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Beyond hemoglobin: Screening for Malaria Host Factors

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Abstract

Severe malaria is caused by the *Apicomplexan* parasite *Plasmodium falciparum*, and results in significant global morbidity and mortality, particularly among young children and pregnant women. *P. falciparum* exclusively infects human erythrocytes during clinical illness, and several natural erythrocyte polymorphisms are protective against severe malaria. Since erythrocytes are enucleated and lack DNA, genetic approaches to understand erythrocyte determinants of malaria infection have historically been limited. This review highlights recent advances in the use of hematopoietic stem cells to facilitate genetic screening for malaria host factors. While challenges still exist, this approach holds promise for gaining new insights into host-pathogen interactions in malaria.

Life Cycle of the Malaria Parasite

Malaria is a parasitic disease that has been described in the medical literature since ancient times, and remains a major public health problem in developing regions of the world where the mosquito vectors that transmit *Plasmodium* spp. parasites thrive [1]. The WHO estimates that there were ~ 214 million cases and 438,000 deaths due to malaria in 2015, primarily among young children, making it one of the leading causes of childhood mortality globally [2]. Although anti-malarial agents are available for treatment, their long-term efficacy is compromised by the emergence of drug resistance by *Plasmodium* parasites [3, 4]. Thus, new approaches to prevention and treatment will be necessary in order to reach the eventual goal of malaria eradication.

Severe malaria is typically caused by *Plasmodium falciparum*, though five different *Plasmodium* spp. can cause disease in humans. *P. falciparum* is transmitted to humans by infected *Anopheles* spp. mosquitoes, which harbor the parasites in their salivary glands and inject it into humans upon taking a blood meal [5]. After deposition in the skin, sporozoite forms enter the bloodstream and travel to the liver, where they infect hepatocytes and multiply, forming merozoites. The invasive merozoites are released back into the blood circulation, where they exclusively invade erythrocytes (RBCs) (Figure 1). Once inside the RBC, the parasite resides within a protective parasitophorous vacuole derived from the erythrocyte plasma membrane. From this protected space, it elaborates organelles and a

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secretory apparatus to support its growth, development, and replication. This process significantly transforms the host erythrocyte over the course of 48 hours, terminating with lysis of the cell and the release of up to 32 daughter merozoites that can go on to infect new RBCs [5].

Innate resistance to malaria

The high mortality associated with untreated malaria has resulted in a strong evolutionary selection for genetic traits that protect humans from severe disease. The “malaria hypothesis”, introduced by Haldane in 1949, proposed that certain deleterious mutations may be under positive selection because they decrease susceptibility to severe malaria [6]. In 1954, A.C. Allison published the first evidence-based study supporting the malaria hypothesis, in which he observed a lower parasite density in individuals heterozygous for hemoglobin S who have “sickle trait”, compared to those with normal erythrocytes [7]. Research over the subsequent half-century has revealed that a variety of RBC genetic variants are protective against malaria, and likely arose as a result of positive selection, such as other hemoglobinopathies (e.g. Hemoglobin C, thalassemia), the enzymopathy *G6PD* deficiency, and surface protein variants such as Southeast Asian ovalocytosis (SAO) and the Duffy null blood type. However, the mechanisms of protection afforded by these erythrocyte variants are complex and likely multi-factorial; readers are referred to several recent excellent reviews [8–12].

The relative impact of host genetics versus environmental factors on the incidence of malaria was assessed using pedigree-based genetic variance component analysis [13]. Long-term monitoring and active surveillance were performed to determine the incidence of severe and mild malaria in cohorts of children in rural Kenya. This study showed that ~25% of variation in the outcomes of mild or severe malaria were explained by host genes with additive effects, though genes with non-additive effects (e.g. dominant effects) were not assessed by this method. Of note, the Hemoglobin S allele accounted for only 2% of the measured variation, highlighting the importance of other host genes, beyond hemoglobin. Household factors explained an additional 29% and 14% of variation in outcomes of mild or severe malaria, respectively. Overall, the results of this study indicate that many host genes influence malaria susceptibility, with each gene causing small effects at a population level, and that household factors also contribute.

Genome Wide Association studies (GWAS) hold potential as a powerful approach to identify genetic loci correlated with natural resistance to severe malaria, but thus far several large studies have yielded few new candidates. An early GWAS searched for loci associated with severe malaria among children in The Gambia using the Affymetrix 500K GeneChip [14], but found that signals even at known loci such as *HBB* were greatly attenuated due to weak linkage disequilibrium. In a subsequent study that employed the Affymetrix Genome-Wide Human SNP Array 6.0 followed by genome-wide imputation using genotype data from 174 individuals of African descent, 5,010,634 single nucleotide polymorphisms (SNPs) were analyzed in severe malaria patients and controls from Ghana [15]. This analysis identified two new candidate loci, including one within the *ATP2B4* gene, the major calcium pump of erythrocytes. A large multi-center study led by the Malaria Genomic Epidemiology Network

(MalariaGEN) confirmed evidence of association at *HBB*, *ABO*, *ATP2B4*, *G6PD* and *CD40LG*, but failed to confirm associations of 22 other candidate loci with severe malaria. These findings highlight how the high level of ethnic diversity in sub-Saharan Africa may lead to false-positive results.

The most recently published GWAS for severe malaria included samples from over 11,000 children in west and east Africa and employed typing of ~2.5 million SNPs using the Illumina Omni2.5M platform followed by imputation analysis [16]. A novel locus of resistance was identified near the glycophorin genes on chromosome 4, but could not be narrowed to a particular gene. By constructing an improved, Africa-centric reference panel, the analysis was subsequently refined and identified large copy number variants affecting *GYP A* and *GYP B* that were associated with protection from severe malaria, including a hybrid *GYP B-A* gene that encodes the previously described Dantu blood group variant [17].

Although many of the host factors known to influence innate resistance to malaria have been identified through population genetic studies, molecular and biochemical approaches have helped validate important host factors for *P. falciparum* such as the glycophorins, and to identify others such as complement receptor I (CR1) and Basigin (BSG) [18–21]. *GYP A*, *CR1* and *BSG* all encode receptors that cooperatively mediate erythrocyte invasion, with BSG being the only known essential receptor. Collectively, the diverse yet small list of host erythrocyte genes with hypothesized or validated roles in malaria susceptibility raises the hypothesis that other host genetic determinants for *P. falciparum* are yet to be discovered. Uncovering such factors is a high priority as they will enhance our understanding of this important global pathogen and may hold promise as targets of new host-directed therapeutics for malaria.

Searching for new host factors: Overcoming the challenge of an enucleate cell

Given the premise that erythrocyte genetic variation can influence malaria susceptibility, screening for additional critical erythrocyte host factors has long been of interest in the malaria field. However, the natural biology of the human erythrocyte has presented a major obstacle to genetic screening because during terminal differentiation these unusual cells shut down protein synthesis and extrude their nucleus and DNA [22]. The erythrocytes found in peripheral blood are thus highly specialized cells that lack genetic material, making them refractory to experimental manipulation. To generate genetically altered erythrocytes, it would be necessary to alter the progenitor cells where the nucleus and protein synthesis machinery are still present, and then differentiate them *ex-vivo*.

Early efforts to generate red blood cells *ex-vivo* from hematopoietic progenitor cells showed promising results in terms of either proliferative capacity or terminal differentiation, but challenges to achieving both goals persisted for many years [23–30]. A major advance came with the 2005 report of an *in vitro* system that supports the large-scale production of mature enucleated red blood cells [31]. Using a three-step protocol, a large expansion of human CD34⁺ cells was achieved, with full maturation into enucleated RBCs with normal hemoglobin levels. In the first step, cell proliferation and erythroid differentiation were

induced in serum-free media supplemented with stem cell factor, IL-3, and erythropoietin, and after eight days the cells were committed to the erythroid lineage. Next, cells were plated on an MS-5 stroma cell line in the presence of erythropoietin. In the third and final step, exogenous cytokines were removed and cells were maintained only in the presence of the MS-5 stroma. The *ex vivo* microenvironment was highlighted for its impact on terminal maturation and hemoglobin synthesis. Subsequent studies have shown that efficient enucleation can occur in the absence of a microenvironment, but full *in vitro* maturation past the reticulocyte stage is harder to achieve [32, 33].

With the advent of a robust erythroid culture system, it became possible to experimentally assess the susceptibility of various progenitor cells to infection by *P. falciparum* [34]. RBC progenitors differentiated *ex vivo* from growth factor mobilized peripheral blood CD34⁺ cells were used to demonstrate that the earliest erythroid progenitors susceptible to *P. falciparum* infection were orthochromatic erythroblasts [34]. Efficient development of *P. falciparum* parasites was observed in late but not early orthochromatic erythroblasts. Attempts to correlate this differential susceptibility to developmental changes in erythroid gene expression were inconclusive.

An additional key piece of groundwork for a malaria host factor screen demonstrated that erythroid progenitors could be genetically manipulated using RNAi prior to terminal differentiation and parasite infection [35]. CD34⁺ HSPCs isolated from human bone marrow were transduced with lentivirus expressing an shRNA targeting *GYPA*, which encodes a known receptor for *P. falciparum* and the most abundant surface protein in human erythrocytes. Cultured red blood cells derived from these transduced cells expressed low levels of GYPA on the plasma membrane, but otherwise appeared to develop normally. In functional assays, a *P. falciparum* strain that uses sialic acid-*GYPA* as a receptor displayed reduced invasion into the GYPA-deficient cultured red blood cells, whereas invasion by sialic acid-independent strains was unaffected. These results provided a proof of principle that knockdown of a host receptor for *P. falciparum* could restrict invasion through a specific invasion pathway.

What to screen? The erythrocyte proteome at a glance

As erythrocytes differentiate, the nucleus condenses, transcription is progressively shut down, and the cells become functionally specialized. These dramatic changes are reflected in a massive constriction of the erythrocyte proteome relative to more typical human cells. Thus, in planning a screen for erythrocyte host determinants for *P. falciparum*, focusing on the mature erythrocyte proteome rather than the entire human genome should maximize sensitivity and specificity. This is particularly important for a screening strategy that relies on robust ex-vivo erythropoiesis, as it could help to avoid unwanted effects of gene knockdown on erythroid development. Using deep quantitative proteomics, the proteome of six partially overlapping stages of erythroid differentiation was determined [36]. Around 5,000 proteins were detected at each stage, demonstrating that the expression of most proteins does not change dramatically over the course of differentiation. In contrast, the proteome of peripheral blood erythrocytes has been shown to be much smaller. A landmark study of the mature erythrocyte proteome reported in 2006 identified 314

membrane and 252 soluble proteins in erythrocytes, reflecting a large increase compared to prior studies due to the technological advance of using a mass spectrometer with very high sensitivity and mass accuracy [37, 38]. This proteome was used as the framework for designing an erythrocyte-specific short-hairpin RNA (shRNA) library to screen for host determinants of *P. falciparum* infection in erythroid cells [39]. In addition to the genes that encode the mature erythrocyte proteome, this library included shRNAs targeting ~100 genes highly expressed in reticulocytes, the last stage before enucleation, as well as some modifying enzymes such as glycosyltransferases and sulfotransferases. Thus, the library had the advantage of being specific and relatively small compared to the human genome, features that could help minimize noise and nonspecific effects.

A genetic screen for malaria host factors

To search for new host factors required by *P. falciparum* for infection of red blood cells, a pooled screening approach was designed in order to minimize well-to-well variation that might be introduced during a prolonged screening period [39]. CD34⁺ cells from human bone marrow were induced to proliferate and differentiate down the erythroid lineage, and transduced with the erythrocyte-specific shRNA lentivirus library, which included a minimum of 5 shRNAs/gene for a total of > 5000 shRNAs. The use of a lentivirus shRNA library as opposed to transient transfection of siRNAs was employed to maintain gene knockdown over the timecourse of erythropoiesis. The multiplicity of infection was kept low to ensure that each cell would be transduced by a single lentivirus, leading to knockdown of a single specific gene. After selecting for transduced cells, erythroid differentiation was continued until the orthochromatic erythroblast stage, at which point *P. falciparum* parasites were introduced. With the use of a GFP-expressing parasite strain, cells in which parasites had successfully invaded were identified and isolated by fluorescence activated cell sorting, and subjected to next-generation sequencing to quantify the relative abundance of each shRNA in the population (Figure 2, Key Figure). To identify genes required for *P. falciparum* invasion, the relative abundance of each shRNA in the pool of infected cells was compared to their relative abundance in control, uninfected cells, thus identifying shRNAs underrepresented in the infected population. In an extreme example, if shRNAs targeting a specific gene were completely absent from the pool of infected cells, one could conclude that knockdown of the gene prevented infection.

Genetic determinants of erythropoiesis

Despite the use of an shRNA library specifically targeting the proteome of the mature erythrocyte, sequencing of proviruses in the control, uninfected pool of cells revealed that many shRNAs changed in abundance over the course of erythroid differentiation [39]. Using the RNAi Gene Enrichment Ranking (RIGER) algorithm to identify gene candidates [40], which ranks shRNAs based on the strength of their phenotype and also accounts for the number of shRNAs per gene with strong effects, it was clear that many (~13%) of genes in the library affected erythropoiesis, some negatively but others positively. These results were unexpected, because the erythrocyte proteome was assumed to be geared towards erythrocyte function, rather than development. Some genes, such as those involved in the heme biosynthetic pathway, could be anticipated to influence erythroid development, but

others had no prior described roles. These findings indicate that the proteome of the mature erythrocyte retains many genes involved in erythroid proliferation and/or differentiation, despite this cell type being terminally differentiated and highly specialized.

The finding that knockdown of many genes in the shRNA library impacted erythropoiesis highlights a challenge inherent to this pooled screening strategy- namely, how does one identify specific effects on *P. falciparum* infection if gene knockdown could be causing detrimental developmental effects on the host cell? To avoid such confounding, the screen was re-focused to specifically evaluate human blood group genes rather than the entire erythrocyte proteome. As all known receptors for *P. falciparum* encode blood group antigens and knockdown of this group of genes did not have strong developmental effects, it was hypothesized that probing this smaller set of genes could prove fruitful while avoiding false positives.

Discovering new host factors for malaria by screening human blood group genes

A lentivirus shRNA library targeting 42 human blood group genes with > 5 shRNAs per gene was used in a focused screen to identify host factors involved in erythrocyte invasion by *P. falciparum* [39]. Blood group proteins are expressed on the plasma membrane, and thus would most likely function during invasion. After differentiating knockdown cells to orthochromatic erythroblasts and infecting with *P. falciparum*, infected cells were sorted by FACS and genomic DNA was isolated. shRNAs underrepresented in the infected cells were identified by next-generation sequencing and compared to the shRNA abundance in control cells. Analysis of the sequencing results using the RIGER algorithm revealed that two known receptors for *P. falciparum*, Basigin and CR1, were among the top candidates. The identification of these positive controls indicated that the screen had sufficient power to detect true host factors. The most highly ranked candidates, CD55 and CD44, did not have previously described roles in malaria infection, but notably they both have precedence as receptors for pathogens on epithelial cells [41, 42].

To further investigate CD55 and CD44 as candidate host factors for *P. falciparum*, cRBCs with specific knockdowns for these genes were generated using individual shRNAs. After differentiation, the mutant cells were confirmed to be deficient in CD55 and CD44, respectively, but otherwise had normal development (Table 1). CD55 and CD44 were validated as host factors required for efficient invasion of cRBCs by *P. falciparum* by performing invasion assays using wt and knockdown cRBCs that had been fully enucleated (in contrast to the orthochromatic erythroblasts used in the screen). The role of CD55 was further investigated using natural erythrocytes from rare CD55-null donors with the so-called Inab phenotype [43]. While individuals with the Inab phenotype have a high prevalence of gastrointestinal disease, they are not anemic and their erythrocytes are believed to be normal (aside from an absence of CD55). CD55-null erythrocytes from two Inab individuals were resistant to invasion by all *P. falciparum* strains tested, demonstrating that this protein is an essential host factor [39].

Concluding Remarks, Challenges and Future Prospects

The development of the first forward genetic screen for erythrocyte host factors for *P. falciparum* introduced a new, unbiased approach to discovery with the potential to significantly advance our understanding of host-pathogen interactions in malaria. This work identified two new host factors that may act as receptors for *P. falciparum* during invasion: CD44 and CD55. At the same time, the screen had several important limitations that narrowed its scope. As reflected by the lack of strong reproducibility between the biological replicates, the screen was somewhat under-saturated [39]. One of the most significant factors contributing to this was the use of nucleated, orthochromatic erythroblasts as the representative host cell (in the pooled format, nucleated cells were employed in order to track the integrated provirus shRNAs by next-generation sequencing). Although *P. falciparum* can clearly invade and develop in these late-stage erythroblasts, the invasion efficiency is low relative to enucleated cells, and it is technically difficult to separate this cell stage from either enucleated cells or from younger progenitors that are inherently resistant to parasitism because ex-vivo erythropoiesis is somewhat asynchronous and there are no molecular markers that discretely define the different stages during terminal differentiation. Together, these limitations ultimately meant that the screen had to be restricted to a relatively small library targeting blood groups in order to limit toxicity and optimize reproducibility. As *P. falciparum* requires host factors throughout its intra-erythrocytic life cycle, future screening efforts that can assess the whole proteome should lead to important insights beyond invasion, e.g. with regard to gene regulation, trafficking, metabolism and nutrient acquisition (See Outstanding Questions).

Results from both transcriptomic and proteomic studies suggest that the overall composition of orthochromatic erythroblasts is significantly different from that of enucleated erythrocytes [36, 44–46]. Future screening efforts might benefit from designing strategies that utilize more mature, enucleated erythrocytes, since these more accurately represent the primary site of infection in clinical malaria. In a pooled format, enucleated cells would need to be “tagged” by a signature that is exported into and stably maintained in the cytoplasm. Alternatively, approaches using arrayed screens, where each well represents a different mutant, could be employed.

The rise of CRISPR-Cas9-based genome editing presents new opportunities for alternative methods of genetic screening in which guide RNA molecules lead the Cas9 nuclease to specific targets in the genome for cleavage or regulation. While CRISPR-Cas9 approaches have revolutionized proteome-wide screening in a variety of systems [47–50], genome editing remains challenging in certain primary human cell types, including the CD34⁺ hematopoietic stem cells from which erythroid cells are generated [51]. Efficient editing has been demonstrated using nucleofection of chemically modified guide RNAs along with Cas9 mRNA or protein, but expanding this methodology to a proteome-wide screen would present challenges in terms of scale. Ultimately, genetic research on the role of the host erythrocyte in *P. falciparum* malaria would greatly benefit from an immortalized cell line that can differentiate to red blood cells. An erythroleukemia cell line termed JK-1 was used to generate knockouts in CD44 and Basigin and study their roles in *P. falciparum* invasion of orthochromatic erythroblast-like cells [52]. The recent report of an erythroid progenitor cell

line able to produce enucleated cells is an exciting development that may prove quite useful for future host-pathogen studies in malaria [53].

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Trends Box

- Susceptibility to malaria is influenced by human genetics.
- Malaria parasites specifically infect enucleated erythrocytes, creating challenges for genetic experimentation.
- Cultured red blood cells (cRBCs) derived ex-vivo from hematopoietic stem cells offer a new tool for forward genetic screening for malaria host factors.

A recent forward genetic screen for erythrocyte determinants of malaria identified new candidates and illuminated challenges.

Outstanding Questions Box

- To what degree do host genetics influence *P. falciparum* invasion and growth in human erythrocytes? The erythrocyte proteome, while dominated by hemoglobin, includes a diverse network of proteins that may be exploited by *P. falciparum* to complete its intra-erythrocytic life cycle. Though GWAS studies have yielded few new candidate susceptibility loci for severe malaria, in vitro screens may lead to insights about host-pathogen interactions important for *P. falciparum* biology.
- How do CD44 and CD55 help mediate *P. falciparum* invasion of human red blood cells? Do they act as receptors, or do they function downstream of the initial parasite-erythrocyte interaction? The use of CRISPR-Cas9 to generate mutant red blood cells from human hematopoietic stem cells will help elucidate the roles of these new host factors.
- What is the potential of host-targeted therapies for treatment or prevention of malaria? Can we identify vulnerable targets that will adversely affect the parasite while sparing host tissues from toxicity?

Figure 1.
Intra-erythrocytic development cycle of *Plasmodium falciparum*.

Figure 2.

Schematic of screening approach to identify erythrocyte host factors for *P. falciparum*.

Lentivirus library was generated from the pLKO.1 vector backbone to express shRNAs from the U6 promoter. *Pf*, *P. falciparum* infection; Mock, mock infection; NGS, next-generation sequencing.

Table 1

Checklist for validating *P. falciparum* host factor candidates identified by pooled genetic screens

Validation Checklist
☐ Generate individual knockdowns with 2 gene-targeting shRNAs
☐ Confirm on-target effects by flow cytometry, immunoblotting, and/or IFA
☐ Assess for effects on RBC development by cytospin and Giemsa staining
☐ Assess for effects on RBC development by molecular markers [54]
☐ Perform invasion/growth assays in enucleated, mutant cRBCs derived from 2 distinct shRNAs