Erythrocyte remodeling by malaria parasites

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Purpose of review

Plasmodium falciparum causes the most virulent form of human malarias. It is a protozoan parasite that infects human erythrocytes and the erythrocytic stages are responsible for all symptoms and pathologies of the disease. Critical to infection is the formation of a parasitophorous vacuolar membrane at the time of entry and within which the intracellular parasite proliferates. Since erythrocytes lack endocytic machinery, it is surprising that they can be infected by pathogens. This review summarizes recent studies of the erythrocyte–malaria interaction that have provided insights into properties of erythrocyte membranes as well as parasite mechanisms that remodel the erythrocyte.

Recent findings

Themes revealed by recent literature suggest that both parasite and erythrocyte components regulate parasite entry and intracellular growth by extensively remodeling host membranes. These remodeling events include the invagination of the host cell membrane during parasite entry that results in the creation and maintenance of a vacuole that surrounds the intracellular organism, and the development of antigenic, structural and transport alterations during intracellular parasite development.

Summary

The implications are that malarial erythrocyte remodeling events occur at a significant cost to the human host since many of the associated virulence events have been linked to severe disease pathologies.

Keywords

infection, *Plasmodium falciparum*, red blood cells

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Abbreviations

β_2AR	β_2-adrenergic receptor
DRM	detergent resistant membrane
HSP	heat shock protein
PfEMP1	*P. falciparum* erythrocyte membrane protein 1

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Introduction

Malaria is an ancient disease that continues to cause enormous human morbidity and mortality [1]. The life-cycle of the causative parasite involves multiple tissues in two distinct host organisms, mosquitoes and humans. All of the clinical symptoms of malaria, however, are a consequence of infection of human erythrocytes. An understanding of the basic mechanisms that govern parasite invasion, remodeling, growth and reinvasion of erythrocytes and the complex events leading to tissue pathology may yield new diagnostics and treatments for malaria. Here, we focus on the most recent understanding of erythrocyte remodeling during malarial infection.

Invasion of erythrocytes by malaria parasites

The mature human erythrocyte is a terminally differentiated cell. It lacks subcellular organelles (such as a nucleus or secretory structures) and de-novo protein/lipid biosynthesis and does not endocytose its plasma membrane [2]. Red cells contains high concentrations of cytoplasmic hemoglobin, prominent solute and ion transport systems and a deformable submembrane cytoskeleton, which reflect the specialized functions of erythrocytes for delivering oxygen to tissues and surviving repeated passage through capillaries. To maintain erythrocyte function in circulation, clearance of older red cells and production of new ones are carefully coordinated to optimize delivery of oxygen to tissues.

Parasite entry into erythrocytes is a complex, dynamic process [3]. The earliest interactions likely require proteins residing on the invasive parasite surface. The invading merozoite subsequently reorients its 'apical' end to direct specialized apical secretory organelles called the micronemes, rhoptries and dense granules toward the junction of invasion. Initial interaction appears to stimulate a rapid 'wave' of deformation across the erythrocyte membrane followed by the formation of a stable parasite–host cell junction [3] (see Fig. 1). Invagination of the erythrocyte bilayer then results in engulfment of the parasite and establishment of the intracellular 'ring' stage parasite surrounded by a vacuolar membrane [4]. Microneme proteins appear to be involved in binding to surface erythrocyte determinants such as host sialic acid (abundant on erythrocyte glycophorin A) and it is possible that micronemal proteins establish the junction while rhoptries drive vacuole formation, leading to cooperation between the two organelles [5].

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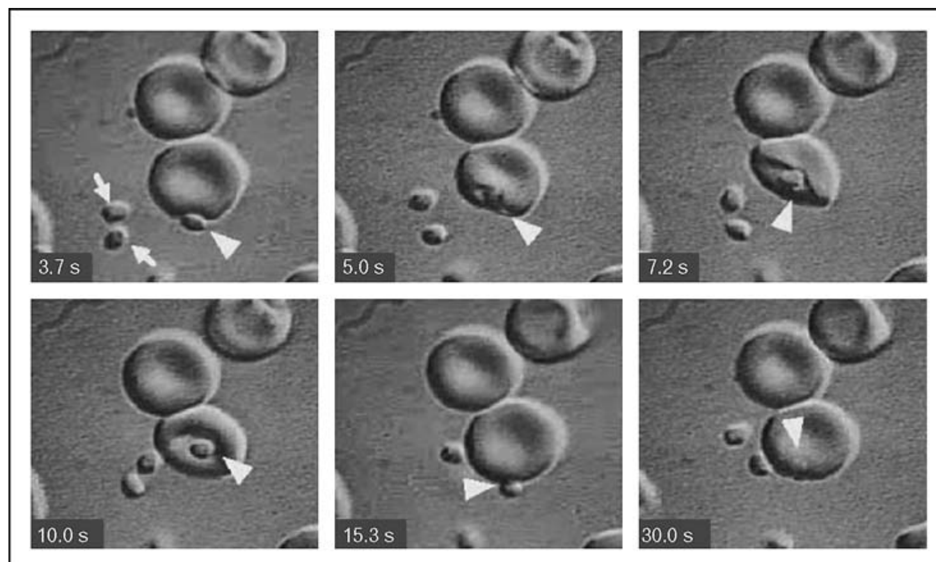
Figure 1 Erythrocyte invasion by malarial merozoites



Still images from real-time video microscopy of *Plasmodium knowlesi* invasion of human erythrocytes; time is denoted in seconds (video courtesy of James Dvorak and Louis Miller, described in [3]).

Although the erythrocyte bilayer envelops the invading parasite in a nascent vacuole [6], major erythrocyte proteins such as band 3, glycophorin A and cytoskeletal proteins are not detected on this vacuole. Instead, our studies [7–10] and those of Nagao and colleagues [11] have shown that erythrocyte detergent resistant membrane (DRM) raft proteins are recruited to the malarial vacuole [10,11]. Proteins isolated in erythrocyte DRMs have provided a rich vein to identify at least 12 host proteins internalized into the plasmodial vacuole [10,12] (see Fig. 2). DRMs contain only a few percent of the total bulk erythrocyte membrane protein [8,10]. This may explain why previous studies failed to detect host proteins in the malarial vacuole.

DRM rafts emerge from cellular membrane microdomains that contain heterotrimeric G proteins and tyrosine kinases on their cytoplasmic face; these proteins associate with rafts because they tend to be acylated [13,14]. Although erythrocytes are not thought to be highly active in signaling, G_s is recruited to the malarial vacuole [7,8] and is functionally important for malaria infection [9,15••]. Peptides designed to disrupt interaction of G_s with its receptor block malarial infection [9,15••]. In addition, agonists of the β_2-adrenergic receptor (β_2AR) stimulate production of cAMP and malarial infection, both of which are blocked by antagonists [9,15••]. Studies from Hines and coworkers [16] demonstrate that red cell β_2AR signaling regulates adhesive properties of erythrocytes taken from patients with sickle cell anemia.

In malarial infection, the target of signaling is expected to be the erythrocyte skeleton, which must be cleared to form the cytoskeleton-free malarial vacuole. Although catecholamines activate β_2ARs to increase cAMP in erythrocytes, however, this alone does not induce endovacuolar formation in the erythrocyte membrane (S. Murphy and K. Haldar, unpublished data). Endovacuolation requires a stimulus from the parasite as well, and a working model proposes that parasite-encoded proteins couple directly to erythrocyte G_s itself or to G_s-containing host–raft complexes that are internalized into the vacuole [17]. In addition to parasite ligands, host catecholamine levels are high during malaria fever periods, the time of parasite emergence and re-invasion into red cells. As described above, catecholamines stimulate erythrocyte signaling pathways that increase red cell flexibility, filterability and deformability [18–20]. Thus, in conjunction with parasite ligands, the host sympathetic response may actually ‘soften’ the erythrocyte membrane, in effect ‘inviting’ the parasite into the host erythrocyte and driving endovacuolation.

An important outcome of these studies has been investigating the erythrocyte G protein as a target for antimalarials [15••]. This was done by preparing erythrocyte ‘ghosts’ whose signaling functions and infection by malaria parasites were the same as normal erythrocytes. These eythrocyte ‘ghosts’ when loaded with a G_s peptide designed to block G_s interaction with its receptors, were found to be blocked in β-adrenergic agonist-induced signaling. This directly demonstrated that erythrocyte G_s is functional and unequivocably that propranolol, an antagonist of G protein-coupled β-adrenergic receptors, dampens G_s activity in erythrocytes. The ghost system was also used to show that in addition to blocking malarial

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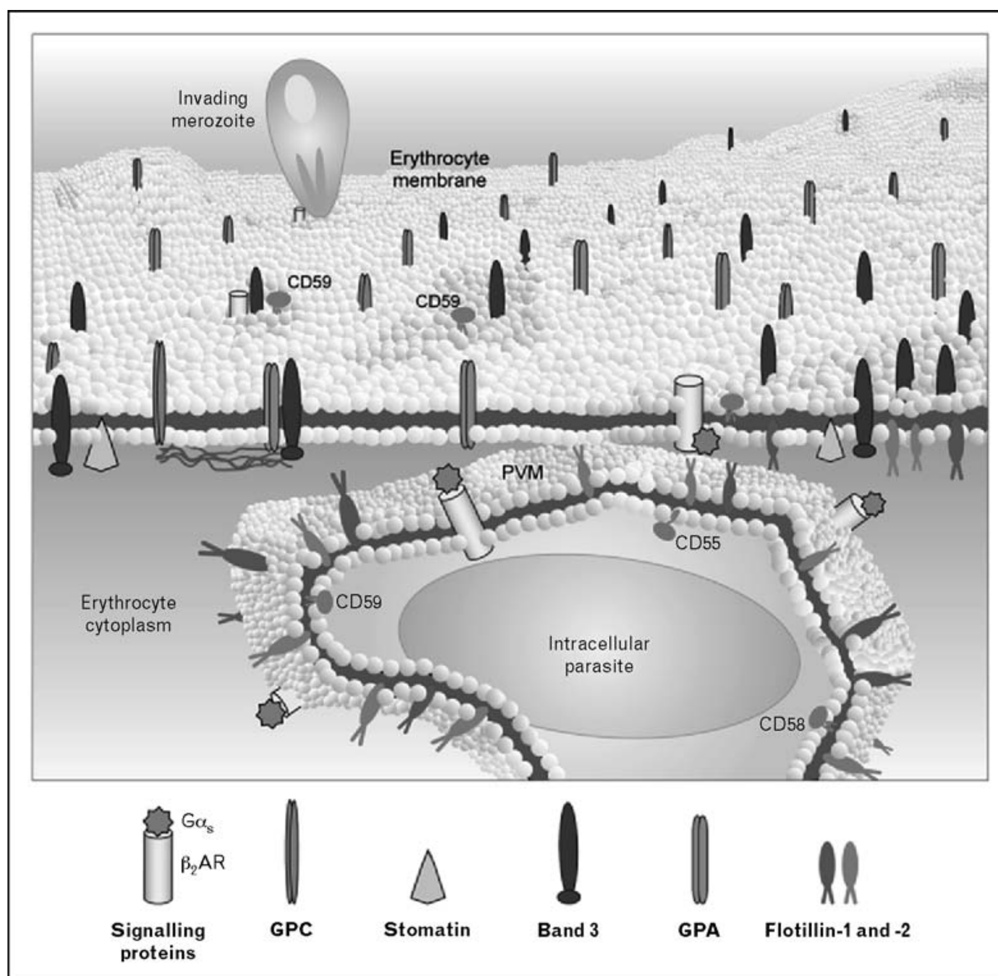
Figure 2 Model of erythrocyte rafts and selective host and parasite raft protein recruitment to the malarial vacuole



The uninfected erythrocyte membrane contains a variety of generalized lipid domains (gray spheres) and raft microdomains (light gray spheres) containing various proteins. Some proteins partition mostly into detergent resistant membrane (DRM) raft domains (i.e. flotillins), while others are only minimally present there (i.e. band 3). During malaria infection, merozoite-stage parasites invade erythrocytes to reside in a membrane-bound parasitophorous vacuole. The parasitophorous vacuolar membrane (PVM) becomes selectively cholesterol enriched, and 10 of the known raft proteins are internalized to the PVM (flotillin-1 and 2, G_s, β_2-adrenergic receptor (β_2AR), aquaporin-1 (AQP1), Duffy, CD55, CD58, CD59, scramblase). Major integral membrane proteins are not internalized to the PVM [i.e. glycophorins A and C (GPA and GPC), cytoskeleton-associated band 3, etc.]. The lower left inset shows the perspective of the model, depicting a single infected erythrocyte with a magnified view of the plasma membrane and PVM. Since the PVM is formed by invagination of the plasma membrane, proteins that are cytoplasmically oriented in uninfected cells remain so upon infection; protein structures exposed to the extracellular space face the vacuolar space upon infection. Reproduced with permission from Murphy *et al.* [10].

invasion, inhibition of host G_s also inhibited blood-stage parasite growth (Fig. 3). This growth inhibition appeared to be due to delay in the terminal 'schizont' stage. Together these data establish that erythrocyte G protein signaling is needed for entry and intracellular parasite proliferation and thus may present a novel antimalarial target. When used in combination with existing antimalarials in cell culture, the β antagonist propranolol reduced the IC_{50} and IC_{90} concentrations for existing drugs against *P. falciparum* by five to 10-fold. These combinations were effective in reducing drug dose in animal models of infection as well. Hence there is now established proof of concept that erythrocyte G_s antagonism offers a novel strategy to fight infection and may be used to develop combination therapies with existing antimalarials.

Erythrocyte remodeling during intracellular parasite growth

In addition to entry, parasite proteins are secreted to the host erythrocyte during intracellular development. The best characterized of these virulence proteins belong to a variant antigen family of surface adhesins called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1 encoded by VAR genes) that has been linked to both cerebral malaria and placental malaria as well as protein

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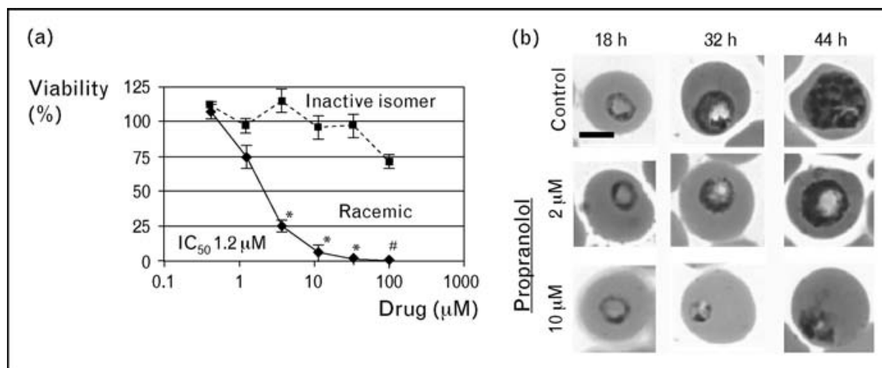
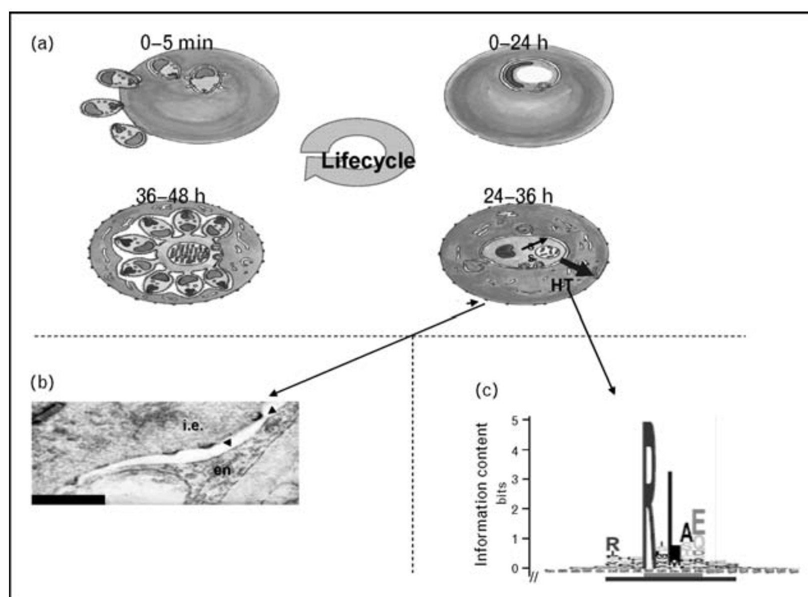
Figure 3 β-blockers target G_s signaling and inhibit maturation of *Plasmodium falciparum* in in-vitro cultures



(a) [^{3}H]-hypoxanthine incorporation of *P. falciparum* 3D7 when treated with racemic propranolol (black diamonds) or its inactive isomer (black squares). Error bars show the standard deviation of triplicate measurements. IC_{50} values were determined by fitting the data to a Lorentz cumulative transition function using TableCurve 2D software; the IC_{50} value obtained for racemic propranolol (1.8 μM) is shown. $n = 3$; $^{\#}P < 0.001$; $^{*}P < 0.05$. (b) Effect of 2 μM propranolol on intracellular growth in normal erythrocytes. Mock and drug-treated cultures were monitored at 15, 32 and 44 h after invasion by examination of Giemsa-stained thin blood smears. Reproduced from [15••].

families like STEVOR, RIFIN, also known for their antigenic variation. Studies (including our own) have identified a critical, conserved, host-targeting signal bearing a distinct, 11-amino-acid motif shared by these and other virulence proteins that enables their export to the erythrocyte [21,22]. This led to identification of a (first) major host-targeting pathway and 'secretome' in eukaryotic pathogens (Fig. 4) [23]. This signal is present

Figure 4 Erythrocytic remodeling upon infection by malarial parasites



(a) Asexual life cycle of *Plasmodium falciparum*. Extracellular merozoite invades mature erythrocyte to form an intracellular ring stage parasite encapsulated in a vacuolar membrane. During ring and trophozoite development numerous parasite proteins are exported to the cytoplasm and membrane of the erythrocyte as well as structures called the Maurer's clefts induced in the erythrocyte. Several hundred parasite proteins [including virulence adhesins such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) delivered to 'knobs'] are predicted to be exported via the host-targeting motif: the host-targeting motif must be embedded in a larger vacuolar translocation sequence (VTS) which is necessary and sufficient for parasite protein transport to the erythrocyte. Logo of host-targeting motif in predicted *P. falciparum* secretome is shown in panel (c). (b) Transmission electron micrographs of electron dense structures called knobs that are induced at the infected erythrocyte membrane. Electron dense knobs (black arrows) contain PfEMP1 adhesins that mediate attachment of infected erythrocytes (i.e.) to endothelial (en) cells, leading to their sequestration in blood vessels of the brain and placenta, the primary injurious event underlying cerebral and placental malaria. (c) Logo representation of the host-targeting motif in *P. falciparum* secretome proteins. On the *x* axis, the longer bar indicates the 11-amino-acid motif recognized by MEME [21], with a five-amino-acid core (shorter bar). Adapted with permission from [23].

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Table 1 In-silico annotation of *Plasmodium* proteins containing a signal sequence and RxLxE/D/Q host-targeting signal

	No. of proteins[a]
P. falciparum	
RIFIN (antigenic family)	165
STEVOR (antigenic family)	32–34
Kinases (FIKK)	18
DNAJ/heat shock (HSP40, 2) (RESA and RESA related, 8) (other, 7)	17
MC-2M (antigenic family)	13
Phosphatases	3
ABC transporter	1
Knob associated histidine-rich protein (KAHRP)	1
Other proteins known to be exported to the erythrocyte (GBP130, 3) (PfEMP2 MESA, 1) (PfHRPII, 1) (PfEMP3, 1)	6
PfEMP1 cell surface adhesin (antigenic family, no leader signal sequence, internal signal sequence)	59
Hypothetical unknown (includes some antigenic families with 10 or few members)	~80–190
P. yoelii	
Hypothetical family-1	39
PfATPase3	1
Plasmodium vivax PV1H14030_P putative	1
DHHC zinc finger domain, putative	1
Merozoite surface protein 4/5	1
Methanol oxidation protein	1
Aminopeptidase-like protein, related	1
Hypothetical, unknown	25

PfEMP1, *P. falciparum* erythrocyte membrane protein 1. Reproduced with permission from [23].
[a] Assignments based on Hiller *et al.* [21], Marti *et al.* [22], Ward *et al.* [24], Sam-Yellowe *et al.* [25], Schneider and Mercereau-Puijalon [26], Sargeant *et al.* [27] and http://fozzie.pathology.northwestern.edu/cgi-bin/PlasmoHT/index.cgi.

in over 400 putative effector proteins (the host-targeting 'secretome'), suggesting that erythrocyte modification is complex. Moreover, parasite-encoded heat shock proteins (HSPs) may play a central role in host remodeling [21].

The overall composition of the host-targeting secretome reveals that three major antigenic families of *P. falciparum* (STEVOR, RIFIN and VAR) comprise approximately half the secretome (Table 1 [24–27]). Approximately 200 additional 'hypothetical' proteins have no recognizable annotation other than some repeats or selective amino acid enrichments [21]. A small proportion (~25 proteins) are predicted by in-silico annotation to include putative HSPs, chaperones, kinases, phosphatases and proteases. Over 50 genes are clearly expressed in blood stages. Bioinformatics suggest that the export signal is also present in sporozoite (i.e. circumsporozoite protein) and liver stage proteins (i.e. liver stage antigen-3), but experimental evidence for its function in these genes is not yet available. Preliminary predictions of rodent malaria species *P. yoelii* and *P. berghei* suggest their host-targeting secretomes are much smaller (~60 total predicted effectors) with little overlap (~10 syntenous orthologues; mostly hypotheticals) with the *P. falciparum* host-targeting secretome [28]. Strikingly, there is *P. falciparum*-specific expansion of proteins, for example amongst proteins containing HSP DNAJ domains, suggesting that expansion may be linked to virulence and disease in human malarial infections.

Early analyses suggest that the host-targeting motif constitutes a new transport signal distinct from the well known examples described for protein transport across cellular membranes in mammalian cells and pathogenic bacteria (i.e. *Salmonella* spp.). Recent studies have indicated, however, that polymorphic avirulence (AVR) proteins in oomycetes, such as *Phytophthora infestans* (which caused the Irish potato famine), contain a functionally equivalent transport signal, even though these signals fall outside of predictive algorithms designed to identify malarial host targeting signals [29•]. There are two important implications with respect to malarial infection. First, recent algorithms that predict malarial host-targeting secretomes on linear motifs [21,22] suffer significant limitations in the absence of three-dimensional, structural information of the active and inactive leader sequences. Second, like bacterial pathogens, divergent eukaryotic microbes may share pathogenic secretion strategies.

Apparatus and machinery for virulence protein export to the erythrocyte surface

Since the erythrocyte lacks a transport apparatus, it is expected that the parasite exports both machinery and cargo for transport into the host cell. For a surface exposed protein like PfEMP1, the host-targeting signal and the transmembrane and cytoplasmic domains of this protein are sufficient to ensure its delivery to the erythrocyte surface [21,22]. High levels of PfEMP1, however, have been shown to reside in cleft structures induced in the periphery of the erythrocyte upon infection, while as little as 10% is exposed at the infected erythrocyte surface [30], suggesting that Maurer's clefts may be intermediates in transport to the erythrocyte surface [30]. Recent studies from Cooke and coworkers [31••] show

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that knocking out a resident Maurer's cleft protein blocks PfEMP1 export to the erythrocyte surface. Trelka, Taraschi and coworkers [32,33] have reported that intraerythrocytic structures traffic PfEMP1 cargo. Knuepfer and coworkers [34] have suggested that PfEMP1 is also transported as soluble protein across the erythrocyte; their idea is based on diffusion rates of green fluorescence obtained in photobleaching studies. There is good evidence that PfEMP1 is trafficked as a soluble protein within the parasite [35]. The soluble protein export in the erythrocyte described above [34], however, could also arise from uniform diffusion of vesicles, and additional extensive controls are needed to confirm that photobleaching itself does not alter the measured rates of transport by liquefaction of cytoplasm despite the use of protective agents (and especially in the hemoglobin-rich environment of the erythrocyte).

An attractive concept is that the parasite reconstitutes the classical secretion machinery in the enucleated host erythrocyte. The predicted host-targeting secretome, however, fails to reveal the export of conserved, parasite-encoded components of eukaryotic secretion (such as SNAREs, SNAPs, Rabs, etc.). There is no evidence from either functional data or primary sequence suggesting that these factors are transported to the erythrocyte. Heterologous antibodies to factors such as COPII, N-ethylmaleimide-sensitive factor (NSF) and Sar1p have been found to label structures in the infected erythrocyte [36–38], but a higher level of proof validating their function in the erythrocyte is not available. Nonetheless, it is still too early to rule out involvement of 'classical secretion' mediated by factors that are exported by as-yet unknown mechanisms to the erythrocyte.

Selective expansion of proteins containing the chaperone heat shock DNAJ domain in the host-targeting secretome of *P. falciparum* suggests that these proteins may regulate virulence mechanisms associated with this malarial species. Some, like ring-infected erythrocyte surface antigens (RESAs) and mature erythrocyte surface antigen (MESA), seem to protect the host skeleton from heat shock [34] and bind junctional complexes in the erythrocyte membrane [39]. Others may directly increase the efficiency of antigen transport. Banumathy and coworkers [40] have shown that host HSP70 complexes with host-targeting cargo such as PfHRPI/knob-associated histidine-rich protein (KAHRP) [40]. The erythrocyte lacks an HSP40, which is frequently needed to couple with HSP70 to facilitate substrate association and ATPase activity [41]. Parasite protein PFE0055c was a predicted host-targeting secretome parasite HSP40. It was also predicted to be in apicoplast [42], but is now demonstrated to in fact be exported to the erythrocyte [21]. Future studies will reveal how this gene as well as possibly other exported HSPs and DNAJ proteins regulate transport of *P. falciparum* antigens in virulence remodeling of human erythrocytes.

Thus, in addition to the exported surface antigens, the actual machinery that regulates transport may also be critical to malaria pathology because it regulates the export virulence determinants at the erythrocyte surface. Chaperones such as HSPs could significantly affect the efficiency of antigen expression by acting at the site of host-targeting exit or the Maurer's clefts. Further, it is now established that knocking out a resident Maurer's cleft protein significantly alters protein export to the host [31••]. This provides persuasive evidence that changes in both the antigenic cargo and the transport apparatus may influence virulence.

Conclusion

Current estimates of erythrocyte raft proteins involved in malarial vacuole formation suggest that approximately 15–120 are likely to be involved in the process. In contrast, estimates of parasite proteins involved in remodeling the erythrocyte during invasion and intracellular growth exceed 400. This suggests that the parasite utilizes multiple strategies to establish infection. Moreover disease pathologies such as cerebral malaria may be linked to multiple parasite factors, illustrating why its pathology is so varied and why sequestration is seen in multiple organ systems [43,44].

Acknowledgements

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 292).

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