Neutrophil Paralysis in *Plasmodium vivax* Malaria

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**Abstract**

**Background:** The activation of innate immune responses by *Plasmodium vivax* results in activation of effector cells and an excessive production of pro-inflammatory cytokines that may culminate in deleterious effects. Here, we examined the activation and function of neutrophils during acute episodes of malaria.

**Materials and Methods:** Blood samples were collected from *P. vivax*-infected patients at admission (day 0) and 30–45 days after treatment with chloroquine and primaquine. Expression of activation markers and cytokine levels produced by highly purified monocytes and neutrophils were measured by the Cytometric Bead Assay. Phagocytic activity, superoxide production, chemotaxis, and the presence of G protein-coupled receptor (GRK2) were also evaluated in neutrophils from malaria patients.

**Principal Findings:** Both monocytes and neutrophils from *P. vivax*-infected patients were highly activated. While monocytes were found to be the main source of cytokines in response to TLR ligands, neutrophils showed enhanced phagocytic activity and superoxide production. Interestingly, neutrophils from the malaria patients expressed high levels of GRK2, low levels of CXCR2, and displayed impaired chemotaxis towards IL-8 (CXCL8).

**Conclusion:** Activated neutrophils from malaria patients are a poor source of pro-inflammatory cytokines and display reduced chemotactic activity, suggesting a possible mechanism for an enhanced susceptibility to secondary bacterial infection during malaria.


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**Introduction**

Malaria is a complex disease that affects approximately 300 million people every year. Among the different *Plasmodium* species that infect humans, *P. falciparum* is the main cause of deaths in sub-Saharan Africa. On the other hand, *P. vivax* is responsible for approximately 60–80% of the malaria cases in the world [1,2], and contributes to significant political, social and economic instability in the developing countries of Latin America and Asia [1].

The innate immune system recognizes *Plasmodium* sp. by different pattern-recognition receptors and initiates a broad spectrum of defense mechanisms that mediate host resistance to infection [3,4]. However, the innate immune response is typically a “two-edged sword”, and clinical malaria is associated with high levels of circulating pro-inflammatory cytokines. The outcome of infection depends on a balance between pro- and anti-inflammatory responses allowing the formation of an effective immune response, while limiting its pathogenic potential [5,6,7].

**Toll-like receptors** (TLRs) play an important role in recognition of pathogens through distinct pathogen-associated molecular pattern (PAMPs). Activation of TLRs on monocytes, dendritic cells, and neutrophils can induce changes in the expression of surface proteins and release inflammatory mediators such as cytokines and chemokines. The production of cytokines amplifies innate immune responses and shapes the development of acquired immunity. In addition, activated myeloid cells release high levels of reactive oxygen species (ROS) and antimicrobial peptides that efficiently kill invading pathogens [8,9].
Author Summary

*Plasmodium vivax* is responsible for approximately 60–80% of the malaria cases in the world, and contributes to significant social and economic instability in the developing countries of Latin America and Asia. The pathogenesis of *P. vivax* malaria is a consequence of host derived inflammatory mediators. Hence, a better understanding of the mechanisms involved in induction of systemic inflammation during *P. vivax* malaria is critical for the clinical management and prevention of severe disease. The innate immune receptors recognize *Plasmodium* sp. and initiate a broad spectrum of host defense mechanisms that mediate resistance to infection. However, the innate immune response is the classic “two-edged sword”, and clinical malaria is associated with high levels of circulating pro-inflammatory cytokines. Our findings show that both monocytes and neutrophils are highly activated during malaria. Monocytes produced high levels of IL-1β, IL-6 and TNFα during acute malaria. On the other hand, neutrophils were a poor source of cytokines, but displayed an enhanced phagocytic activity and superoxide production. Unexpectedly, we noticed an impaired chemotaxis of neutrophils towards an IL-8 (CXCL8) gradient. We propose that neutrophil paralysis is in part responsible for the enhanced susceptibility to bacterial infection observed in malaria patients.

It is noteworthy that glycosylphosphatidylinositol anchors and DNA from *Plasmodium* parasites are important PAMPs that activate TLRs during malaria [10,11,12,13]. Some in vitro studies show that phagocytosis of opsonized hemoglobin (Hb) decreases expression of HLA-DR in monocytes [14,15]. On the other hand, study has demonstrated that DNA bound to Hb induces monocytes to produce high levels of cytokines and contribute to dendritic cell maturation [16]. While other report evaluated activation of polymorphonuclear cells and observed elevated levels of myeloperoxidase, lysozyme and lipocalin in patients with severe malaria [17], the involvement of neutrophils in malaria pathogenesis has been poorly investigated.

Our findings show that both monocytes and neutrophils are highly activated during malaria. Monocytes produced high levels of IL-1β, IL-6 and TNFα in response to TLR agonists during acute malaria and seem to be the main source of pro-inflammatory cytokines in the blood. On the other hand, neutrophils were a poor source of cytokines, but displayed an enhanced phagocytic activity and superoxide production. Interestingly, we noticed an enhanced expression of G-protein receptor protein kinase (GRK2) associated with decreased levels of CXCR2 and impaired chemotaxis of neutrophils towards an IL-8 (CXCL8) gradient. Our findings indicate a mechanism by which malaria patients may become more susceptible to bacterial infection.

**Methods**

**Ethics statement**

All protocols and consent forms were approved by the Institutional Research Board from University of Massachusetts Medical School (IRB-UMMS 10260), the Ethical Committees on Human Experimentation from Centro de Pesquisa em Medicina Tropical (CEP-CEPEM 005/2009) and Centro de Pesquisas René Rachou – Fundação Oswaldo Cruz (CEP-CFRR 2004), as well as by the National Ethical Committee (CONEP 15652) from Ministry of Health, Brazil. A signed informed consent was obtained from each subject prior to enrollment in the study.
pro-inflammatory cytokines during *P. vivax*-malaria? We found that in the blood, monocytes are an important source of cytokines compared with neutrophils. Despite the fact that neutrophils are an important source of cytokines [25], we found that except for IL-8 (CXCL8), the neutrophils from malaria patients produced none or very small amounts of pro-inflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) in response to TLR agonists. Importantly, PBMCs from individuals experimentally or naturally infected with *P. falciparum* are hyperresponsive and produce high amounts of pro-inflammatory cytokines once activated with TLR agonists [6,7]. Here, we observed that highly purified monocytes (but not neutrophils) derived from *P. vivax* malaria patients were primed and produce high levels of IL-1β, IL-6 and TNF-α upon TLR stimulation. In contrast, the monocytes from malaria patients produced low levels of IL-10, even when activated with TLR agonists. Thus, we favor the hypothesis that during malaria monocytes differentiate into an inflammatory stage producing high levels of pro-inflammatory cytokines and low levels of IL-10.

On the other hand, circulating neutrophils from malaria patients displayed enhanced phagocytic activity and constitutively released high levels of superoxide. The process of neutrophil activation could involve phagocytosis of opsonized parasites, which would in turn trigger the antibody dependent respiratory burst [26]. Alternatively, phagocytosis of the malaria pigment hemozoin may also activate neutrophils [27]. The enhanced effector function of neutrophils may account for a more efficient uptake and destruction of free parasites and infected erythrocytes [26]. On the other hand, activated neutrophils have been shown to cause damage of endothelial cells, in a process that is mediated by scree of malaria patients [26]. Therefore, enhanced effector functions in neutrophils could be involved in host resistance and pathogenesis of *P. vivax* malaria.

Unexpectedly, we found an altered migration towards IL-8 gradient, which was associated with a decreased expression of CXCR2 on neutrophils from *P. vivax*-infected patients. Functional studies showed that upon phagocytosis of bacteria by neutrophils there is induction of expression of CXCR1 and CXCR2 [29]. Down-regulation of CXCR2 in severe sepsis also results in failure of neutrophil migration that is associated with enhanced susceptibility to bacterial infection [30]. Furthermore, expression of CD18 (β2 integrin) and CD62L, L-selectin is decreased on the surface of neutrophils from *P. vivax*-malaria patients. CD18 is a G protein-coupled receptor involved in recruitment [19,31], whereas CD62L is a key molecule that mediates cytoadherence of leukocytes [32]. Thus, altogether, our data strongly suggest that systemic activation of neutrophils, lead to failure of extravasation and chemotaxis from blood to the tissues. Since IL-8 mediates chemotaxis and stimulate neutrophils to release specific granules and proteases to fight microbial infections [33,34], the impairment of neutrophil migration to the site of infection would prevent from line cells to promote an infection to effectively kill infectious pathogens allowing secondary infections.

Production of IL-8 has been assessed in several cell type upon stimulation, several medical conditions and even constitutively [35,36,37]. The observed high levels of circulating IL-8 may mediate desensitization and/or down-regulation of CXCR2 in acutely infected malaria patients. In addition, TNF-α [38], nitric oxide [39], heme oxygenase products [39] and TLR ligands [20], cause the heterologous desensitization of CXCR2 via G protein induction. As previously described in *vivax* malaria [10,11], we did not find a high level of TNFα in serum, the monocyte stimulated with TLR agonists produced high amounts of TNF-α.

![Figure 3. TLR agonists induce production of high IL-1β, IL-6, and TNF-α levels by monocytes from *P. vivax*-infected subjects. Purified monocytes (A) or neutrophils (B) from *P. vivax*-infected subjects before (closed circles: n = 13) and 30–45 days after treatment (open circles: n = 13) were cultured for 48 hours in the absence or presence of LPS or Pam. Levels of IL-1β, IL-6, IL-10, TNF-α, and IL-8 (CXCL8) were measured in supernatant of monocyte (A) and neutrophil (B) cultures. Levels of cytokines were measured employing the Cytometric Bead Array (CBA). Significant differences are indicated with values using paired t test or Wilcoxon signed rank test when a normality test failed.](https://journal.pmds.org/doi/10.1371/journal.pmds.0001710.g003)

![Figure 4. Neutrophils from *P. vivax*-infected patients produce high levels of superoxide and display enhanced phagocytic function. Neutrophils were isolated from *P. vivax*-infected patients (closed circles: n = 19) or healthy donors (open circles: n = 15), and the frequencies of neutrophils reducing NBT (left panel) as well as cell containing myeloperoxidase (right panel) were quantified. Significant differences are indicated with *p*-values using unpaired t test or Mann-Whitney test when a normality test failed.](https://journal.pmds.org/doi/10.1371/journal.pmds.0001710.g004)
that may contribute to CXCR2 desensitization. In addition, *Plasmodium* can be recognized by TLR2, TLR4 and TLR9 [10,42] and induce down-regulation of CXCR2 via GRK2. Importantly, CD88 is also desensitized via GRK2 [43]. Thus, decreased expression of CXCR2 and CD88 on neutrophils from malaria patients may be a consequence of an enhanced expression of GRK2.

GRKs constitute a group of serine/threonine protein kinases that are key modulators of protein-coupled receptor signaling (GPCR) [44]. A major mechanism for desensitization of activated GPCR is their phosphorylation by GRKs [45]. Of note both CD88 and CXCR2 are GPCRs. Deficient expression of GRK and regulation of chemokine receptors in GRK2−/− mice results in enhanced migration of lymphocytes and chemotaxis toward

Figure 5. Malaria impairs neutrophils response to CXCR1 and CXCR2 ligand. Neutrophils were isolated from *P. vivax*-infected patients (closed circles; n = 15) or healthy donors (open circles; n = 15), and chemotaxis towards IL-8 (CXCL8) and CCL2 was assessed (A). MFI of CXCR1, CXCR2 and CCR2 on neutrophils were evaluated by flow cytometry and representative histograms of CXCR2 expression are shown (B). CXCR2 message was measured by qPCR (C). Significant differences are indicated with p-values using unpaired t test or Mann-Whitney test when a normality test failed. doi:10.1371/journal.pntd.0001710.g005

Figure 6. GRK2 expression is enhanced in neutrophils during acute malaria. Neutrophils isolated from *P. vivax*-infected patients (closed circles; n = 11) or healthy donors (open circles; n = 12) were stained for GRK2 and mean fluorescence intensity (MFI) of GRK2 was quantified (A). Representative fluorescence microscopy illustrating GRK2 expression in neutrophils from a healthy donor and a *P. vivax*-infected patient (B). Significant difference is indicated with p-values using unpaired t test. doi:10.1371/journal.pntd.0001710.g006
CCL4, the GCR5 ligand [16]. It was also described that transcription of GRK2 and GRK3 is upregulated upon LPS-mediated activation, leading to reduced expression of chemokine receptor and neutrophils chemotaxis [47]. GRK2 and GRK3 expression are enhanced in sepsis patients [48] and in rodent models of severe sepsis [29], which are associated with impaired migration of neutrophils and enhanced susceptibility to secondary microbial infection.

For many years, P. tertio malarial was considered a benign and self-limited disease, especially when compared to P. falciparum infection [19]. However, recent studies highlighted the association of P. tertio malarial with life-threatening manifestations, such as respiratory distress, severe thrombocytopenia, and anemia, as well as neurological manifestations [1, 22, 50, 51, 52, 53, 54]. A main hypothesis of our research group is that secondary infections, in malaria primed individuals, is a main cause of severe disease. In this regard, pro-inflammatory priming during malaria would result in dramatic decrease in the threshold to initiate a septic shock [7], due to an over-reaction to secondary infections, particularly in the case of bacteria that have extremely potent TLR agonists.

Importantly, areas of the world with the highest incidence and prevalence of malaria also have a high incidence of bacterial infections, including *Staphylococcus*, *Pseudomonas*, and *Mycobacterium* [23, 55]. Furthermore, a recent study highlights that severe malaria as indicated by respiratory distress, anemia and mortality, is 3.5 times more elevated in children with both malaria and bacteremia as compared to infection with *P. falciparum* alone [24]. Thus, co-infection with bacteria is not only common, but as we might predict, it is an important factor influencing outcome of disease and development of severe disease [24, 35, 36]. Here, we demonstrate for the first time that circulating neutrophils from malaria patients display a decreased expression of chemokine receptors and adhesion molecules, which culminates in impaired chemotaxis. Hence, our results suggest that a failure of these PMNs to migrate to peripheral tissues is an important mechanism leading to enhanced susceptibility to bacterial infection during malaria.

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**Author Contributions**

Conceived and designed the experiments: FMSL, LRVA, RTG. Performed the experiments: FMSL, LRVA. Analyzed the data: FMSL, LRVA. Contributed reagents/materials/analysis tools: FMSL, LRVA. DTG, FQG. Wrote the paper: FMSL, LRVA, RTG. Assisted with patient care and case identification: MIST, DBP.

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