red cells a junctional complex of 14-16 actin monomers help to stabilize spectrin tetramers within the cytoskeleton. However, following invasion, host actin protofilaments are hijacked in order to form elongated filaments that tether a parasite compartment known as Mauer’s clefts to the knob-like protrusions of the plasma membrane that, as mentioned above, are essential for adherence to the post-capillary endothelium. Stunning high-resolution tomograms of Cyrklaff et al show that this conduit is disrupted in parasitized SS and SC red cells and thus provides an elegant molecular explanation for the decrease in the expression of PfEMP-1 and its abnormal distribution.
previously observed in parasitized AS\textsuperscript{42} and AC\textsuperscript{48} red cells. It is a puzzle why Cyrklaff et al\textsuperscript{14} chose to study SC and CC red cells rather than epidemiologically relevant and more accessible AS and AC red cells. SC and CC cells share a number of abnormal properties that impact on membrane structure and function and thus could confound experimental interpretation. These abnormalities include enhanced potassium efflux, a marked elevation of intracellular hemoglobin concentration, and intracellular formation of hemoglobin aggregates. These features are much less pronounced in AS and AC red cells.

**Animal models and immune tolerance** There is a pressing need for the development of small animal models of falciparum malaria in order to better understand the pathophysiology of the disease as well as to test novel drugs and vaccines. The major obstacle has been the limited host range of different malaria species. Rodent studies have been largely restricted to *P. berghei*, *P. yeolii*, and *P. chabaudi*, pathogens that have limited relevance to human disease. It is possible to study *P. falciparum* infection in mice, but it requires intravenous infusion of parasitized human red cells into strains with ablated adaptive immunity (SCID, Nude, NOD/SKID, NSG) combined with measures to suppress innate immunity such as administration of clodronate loaded liposomes.\textsuperscript{45}

Despite these concerns, Ferreira et al\textsuperscript{15} have recently made clever use of a mouse model to study the mechanism by which sickle hemoglobin confers resistance against severe forms of malaria. They utilized the SAD transgenic mouse model of sickle cell disease\textsuperscript{46} and compared it to normal mice of the same strain infected with *P. berghei*. The SAD transgene expresses human $\beta$-globin that contains not only the $\beta$6 valine sickle mutation but also two other mutations that are known to enhance Hb S polymerization. As mentioned above *P. berghei* infection in mice is of limited relevance to human infection with *P. falciparum*. Importantly however, cerebral malaria is a cardinal disease manifestation in both. All wild type C57BL/6 mice died 10 days after inoculation with *P. berghei* whereas 75% of SAD mice of the same strain survived. Moreover all of the wild type animals developed severe pathologic hallmarks of
cerebral malaria whereas the SAD mice had minimal involvement. The fact that the level of parasitemia was the same in both groups led the authors to conclude that protection conferred by sickle hemoglobin entailed enhanced tolerance to the pathogen, mediated in part by inhibition of immune attack by CD8+ T cells."** 47

In addition the protection in the SAD mice appeared to be dependent on induction of heme oxygenase at sites of uptake of parasitized cells resulting in rapid and efficient degradation of heme. SAD mice that also harbored a deletion in one heme oxygenase-1 allele (SAD/Hmox1+/−) had decreased survival and cerebral malaria comparable to the wild type mice. Ferreira and colleagues propose that the release of carbon monoxide during heme catabolism protects hemoglobin from further oxidation and release of heme and its toxic metabolites. A key question is whether the up-regulation of heme oxygenase in the SAD mouse is relevant to the protection of humans with sickle trait against *P. falciparum*. Because of the additional β-globin mutations mouse red cells containing low levels (19-26%) of SAD hemoglobin undergo hemolysis and have much more sickling than human AS red cells containing 30-40% Hb S. It would be worthwhile repeating the experiments of Ferreira et al with a mouse model expressing only human globins48 (α, βA and βS) and therefore a much more faithful mimic of sickle trait.

**Role of host microRNA** Recently Lamonte and his colleagues17 have presented a comprehensive and coherent body of evidence that microRNAs play a significant role in mediating the reduced growth rate of *P. falciparum* in AS red cells. At roughly 1000 sites on the human genome RNA transcripts are expressed that are then truncated by “dicer” ribonucleases into small RNA species with an average length of 22 nucleotides. These microRNA species fold into stable hairpin structures and are post-transcriptional regulators. They bind to complementary sequences on target mRNAs and either suppress translation or trigger degradation.

*In contrast Shear et al47 had previously inoculated transgenic mice with *P. berghei* and *P. chabaudi* and reported that those expressing Hb S had markedly delayed and diminished increases in parasitemia.*
LaMonte et al\textsuperscript{17} found that following infection of normal human red cells with \textit{P. falciparum} several microRNAs are enriched and translocate to the cytosol of the parasite. The levels of two of these (miR-451 and miR-223) were markedly elevated in non-infected SS red cells, and to a somewhat lesser extent in AS red cells. Parasite growth rate in SS was markedly (~80\%) lower than that in AA red cells, while in AS red cells the growth rate was moderately lower (~60\%). This protection was significantly abrogated by miR-451 and miR-223 antisense oligonucleotides. Moreover transfection of miR-451 and miR-223 into normal AA red cells prior to \textit{P. falciparum} infection resulted in ~ 50 \% reduction in parasite proliferation. Surprisingly these miRNA species inhibited growth of \textit{P. falciparum} not by impacting mRNA translation or stability but by linear integration of into key parasite mRNAs thereby inhibiting their translation (Figure 2). This remarkable discovery introduces a novel mechanism by which the host genome can modify the virulence of a microbial pathogen.

In considering the role of these microRNAs in conferring protection against \textit{P. falciparum}, two deeply perplexing questions need to be addressed. What accounts for the differential expression of microRNAs in AS versus AA red cells? Certain microRNAs (including miR-451) are expressed in erythroid progenitor and precursor cells prior to enucleation and are crucial for their orderly development.\textsuperscript{49} It is surprising that they persist in mature anucleated red blood cells. Why are selected miRNAs strongly enriched in mature circulating AS red cells? There is no evidence that erythropoiesis is perturbed in sickle trait individuals. Unlike patients with SS disease, refined laboratory parameters in AS individuals indicate neither hemolysis nor ineffective erythropoiesis. Secondly the proposed protection via specific species of microRNAs appears to involve a process completely independent of the other mechanisms outlined in this Perspective, all of which, one could plausibly argue, are predicated on polymer formation in parasitized, deoxygenated AS red cells.
Conclusions

The enormous selective pressure imposed by falciparum malaria has engendered commonly encountered polymorphisms in genes encoding globin, an enzyme (G6PD) and a critical protein in the red cell cytoskeleton (band 3). These mutations cause no significant impairment in the fitness of heterozygotes but confer robust resistance to the disease. Thus the mechanisms underlying this protection must be subtle.

The impact of generating reactive species (ROS) and possibly hemichromes appears to be a unifying theme for most if not all of these mutations. In the case of sickle trait, the path to enhanced ROS production is certainly subtle indeed. As mentioned above, AS individuals have normal reticulocyte counts and normal red cell life span. Oxidation of the heme iron to methemoglobin is the proximal step in hemichrome formation. Purified Hb S has a slightly higher rate of auto-oxidation than that of Hb A. However, in AS red cells this small difference would not lead to methemoglobin accumulation because of the high activity of red cell cytochrome b5 reductase. In fact there is no convincing evidence of methemoglobinemia in AS or AC individuals or indeed even in SS disease. Moreover there is no structural rationale for why mutations at beta 6 should impinge on the beta chain heme environment to promote hemichrome formation in heterozygotes. In contrast, distortion of the SS red cell membrane, along with iron accumulation and superoxide formation from the low oxygen affinity of SS red cells, could and does lead to hemichrome formation. This conundrum is addressed and perhaps resolved by the early in vitro studies demonstrating that under low oxygen tension parasitized AS red cells readily sickle. Extensive polymer formation in these cells would be expected to distort the plasma membrane similar to what occurs in SS red cells. It seems plausible that oxidant-dependent perturbations of the red cell membrane in AS red cells and well as in thalassemic and G6PD deficient red cells could underlie several of the mechanisms proposed for protection against P. falciparum: enhanced macrophage uptake, impaired growth
and maturation of parasite and decreased deposition of parasitized red cells in deep post-capillary beds.

The impact of polymer formation in parasitized AS red cells, as summarized in the above paragraph, must be weighed against the startling and heuristic discovery of preferential expression of selected microRNAs in AS red cells that integrate into parasite mRNAs and inhibit growth. One’s imagination must be stretched to imagine how a single mutation in the host genome can confer resistance to a microbial pathogen by such divergent mechanisms. Resolving the relative importance of these apparently independent mechanisms of parasite resistance will provide fresh insights into the complex interrelationship between the genome of the host and that of threatening microbial pathogens.
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Authorship

H.F.B. did the relevant background literature review and wrote the article.

Conflict-of-interest disclosure

H.F.B. declares no competing financial interests or any other conflicts of interest.

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References


40. Fairhurst RM, Bess CD, Krause MA. Abnormal PfEMP1/knob display on
Plasmodium falciparum-infected erythrocytes containing hemoglobin variants: fresh
insights into malaria pathogenesis and protection. *Microbes and infection / Institut
41. Luse SA, Miller LH. Plasmodium falciparum malaria. Ultrastructure of parasitized
falciparum-infected erythrocytes containing sickle hemoglobin. *Proceedings of the
43. Fairhurst RM, Baruch DI, Brittain NJ, et al. Abnormal display of PIEMP-1 on
erthrocytes carrying haemoglobin C may protect against malaria. *Nature.*
44. Krause MA, Diakite SA, Lopera-Mesa TM, et al. alpha-Thalassemia impairs the
46. Trudel M, Saadane N, Garel MC, et al. Towards a transgenic mouse model of
47. Shear HL, Roth EF, Jr., Fabry ME, et al. Transgenic mice expressing human
48. Paszty C, Brion CM, Manci E, et al. Transgenic knockout mice with exclusively
49. Duraisingh MT, Lodish HF. Sickle Cell MicroRNAs Inhibit the Malaria Parasite.
50. Sheng K, Shariff M, Hebbel RP. Comparative oxidation of hemoglobins A and S.
51. Hebbel RP, Eaton JW, Balasingam M, Steinberg MH. Spontaneous oxygen
Figures and Legends

Figure 1  A. Parasitization of AS red cells causes increased oxygen consumption, a decrease in pO$_2$ and sickle hemoglobin polymerization. The membranes of these cells are further modified by oxidant stress, resulting in uptake by macrophages, impaired parasite growth and development and decreased adherence to endothelium. B. Parasitization of AS red cells leads to a decrease in the display of knobs of the cell surface along with uneven distribution. It is likely, though unproven, that hypoxia-induced sickling would aggravate this abnormal topology and further weaken interactions between the parasite protein PfEMP-1 and cognate receptors on endothelial cells such as ICAM-1 in the brain.
Inhibition of translation of parasite mRNAs by microRNAs in AS red cells. Both miR-451 and miR-223 are highly enriched in AS red cells. Following invasion by *P. falciparum*, these miRNAs enter the parasite cytoplasm by penetrating the parasitophorous vacuolar membrane (dashed blue circle), the vacuole (white space) and the parasite’s plasma membrane (solid blue circle). The miRNAs are then trans-spliced onto the 5’ ends of specific *P. falciparum* mRNAs. Translation is blocked in these chimeric RNAs, leading to impaired parasite growth. Adapted from Duraisingh and Lodish.  

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**Figure 2** Inhibition of translation of parasite mRNAs by microRNAs in AS red cells. Both miR-451 and miR-223 are highly enriched in AS red cells. Following invasion by *P. falciparum*, these miRNAs enter the parasite cytoplasm by penetrating the parasitophorous vacuolar membrane (dashed blue circle), the vacuole (white space) and the parasite’s plasma membrane (solid blue circle). The miRNAs are then trans-spliced onto the 5’ ends of specific *P. falciparum* mRNAs. Translation is blocked in these chimeric RNAs, leading to impaired parasite growth. Adapted from Duraisingh and Lodish.
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