The molecular basis of paediatric malarial disease

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Abstract

Severe falciparum malaria is an acute systemic disease that can affect multiple organs, including those in which few parasites are found. The acute disease bears many similarities both clinically and, potentially, mechanistically, to the systemic diseases caused by bacteria, rickettsia, and viruses. Traditionally the morbidity and mortality associated with severe malarial disease has been explained in terms of mechanical obstruction to vascular flow by adherence to endothelium (termed sequestration) of erythrocytes containing mature-stage parasites. However, over the past few decades an alternative 'cytokine theory of disease' has also evolved, where malarial pathology is explained in terms of a balance between the pro- and anti-inflammatory cytokines. The final common pathway for this pro-inflammatory imbalance is believed to be a limitation in the supply and mitochondrial utilisation of energy to cells. Different patterns of ensuing energy depletion (both temporal and spatial) throughout the cells in the body present as different clinical syndromes. This chapter draws attention to the over-arching position that inflammatory cytokines are beginning to occupy in the pathogenesis of acute malaria and other acute infections. The influence of inflammatory cytokines on cellular function offers a molecular framework to explain the multiple clinical syndromes that are observed during acute malarial illness, and provides a fresh avenue of investigation for adjunct therapies to ameliorate the malarial disease process.

Introduction

Although many species of malarial parasite exist, only *Plasmodium falciparum*, *vivax*, *ovale*, and *malariae* are classically associated with human infection. The former two species are most frequently associated with malarial disease in humans, with severe malarial disease almost exclusively associated with *P. falciparum* infection. Falciparum malaria is responsible for considerable morbidity (300–500 million annual clinical cases) and death across the globe, with a particular burden of mortality among children in sub-Saharan Africa. Infection with *P. vivax* is rarely fatal, but is associated with considerable morbidity outside the African continent. It should also be recalled that malaria causes social and economic disruption on a uniquely large scale [1].

Severe adult malaria is a clinical syndrome originally classified using 10 defining and 5 supportive (often overlapping) clinical features unified by the presence of asexual malarial parasites in the peripheral blood smear [2]. Based on observations of children in coastal Kenya, paediatric severe malaria has similarly been distilled into three main (again often overlapping) clinical syndromes, anaemia, respiratory distress (an indicator of an underlying metabolic acidosis) and impairment of consciousness [3]. These clinical syndromes are discussed below.

In the review mentioned above [3], the authors' judicious use of the term impaired consciousness, rather than cerebral malaria (CM), promoted the useful concept that the neurological features (and in-turn the underlying mechanisms) associated with severe malaria are not necessarily unique to malarial disease. Indeed, over 60 years ago, it was noted that the clinical features of malaria can resemble those exhibited in patients with fulminant bacterial or viral infections [4].

Severe malaria has been intensively studied, and there appears to be a complex interplay between host infection and disease. This is highlighted by the different clinical manifestations of severe malaria exhibited by children and adults. These differences are undoubtedly, in part, a function of patient age. However, age is just one of a series of interacting factors, e.g. geographical region, level of malaria transmission, degree of previous malaria exposure, length of illness prior to treatment and host immunity that may influence the clinical presentation of severe malaria. This variation in clinical presentation has been mirrored by a similar multitude of proposals regarding the functional mechanisms underlying pathogenesis of severe malaria.

One concept of pathogenesis consistently articulated has been the 'mechanical theory'. Historically, this theory was developed from two fundamental differences between *P. falciparum* and *P. vivax* infection. Firstly, erythrocytes parasitised with *P. vivax* do not sequester. Secondly, death following *P. vivax* infection is rare. Consequently, pathogenesis is believed to be due to obstruction of micro-vascular flow by erythrocytes containing mature-stage falciparum parasites adhering to the endothelium (termed sequestration).

More recently the 'cytokine theory of disease' has also gained credence. This theory can be applied to disease following both falciparum and vivax infection. The lower mortality associated with *P. vivax* being explained by a relatively milder degree of pro-inflammatory imbalance during the host's response to *P. vivax* infection.

The main theme of this chapter is to examine the increased understanding of the functions of inflammatory cytokines gained over the past 15 years, and explore how these insights are changing attitudes in malarial disease research. We also discuss how two theories (mechanical and cytokine) can, as proposed first in a recognisable form at least 65 years ago [5], be complementary.

Table 1. Comparison of Kenyan children and Papua-New Guinea adults admitted to hospital using the WHO classification

	Kenyan children [7]		Adults Papua-New Guinea [19a]	
	Prevalence	Mortality	Prevalence	Mortality
Defining criteria				
Coma*	10.0	16.8	17.1	41.7
Severe anemia**	17.6	4.7	10	0
Pulmonary oedema				
Hypoglycaemia	13.2	21.7	5.7	75
Circulatory collapse	0.4	71.4	0	
Renal Failure	0.1	0	22.9	37.5
Spontaneous bleeding	0.1	0	0.1	100
Haemoglobinaemia	0.1	50	0.1	
Acidosis	63.6	21.4		
Repeated convulsions	18.3	6.8	0.3	0
Supporting criteria				
Impaired consciousness	8.2	6.0	37.1	11.5
Jaundice	4.7	11.9	45.7	25
Prostration	12.2	5.2		
Hyperpyrexia	10.6	1.6	20	7.1
Hyperparasitaemia	8.9	4.3	40	28.6

* Childhood coma is defined by a Blantyre coma score ≥ 2.
** The childhood definition for severe anemia does not include a cut off for parasitaemia.
Modified from [11].

Severe malaria in children compared to adults

The majority of the clinical cases of malaria occur in sub-Saharan Africa. Nevertheless, malaria also accounts for considerable morbidity and mortality in other continents particularly South East Asia [6]. In malaria-endemic regions (e.g. sub-Saharan Africa), where the resident population have continuous exposure to malarial parasites, most of the severe cases are seen in children [7]. In hypoendemic regions (e.g. South East Asia), where parasite exposure is more intermittent, cases of severe malaria are also common in adults (Tab. 1).

Clinical features associated with malaria mortality vary between children and adults, but acidosis and coma are associated with malarial mortality in both populations [7, 8]. Acute renal failure (ARF) and pulmonary oedema,

a marker for adult respiratory distress syndrome (ARDS), are almost exclusively reported among adults [9, 10], whereas mortality associated with hypoglycaemia is frequently reported among children [11].

Why malarial disease displays such age-related differences in pathophysiology is unclear. However, these differences are not exclusive to malaria. ARDS, which is more frequently observed as a complication of trauma in adults compared with children [12], is believed to reflect an exaggerated pro-inflammatory response within the lung [9]. A possible lead for future studies on these age-related differences in malaria is suggested by a report of peritoneal macrophages collected from healthy adults producing much less interleukin (IL)-10 (an anti-inflammatory cytokine), but the same levels of pro-inflammatory cytokine, than those from healthy children, giving adults a much higher pro-inflammatory status [13, 14].

The mechanism of malarial ARF pathogenesis is postulated to be multifactorial, involving mechanical, haemodynamic, and immunological factors [15]. The observation that ARF is more frequently observed as a complication of trauma in adults than children [12] suggests that age-related variations in cytokine response may again influence pathogenesis.

Hypoglycaemia is regarded as a more frequent complication of sepsis in paediatric populations compared with adults [16]. Hypoglycaemia in children may, in part, be associated with a higher basal metabolic rate, and lower glycolytic [17] and gluconeogenic substrate reserves compared to adults [18]. However, these substrates are not always limiting during acute paediatric malaria, suggesting functional impairments of glucose metabolism may also occur [19]. Such functional impairments may, in part, be influenced by increases in inflammatory cytokines as the infection progresses.

How might *P. falciparum* cause this complex disease?

Once the malarial parasite was identified as the cause of disease, it quickly became apparent that illness and death were linked with parasite invasion into bloodstream and subsequent parasite growth within (and release from) the erythrocytes. By the start of the 20th century, two major theories, capillary blockage and toxicity of the parasites themselves, had been proposed to explain morbidity and mortality. Thus, the study of malarial disease is not a settled story requiring regular updates, but one containing, from its beginning, an unresolved tension. Vascular occlusion and malarial toxin (nowadays vascular occlusion and inflammatory cytokines) have been alternative approaches to understanding malarial disease as a whole, as well as the coma, for over a century, and the two have often been discussed side by side [5, 20, 21]. The presence of hyperlactataemia, hypoglycaemia, and metabolic acidosis, all three consistent with a patient being forced to rely on anaerobic glycolysis for energy production, have provided a consensus that hypoxia is central to disease pathogenesis in falciparum malaria. As sum-

marised below, the modern literature offers two main theories for cellular hypoxia during infection; insufficient oxygen delivery to cells and impaired oxygen utilization within the cells. Both mechanisms may be governed by the host inflammatory cytokine response to infection. This chapter focuses on how an increased understanding of the molecular functions of cytokines during disease demonstrates a closer alignment between the pathogenesis of falciparum infection and other systemic infectious diseases.

Inflammatory cytokines and malarial disease

One hundred and twenty years ago, Golgi (of the Golgi apparatus [22]), noted onset of malarial fever and illness at a predictable short interval after the regular shower of new parasites were released from bursting red cells. The nature of the putative toxin so released was much discussed in the first decade of the 20th century [23]. It was assumed to be directly toxic, in the manner of tetanus toxin. The proposal that malarial products were not harmful in themselves, but only through causing the infected host to harm itself through generating toxic amounts of molecules (pro-inflammatory cytokines) that, in lower concentrations, inhibit growth of malarial parasites did not arise until 1981 [24]. Indeed, acceptance of the broad applicability of this concept to infectious disease in general is now sufficient for its evolution to be a subject for research [25]. Tumour necrosis factor (TNF) is regarded as a major player, malaria being the first disease in which it was proposed to cause systemic illness and pathology [24]. Multiple TNF promoter polymorphisms have since been independently associated with severe malaria across several geographical populations [26]. A longitudinal study in Burkino Faso has also demonstrated several TNF promoter polymorphisms associated with the regulation of host-parasite density [27]. The TNF concept has since begun to dominate the sepsis literature [28], and the virulence of different strains of influenza, a disease that is a standard clinical misdiagnosis for imported malaria, has recently been expressed in terms of their capacity to induce TNF [29]. The critical role of TNF in both malaria and influenza pathogenesis is consistent with the clinical similarities between the diseases. Indeed, TNF infusions in tumour patients produce side effects mimicking both diseases [30], as discussed below.

Although TNF is the prototype pro-inflammatory cytokine linked with severe malaria, other cytokines (and mediators) including interferon (IFN)-γ [31], its corresponding receptors IFN-γ receptor-1 [32] and IFN-α receptor-1 [33], IL-1 [34], IL-4 [35] and IL-10 [36] have all be identified through genetic association analysis to be linked with their potential regulation of malarial disease severity.

All the above cytokines typically act as homeostatic agents, but can cause pathology if produced excessively. When this happens they also induce a late-onset, but long-acting cytokine termed the high mobility

Table 2. Some changes common to systemic inflammatory states, including falciparum malaria

Cytokines	– TNF, IL-1, iNOS, IFN-γ raised
	– MIF, IL-10, and HO-1 raised
	– γ/δ T cells increased
	– S100A8–S100A9 complex raised
	– Procalcitonin raised
	– S100A12 raised
	– HMGB1 raised
	– ICAM, VCAM and p-selectin raised
Consequences	– Insulin resistance
	– Hyperlactataemia
	– Hypertriglyceridaemia
	– Hypoglycaemia
	– Metabolic acidosis
	– Hyponatraemia
	– Coagulopathy
	– Thrombocytopaenia
	– Decreased red cell deformability

group box 1 (HMGB1) protein, which prolongs and amplifies inflammation [37, 38]. This molecule, normally in the cellular nucleus and previously known only for several physiological functions, now shows great promise as a therapeutic target in sepsis, in that countering it after the onset of illness protects well in experimental sepsis [39, 40]. It accumulates, in proportion to degree of illness, in serum from African children infected with falciparum malaria [41].

Once neutralising anti-TNF antibodies became available for human use, they were tested for efficacy against malarial disease. Unfortunately, a central tenet of the cytokine concept of infectious disease (that the pro-inflammatory cytokines that cause disease are the same mediators that, in lower concentrations, are responsible for the innate immunity that controls parasite growth) was not taken into consideration. TNF has been shown to inhibit a mouse malarial parasite *in vivo* [42], and *P. falciparum in vitro*, provided white cells to generate the next down-stream mediator, possibly nitric oxide (NO) [43], were present [44]. This is consistent with findings in human subjects [45]. Thus, it is not surprising that anti-TNF antibody, by removing inhibitory pressure from the pathogen, can enhance the disease in falciparum malaria [46], as shown 5 years earlier in human sepsis [47].

Cytokines as a disease mechanism extends beyond malaria

As noted above, the idea that excessive production of inflammatory cytokines underlies the pathology of illness is used widely, from malaria across a range of conditions, infectious or otherwise. As reviewed recently [48], this now includes the illnesses caused by rickettsias, protozoa other than malaria, and viruses. Increased circulating levels of these cytokines have been detected in the serum very soon after onset of illness in virtually all those infectious diseases in which they have been sought. Some cytokine increased, and consequences are shown in Table 2. When rTNF was under trial in volunteers as an anti-tumour agent [49, 50] nearly 20 years ago, virtually all of the symptoms and signs they share were reproduced as side effects. This includes headache, fever and rigours, nausea and vomiting, diarrhoea, anorexia, myalgia, thrombocytopaenia, immunosuppression, and central nervous system manifestations, all of which have been shown to be caused by a mechanism involving inflammatory cytokines. The rate, timing and intensity of cytokine release vary in different disease states, and provide them with somewhat individual clinical pictures, but the fundamentals remain. Nevertheless, the clinical patterns generated are remarkably close, in that, at least in some populations, clinical features cannot predict a diagnosis of malaria from other causes of fever [51].

Inflammatory cytokines acting indirectly to cause disease

Vascular occlusion

Mature erythrocytic forms of *P. falciparum* are not seen in peripheral blood smears, and cause the erythrocytes they inhabit to adhere to the walls of venules and capillaries. From this observation arose the widely held view that much of the pathology following malarial infection is explained through parasite sequestration causing impairment of microvascular flow. Sequestration certainly occurs, since the life cycle dictates this. However, whether the temporal and anatomical patterns of sequestration are the same in both individuals with fatal disease and in parasite tolerant individuals has not been ascertained. Consequently, whether sequestration is the principal instigator of local pathology, or whether sequestration is an associated feature of all malarial infections with local pathology determined by other factors in the host response to the infection, e.g. a local imbalance of inflammatory mediators, has not been fully elucidated.

Erythrocyte cyto-adherence (irrespective of whether this adhesive process is directly or indirectly due to parasite sequestration) has repeatedly been shown to be mediated through a series of host-derived ligands. CD36 and thrombospondin were the first described endothelial receptors that bound infected red blood cells (RBCs) [52, 53], with most studied wild

parasite isolates demonstrating adhesion to CD36 [54]. More recently, it has been shown that *P. falciparum* also interacts with other host adhesion receptors, i.e. intercellular adhesion molecule-1 (ICAM-1 CD54), vascular cell adhesion molecule-1 (VCAM-1 CD106) and E-selectin [55, 56]. Certain adhesive phenotypes, such as rosetting (the spontaneous tethering of infected and non-infected RBCs) and clumping (tethering of infected RBCs through platelets) have been preferentially associated with severe malarial disease [57, 58]. CD36 is involved in both mechanisms of adhesion, and a non-sense mutation in the gene encoding for CD36 has also been associated with protection from severe malaria [59]. Polymorphisms in the gene encoding ICAM-1 have also been associated with susceptibility to severe disease [27]. Furthermore, ICAM-1, together with VCAM and E-selectin, are up-regulated by TNF, with circulating levels of these ligands shown to be increased in severe malaria compared to uncomplicated infection [60].

Sequestration during falciparum malaria appears to be concentrated in the brain and placenta. There is some evidence to suggest that the propensity of inflammatory cytokines to up-regulate cell adhesion molecules, secondary to local variation in the density of thrombomodulin, is potentially higher in the microvasculature of the brain and placenta compared to other tissues. As reviewed [61], TNF and IL-1 increase tissue factor expression on endothelial cells, thereby initiating pathways that generate thrombin [62]. When thrombin binds to thrombomodulin on the endothelial cell surface, protein C is activated, which in turn can lead to further downstream activation of the coagulation cascade. Therefore vasculature with lowest thrombomodulin densities on the endothelial cell surface (brain least, placenta next least, and other organs more [63]) will have more unbound thrombin available for its other functions on activated endothelium. These other functions include up-regulation of adhesion molecules such as selectins, ICAM-1, VCAM-1 [64] and monocyte chemotactic protein-1 (MCP-1) [65]. Therefore, up-regulation of adhesion molecules within the cerebral vessels may occur as a local endothelial response to systemic inflammation and may not necessarily be precipitated by parasite sequestration.

Anaemia

Anaemia is another obvious way in which too little oxygen reaches cells, and thus their mitochondria [66]. As recently reviewed [67], critical illness associated with an inflammatory response invariably causes multifactorial anaemia. Obviously a high parasite load in malaria indicates that the infected RBCs will soon burst when the next generation of erythrocytic forms escapes, but anaemia does not correlate with parasitaemia, and sometimes is extreme when very few parasites are, or have been, present. The severe anaemia in transgenic mice expressing human TNF [68] incriminates the

inflammatory response itself, so anaemia and mitochondrial dysfunction (see Mitochondrial dysfunction section below), both consequences of systemic inflammation, can be expected to coexist, and both contribute to total energy depletion.

Poor red cell membrane deformability

The lifespan of an RBC is, in part, limited by how long it can remain flexible enough to squeeze through fenestrations in specialised vessels in the red pulp of the spleen, and thus avoid phagocytosis by adjacent macrophages. Normally this loss is balanced by erythropoiesis, and haematocrit remains normal. If RBCs develop a premature loss of deformability they are removed from the circulation earlier. This loss of deformability happens to both infected and non-infected red cells in malaria, whether caused by *P. vivax* or *P. falciparum*.

Under physiological conditions, erythrocytes (and other cells) control the passive influx of osmotic active solutes (especially Na^+) *via* an active, energy-dependent elimination of these solutes using Na^+/K^+-ATPase. This prevents intracellular accumulation of osmotically active solutes, preventing a subsequent influx of water, cell swelling and loss of cell integrity. During human [69] and monkey [70] malaria infection, intracellular Na^+ accumulates within erythrocytes (both parasitised and non-parasitised) implying that this Na^+/K^+ pump is impaired during the disease process. Parallel changes in the ionic content of erythrocytes have been documented in a sepsis model of infection [71]. Similarly, reduction in erythrocyte deformability was shown to be associated with increased NO, an inhibitor of this membrane pump [72], in another sepsis model [73]. Since inhibition of the Na^+/K^+ pump *in vitro* correlates with both reduced red cell deformability and decreased red cell filterability [74], any factor that inhibits the Na^+/K^+ pump could potentially worsen anaemia. Identification of inducible NO synthase (iNOS) activity, as one factor influencing red cell deformability, suggests that a pro-inflammatory milieu [75] may again govern the reduction in red cell deformability observed during malaria infection.

Originally observed in uraemic patients, poor red cell deformability was recognised in a small pilot study of malaria patients in 1985 [76]. It was reported soon afterwards in sepsis [77, 78], and subsequently studied in falciparum malaria with a view to understanding both circulatory obstruction [79] and anaemia [80]. It seems clear that a short life (poor deformability), and a slow replacement rate (dyserythropoiesis, below) can combine to cause severe anaemia in various diseases, particularly in chronic infections such as malaria.

Dyserythropoiesis

When red cells have a shortened lifespan, e.g. secondary to reduced erythrocyte deformability, replacement by new recruits is vital to avoid anaemia. Unfortunately, the same inflammatory cytokines that shorten lifespan also

retard replacement. Some years ago researchers began to stress the contribution of bone marrow dyserythropoiesis to the anaemia of falciparum malaria [81, 82]. A group in Oxford [83], seeking an explanation for this dyserythropoiesis through an electron microscopy study of bone marrow, observed sequestration of parasitised red cells and argued that this caused the bone marrow dysfunction in falciparum malaria by restricting blood flow and thus inducing hypoxic changes. This idea proved inadequate, however, when this same group subsequently reported dyserythropoiesis and erythrophagocytosis in vivax malaria, in which parasitised red cells do not sequester [84].

Some time ago an undefined product in macrophage supernatants [85], later identified as TNF [86], was found to inhibit the growth and differentiation of erythroid progenitor cells. When rTNF became available, the dyserythropoiesis and erythrophagocytosis seen in terminal *Plasmodium vinckei*-infected mice was reproduced by giving a single injection early in the course of the infection [87]. Phagocytosis of erythroblasts in bone marrow, a phenomenon also reported by Wickramasinghe et al [83, 84] in human malaria, also occurred. Decreased erythropoiesis was subsequently reported in mice receiving continuous TNF infusions *via* implanted osmotic pumps, and mice expressing high levels of human TNF have been shown to become markedly anaemic during malaria infections [68], even though parasite numbers, and therefore red cell loss post-schizogony, are considerably reduced.

The past decade has seen an expansion of this line of enquiry into human malaria, and also the number of cytokines, both pro-inflammatory and anti-inflammatory [88, 89] in absolute amounts and ratios [90, 91], that have been investigated in this context. Investigations have been extended to include other pro-inflammatory cytokines, such as IL-12 [92] and FasL [93], and examined the role in anaemia of the persistence of cytokine production during malaria infection [94]. Another inflammatory cytokine, macrophage inhibitory factor (MIF) that is increased in malaria, and induced by TNF, has been shown to cause dyserythropoiesis in *in vitro* studies on bone marrow cells [95, 96]. Thus, inflammatory cytokines generated during malaria are a major determinant of the degree to which anaemia influences the amount of oxygen that reaches tissues in malaria.

Inflammatory cytokines acting directly to cause disease

Mitochondrial dysfunction

Mitochondria are vital to energy (ATP) generation through cellular respiration. Cellular respiration requires oxygen and pyruvate, as well as multiple cofactors and active transport molecules. Within the matrix of the mitochondrion organelle, pyruvate is catabolised *via* the Krebs cycle and oxidative

phosphorylation (involving NADH and FADH2) to generate ATP. When this series of reactions are 100% efficient (unlikely *in vivo*), 1 molecule of glucose generates 2 molecules of pyruvate, which are further catabolised to water and carbon dioxide with the concomitant generation of 36 molecules of ATP. In comparison, during anaerobic glucose catabolism, pyruvate is converted to lactate with the concomitant generation of 2 molecules of ATP, a process that also facilitates regeneration of NADH and FADH2.

Evidence is accumulating that inflammatory cytokines, as released in malaria, sepsis, and viral diseases, induce mitochondrial dysfunction and dysregulate cellular respiration, resulting in the incomplete catabolism of pyruvate. The process, termed 'cytopathic hypoxia'[97], mimics cellular hypoxia, in that it results in the incomplete catabolism of pyruvate and accumulation of lactate. Awareness of this mechanism began with oxygen tension being shown to be increased in septic rats [98] and patients [99]. A cytokine model of mitochondrial dysfunction has since been developed in which impairment of cellular respiration occurs following induction of sepsis (or exposure to pro-inflammatory cytokines), despite sufficient oxygen supply [97, 100, 101]. More recently, impairment of enzyme activity associated with the mitochondrial complexes has been demonstrated in muscle biopsies retrieved from rodent models of sepsis [102] and septic patients [103, 104]. The observation that the inflammatory cytokines implicated in mitochondrial shutdown are prominent in both sepsis and malaria [105, 106] supports such organelle dysfunction being equally plausible in malaria.

Researchers are also becoming aware that, beyond energy production, mitochondria also play a vital role in cell homeostasis through generation and detoxification of reactive oxygen species [107]. The accelerated oxidative damage that accompanies sepsis could be both a cause and a consequence of cytokine-induced mitochondrial dysfunction. Interestingly, the ultrastructural damage reported to accompany mitochondrial dysfunction in sepsis [102] reflects Maegraith's observations in monkey malaria [108–110] decades ago.

Metabolic acidosis in falciparum malaria

Metabolic acidosis, often associated with hyperlactataemia, has been described in African children with severe falciparum malaria [111, 112]. It is not unique to this disease, being seen in viral, rickettsial and bacterial infections [113] as well as acute gastroenteritis, where its prevalence is higher than in malaria [114]. The terms hyperlactataemia and lactic acidosis are often mistakenly used interchangeably in the malaria literature. As often reviewed in the basic literature [115–118], protons (H^+, the basis of acidosis) are not formed when ATP and lactate are generated during glycolysis, but on the subsequent hydrolysis of ATP in tissues. Every time a molecule of ATP undergoes hydrolysis, a proton is released. If this occurs under aero-

bic conditions, these protons are consumed within ATP regeneration from ADP, and pH remains normal, i.e. acidosis does not occur. In contrast, if the mitochondria are not functioning adequately, whether through insufficient oxygen supply or an inability to use it, ATP regenerates under anaerobic condition, and the protons are not consumed. Hence, once the buffering capacity of the body is exceeded, acidosis occurs. In short, metabolic acidosis requires the ratio of glycolytic (i.e. anaerobic) ATP hydrolysis to mitochondrial (i.e. aerobic) ATP hydrolysis to reach a point at which the buffering systems can no longer cope. Pathological changes in the buffering system can be a major determinant of when this occurs.

Is hyperlactataemia a cause or marker of the acidosis of malaria?

High lactate levels have traditionally been seen not only as a marker for poor oxygen delivery in disease states, but also a consequence of it, and the cause of the acidosis. For some time hyperlactataemia has been regarded as a functionally relevant marker for a poor prognosis in both sepsis [119] and malaria [66, 112, 120]. Although the sepsis world now discusses several origins for the lactate increase, including inflammation-induced mitochondrial dysfunction [97], in falciparum malaria it is still generally attributed to a reduced oxygen supply, mostly through microvascular occlusion by sequestered parasitised erythrocytes [121]. Other mechanisms are known to contribute to acidosis in malaria, independent of lactate production, e.g. acute renal failure [8]. Impaired hepatic clearance [8, 112], production by parasites, and, in some areas, thiamine deficiency [122] are also argued to contribute to lactate accumulation independent of impaired cellular respiration. Thus, as described below, although acidosis and hyperlactataemia can be associated, they are independent cellular mechanisms.

Lactate anion has complex roles in biology. Hyperlactataemia may be associated with acidosis, a normal pH, or alkalosis [123]. A recent editorial in Critical Care Medicine [124] has lucidly summarised the key points of the mechanism of metabolic acidosis in sepsis, a condition that shares systemic inflammation and a range of its consequences with severe malaria (Tab. 2). These authors argue against lactate as the cause of the acidosis associated with hypoxia. Instead, they note the evidence that during hypoxia, be it from limited oxygen supply or utilisation, the unconsumed protons that cause acidosis arise from the hydrolysis of non-mitochondrial ATP. Since these reactions are independent of lactate levels, it is difficult to see how therapeutically reducing levels of this anion, as has been proposed [125], could increase survival rate in falciparum malaria any more than in sepsis [126]. Indeed, in theory it could harm comatose patients, since there is evidence that lactate helps brain tissue survive hypoxic and hypoglycaemic episodes [127–129], and the lactate shuttle is proving to be how astrocytes protect neurons from metabolic stress [130].

Even when considerable lactate is generated in acute inflammatory states, other, unidentified, anions contribute much more than it does to the strong ion difference that, through influencing the body's buffering capacity, influences acidosis in sepsis [131, 132] and falciparum malaria [114, 133]. Thus, lactate accumulation can only partially account for the high anion gap observed during the metabolic acidosis associated with severe malaria.

In summary, lactate is an imprecise but useful marker for metabolic acidosis in malaria. In turn, acidosis is an imprecise but useful marker of impaired cellular respiration. Whether impaired cellular respiration arises from (a) poor supply of oxygen to mitochondria (through vaso-occlusion, low circulating volume, anaemia or cardiac insufficiency) or impaired mitochondrial function (in response to severe systemic inflammation) the outcome is essentially the same. The resulting high anion gap metabolic acidosis is strongly predictive of death in severe malaria. Greater understanding of the multiple factors influencing the metabolic acidosis could provide further insight into the underlying pathophysiological process and may provide additional therapeutic options.

Hypoglycaemia in paediatric malaria

When glycolysis is enhanced for any period glycogen stores are soon depleted, and gluconeogenesis supervenes. However, its substrate supplies are limiting [134], and the hypoglycaemia often reported in severe malaria [135] and sepsis [19, 136] occurs. Hypoglycaemia is therefore a secondary cause of harm in these diseases, and is an inevitable consequence of exuberant, mostly anaerobic, glycolysis.

Neurological involvement in malaria

CM is a clinical syndrome characterised by coma (inability to localise a painful stimulus) at least 1 h after termination of a seizure or correction of hypoglycaemia, detection of asexual forms of *P. falciparum* malarial parasites on peripheral blood smears, and exclusion of other causes of encephalopathy [137].

Energy depletion and cerebral oedema

A relatively consistent feature of acute CM in children is raised intracranial pressure (ICP). Studies in African children have demonstrated a raised cerebrospinal fluid (CSF) opening pressure during lumbar puncture in 80% of CM children [138], raised ICP during intracranial pressure monitoring

(23/23 ICP > 10 mmHg) [139]and papilloedema (a late sign of raised ICP) in 44% of CM patients who died [140]. Where computer tomography has been performed, there was evidence of diffuse brain swelling in 40% of patients [139]. The cause of the raised ICP is likely to be multi-factorial and has been postulated to involve both vasogenic and cytotoxic patterns of cerebral oedema.

Vasogenic oedema is characterised by accumulation of interstitial fluid within the brain secondary to increased permeability of the blood-brain barrier (BBB). It has been demonstrated in bacterial cerebral infections, but evidence of significant disruption of the BBB is not conclusive in CM [141]. Others have proposed that ICAM-1 binding by infected erythrocytes may generate a cascade of intracellular signalling events that disrupt the cytoskeletal-cell junction structure and cause focal disruption to the BBB [142]. Adult post-mortem analysis has shown cerebrovascular endothelial cell activation (increased ICAM-1 endothelial staining, reduction in cell junction staining, and disruption of junction proteins), particularly in vessels containing infected erythrocytes [143]. However, disruption of intercellular junctions is not associated with significant leakage of plasma proteins (fibrinogen, IgG, or C5b-9) into perivascular areas or CSF [143]. In Thai adults, transfer of radioactively labelled albumin into CSF was not raised during unconsciousness compared with convalescence [144]. Similarly, the albumin index (ratio of concentrations of albumin in CSF to those in blood) was not altered significantly in Vietnamese adults [145] or significantly different between Malawian children with CM who died and those who survived [143].

Cytotoxic oedema is increasingly being recognised as an important mechanism of cerebral oedema in traumatic brain injury [146]. As previously discussed, this type of cell swelling involves disturbance of the "pump-leak equilibrium" maintained, under physiological conditions *via* active elimination of osmotically active solutes through the energy-dependent Na^+/K^+-ATPase. Thus, cytotoxic oedema can occur secondary to an imbalance in supply and demand of energy within the cells. Several mechanisms, such as sustained increase in neuronal activation, impaired substrate delivery (structural and functional) and impaired mitochondrial utilisation of available substrates, including oxygen, may coexist to generate this imbalance. All these mechanisms could contribute to ATP depletion and Na^+/K^+ ATPase failure, leading to cytotoxic oedema in CM.

CM is clearly associated with increased neuronal activity. A recent review identified that 80% of African children with CM have a history of seizures, with prolonged and recurrent seizures associated with a poor outcome [147].

Impaired vascular flow during acute CM may limit substrate delivery within the brain and contribute to energy imbalance. In the past, a common premise was that parasite sequestration precipitated cerebral vaso-occlusive/ischaemic (i.e. stroke-like) events that manifested clinically as CM. However, CM demonstrates several features that are atypical for stroke. In

children, focal neurological signs do not tend to accompany coma, although a sub-set of patients do exhibit hemiparesis or focal brainstem deficits during the agonal period [148]. The incidence of residual neurological deficits following recovery from coma is relatively low (11% [147]) when compared to childhood stroke (93% had residual neurological deficit [149]). Where computer tomography has been performed in children, diffuse brain swelling was observed [150] rather than focal lesions more typical of stroke. Although retinal haemorrhages have been observed in 46% of Malawian children with CM (and in 63% of patients who died), these lesions were also seen in 30% of children with SMA in the same study [140]. Consequently, although associated with CM, retinal haemorrhages do not confirm that focal cerebral vaso-occlusive/ischaemic events underlie CM. Similarly, histological examination of 32 fatal CM cases of African children at autopsy demonstrated that one third had little or no evidence of local vascular change in the brain, as indicated by sequestered parasites, monocyte clusters, micro-haemorrhages, local vascular iNOS [151] or haemoxygenase -1 (HO-1) [152] staining. Accepting that CM may occur without ischaemia does not exclude temporary or less severe reductions in vessel flow occurring during acute CM (associated or independent of parasite sequestration) that may contribute to impaired substrate delivery and lead to energy imbalance.

As previously discussed, energy imbalance may also be impaired due to the uncoupling action of inflammatory cytokines on mitochondrial ATP production. In Gambian and Ghanaian children, concentrations of TNF and its receptor were higher in those with CM than in those with mild or uncomplicated malaria [153, 154]. Polymorphisms in the TNF promoter region have also been associated with increased risk of CM and death [155] or neurological sequelae [156]. Cytokines may also up-regulate iNOS in brain endothelial cells, increasing production of NO, which could then diffuse into brain tissue and disrupt neuronal (and/or mitochondrial) function [157, 158].

In the brain, mitochondrial function may also be influenced by neuronal excitotoxins. Within the simplified model of dissociated neuronal culture, mitochondria appear to play a critical role in neuronal homeostasis during excitotoxin exposure. Mitochondria are not only involved with maintaining ATP production but also calcium homeostasis, and generation and detoxification of reactive oxygen species [107]. Excitotoxin production may also be influenced by cytokine release. TNF administration has been shown to alter brain metabolism of tryptophan to produce more kynurinine [159, 160]. Thus, as part of a general inflammatory reaction, increased excitotoxin generation during acute malaria may contribute to cellular energy imbalance. Elevated levels of neuronal excitotoxins (quinolinic and picolinic acid) in the CSF have been associated with a fatal outcome in Malawian children with CM [161]. Similarly, a graded increment of quinolinic acid concentration in CSF was observed across patient outcome groups of increasing severity in African children [162].

Encephalopathy with systemic inflammation but without sequestration

Although a subset of the Malawaian autopsy patients [163] demonstrated negligible histological change in their brains, they did demonstrate inflammation, as indicated by iNOS, MIF [151] and HO-1 [152], staining in other tissues. These systemic changes were shared with the comatose sepsis cases in the study, and therefore are consistent with the premise that coma may in part be secondary to a host inflammatory response to systemic infection. Below are further examples of systemic responses to infection that present with diffuse cerebral syndromes, including coma.

Cerebral malaria manifesting with *P. vivax* infection

In the past, the term CM has been restricted to falciparum malaria, and patients with *P. vivax* infection exhibiting symptoms of severe malaria, including coma, have been dismissed as undiagnosed falciparum co-infections. However, the use of more sensitive diagnostic techniques makes such dismissal less tenable. Two such studies report adults exhibiting severe malaria with *P. vivax* (but not *P. falciparum*) infection detectable on PCR and serological and testing [142, 143]. The patients exhibited multiple organ failure including cerebral symptoms, renal failure, circulatory collapse, severe anaemia, haemoglobinuria, abnormal bleeding, acute respiratory distress syndrome, and jaundice. Vivax malaria has been associated with a strong systemic inflammatory response [164], but this was not investigated in the above studies.

Sepsis-associated encephalopathy

Sepsis-associated encephalopathy (SAE) syndrome has multiple features that resemble CM. It is characterised by a diffuse disturbance of cerebral function (typically impairment of consciousness) that occurs in the context of systemic response to infection without direct neuroinvasion (i.e. meningitis, macroscopic cerebritis and brain abscesses are excluded). SAE is associated with generalised slow waves on the electroencephalogram (EEG), with the depth of coma linked with mortality. Mild SAE cases often recover completely, while survivors of severe SAE may have persistent neurological deficit [165]). In line with adult CM, the severity of encephalopathy parallels the severity of systemic organ failure [141]. Inflammatory cytokines have been demonstrated to be higher in the serum than in the CSF, suggesting that sepsis encephalopathy is a consequence of the systemic inflammatory response to infection [141]. An animal model in which prior administration of a neutralising antibody to TNF prevented the sepsis encephalopathy of pancreatitis [166] is consistent with this. Further postulated reversible

Table 3.

	Influenza encephalopathy	Cerebral malaria
Seizures/coma after high grade fever	+	+
Metabolic acidosis	+	+
Hyperlactataemia	+	+
Serum TNF, IL-6, sTNFRI up	+	+
Serum nitrite/nitrate up	+	+
CSF TNF, IL-6, sTNFRI up	+	+
Multiple organ failure	+	+
Residual neurological deficit	+	+
Thrombocytopaenia	+	+
Damage to vascular endothelial cells	+	+
Brain oedema/damage to BBB	+	+
Apoptosis in neurons/glial cells	+	+
Evidence of active caspase-3 (brains)	+	+
Caspase-cleaved PARP (brains	+	+

mechanisms of pathogenesis include changes in regional cerebral blood flow, neurotransmitter imbalance, mitochondrial dysfunction, BBB impairment and oxidative stress [167].

Influenza encephalopathy

Severe influenza infection can present with encephalopathy, yet as in malaria, the pathogen is not neuroinvasive [168]. Seizures and coma occur after high fever [169], commonly accompanied by thrombocytopaenia [169], with metabolic acidosis and hyperlactataemia in severe cases (T. Ichiyama, personal communication). Similar to adult malaria, neurological sequelae occur concurrently with multiple organ failure [170]. TNF, IL-6, sTNFRI, and soluble E-selectin are increased in serum and CSF [171, 172], and serum nitrite/nitrate levels are increased [173]. Detailed examination of brain has revealed apoptosis of neurons and glial cell, histological evidence of active caspase-3 and caspase-cleaved PARP, cerebral oedema, and BBB impairment [174]. These parallel changes are set out in Table 3. It is clear, therefore, that the presence of sequestering parasitised red cells is not necessary to generate these changes, which are also demonstrable in the falciparum malaria encephalopathy. Notably, high levels of inflammatory cytokine are present in each disease.

Seizures and malaria

Seizures are a very common component of acute malaria illness in children. A recent review documented that 80% of African children had a history of seizures, with 60% exhibiting seizures during hospital admission [175]. The molecular basis of the seizures is unclear. Multiple mechanisms have been postulated, including fever, hypoxia and/or cytokine stimulation leading to an imbalance of neurotransmitters and excitotoxins or neuronal damage [11, 148]. Recently, Lang and co-workers [176] demonstrated that falciparum parasitaemia is associated with the generation of specific antibodies for voltage-gated calcium channels directed against neurones. Higher antibody concentrations were detectable in sera from patients exhibiting CM or malaria with seizures than uncomplicated malaria, suggesting that these antibodies may influence seizure propensity.

Red cell abnormalities and malaria

Only the erythrocytic form of malaria is associated with disease, so valuable information about which African children are likely to have more, or less, severe malaria has inevitably been obtained from examining the inborn RBC abnormalities that endemic malaria has selected across the tropics.

The coinciding geographic distributions of malaria transmission and the thalassaemias prompted Haldane to put forward the 'malaria hypothesis', which proposed that common erythrocyte abnormalities are selected because of the fitness advantage they confer against malaria [177]. Sickle cell haemoglobin (HbS) has also been repeatedly shown to be associated with malaria resistance, with heterozygotes for the HbS trait demonstrating 10% of the population at risk for severe malaria in certain populations [178]. Other haemoglobinopathies (e.g. HbC [179, 180] and HbE [181]) and deficiencies in RBC enzymes (e.g. glucose-6-phosphate dehydrogenase deficiency [182]) have also been linked with protection against severe malaria. The mechanisms of protection afforded by haemoglobinopathies are likely to be multi-factorial. Studies have demonstrated evidence to support several independent mechanisms including: reduced parasite invasion of RBCs and diminished intraerythrocytic growth of parasites in patients with the HbS trait [183], enhanced phagocytosis of parasite-infected erythrocytes (IEs) [184] and enhanced immune responses against IEs [185].

Recent *in vitro* studies observed that HbC modifies the quantity and distribution of the variant antigen *P. falciparum* erythrocyte membrane protein 1(PfEMP1) on the IE surface. PfEMP1 has been implicated in numerous IE adhesive interactions. In the latter study the authors demonstrated that HbC reduces the level of IE adhesion to endothelial monolayers, in addition to IE rosetting (the adhesion of IEs to uninfected erythrocytes) and IE agglutination by sera. These findings provide the prospect that HbC pro-

tects against severe malaria by mitigating the obstruction and inflammation caused by the PfEMP1-mediated adherence of IEs [186]. However, sequestration is believed to enhance parasite survival by enabling IEs to avoid splenic clearance, so any reduction of sequestration by HbC can be expected to limit parasite fitness. Multiple epidemiology studies (e.g. [179, 187, 188]) have failed to identify any significant impact of HbC on the frequency or density of parasitaemia in naturally exposed populations. Consequently, the influence of the changes in IE surface conformation needs to be confirmed and further examined *in vivo* [189].

A recent study re-confirmed that African children with α-thalassaemia trait are significantly less likely to be hospitalised with severe malaria, particularly with coma or severe anaemia (Hb<5 g/100 ml). It is intriguing that the α-thalassaemia patients did not demonstrate a lower incidence of uncomplicated malaria nor any reduction in peripheral parasite density [190]. Thalassaemia has also been associated with increased incidence of clinical vivax and falciparum malaria during early life [191]. The findings raise speculation that the trait may indirectly afford enhanced immunity through increased non-lethal exposure to malarial parasites. Such a mechanism is appealing, since it would be equally plausible across a range of haemoglobinopathies, including HbC.

Variations in erythrocyte membrane proteins also have a profound influence on malaria susceptibility. Most notably the absence of Duffy antigen protein confers absolute protection to *P. vivax* infection. More recently, the Duffy antigen has also been associated with a protection against falciparum malaria [192].

Enzymes involved with iron handling may also have a critical influence on malaria morbidity. A recent study from the Gambia demonstrated that children in an endemic malaria area possessing the haptoglobin 2,2, isotype had a significantly increased risk of anaemia [193]. However, a lack of parallel alterations in other haematinic indices leaves the mechanism of this process unclear.

Malarial protection within individuals exhibiting multiple RBC abnormalities appears even more complex. A recent study observed that the concurrent presence of sickle cell and α-thalassaemia trait among African children had a negative influence on the risk of malaria infection [194]. The results warn geneticists that gene epistasis may have a profound influence on overall malarial susceptibility.

Potential therapies directed at disease mechanisms

In tropical countries many hospital deaths from falciparum malaria happen before anti-malarial drugs have had time to kill the parasites. Two approaches could help rectify this – addressing public-health problems resulting in delayed presentation, and identifying the physiological processes and

molecular pathways that lead to these early deaths, with a view to developing evidence-based adjunct therapies.

Therapies being explored in sepsis, and based on disease pathogenesis data common to sepsis and malaria, may prove to be transferable from either of these diseases to the other. As noted above, circulating levels of a late-appearing inflammatory cytokine, HMGB1, are increased in falciparum malaria [41] as well as in sepsis. Results from animal models on the role of HMGB1, although untested in humans, have inspired enthusiasm for inhibition of this molecule as a potential intervention for human sepsis. For instance, anti-HMGB1 antibodies provided dose-dependent protection [37] and reduced mortality [195] against experimental sepsis in mice. Late administration of ethyl pyruvate, which inhibits HMGB1 release from macrophages, also conferred protection against endotoxaemia in mice [196].

Treatments directed towards critical downstream consequences of malaria infection and inflammation, such as those intended to limit acidosis, are also a focus of investigation. One current approach is to identify which acute malaria patients most benefit from early volume expansion [197]. Controlling lactic acidosis *via* sodium dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenate kinase (maintaining pyruvate dehydrogenase in its active form), is also being examined. DCA reduced lactate levels in acute malaria patients [198], although the study was unable to determine whether treatment improved outcome. An earlier large sepsis study also demonstrated that DCA reduced lactate, but again with no improvement in outcome [126]. As outlined in the section 'Is hyperlactataemia a cause or marker of the acidosis of malaria?', some researchers argue, in view of the strong ion difference contributing to acidosis and the postulated mitochondrial dysfunction during acute malaria infection, that lactate reduction *per se* may have limited impact on prognosis.

Other adjunct therapies are also being examined. Improving RBC deformability provides one potential therapeutic approach. *In vitro* studies with *N*-acetylcysteine (NAC), reported to scavenge free radicals, showed improvement in red cell deformability through *in vitro* studies [199]. Unfortunately, an initial *in vivo* trial of NAC in malaria patients had no effect on mortality [200]. Blocking endothelial activation is also a focus of research, with initial *in vitro* studies providing some encouraging results [201].

In conclusion, continuing to identify the host responses to malaria infection that lead to disease is providing insights into novel molecular mechanisms. This information is beginning to guide the design of much needed additional therapies against this disease. There is little doubt that poor oxygen supply through vascular occlusion or anaemia could contribute to the body relying on excessive glycolysis to generate energy, resulting in hyperlactataemia, hypoglycaemia, and metabolic acidosis, and altered consciousness. However, inflammatory cytokines control these changes, as well as inhibit the capacity of mitochondria to use oxygen. Thus, as described
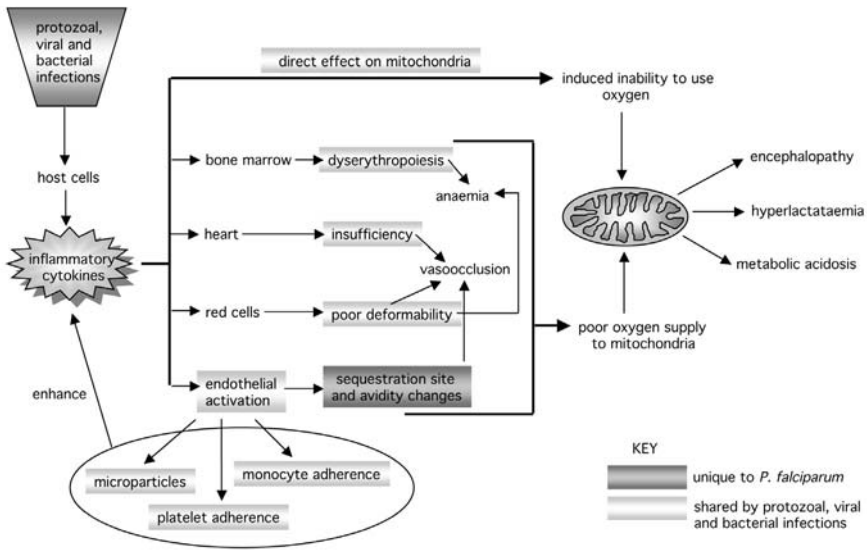
Figure 1. The wide-ranging influences of inflammatory cytokines in severe malaria.

throughout this review, inflammatory cytokines are likely to have various pivotal roles across the multiple pathological processes involved in malarial disease (Fig. 1).

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