Oxidative Stress, Malaria, Sickle Cell Disease, and Innate Immunity

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Abstract

Plasmodium falciparum shields from adaptive immunity in erythrocytes, but how might the innate immune system recognize infected cells? Replication by the parasite results in oxidative stress, which causes surface expression of high mannose glycans. These should act as pathogen associated molecular patterns to stimulate phagocytosis in the spleen. The sickle cell allele enhances these responses.

Malaria and Immunity

Plasmodium falciparum, the main cause of severe malaria, infects ~200 million and kills ~400,000 annually worldwide [1, 2]. Its life cycle in humans is mainly intracellular, which helps avoid binding of circulating antibodies and innate immune adaptor molecules to the parasites' cell walls. The presence of intracellular pathogens is generally signaled to the immune system by pathogen-associated molecular pattern (PAMP) receptors and presentation of foreign antigens on MHC molecules. However, to improve oxygen delivery for maintenance of warm blooded temperatures, mammalian RBCs dispensed with nuclei and most cellular functions, including MHC molecules and most PAMP receptors. *P. falciparum's* success in evading adaptive immunity is illustrated by a lack of MHC associations [3]. After replication in RBCs, **merozoites** are released into plasma, so are susceptible to host adaptive and innate immune mechanisms. They therefore enter another red blood cell (RBC), usually within 10 minutes [4]. Subsequently invisible to adaptive immunity, how can the innate immune system detect and mark infected RBC for destruction?

Several innate immune receptors are implicated in host responses to malaria, although most induce inflammatory responses rather than target infected cells for destruction [5]. The spleen removes damaged RBCs and plays a crucial role in clearing cells infected by malarial parasites [6]. In the absence of evasive strategies, a human RBC would pass through the spleen around 72-288 times per replicative cycle (5-10% of cardiac output, circulation time 1 minute, life cycle 24-48 hours) [2]. To traverse the spleen, infected RBCs must pass through macrophage rich red pulp sinusoids, then squeeze through narrow slits lined by specialized endothelial cells before re-entering the systemic circulation. Failure to navigate through this hostile environment results in phagocytosis. How infected are discriminated from healthy cells remains uncertain, although the increased rigidity known to be caused by the parasites is thought to be important [6].

Malaria and Oxidative Stress

RBCs are particularly susceptible to oxidative stress (OS) due to their high concentration of heme moieties containing iron atoms in a state that, free from their binding sites in hemoglobin, catalyze the formation of reactive oxygen species. Mammalian plasmodia took advantage of MHC-free RBCs, but subsequently had to deal with this toxicity. Despite sequestering heme into specialized organelles, infection is still associated with profound OS [7]. Therefore, the discovery of a mechanism inducing an RBC surface **'eat me' signal** in response to OS [8] raised the possibility that it might be used as a pro-phagocytic signal in the context of malaria. Specifically, OS causes cross-links between macromolecules. Most such damaged proteins cannot be repaired and must be removed. Cytoplasmic proteins are mainly degraded by the proteasome. Damaged membrane proteins tend to be delivered to

lysosomes, which RBCs are the only mammalian cells to lack. OS in RBCs results in the accumulation of plasma membrane high molecular weight aggregates, which carry N-linked high mannose glycans [8]. These motifs could be recognized by the mannose receptor (CD206), a well characterized immune receptor that belongs to the C-type lectin family [9]. It is able to mediate phagocytosis of high mannose bearing RBCs by macrophages [8]. Infection of RBCs *in vitro* by *P. falciparum* was shown to induce these high mannose bearing aggregates [8], which are therefore putative parasite-specific 'eat me' ligands. Which phagocytic cells might use these motifs as pro-phagocytic signals remains be established. Most phagocytosis of RBCs infected with malarial parasites takes place in macrophages surrounding splenic red pulp sinusoids, certainly in mice and supported by more limited studies from humans [6]. In mice, CD206 is expressed by both red pulp macrophages and endothelial cells [9]. However, in humans CD206 is expressed only on the specialized Lyve-1^{+} endothelial cells that line the slits through which red cell must pass to exit the splenic red pulp [9]. Thus CD206 may be a more important ligand in mice than humans. In humans, perhaps the endothelial cells mediate high mannose mediated phagocytosis, while other lectins, like DC-SIGN, together with high degrees of cellular rigidity, are used by macrophages. Further uncertainty surrounds the question of how the macrophages react to phagocytosing parasitized cells. Malaria is an inflammatory disease and higher degrees of inflammation are associated with a worse prognosis [2]. The parasite does not induce inflammatory cytokine responses in many macrophages in vitro and the source of inflammation in patients remains unclear, although several mechanisms have been proposed [5]. CD206 is generally regarded as as an anti-inflammatory receptor [9], so perhaps is used preferentially in cases of less severe disease.

Malaria and the sickle cell allele

Relationships between hosts and pathogens are characterized by an evolutionary arms race, with each side's response begetting retaliatory responses. Human hosts have evolved to acquire protective mutations against malarial parasite infections [3]. The best known of these is the sickle cell mutation (*HBB* E6V), heterozygosity for which, sickle cell trait, results in a mixture of hemoglobins (HbAS) compared to wild type (HbAA) [10]. Sickle cell trait confers >90% protection against severe malaria [11], particularly cerebral disease and hemolysis [3]. The mutant protein can polymerize and inhibit parasite replication under hypoxic conditions that plausibly pertain following cytoadherence and sequestration into certain organs [12].

To avoid passage through the spleen*, P. falciparum* harbors the var gene family, encoding the PfEMP1 family of adhesin proteins, which mediate adherence of infected cells to the endothelium of blood vessels in the systemic circulation [2]. Transcription in any single parasite is restricted to only one of about 60 var genes, with both heterogeneity of expression and a degree of switching after infection contributing to avoidance of adaptive immune responses [2]. Following infection of human RBCs with *P. falciparum*, the sickle mutation exacerbates the redox imbalance of erythrocytes, causing aberrant actin remodeling and downregulating PfEMP1 protein expression relative to RBCs with wild type hemoglobins [13]. Passage through the spleen is thereby enhanced by the mutation [14]. The polymorphism might also potentially enhance the expression of high mannose glycans induced by *P. falciparum* infection. This hypothesis was validated when a striking inverse correlation was observed between high mannose glycan and PfEMP1 expression in infected RBC from donors with sickle cell trait relative to controls [8]. Thus the redox state of infected cells both upregulates 'eat me' signals and downregulates ligands that prevent interaction of these

signals with their cognate pro-phagocytic receptors [8,14]. The observation that the sickle cell mutation protects more strongly against severe anemia than cerebral malaria caused by intracerebral sequestration indicates that the beneficial effects of the mutation might be mediated more strongly through induction of 'eat me' signals than inhibition of sequestration signals [3].

Of note, other immunological mechanisms have been invoked to explain, at least in part, how the sickle cell mutation might protect against severe malaria [12]. Immunologically, free plasma heme induces heme oxygenase-1 (HO-1), and via cascading events, protects mice against cerebral malaria [15]. However, we posit that this mechanism is unlikely to be important in humans as it depends on intravascular hemolysis, which is not observed in individuals with sickle cell trait, yet is prominent in those with sickle cell anemia, who have no particular protection against severe malaria. The sickle cell allele might also enhance adaptive immune responses [10], although this remains to be fully assessed and the putative molecular mechanisms for such responses remain elusive.

Summary

Infection of mammalian RBC with malarial parasites leads to OS. This is known to inhibit the mechanism whereby the parasite trafficks adhesins to the RBC surface, so increasing their passage through the spleen (Figure 1). OS is also now known to cause surface expression of oxidatively cross-linked protein aggregates bearing N-linked high mannose glycans [8]. These are likely to act as 'eat me' signals in the spleen, mediated by mannose receptors that include the mannose receptor (CD206). Thus high mannose glycans orignally acted physiologically as

damage associated molecular patterns (**DAMPs**), but in the context of malaria, have been recruited to act as pathogen associated molecular patterns (**PAMPs**).

In humans, these effects of OS are enhanced if infected cells express sickle cell hemoglobin in a heterozygous context. We find it intriguing that so many anti-malarial drugs (including chloroquine, primaquine, artemisinin, dapsone, sulfadoxine, pyrimethamine) have prominent oxidative properties [7]. Although conjectural, we propose that these drugs might work not only by inhibiting parasite growth directly, but perhaps also as enhancers of innate immunity, in an analogous way to the multi-effect sickle cell mutation. Nevertheless, these hypotheses remain to be tested. Incorporating phenotypes of OS into drug screening assays might help inform the development of more effective anti-malarial drugs in the future.

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Authors' contributions

HC and MAV wrote the article.

Declaration of Interests

The University of Aberdeen has been granted a patent based on the work described in reference 8.

Glossary

Damage-associated molecular pattern (DAMP)

Ligands carried on the surface of, or secreted by, cells in distress. They are recognized by pattern recognition receptors to provoke repair and inflammatory responses.

Eat me signal

Ligands expressed on the surface of cells that interact with potentially phagocytic cells to stimulate their uptake

Heme

A tetradentate ligand comprising porphyrins that chelates iron atoms in a form that can reversibly bind oxygen molecules. Heme is tightly bound to hemoglobin molecules, as the iron atoms are held in a valency state that stimulates the production of reactive oxygen species and thus oxidative stress.

Merozoites

The form of *P. falciparum* released from hepatocytes and RBCs that has the ability to enter into, and replicate within, RBCs.

Pathogen-associated molecular pattern (PAMP)

Ligands carried on the surface of potentially pathogenic infectious agents. They are recognized by pattern recognition receptors to provoke immune responses.

Schizonts

The last stage of replication of *P. falciparum* in RBCs before formation and release of merozoites.

Trophozoites

The stage of replication of *P. falciparum* in RBCs formed after merozoites begin to replicate and before schizont formation. They account for the majority of parasites in clinical malaria.

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Figure 1: sickle cell hemoglobin inhibits the replication of *P. falciparum* in and surface expression of adhesins on human red blood cells (RBCs), but enhances the surface expression of high manose glycans, which likely mediate RBC phagocytosis in the spleen.

P. falciparum merozoites, shown in blue on the left, invade red blood cells. Wild type cells, shown above, contain normal hemoglobin (HbAA), while those with sickle cell trait, shown below, contain a mixture of hemoglobins (HbAS). As replication proceeds through trophozoite and schizont stages, in cells with sickle cell hemoglobin oxidative stress is greater, resulting in (i) slower parasite replication, (ii) fewer/later adhesin expression on RBCs [13,14] (shown in yellow), (iii) a degree of sickling [12], and (iv) greater/earlier expression of cross-linked membrane protein aggregates bearing high mannoses [8] (shown as dark green circles). Fewer adhesins result in less binding to ligands expressed on peripheral vascular endothelium (shown as light green), so less sequestration in hypoxic tissues and more frequent passage of infected RBCs through the spleen [14], where recognition of high mannoses by mannose receptors results in more efficient uptake by phagocytic cells [8] (shown in purple).