Plasmodium vivax in haematopoietic niches: hidden and dangerous

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Abstract

A series of recent studies have suggested the haematopoietic niche of the bone marrow as a major reservoir for parasite replication and the development of transmission stages. However significant knowledge gaps remain in our understanding in the host parasite interactions, pathophysiology and implications for treatment and diagnosis of such reservoir. Here, we discuss the current status of this emerging research field in the context of *Plasmodium vivax*.

Introduction

Outside of Sub-Saharan Africa, *Plasmodium vivax* dominates the malaria public health burden. In these regions it accounts for 41% of all malaria cases resulting in 35% of the global population living at risk of *P. vivax* infection [1, 2]. Even in Sub-Saharan Africa, an increase of *P. vivax* cases has been observed despite high frequency of Duffy-negative alleles [3]. In the Brazilian Amazon region, *P. vivax* is the main species causing malaria and responsible for more than 85% of all cases [1]. In general, *P. vivax* persists in areas that succeeded to eliminate *P. falciparum* by malaria control programs [4]. However, major knowledge and tool gaps remain in *P. vivax* research as the focus so far has been on *P. falciparum*.

For a long time, *P. vivax* research was neglected due to failure to establish an *in vitro* culture system and apparently low prevalence of severe cases compared to *P. falciparum* [5-7]. Presence of all stages in the blood circulation, and therefore assumed lack of sequestration, has contributed to the long-standing misconception that *P. vivax* is a benign parasite [8]. However, recent data have demonstrated that late asexual blood stage *P. vivax* parasites are capable of cytoadhering to endothelial host receptors [7, 9], and that they are less abundant in blood circulation than younger stages in *P. vivax* patients [7, 10]. Estimation of parasite biomass based on circulating biomarkers indicates existence of a predominant parasite biomass outside of circulation that is not captured by peripheral *P. vivax* parasitemia, in particular in patients with complicated outcomes [10]. Moreover, a series of recent histological studies in *P. vivax* patients and experimentally infected non-human primates (NHP) provides direct evidence for the existence of a major reservoir of *P. vivax* blood stage parasites, both asexual and sexual (gametocytes) in the haematopoietic niche of bone marrow and possibly spleen. These recent findings, together with more stringent diagnosis techniques of *P. vivax* infection suggesting a similar risk of severe disease and death as *P. falciparum* infection [6], strongly argue against the benign nature of *P. vivax* malaria, especially in patients with other comorbidities.

P. vivax biomass in peripheral circulation: the tip of the iceberg?

P. vivax parasites exhibit a narrow tropism by strictly infecting young reticulocytes. In contrast, *P. falciparum* can infect normocytes even though it also prefers to infect young reticulocytes [11-14]. The restriction of *P. vivax* for young reticulocytes that are exceedingly rare in circulation (<2% of all circulating red blood cells) means parasitaemia is greatly limited by the abundance of available host cells [6, 15]. Low peripheral parasitaemia and

apparent presence of all parasite stages in the blood contradicts numerous reports of vivax malaria with severe illness and deaths due to *P. vivax* infection in all endemic regions [16-20]. It has been suggested that *P. vivax* parasites have a lower pyrogenic threshold and hence induce a stronger inflammatory response compared to other *Plasmodium* infections with similar or greater parasitaemia [15]. Indeed, the host inflammatory response and endothelial activation are greater in patients infected with *P. vivax* than with other malaria infections [19-21].

It has also been suggested that the peripheral parasitaemia represents only a fraction of the total *P. vivax* parasite biomass. Various indirect lines of evidence support this hypothesis. First, several reports from *P. vivax* patients have shown that the total parasite biomass, as define by pvLDH levels in blood, is underestimated by microscopic analysis of peripheral blood smears [6, 21]. Second, there is no clear correlation between the burden of peripheral parasitaemia and disease severity. Accordingly, a wide range of clinical syndromes occurs in *P. vivax* patients even with modest peripheral parasite counts, in contrast to *P. falciparum*-infected individuals [6, 21-31]. Third, NHP models susceptible to *P. vivax* infection have been very informative in inferring sequestered parasite biomass and correlations with pathogenesis [31-34]. A computational model capable to quantify the parasite biomass concealed in a tissue reservoir by measuring blood parasitaemia was designed by observing the longitudinal dynamics of *P. cynomolgi* parasitaemia in infected *Macaca mulatta*, a *P. vivax* simian malaria model [31]. Through the application of this model and additional observations made in vivax malaria patients it was inferred that a large fraction of parasites is withdrawn from the peripheral circulation early during blood stage infection and hidden in a reservoir, with potential role in disease pathogenesis [31, 34]. Fourth, clinical studies in *P. vivax* patients and in NHP models demonstrate that this hidden parasite population seemingly expands without detection and contributes to disease severity [6, 21-34], systemic inflammation [15, 21, 28] and intravascular accumulation of immune cells in pulmonary pathologies [28].

Finally, several studies have reported a biased distribution of asexual forms in blood smears of *P. vivax* patients, with higher prevalence of ring stages compared to trophozoites and schizonts in peripheral blood [7, 10]. Likewise, transcriptomic analysis from *P. vivax* blood samples demonstrated a quantitative depletion of transcripts from late asexual and immature sexual stages, or gametocytes, in the blood of *P. vivax*-infected patients [32], similar to observations with *P. falciparum* [35]. At the same time these later asexual stages display a higher adhesive capacity compared to young stages, indicating that the latter part of

the asexual *P. vivax* cycle could occur in deep tissues and outside of peripheral circulation [6, 7, 10]. Specifically, late asexual parasites are able to cytoadhere *in vitro* to endothelial receptors, such as ICAM-1, CD36 and chondroitin sulfate A (CSA), receptors expressed in cerebral, pulmonary and placental microvasculature, with a similar strength but lower frequency than red blood cells (RBCs) infected with *P. falciparum* [7, 9, 36, 37].

Emerging evidence for a *P. vivax* reservoir in the hematopoietic niche of the bone marrow

The reticulocyte population makes only 1–2% of all circulating RBCs [13, 14]. Immature reticulocytes are largely confined to the bone marrow (BM, ~0.016% of all enucleated erythroid cells in the circulation) and more cytoadhesive (higher expression of adhesion molecules such as CD49d and CD44) than circulating reticulocytes. Reticulocytes are formed from haematopoietic stem cells and released from the bone marrow niche for final maturation in the spleen [11-14, 38, 39]. *P. vivax* preferentially invades BM resident immature reticulocytes making this niche highly advantageous for the parasite. Multiple case reports have detected *P. vivax* at higher parasite biomass in the BM compared to blood, or exclusively in BM [12, 25, 40-43]. *P. vivax* infections after BM transplantation have also been reported [27, 44-46], suggesting that BM may represent a pivotal tissue reservoir in *P. vivax* infection.

A systematic analysis of *P. vivax* distribution in tissue samples from infected splenectomized *Aotus* and *Saimiri* monkeys revealed enrichment of gametocytes and schizonts in the BM and liver [32]. Together, these organs accounted for about 30% of the total parasite burden. 70% of the gametocyte load and 90% of the schizont load was accumulated in the BM and liver, suggesting that these tissues are major parasite reservoirs. Importantly, in the BM the vast majority of parasites were located in the parenchyma, where haematopoiesis takes place. Immunohistochemistry (IHC) analysis revealed that the majority of parasites detected by the constitutive marker pLDH (*Plasmodium* lactate dehydrogenase) were negative for antibodies against late sexual (PvLAP5) and asexual stages (PvAMA1) markers, indicating the enrichment of early ring stages and immature gametocytes in the BM parenchyma [32]. In agreement with these data a recent case report demonstrated enrichment of rings, schizonts and gametocytes in BM compared to blood [47]. Together, these studies suggest that the BM contributes significantly to the total *P. vivax* biomass, providing a niche for asexual growth and development of gametocyte stages (Figure 1).

These findings are in line with similar observations in *P. falciparum* and the rodent malaria parasite *P. berghei*. Autopsy case studies and analyses of biopsies and aspirates have consistently revealed a significant enrichment of *P. falciparum* immature gametocytes in the BM and spleen of infected patients [48-50]. In the BM parenchyma, gametocytes were enriched at erythroblast islands before re-entering the circulation [49]. Quantitative imaging experiments in the rodent malaria model *P. berghei* also demonstrated gametocyte development in the extravascular niche of the BM and spleen, involving selective tissue homing, transmigration across the endothelial barrier and mobile behaviour of mature gametocytes [51]. In addition, asexual parasite stages were observed in the extravascular environment both in *P. falciparum* (in human autopsies) and in *P. berghei* (in infected mice), suggesting existence of a genuine extravascular replication cycle in both *Plasmodium* species [51]. Altogether these observations establish infection of the BM haematopoietic niche as a new paradigm in *Plasmodium* biology.

What is the role of the spleen as a parasite reservoir?

Experimental and clinical studies have demonstrated that *P. vivax* infection induces a marked splenomegaly, with incidence of splenic rupture and death higher than in other malaria infections [52-58]. In humans, the spleen contributes to the clearance of damaged and infected RBCs, generation of immunity and it changes to parasite antigens expressed on the surface of infected RBCs [54, 55]. Examinations of spleen samples from *P. vivax* patients revealed extensive remodelling, enlargement of the white pulp, increased cellularity and large numbers of intact *P. vivax*–infected reticulocytes in the red pulp [22, 54, 55]. In one case report, confocal microscopy analysis showed macrophages containing large amounts of parasite pigments, but no intact RBCs were detected in macrophages. Interestingly, intense proliferation of B cells, plasma cells and plasmablasts in extrafollicular compartments, which resembled a B-cell lymphoma phenotype was also observed [22], suggesting that alterations in the spleen are linked to acquisition of anti-parasite immunity.

Initial investigation of *P. vivax* sequestration in spleen-intact common squirrel monkeys (*Saimiri sciureus*) and night monkeys (*Aotus lemurinus lemurinus*) identified the splenic vasculature as the primary site of *P. vivax* asexual development, with a high proportion of schizont-infected RBCs [33]. The liver and BM appeared as secondary sites for trophozoite and schizont accumulation [33] while gametocytes were not analysed. Although these observations were based on organ crushes only and the organs were not perfused, a recent study in *P. vivax*-infected *Saimiri* included one spleen-intact control animal that

showed a similar pattern of parasite distribution [34]: spleen contained the highest parasite counts followed by the liver, lung and BM. In agreement with the work by Obaldia *et al*. [32], parasites were enriched in BM and liver in the splenectomised animals [34] (Figure 1). Splenectomy before infection is an important limitation in these studies, as the significant parasite load observed in BM and liver could mask a significant reservoir in the spleen. So far, no systematic autopsy case or other tissue biopsy studies of *P. vivax*-infected patients comparing the role of both spleen and BM as potential parasite reservoirs have been conducted. In *P. falciparum*, high numbers of parasites were found in spleen samples from autopsies cases [49], however it remains to be determined whether these are viable or present within macrophages. In the rodent malaria model the spleen represents the major parasite reservoir outside of circulation with significant levels of asexual and gametocyte stages [51].

In contrast to humans and primates, the adult murine spleen is haematopoietically active. However, splenic extramedullary erythropoiesis can also occur in humans during specific pathological conditions including malaria. Such mechanism has been suggested to be stimulated during vivax malaria to compensate anaemia [59], and it would further support the role of the spleen as a parasite reservoir [60-62]. In this scenario, haematopoiesis would take place in the red pulp of the spleen before the release of reticulocytes into the circulation. In addition, *P. vivax* infection may also induce a remodelling of uninfected RBCs, resulting in their arrest in the spleen. In turn, this could generate a reservoir for parasite invasion. These *P. vivax*-infected reticulocytes may remain trapped in the red pulp by interacting with contractile fibroblasts, cells that proliferate during splenic erythropoiesis and surround reticulocytes [59, 61].

Sub-patent infection in the hematopoietic reservoir as a source of recurrences?

The observed concealment of infected RBCs in the haematopoietic system is likely to be critical for the parasite to evade immunity and drug pressure, and this reservoir may contribute to the observed recurrence patterns in *P. vivax* infection. Proof-of-principle support of this hypothesis comes from the *P. berghei* model, which – as *P. vivax* - has a preference for young reticulocytes. Mice infected with *P. berghei* and treated with at least 10 mg/kg of artemisinin clear peripheral parasitaemia but maintain low level infection rates in BM and spleen that initiate recurrence of peripheral parasitaemia [63].

Case reports also support the hypothesis of *P. vivax* recurrence from the haematopoietic niche. For example, one report from Brazil documented a patient with persistent thrombocytopenia and an enlarged spleen who was diagnosed with chronic *P. vivax*

malaria after discovering schizonts in BM aspirate [24]. In another case report, a patient developed vigorous vivax malaria with relatively high parasitaemia (1%) 40 days after a donor BM transplant [27]. Investigations revealed that the donor was diagnosed with malaria 11 months before BM collection, and had an asymptomatic recurrence following treatment of the first infection [27]. The uncertain origin of homologous vivax recurrences [64], case reports of *P. vivax* parasites detected only in BM aspirates without peripheral blood parasitaemia [24, 27, 40-44, 65], reports of vivax infection following sibling allogeneic BM transplants [45], and recurrence after autologous BM transplantation [46] further suggest presence of sub-patent *P. vivax* infections in the BM that can lead to recurrence. Hence BM could be an alternative source of parasite recurrence upon drug treatment, as opposed to liver relapse from quiescent hypnozoite stages. Notably, primaquine and related 8-aminoquinolines, the first line treatment against hypnozoites, are prodrugs that require activation through an enzymatic pathway that is predominant in liver and BM tissue [66].

Analysis of recurrence patterns in neurosyphilis patients who underwent malaria therapy either through inoculation by *P. vivax* blood stage or sporozoites provide interesting information in that regard [67]. While there is wide variation in recurrence patterns across individual patients, parasite dynamics are not significantly different during the first 2 months post infection [67], whereas only sporozoite-inoculated infections seem to exhibit recurrent parasitaemias weeks after absence of peripheral blood parasites, indicative of relapses from liver hypnozoites (Figure 2). Data from other studies performed around the same time as the malaria therapies confirm these observations. For example, a study covering around two years of observations of general paralysis in patients inoculated with *P. vivax* trophozoites and treated with quinine (30g, 2-4 days), revealed that 2% relapsed up to a month after end of treatment [68, 69]. In patients who survived infection after mosquito bites, 18% recurred between two to six months and 33% of these recurred more than once [68]. Another study comparing *P. vivax* blood stage versus sporozoite infection reported that both inoculation routes lead to 35-40% of recurrences within the first two months after termination of the primary infection, while only sporozoite infections recurred beyond that point [70]. Similar to the malaria therapy studies, a longitudinal study in rhesus monkeys infected with *P. cynomolgi* showed recurrence in 48% of the monkeys inoculated with trophozoites while 79% of those infected with sporozoites recurred [71]. In this study, animals who were negative by thick smear for 60 days or more underwent splenectomy: 25% of sporozoite-infected animals relapsed up to two weeks after splenectomy, while none of the trophozoite-infected monkeys did [71].

Taken together, comparative data from experimental infections in humans and animals indicate that both the blood stage and sporozoite infection routes can exhibit similar recurrence patterns, at least during the first 2 months post infection and following (sub-curative) drug treatment. On the other hand, the limited data available indicates that hypnozoite relapse have a much greater contribution during later phases of infection, which together suggests that the BM reservoir and liver hypnozoites are distinct but synergistic strategies by which the parasite prolongs infection and thus enhances its transmission success.

What are the implications of *P. vivax* development in the BM for the host?

Parasite infection in the haematopoietic niche has implications for malaria pathogenesis, diagnosis and treatment. The BM parenchyma is a specialized and complex microenvironment that provides a set of molecular, structural and physical cues to regulate hematopoietic stem and progenitor cell (HSPC) production [72]. Haematopoiesis is a dynamic biological process that can also be responsive and shaped by pathogens during infection. HSPCs are capable of responding to pathogens by directly sensing pathogen-associated molecule patterns (PAMPs) through their respective pathogen-recognition receptors (PRRs). They also express a broad range of cytokines/chemokines receptors, which allows them to detect pro-inflammatory signals (DAMPs).

Recent studies investigating the impact of parasite infection in the BM have focused on potential changes in erythropoiesis to understand the pathophysiology of vivax malaria anaemia [47, 73]. These changes include altered levels of miRNA transcripts [48] and impaired activity of transcription factors, such as GATA1/GATA2 [74, 75], regulating erythropoiesis. GATA1/GATA2 changes are mediated by intermediate and non-classical monocytes and activation of IFN type I and II signalling pathways in the BM [74]. Similarly, IFN-γ is implicated in malarial anaemia in rodent malaria models and can directly cause apoptosis of erythroid progenitors *in vitro* [76]. It has also been suggested that inflammatory cytokines produced by macrophages and monocytes in the BM in response to parasite by-products promote the dyserythropoiesis observed during malarial anaemia [77]. In *P. vivax*-infected patients lymphopenia and thrombocytopenia are common clinical signs of infection, while myeloid cell count (e.g., monocytes and neutrophils) often remain unchanged in the peripheral blood [6, 15, 21, 24, 73, 78-81]. Although the level of cytokines implicated in the expansion of the megakaryocyte lineage and myelopoiesis in the BM remain to be determined, these molecules (e.g., IL-1α, IL-1β, IL-6, IL-8, TNF-α, IFN-γ, thrombopoietin [TPO] and G-CSF) are increased in the plasma of *P. vivax* patients [15, 21, 24, 73, 78, 82-84].

Increased cytokine levels inducing myeloid-biased HSC differentiation while reducing lymphopoiesis could explain the normal counts of myeloid cells and decrease of lymphocytes in the circulation in *P. vivax* patients [21, 24, 73, 78]. Indeed, less differentiated neutrophils (band cells) in peripheral blood are increased in *P. vivax* patients during acute infection, possibly as a result of rapid neutrophil production and/or their premature release from the BM [78, 79]. Likewise, elevated levels of cytokines inducing megakaryocyte differentiation indicates that the BM mounts a response to compensate reduced platelet counts in peripheral blood. Analysis of a BM biopsy from a patient with chronic vivax malaria revealed hyperplasia of myeloid and megakaryocytic cells [24], and a similar phenotype has been described in *P. cynomolgi*-infected monkeys [74]. In the rodent malaria model *P. chabaudi* it was demonstrated that IFN-γ signalling in hematopoietic progenitors induces myeloid-biased differentiation and myeloid cell numbers, which appeared to be associated with parasite clearance [85]. A similar mechanism was also observed in the *P. berghei* rodent malaria model [86]. The analysis of *P. cynomolgi* infection in *Rhesus macaques* also demonstrated transcriptomic changes in the BM including upregulation of *IFN-γ* and *IL-27*, as well as pathways related to pathogen recognition, such as TLRs, NOD-like receptors and RIG-1/MDA5 [74]. Of note, reticulocytes express parasite antigens via human leukocyte antigen class I (HLA-1), which are recognized by antigen-specific $CD8^+$ T cells, resulting in the formation of immunological synapses and killing of the *P. vivax*–infected reticulocytes [87].

Collectively, these observations suggest that *P. vivax* antigens can be presented and induce immune responses in the extravascular niches of the BM. Resident cells including haematopoietic progenitor/stem cell (HPSCs) could adapt to these signals through proliferation, mobilization from the BM and skewing toward the myeloid lineage, at the expense of lymphopoiesis [82-84] (Figure 3, Key Figure). However, this infection-induced adaptation toward enhanced myelopoiesis might also perpetuate inflammation in chronic or repeated infections by generating a feed-forward loop between myeloid-biased HSPCs and the inflammatory response. Indeed, chronic HSPC activation by infection and/or inflammatory stimuli causes impairment of function and exhaustion, alters global patterns of gene expression skewing hematopoietic potential and further perpetuates inflammation, which leads to BM remodelling and potentially myelodysplastic syndromes [84]. It will be important to investigate acute and long-term impacts of *P. vivax* infection in the BM, in particular in patients continuously exposed to the parasite.

Concluding remarks

Recent studies have demonstrated that the haematopoietic niche represents a major reservoir for *P. vivax* that is subject to significant changes during infection. These observations raise a series of questions with regards to parasite biology (see Outstanding questions). In addition, the host-parasite interactions established in the different reservoirs and their clinical implications represent key knowledge gaps. Because *P. vivax* develops/accumulates in the BM parenchyma, its antigens and parasite-induced cytokines can potentially be sensed by HSPCs, MSCs, ECs and mature immune cells in the BM parenchyma and shape its function. This raises questions about the underlying host-parasite interactions in hematopoietic stem cell niche environments (see Outstanding questions). Understanding the acute and long-term effects of the hidden parasite biomass in haematopoietic reservoirs is relevant to the study of acute and chronic *P. vivax* infection. In addition, NHP models and human cohort studies (autopsies, BM and/or spleen aspirates – see Box 1) will be of great value to further evaluate the importance of the haematopoietic reservoirs for *P. vivax* survival, recurrence, transmission and pathogenesis.

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Box 1: BM aspiration in the clinical routine

BM biopsy is performed routinely as part of the clinical management of malaria patients with anaemia (Hb<7g/dl) to exclude other aetiologies, such as erythroid hyperplasia. The current knowledge gaps reported in this review are due, in part, to the fact that BM biopsy is often perceived as being associated with unnecessary risks for the patients and is therefore seldom performed on conscious individuals with mild illness. A study conducted in 2001-2003 and surveying about 20,000 BM aspiration/biopsy procedures across 63 hospitals reported only sixteen adverse events, representing 0.08% of total reported procedures and suggesting that risks are, in fact, minimal [88]. Larger studies with serial BM biopsies and/or aspirations from patients infected with *P. vivax* (pre/post treatment, for example) should therefore be considered in the future to shed more light on the BM "ecosystem" in vivax malaria.

Figure 1: *P. vivax* tissue distribution in non-human primates (NHPs). (A) Representative images obtained by Obaldia et al. [32] of parasites in the immunohistochemistry (IHC) analysis of bone marrow, liver, lung and brain. pLDH (total), PvLAP5 (gametocytes), and PvAMA1 (schizonts) antibodies were used to detect stage-specific parasites; CD31 antibodies stained the endothelium. Black arrowheads mark parasites. (B) Representative images obtained by Peterson et al. [34] of H&E-stained sections of bone marrow from a splenectomized animal and the spleen from the intact animal indicating the distribution of parasites (black arrows). (C-E) Heatmaps representing total, schizonts or gametocytes distributions in similar organs analyzed in 3 different studies: (C) Freemont et al. [33], (D) Obaldia et al. [32] and (E) Peterson et al. [34].

Figure 2: Comparison blood- and sporozoite-inoculation on experimental *P. vivax* infection dynamics. (A) Average parasitaemia curves of blood- (red line) and sporozoite-inoculated (blue line) *P. vivax* infections (St. Elizabeth strain) are highly similar for the first 1-2 months (solid lines) before they start to diverge, partially driven by relapses in sporozoite-inoculated individuals (N_{blood}=92, $N_{sporozoite}$=88). (B) Individual infection timeseries of *P. vivax* (St. Elizabeth strain) infected individuals, illustrating recurrent parasitaemias even in blood-inoculated infections (patient numbers S12 and S273, top and middle graph), especially following sub-curative drug treatment (arrows), and liver relapse following absence of peripheral parasites for ~ 2 weeks (patient number S484, bottom graph). Data courtesy of G. M. Jeffery and W. E. Collins.

Figure 3, Key Figure: Potential *P. vivax*-induced immune responses in the bone marrow. Resident bone marrow cells, including hematopoietic stem cells (HSCs), multi-potent progenitors (MPPs) and endothelial cells express pathogen-recognition receptors (PRRs), such as Toll-like receptors (TLRs). This allows them to directly sense parasite-derived products presented by local antigen-presenting cells (APCs) or even infected immature reticulocytes, which still retain the capacity to present antigens via human leukocyte antigen class I. This would stimulate the release of cytokines such as IL-6 and G-CSF, which along with other cytokines that are produced during the course of infection, such as IL-1, IFNs and M-CSF, could act directly in the BM cells. This would promote HSC proliferation, myeloid-biased differentiation and also act in the granulocyte-monocyte progenitors (GMPs) and promote generation of myeloid cells.