Innate Immunity to Malaria

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Contents

Abbreviations

DCs	Dendritic cells
GPI	Glycosylphosphatidylinositol
IFN	Interferon
Ifnar	Interferon alpha receptor
ISG	Interferon-stimulated gene

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MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation-associated protein 5
NK	Natural killer cells
NKT	Natural killer T cells
PBMC	Peripheral blood mononuclear cells
ROS	Reactive oxygen species
TLR	Toll-like receptors

1 *Plasmodium* Infection and Disease

Malaria infection starts when an infected *Anopheles* mosquito injects *Plasmodium* sporozoites into the skin of the vertebrate host. After traversing the dermis, the parasites enter the circulation and home to the liver, initiating the hepatic stage of infection. Within the liver, hepatocyte traversal precedes parasite invasion and replication in the host cell, ultimately maturing into erythrocyte-infectious merozoites [1]. These are later released into the lumen of liver sinusoids [2] invading, developing, and multiplying inside erythrocytes, the blood stage of infection, associated with the establishment of disease and all its complications.

The blood stage of malaria is characterized by high cyclical fevers and elevated levels of inflammatory mediators in the circulation. Excessive and persistent inflammation during *P. falciparum* infections contributes to severe pathology and to the development of associated complications such as cerebral malaria and severe malarial anemia [3].

Acute malaria is always characterized by high fevers, but it is known that different species of *Plasmodium* need to reach higher parasitemias to induce inflammatory responses in the host. Although the mechanisms underlying this effect are not clear, it seems apparent that *P. vivax* is more effective at inducing inflammation and therefore needs lower parasitemias to induce a high fever response compared to *P. falciparum* [4]. This difference has also been observed in the levels of TNF compared to parasitemia in patients, where *P. vivax* or *P. ovale* induce much higher levels of inflammation per infected erythrocyte compared to *P. falciparum* [5, 6], although this observation has been recently challenged [7, 8].

2 Parasite- and Host-Derived Inflammatory Molecules

The asymptomatic nature of the liver stage, the first step of *Plasmodium* infection in the mammalian host, has led to the long-lasting view that the parasite can establish and replicate within the host hepatocyte without being detected [1]. However, such notion has been challenged by the presence of inflammatory cell foci in the liver during exoerythrocytic parasite development [9–12], as well as by the mounting of a strong inflammatory reaction aiming at controlling *Plasmodium* hepatic burden,

as is the case of the rate-limiting enzyme of heme catabolism heme oxygenase 1 [9]. Taken together, these findings suggest that the host is able to sense *Plasmodium* hepatocyte infection and respond to it.

In contrast, the inflammatory nature of the blood stage of infection has long been recognized. As described by Golgi [13], the synchronized rupture of infected erythrocytes in the peripheral circulation is followed by a peak of fever in malaria patients. This observation led to the hypothesis that the high levels of inflammation in malaria during the blood stage of infection were caused by molecules released from infected erythrocytes during parasite egress, including merozoites and erythrocyte cellular contents. The search for these molecules for the past century has led to the identification of several pro-inflammatory molecules that are either derived directly from *Plasmodium* or generated as a result of infection from erythrocyte components.

Among the molecules generated by the parasite, glycosylphosphatidylinositol (*GPI*) anchors were identified early as inflammatory mediators [14]. These glycolipid structures anchor parasite proteins to the plasma membrane in the merozoite [15] and activate toll-like receptors (TLR), preferentially TLR-2/TLR-6 and TLR-2/TLR-1 heterodimers, but also TLR-4 homodimers [16]. Although purified GPI anchors induce an inflammatory response in mice [14], in vitro this response is downregulated in the presence of other *P. falciparum* lipids [17], which may explain why in vitro inflammatory responses do not require TLR-2 or TLR-4 [18]. The association of TLR-4 [19], TLR-2, TLR-1, and TLR-6 [20, 21] polymorphisms with malaria severity may be compatible with a role for GPI anchors in malaria inflammation, but this association may also be caused by other *Plasmodium*-derived inflammatory mediators. The role of GPI in malaria-induced inflammation in patients remains unclear.

Another parasite-derived molecule with inflammatory effects is *hemozoin*, a crystal polymer of heme that *Plasmodium* generates after degradation of hemoglobin within infected erythrocytes. Hemozoin is generated in the parasite's food vacuole and is released after erythrocyte rupture and merozoites egress. It is important to consider than the inflammatory properties of crystals, such as hemozoin, depend on the size, charge, and association with protein, lipids, or other elements of the crystal [22]. Different groups have found conflicting results regarding the inflammatory effects of hemozoin, which are probably due to the variations in the protocols used to obtain hemozoin that would result in crystals with different inflammatory characteristics. When synthetic hemozoin, or also hemozoin purified from infected erythrocytes and stripped of any binding molecules, was used as starting material, activation and binding to TLR-9 [23, 24] was observed. Inflammatory pathways dependent on nitric oxide and NF-kB [25], as well as activation of the NLRP3 inflammasome [26, 27], were also identified.

It is likely that hemozoin obtained from cultured parasites that naturally rupture and release their contents would more accurately resemble the characteristics of the hemozoin generated during disease. This kind of hemozoin was used in studies that found parasite and host components bound to it, including *Plasmodium DNA* [28] and host fibrinogen [29]. The bound materials DNA and fibrinogen, not the hemozoin per se, were found to mediate the inflammatory response observed in vitro

through the activation of TLR-9 and TLR-4 or the integrin CD11b/CD18, respectively [28, 30]. Indeed, the inflammatory activity of hemozoin was lost after treatment to remove associated proteins or DNA, probably because the proteins provide a link to bind DNA to the hemozoin crystal [31]. These results indicate that hemozoin could act as a "carrier" for other molecules, increasing their inflammatory potential. Another effect of hemozoin, also caused by other crystals, is the destabilization of the phagosome [32], which results in the release of hemozoin and DNA to the cytosol of the phagocytic cell and in the activation of the AIM2 and NLRP3 inflammasomes, respectively [31]. Accordingly, synthetic hemozoin is being developed as a vaccine adjuvant for other diseases [33, 34].

The "carrier" effect was also proposed for *Plasmodium* histones, which are bound to parasite DNA and mediate inflammation in vitro [35]. Additionally, immune complexes formed by DNA and anti-DNA antibodies, which are found in high concentrations in the sera of malaria patients, were found to induce cytokine secretion from immune cells in vitro [36], suggesting that another *Plasmodium* DNA-carrier complex is also contributing to the inflammatory pathway. *Plasmodium* DNA can activate not only TLR-9, which recognizes CpG motifs, but also an alternative inflammatory pathway that recognizes AT-rich hairpin motifs, involves STING/TBK1/IRF3 signaling, and results in the production of type I interferon [37].

Interestingly, whole lysates of *P. falciparum*-infected erythrocytes injected into mice are more immunogenic in *wt* compared to TLR-9-deficient mice, independently of *Plasmodium* DNA [24]; however, infection of TLR-9-deficient mice with *P. berghei* did not show a reduction in inflammatory cytokine response [38, 39]. Studies in malaria patients have found an association of TLR-9 polymorphisms with malaria susceptibility and development of anemia [40, 41], but not with malaria severity [19, 40, 42]. The role of hemozoin and *Plasmodium* DNA in the inflammatory response in malaria patients is still not well defined.

Plasmodium RNA is probably also an inflammatory activator during the blood stage of infection, since TLR-7, which recognizes ssRNA, was found to be essential for cytokine production in mice [39]. Since TLR-7 and 9, which recognize ssRNA and dsDNA, respectively, are not found in the plasma membrane of immune cells, the "carrier" effect is thought to facilitate phagocytosis of the nucleic acids, allowing the contact with the endosomes in the phagolysosome. In the liver stage, however, it is established that *Plasmodium* dsRNA is sensed by the host hepatocyte by a mechanism involving the intracellular RIG-I-like receptor melanoma differentiation-associated protein 5 (MDA5 also known as IFIH1), its adaptor protein mitochondrial antiviral-signaling protein (MAVS), and the transcription factors IRF3 and IRF7. Notwithstanding, the ensuing immune response is partially MDA5 independent, supporting the idea that several parallel mechanisms could contribute to *Plasmodium* recognition by the host during the liver stage of infection [43]. The question remains, however, on how *Plasmodium spp*. dsRNA becomes accessible to the host cytosolic receptors. Several hypotheses can be put forward including: (1) the active transport of this ligand across the parasitophorous vacuole, (2) passive release of dsRNA through vacuolar membrane pores, (3) leakage of dsRNA into the cytosol from nonviable parasites, and/or (4) vesicular transfer of parasite dsRNA

from infected cells to liver-resident immune cells, such as Kupffer cells. Overall, we cannot exclude that several of these mechanisms operate simultaneously to trigger the immune response against *Plasmodium* during the liver stage of infection.

Another pro-inflammatory mediator that could contribute to the inflammatory response to *Plasmodium* infection is precipitated *uric acid. Plasmodium*-infected erythrocytes import hypoxanthine, which is the precursor of uric acid, for the synthesis of nucleic acids required during parasite replication. After requirements for hypoxanthine decrease at the end of the replication cycle, hypoxanthine accumulates in the infected erythrocyte [44, 45]. Precipitates of uric acid are also observed in the cytoplasm of the *Plasmodium* parasitophorous vacuole [46]. Upon rupture of infected erythrocytes, precipitates of uric acid and soluble hypoxanthine, which would be degraded into uric acid in the tissues, are released and can become inflammatory [44–46]. Indeed, activation of the NLRP3 inflammasome by uric acid precipitates is well characterized [47], and activation of both NLRP3 and NLRP12 inflammasomes has been observed in infected mice and malaria patients [48], possibly caused by hemozoin and/or uric acid crystals. Uric acid was also found to mediate the activation of mast cells in a mouse model of malaria, leading to the regulation of a subset of dendritic cells, which then activate pathogenic $CD8^+$ T cell responses directed against the parasite [49]. Interestingly, treatment of malaria patients with an inhibitor of xanthine oxidase, an enzyme that produces uric acid, results in a more rapid decrease of the inflammatory response [50], suggesting that uric acid may be involved in the inflammatory response in patients.

Microvesicles are shed by almost all cell types in response to different stimuli, such as activation or response to environmental stress [51]. Microvesicles derived from *Plasmodium*-infected erythrocytes, induced probably in response to oxidative stress during infection [52], can activate macrophages and trigger the secretion of inflammatory cytokines in vitro through the activation of TLR-4 [53, 54]. Endothelial microvesicles can also induce the proliferation of T cells [55]. In malaria patients, increased levels of circulating microvesicles are derived preferentially from uninfected erythrocytes, but also infected erythrocytes, lymphocytes, platelets, and endothelial cells [52, 56, 57]. Correlations of microvesicle levels with inflammatory markers or disease severity has been observed in malaria patients. Microvesicle levels correlate with TNF in cerebral malaria patients [58], erythrocyte [52] and endothelial [57] microvesicles correlate with the severity of *P. falciparum* malaria, and platelet microvesicles also correlate with fever in *P. vivax* infections [56]. It is still not clear whether microvesicles are causing inflammation and malaria severe pathology in patients or their formation is induced as a consequence of the high inflammation [59], which is characteristic of severe malaria.

Although *Plasmodium* converts *heme* derived from hemoglobin degradation into nontoxic hemozoin, which in itself can be inflammatory (see below), up to 40 % of the hemoglobin of an infected red blood cell can be released and oxidized. This leads to the formation of toxic heme in the circulation of infected individuals [60]. In vitro and in vivo experiments suggest that heme induces apoptosis of brain vascular endothelial cells, which affects the stability of the blood-brain barrier in experimental cerebral malaria [61, 62]. Heme-induced apoptosis of brain vascular

endothelial cells or endothelial progenitor cells was shown to be mediated by the transcription factor STAT3 [61, 62], the tumor protein p73 [63], or TLR-4-induced CXCL10 [64].

Another source of inflammation in malaria appears to be *oxidative stress* that is generated during infection. Malaria patients exhibit high levels of oxidative stress, as measured by lipid peroxidation, and at the same time lower anti-oxidative factors compared to healthy controls [65–68]. The source of increased reactive oxygen species (ROS) leading to oxidative stress has been subject of speculation. While some reports suggest that ROS might be produced by the parasite [69–71], others indicate that the human host can be a potent source of ROS to combat *Plasmodium*, most notably through the oxidative burst of phagocytes [72, 73] and ROS-producing enzymes like xanthine oxidase [74]. This implicates that ROS production by the host might be an important inflammatory response to control parasitemia; however, elevated levels of oxidative stress also correlate with increased disease severity during the infection [75, 76], suggesting a role in pathology. It is not clear whether oxidative stress is a cause or consequence of inflammation during malaria. Given the severe nature of complications often leaving traces of oxidative damage like impaired memory after cerebral malaria, the use of antioxidants as adjunctive therapy has been discussed. Although of great potential benefit, antioxidants could increase parasite survival by interfering with the host inflammatory response or the action of antimalarial drugs (reviewed in [77]).

All the parasite and host components described above are able to induce inflammatory reactions in vitro and/or when injected in mice, but their relative importance in the inflammation observed in malaria patients remains unclear. Assessing their real contribution during disease has not been possible because of the difficulties in specifically inhibiting each of them in vivo; therefore, most of the available evidence comes from correlations of inflammatory parameters in the blood of malaria patients that cannot establish a causative relation. In vitro inhibition of DNA and/or uric acid is possible using *P. falciparum* lysates or merozoites treated with DNAse and/or uricase, which suggest an important role for these molecules in the activation of human dendritic cells [18, 46]. The role of hemozoin in the inflammatory response in vivo may now be tested in mice using *P. berghei* parasites that produce very low levels of hemozoin [78]. Inhibition of uric acid formation in vivo is possible by treatment of malaria patients with allopurinol, an inhibitor of the enzyme that produces uric acid. In this case, a more rapid decrease of the inflammatory response was observed in treated patients [50].

There is an unsolved paradox in the study of the innate immune response to malaria blood stage, where patients present all signs of an intense inflammatory response, including high fevers and circulating inflammatory cytokines, but innate immune cells in vitro respond weakly to the parasite when incubated together. When naïve peripheral blood mononuclear cells (PBMCs), isolated dendritic cells, or macrophages are incubated with *P. falciparum*-infected red blood cells in vitro, the levels of inflammatory cytokines released, such as TNF, are undetectable or substantially lower than responses triggered by well-characterized activators (LPS, CpG, or β-hematin [79, 80]) or inflammasome activators (uric acid). Conversely,

cells from infected individuals were much more responsive to parasite stimuli in the secretion of cytokines [81, 82] when compared to cells from healthy individuals or recovered patients. These results suggest that there still may be components of the innate immune response to blood-stage *Plasmodium* infection that have not been identified yet and play an important role during infection.

3 Cellular Responses During Malaria

3.1 Hepatocytes

Within hepatocytes, *Plasmodium* parasites settle inside a parasitophorous vacuole where it undergoes a remarkable transformation differentiating into highly metabolically active merozoites. By the end of the liver stage, as the vacuole expands, a single parasite generates thousands of merozoites. During this process plasmodial dsRNA gains access to the host cell cytosol and activates the MDA5/MAVS/IRF3/7 cytosolic signaling pathway leading to the release of type I interferon (IFN) into the extracellular environment. The initial signal is consequently propagated in an autocrine and paracrine manner through the activation of the interferon alpha receptor (Ifnar) in neighboring hepatocytes via the production of interferon-stimulated genes (ISGs). This type I IFN-mediated response enables the host to control hepatic parasite burden. However such control does not seem to be mediated directly by hepatocytes but rather relies on the recruitment of accessory immune cells [43, 83]. In fact, liver-stage infection results in the recruitment of natural killer T (NKT) cells in an IFNAR-dependent manner, that through an IFNγ-mediated mechanism are responsible for the control of *Plasmodium* liver infection [83]. Although IFNγ has been shown to directly kill liver-stage parasites [84, 85], it is possible that an IFNγ-independent killing mechanism within the hepatocyte could also take place.

3.2 Granulocytes

The levels of circulating *neutrophils* during malaria are significantly increased [86] and correlate with inflammation and severe disease [87, 88]. Adoptive transfer of neutrophils from infected rats provided partial protection against infection, suggesting that they play a role in protection against malaria [89]. However, the chemokine CXCL10 that is secreted by neutrophils during *P. berghei* infection in mice inhibits the control of blood-stage parasitemia and is required for the development of experimental cerebral malaria [90], although depletion of neutrophils did not prevent the development of this pathology [91]. Interestingly, activated neutrophils correlate with cerebral malaria vasculopathy, which presents with higher cytoadhesion levels of infected erythrocytes to endothelial cells in the brain [92], suggesting a role for neutrophils in *P. falciparum* malaria-induced pathology. In *P. vivax* infections

expression of type I interferon in neutrophils was correlated with liver damage [93]; however, neutrophils present an atypical activation profile since phagocytic activity and superoxide production were increased but molecular markers of activation and secretion of cytokines are very low in response to stimulation [94].

Mast cells contribute to parasite clearance and TNF production in rodent malaria [95] and appear to have a role in promoting innate immune activation since they produce Flt3 ligand during malaria in mice, which, in turn, induces proliferation of a subpopulation of dendritic cells. Both Flt3 ligand and this subpopulation of dendritic cells are also elevated in malaria patients [49].

3.3 Monocytes and Macrophages

Macrophages are essential for clearance of infected erythrocytes as observed in mouse models of infection [96, 97]. Macrophages efficiently phagocytose infected erythrocytes, as early as ring stage [98], which are subsequently degraded in acidic phagosomes [99]. Non-opsonic phagocytosis is mediated by binding of infected erythrocytes to CD36 [100], while opsonic phagocytosis is mediated by complement receptor-1 [101, 102] and Fc-γ receptors [103].

3.4 Dendritic Cells

Dendritic cells (DCs) are crucial for the initiation of the adaptive immune responses and regulate both innate and adaptive immunity to infections. DCs activate, or mature, in response to different pathogen signals, enabling their capacity as antigen-presenting cells that efficiently activate naïve T cells [104]. In vitro studies incubating DCs with Plasmodium showed efficient phagocytosis and phagosomal maturation of infected erythrocytes [99], but demonstrate that there is a dose-dependent inhibition of DC maturation [105] that takes place only at high concentrations of infected erythrocytes, where DCs do not upregulate co-stimulatory molecules [106]. Studies using isolated human DCs and *Plasmodium* lysates have found upregulation of classical co-stimulatory molecules in DCs [46, 79]. Also, plasmacytoid DCs are activated through TLR-9 by infected erythrocytes [79]. During early infection [107] and in asymptomatic patients [108], the level of expression of surface HLA-DR in circulating DCs maintains normal levels, but during the acute phase of disease, these levels are reduced [49, 109, 110]. The decreased levels of HLA-DR could affect antigen presentation and T cell activation during disease, although their role in malaria immune response remains unclear.

The dendritic cell cytokine response to *Plasmodium* in vitro was found to be low for common cytokines such as IL-12, IL-8, IL-6, IL-1β, IL-10, and TNF [80, 105], with the exception of IFN-α that is secreted by plasmacytoid DCs [79]. However, other authors found upregulation of inflammatory cytokines upon incubation with infected erythrocytes [111, 112].

When DCs were extracted from malaria patients, they show an impaired capacity to mature, capture, and present antigens to T cells. They also undergo high levels of apoptosis probably as a result of increased IL-10 during infection [113, 114]. It appears that malaria, despite the high levels of inflammation, does not induce classical activation of DCs. However, studies in patients are limited to circulating DCs, and it is possible that effective, mature DCs are migrating into tissues and lymphatic organs and are not being detected in the studies.

During severe malaria, the numbers of BDCA3 DCs, which are a minor subset of myeloid DCs, are increased in peripheral blood [115] and correlate with high levels of Flt3L, a factor that induces expansion of DCs. Other DC populations, BDCA1 and plasmacytoid DCs (BDCA2), were not increased in malaria patients [49]. Conversely, in early uncomplicated human malaria infections, the frequency of BDCA3 DCs was not increased, but plasmacytoid DCs were [116], suggesting that the DC response may vary at different stages of infection.

3.5 NK Cells

In vitro studies show that incubation of PBMC with *P. falciparum*-infected erythrocytes results in the rapid activation of natural killer (NK) cells to secrete IFN-γ [117]. This activation requires the help of cytokines such as IL-2, IL-12, and IL-18 [117–119] from T cells but also contact-dependent signals from monocytes and dendritic cells [120]. In mice, NK cells are important for the control of parasitemia during early infection probably through the production of IFN-γ [121, 122]. Also in human malaria infections, restimulation of PBMCs show that NK cells contribute moderately to the production of IFN-γ [107].

3.6 γδ T Cells

γδ T cells are a minor population of T cells in the peripheral circulation that recognize self and non-self antigens without the restriction of MHC antigen presentation. γδ T cells expand during malaria to constitute a high percentage of circulating T cells in humans infected with *P. falciparum* [123, 124]. They get activated by parasite phosphoantigens produced by *Plasmodium* apicoplast [125] and release cytotoxic granules containing granulysin that are effective against merozoites [126]. γδ T cells proliferate and produce IFNγ and TNF after in vitro stimulation with *P. falciparum*-infected red blood cells which is dependent on IL-2 or autologous irradiated PBMC [127–131]. γδ T cells are the main producers of IFNγ in response to *P. falciparum* in vitro [127] and upon in vitro restimulation of PBMCs from humans infected with *P. falciparum* [107]. In mice, γδ T cells contribute to parasite clearance [132–134], probably due to the high production of IFNγ and their role in activation of dendritic cells [132].

In human malaria patients, γδ T cells are important contributors to inflammatory cytokines and have been associated with severe malaria [129]. When single experimental *P. falciparum* infections were analyzed, both γδ T cells and NK cells showed an enhanced IFNγ response upon restimulation with *P. falciparum*-infected red blood cells, even several weeks after the parasite clearance, indicating a memory-like activation [107, 135]. However, when patients with repeated exposure to malaria in endemic areas were analyzed, loss and dysfunction of γδ T cells was observed in the most exposed patients, which is associated with reduced symptoms and clinical tolerance upon reinfection [136]. This suggests that γδ T cells have roles in both clearance of the parasite as well as pathology.

4 Cytokine Responses in Malaria

The inflammatory response during acute malaria is often described as a "cytokine storm" to convey that there are high levels of a broad range of cytokines in the circulation. Earlier studies already correlated the levels of IFN-α and IFN-γ with levels of parasite [137, 138] that were followed up by the confirmation that plasma levels of inflammatory cytokines—IFN-γ, TNF, IL1β, and IL-6—are elevated in patients with malaria and directly correlate with disease severity in *P. falciparum* and *P. vivax* infections [139–150]. Gene expression profiles also confirm high levels of inflammatory cytokines in peripheral blood mononuclear cells [148] and in tissues such as the brain of cerebral malaria patients [151, 152]. The use of mice models of malaria has allowed the continued evaluation of cytokine production during a self-resolving infection, where it was observed that there is an early production of pro-inflammatory cytokines that start decreasing before the parasitemia [153].

TNF is a pro-inflammatory cytokine produced early in mouse *Plasmodium* infection that is important in the clearance of parasites both in the liver and blood stages [154–157]. This protection is induced through the generation of nitric oxide [158]. Studies on malaria patients showed that TNF not only correlates with disease severity but was ten times as high in fatal cases of cerebral malaria [144]. Further studies confirmed the importance of TNF in malaria pathology showing that different alleles of the promoter region of TNF confer either decreased or increased susceptibility to cerebral malaria and severe anemia in populations of children in endemic areas [159–163]. High levels of TNF are also correlated with a rapid parasitological cure in patients supporting the hypothesis that inflammatory cytokines were effective and necessary for clearance of the parasite, but could also lead to severe forms of the disease [143]. Based on the evidences for the strong association of TNF with malaria severity, a clinical trial for the use of anti-TNF antibodies as adjunctive treatment for cerebral malaria patients was performed. However, no improvement in survival was found in the patients [164], a finding that revealed the complexity of the antimalarial immune response and our limited knowledge in the mechanisms underlying immune-mediated pathology.

Another inflammatory cytokine that has been implicated in parasite clearance and is highly increased during acute malaria is *IFN-γ*, with the particularity that

increased levels of this cytokine during liver-stage infection were correlated with lack of blood-stage development in humans [165] and monkeys [166], suggesting that this cytokine may be important to inhibit progression of the disease, a finding that is specially relevant for vaccines targeting the liver stage. Studies in mice have shown that IFN-γ is key in the elimination of *Plasmodium*, since infected mice given exogenous IFN-γ showed lower parasitemias and delayed mortality, while mice deficient in the IFN-γ gene or treated with anti-IFN-γ monoclonal antibodies had higher parasitemia and increased mortality [153, 155, 156, 167–175].

Mouse studies on *IL-12* and *IL-18* have established that these cytokines contribute to parasite clearance [176]. The increased protection granted by these cytokines is probably mediated by the positive effect on IFN-γ production [175, 177, 178]. However, high IFN-γ levels can be dangerous, since experimental *Plasmodium* infections also induce liver injury mediated by IL-12-dependent IFN-γ production [179, 180].

Regulatory, or anti-inflammatory, cytokines such as *IL-10* are also highly increased during malaria and correlate with severe disease [149, 181, 182]; although in fatal severe cases, low IL-10 was observed in late stages as death approached [183]. Since regulatory cytokines are probably elevated as a response to the high levels of pro-inflammatory cytokines, it is considered more informative to study the ratio between both types of cytokine responses rather than the absolute levels of each specific cytokine. Different studies have confirmed that, in general, high pro-inflammatory versus regulatory cytokine ratios are indicative of severe disease, with specific examples such as high ratios of IL-6/IL-10 or IFN-γ/IL-10 being associated with severe *P. falciparum* malaria [139, 183]. However, the ratios of TGF-β1/IL-12 and IL-10/IL-12 were significantly higher in the severe malaria patients, suggesting that the generally considered pro-inflammatory cytokine IL-12 could have protective effects [184]. Further studies have shown that high IL-10/TNF ratios were found in children with uncomplicated malaria, while a low IL-10/TNF ratio is associated with malarial anemia in falciparum patients [184–187]. The role of IL-10 in malaria anemia is also supported by the finding that different IL-10 promoter haplotypes that result in low levels of IL-10 increase susceptibility to severe anemia in falciparum patients [188]. As observed before for inflammatory cytokines that contribute to parasite clearance but can promote malaria-associated pathologies, high levels of regulatory cytokines appear to be protective against severe malaria complications but induce a less effective clearance of *P. falciparum* parasites [189, 190].

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