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MODULAÇÃO DA VIA DA QUINURENINA NA MALÁRIA

RAFAELLA OLIVEIRA DOS SANTOS

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Imunologia Básica e Aplicada do Instituto de Ciências Biológicas da Universidade Federal do Amazonas, como requisito final para obtenção do título de Doutora em Imunologia

Orientador: Dr. Pritesh Jaychand Lalwani

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DEDICATÓRIA

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RESUMO

A malária ainda é considerada um problema de saúde pública mundial. No Brasil, a espécie predominante é o *Plasmodium vivax (*Pv), responsável por mais de 80% dos casos. De acordo com estudos publicados, a resposta imunológica inapropriada contra infecções parasitárias é considerada um dos principais impulsionadores da patogênese da malária, e isso pode ser consequência de uma resposta imune suprimida. Pesquisas mostraram que a indoleamina 2,3-dioxigenase (IDO), enzima que converte triptofano (TRP) em quinurenina (KYN), é impulsionada em parte pela inflamação e que sua alta expressão tem papel fundamental no aumento exacerbado da KYN. A KYN, metabólito imunossupressor, se liga ao fator de transcrição AhR presente nas células T, induzindo uma maior frequência de células T reguladoras (Tregs) $CD4^{+}CD25^{+}FoxP3^{+}$ e a supressão, anergia e morte de células T efetoras. No entanto, o papel da via da KYN na patogênese da malária ainda não foi completamente esclarecido. Para isso, o objetivo do estudo é investigar o funcionamento da resposta imunológica frente a elevados níveis de KYN e demonstrar a influência desse metabólito na maior expressão de células Tregs na malária vivax. TRP, KYN e citocinas pró-inflamatórias foram quantificadas por HPLC e CBA, em pacientes infectados com Pv. Para avaliar a expressão de células Tregs, os PBMCs dos pacientes foram submetidos a imunofenotipagem por citometria de fluxo. Com intuito de compreender a atividade da IDO na malária, os PBMCs de doadores saudáveis foram estimulados *in vitro* com lisados de eritrócitos infectados por Pv (iPV-RBC) na presença ou ausência de inibidores da MyD88, enzima IDO e do AhR. Em nossos resultados, mostramos o aumento nos níveis de KYN, expressão de AhR e maior proliferação de células Tregs $CD4^{+}CD25^{+}FoxP3^{+}$. Adicionalmente, esses dados foram acompanhados do aumento de citocinas pró-inflamatórias em especial IFN-y o qual possui uma correlação positiva com a razão de KYN/TRP, em pacientes com malária antes do tratamento, em comparação com pacientes após o tratamento, o mesmo acontece em pacientes que apresentaram apenas um episódio de malária em comparação a indivíduos com malária prévia. A maior frequência de células Tregs vem acompanhada de maior expressão de AhR e KI-67 nessa população celular. Da mesma forma, ao avaliarmos *in vitro*, os níveis de KYN se mantiveram elevados com maior expressão de células Tregs, quando inibimos a IDO ou o AhR a frequência de células Tregs reduziu. Com isso, nossos dados reforçam que as células Tregs estão mais expressas na malária e que esse aumento pode estar relacionado a atividade da KYN ao se ligar ao receptor AhR.

Palavras-Chave: Malária; Indoleamina 2,3 – dioxigenase; triptofano; quinurenina; AhR; células T reguladoras

ABSTRACT

Malaria is still considered a worldwide public health problem. In Brazil, the predominant species is *Plasmodium vivax* (Pv), responsible for more than 80% of cases. According to published studies, the inappropriate immune response against parasitic infections is considered to be a major driver of the pathogenesis of malaria, and this may be a consequence of a suppressed immune response. Research has shown that indoleamine 2,3-dioxigenase (IDO), an enzyme that converts tryptophan (TRP) to kynurenine (KYN), is driven in part by inflammation and that its high expression plays a key role in the exacerbated increase in KYN. KYN, an immunosuppressive metabolite, binds to AhR transcription factor present in T cells, inducing a higher frequency of regulatory T cells (Tregs) $CD4^+CD25^+FoxP3^+$ and suppression, anergy and death of effector T cells. However, the role of the KYN pathway in the pathogenesis of malaria has not been fully clarified. For this, the objective of the study is to investigate the functioning of the immune response to high levels of KYN and to demonstrate the influence of this metabolite on the greater expression of Treg cells in vivax malaria. TRP, KYN and pro-inflammatory cytokines were quantified by HPLC and CBA, in patients infected with Pv. To evaluate the expression of Treg cells, the PBMCs of the patients were submitted to immunophenotyping by flow cytometry. In order to understand the activity of IDO in malaria, PBMCs from healthy donors were stimulated *in vitro* with red cell lysates infected with Pv (iPV-RBC) in the presence or absence of inhibitors of MyD88, enzyme IDO and AhR. In our results, show an increase in KYN levels, AhR expression and increased proliferation of $CD4^+CD25^+FoxP3^+$ Treg cells. Additionally, these data were accompanied by an increase in proinflammatory cytokines, especially IFN-y which has a positive correlation with the KYN/TRP ratio, in patients with malaria before treatment, compared with patients after treatment, the same it happens in patients who had first malaria episode of compared to individuals with previous malaria. The higher frequency of Treg cells is accompanied by higher expression of AhR and proliferation in this cell population. Likewise, when evaluating in vitro, KYN levels remained high with greater expression of Treg cells, when we inhibited IDO or AhR the frequency of Treg cells decreased. Thus, our data reinforce that Treg cells are more expressed in malaria and that this increase may be related to KYN activity when it binds to the AhR transcription factor.

Key words: Malaria; indoleamine 2,3-dioxygenase; tryptophan; kynurenine; AhR; regulatory T cells.

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LISTA DE ABREVIATURAS E SIGLAS

AhR	Receptor de Hidrocarboneto Aromático
APCs	Células apresentadoras de antígeno
CD	Grupos de diferenciação
CTLA-4	Linfócito T Citotóxico Associado à Proteína 4
DC	Célula Dendrítica
DNA	Ácido Desoxirribonucleico
ELISA	Ensaio imunoenzimático
FACS	Fluorescence Absorbing Cell Sorting
FITC	Isocianato de Fluoresceína
FMT	Fundação de Medicina Tropical
FoxP3	Fator de Transcrição Caixa Forkhead P3
GPI	Glicosilfosfatidilinositol
HIV	Vírus da Imunodeficiência Humana
HPLC	Cromatografia líquida de alta eficiência
IDO	Indoleamina 2,3 dioxigenase
IL	Interleucina
ILMD	Instituto Leônidas e Maria Deane
IPA	Incidência Parasitária Anual
IFN-γ	Interferon-gama
KYN	Quinurenina
mL	Mililitro
NLR	Receptor do tipo NOD
NK	Natural Killer
OMS	Organização Mundial de Saúde
PAMP	Padrão Molecular Associado ao Patógeno
PBS	Solução Tamponada com Fosfato
PE	Ficoeritrina
PerCP	Clorofilpiridina
Pf	*Plasmodium falciparum*
PRR	Receptor de Reconhecimento Padrão
Pv	*Plasmodium vivax*
RLR	Receptor do tipo Rig
SIVEP	Sistema de Informação de Vigilância Epidemiológica
TCLE	Termo de consentimento Livre e Esclarecido
TGF-β	Fator transformador de crescimento beta
TLR	Receptor do tipo Toll
TNF-α	Fator de Necrose Tumoral alfa
Tregs	Linfócitos T reguladores
TRP	Triptofano
UFAM	Universidade Federal do Amazonas

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MyD88-IDO1-AhR axis increases T regulatory cells in vivax malaria infection

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Capítulo 1

INTRODUÇÃO

A infecção por *Plasmodium sp.* ainda é considerada um importante problema de saúde pública, principalmente, nos países em desenvolvimento. Acarreta em uma morbimortalidade importante, tendo sido responsável, em 2018, por 228 milhões de casos em todo o mundo, com 405.000 pessoas indo a óbito (WHO, 2019).

No Brasil, mais de 84% dos casos são causados pelo *Plasmodium vivax* (Pv). A maioria dos episódios está confinada a regiões da Amazônia, com casos isolados ocorrendo em outras localidades do país (DA SILVA-NUNES *et al.*, 2012; OLIVEIRA-FILHO; MARTINELLI, 2009; PARISE, 2009). Nas infecções por Pv, a carga parasitária é menor e os casos complicados são mais raros. No entanto, estudos realizados na Amazônia brasileira e em outros locais do mundo, reforçaram a associação entre casos graves de malária e morte por Pv (ANDRADE *et al.*, 2010; GETHING *et al.*, 2012).

A transmissão da malária ocorre a partir da inoculação do *Plasmodium sp.* através da picada pelo mosquito vetor anofelino. Durante as diferentes fases evolutivas, os parasitos da malária podem ativar a resposta inata e adaptativa. Na malária as respostas imunológicas têm papel fundamental na eliminação do parasita ou diminuição da carga parasitária (MEDZHITOV, 2007; OLIVEIRA-FILHO; MARTINELLI, 2009; ORGANIZATION, 2013).

A imunidade inata, em infecções por *Plasmodium sp.,* funciona como a primeira barreira de defesa do organismo ao parasito, quando os antígenos do parasito ativam monócitos/macrófagos e células dendríticas (CDs). Esta ativação se dá por meio do reconhecimento dos antígenos do plasmódio como âncoras de glicosilfosfatidilinositol (GPI), hemozoína, DNA e RNA derivados do plasmódio por receptores de reconhecimento padrão (PRRs) que participam na produção de derivados da resposta imunológica, como, citocinas TNF-α, IL-1β, IL-6, IL-12, gerando uma resposta inflamatória rápida (CHAVES *et al.*, 2016; CHUA *et al.*, 2013; GUIMARÃES DA COSTA *et al.*, 2014; MEDZHITOV, 2007; ROPERT; FRANKLIN; GAZZINELLI, 2008; TAKEDA, 2004; TAKEUCHI; AKIRA, 2010).

A infecção por *Plasmodium sp.* pode causar doença grave, devido a uma resposta insuficiente para eliminar o parasita ou a incapacidade do hospedeiro em controlar a inflamação (BUENO *et al.*, 2010a; KHO *et al.*, 2016; LAU *et al.*, 2014;

TORRES *et al.*, 2014; WAMMES *et al.*, 2013). A resposta imune adaptativa é desencadeada devido a não eliminação do parasita pela resposta imune inata. Desta forma, a participação das células T se faz necessária para a produção de citocinas (BUENO *et al.*, 2010a; HAFALLA; COCKBURN; ZAVALA, 2006).

Estudos apontam que uma população de células tem a capacidade de criar um equilíbrio entre o controle da infecção e a prevenção da imunopatologia, as chamadas células T reguladoras (Tregs) $CD4^{+}$ $CD25^{high}$ $FoxP3^{+}$ $CD127^{low}$ (ARTAVANIS-TSAKONAS; TONGREN; RILEY, 2003; SAKAGUCHI, S.; POWRIE, 2007). No entanto, na infecção pelo *Plasmodium sp.*, uma maior expressão dessas células Tregs vêm sendo apresentada, e isso contribui para uma resposta inversa, colaborando para o desenvolvimento da infecção, levando a taxas elevadas de citocinas reguladoras como interleucina 10 (IL-10), que conduzem a uma diminuição das respostas inflamatórias mediadas por interferon gama (IFN-γ) (FINNEY; RILEY; WALTHER, 2010; TODRYK *et al.*, 2008; TORRES *et al.*, 2014; WALTHER *et al.*, 2005).

Estudos têm mostrado o papel fundamental da enzima indoleamina 2,3-dioxigenase (IDO) no mecanismo de regulação da resposta imune nas infecções. A IDO está envolvida no catabolismo do triptofano (TRP) em um metabólito imunossupressor chamado quinurenina (KYN), e essa conversão tem sido demonstrada como a principal atividade realizada pela enzima (FRIBERG *et al.*, 2002; LABADIE; BAO; LUKE, 2018; MELLOR; MUNN, 2004; MUNN, David H.; MELLOR, 2007; MURAKAMI *et al.*, 2013; POTULA *et al.*, 2005; VACCHELLI *et al.*, 2014)

Na malária, estudos com camundongos infectados com *P. yoelii* e *P. berguei* mostraram um aumento na produção da enzima IDO, relacionado a altos níveis de IFN-γ (HANSEN, D. S.; SCHOFIELD, 2010; TETSUTANI *et al.*, 2007). A enzima IDO modifica as respostas imunes por meio da produção de KYN, que se liga ao receptor de hidrocarboneto aromático (AhR), levando à hiper-ativação de células Tregs e supressão, anergia e morte de células T efetoras (LABADIE; BAO; LUKE, 2018; MUNN, D H *et al.*, 1999; MUNN, David H.; MELLOR, 2013; PALLOTTA *et al.*, 2011).

A diferenciação de células T em Tregs através da ligação da KYN ao AhR já foram descritas em estudos com outras doenças, inclusive possui um papel de igual importância na indução da expressão da IDO (CAMPESATO *et al.*, 2020; NGUYEN *et al.*, 2010). O catabolismo do TRP induzido por IDO promove a diferenciação de células T em Tregs $CD4^{+}$ $CD25^{+}$ $CD127^{-}$ $FoxP3^{+}$ (LABADIE; BAO; LUKE, 2018; MELLOR;

MUNN, 2004; ORABONA; PALLOTTA; GROHMANN, 2012). Essas células estão em níveis elevados na malária humana e limitam a amplitude da resposta imune celular, devido à produção de fatores solúveis, como IL-10 e TGF-β; e podem resultar na incapacidade de controlar a infecção adequadamente (BELKAID; TARBELL, 2009; BUENO *et al.*, 2010a; HANSEN, D. S.; SCHOFIELD, 2010; HISAEDA *et al.*, 2004; SEHRAWAT; ROUSE, 2011).

A atividade de IDO já foi relacionada a outras doenças graves, como o HIV (FAVRE *et al.*, 2010; PLANÈS; BAHRAOUI, 2013) e o câncer (LABADIE; BAO; LUKE, 2018; MOON *et al.*, 2015). Um trabalho publicado por (WOODBERRY *et al.*, 2017), mostrou que em infecções por *P.vivax* as células Tregs estão aumentadas e que isso se deve a uma maior atividade da enzima IDO e consequente aumento de KYN.

Em trabalhos publicados, mostramos que a KYN está elevada em pacientes com malária, tendo maiores níveis de KYN em pacientes primo-infectados. Da mesma forma, em um estudo não publicado, mostramos que *in vitro* as células Tregs estão mais frequentes, acompanhadas de níveis elevados de KYN e maior expressão da IDO. Ao inibir o AhR observamos uma redução de Tregs que foi acompanhada de uma diminuição no catabolismo do TRP. Tivemos como objetivo mostrar essa atividade da resposta inata associada a maior expressão da IDO, com consequente atividade dessa enzima no catabolismo do TRP e aumento de Tregs através da sinalização do AhR em malária. A hipótese do nosso estudo é de que a resposta inata específica do *P. vivax* está levando a uma maior atividade da enzima IDO e consequente aumento de KYN, e esse metabólito está relacionado a uma maior expressão de Treg na malária, através da ligação KYN – AhR (**Figura 1**).

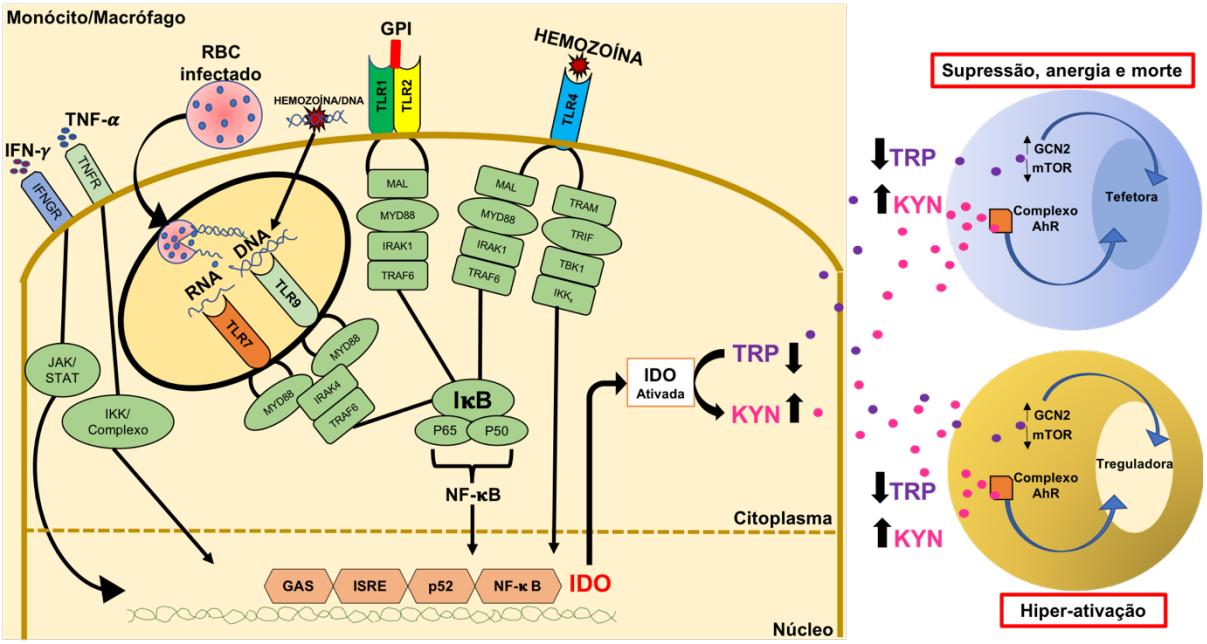

Figura 1. Ativação da enzima IDO leva a um controle metabólico de células T.
O Monócitos/Macrofágos reconhecem PAMPs do plasmódio (RBC infectado, GPI, Hemozoína, DNA, RNA e hemozoína complexada ao DNA) atráves de receptores de reconhecimento padrão PRRs e ativam a resposta imune inata. As ativações das vias de sinalização induzem fatores de transcrição que se ligam *upstream* a sequencias específicas de nucleotídeos, como, como os *interferon sequence response-like elements* (ISRE), *palindromic gamma-activated sequences* (GAS), além da via não canônica do NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells), presentes na região promotora da IDO-1 que controlam a versão ativa da enzima. A IDO ativa faz a conversão do TRP em KYN, em infecções por *P.vivax* ela está mais expressa, isso induz uma maior produção da KYN. A KYN por sua vez se liga ao AhR levando a supressão, anergia e morte de T efetoras e híper-ativação de Tregs. A depleção de TPR ativa a cinase GCN2 e o alvo de rapamicina em mamíferos (mTOR), que responde a retirada dos aminoácidos, que atua também como um sinal regulatório.

2. REVISÃO BIBLIOGRÁFICA

2.1 Malária

Apesar de ser uma doença considerada muito antiga, a malária continua sendo um dos principais problemas de saúde pública ao redor do mundo. Essa parasitose ocorre principalmente em regiões tropicais e subtropicais, abrangendo em torno de 100 países, desses a maioria com problemas econômicos e sociais. A transmissão da malária na maioria das vezes ocorre em áreas rurais, com poucas notificações em áreas urbanas. Até o momento inúmeras medidas de controle são elaboradas por equipe da organização das nações unidas (WHO) para tentar parar a transmissão dessa parasitose, não obstante, muito esforço ainda se faz necessário para o controle completo da doença (ALONSO; TANNER, 2013; MINISTÉRIO DA SAÚDE, 2020; ORGANIZATION, 2013; PARISE, 2009).

São diferentes medidas estratégicas utilizadas para o controle tanto vetorial como do parasita, sendo elas, através da utilização de rede de mosquiteiros

impregnados com inseticida, uso de telas em todas as áreas dos domicílios, conscientização da população para os principais horários de transmissão da doença, aumento de equipes capacitadas para diagnosticar e tratar essa parasitose, acesso facilitado aos medicamentos utilizados no tratamento, entre outras medidas (FERREIRA; DA SILVA-NUNES, 2010; OLIVEIRA-FERREIRA *et al.*, 2010). No entanto, mesmo com todas essas medidas de controle ainda há inúmeros obstáculos que precisam ser superados, um deles é a resistência dos parasitas aos antimaláricos. Como por exemplo a utilização da Cloroquina, que tem se tornado fonte de resistência dos parasitas em infecções por *P. vivax*, sendo identificada pela primeira vez em Papua Nova Guiné, mas mais casos estão ocorrendo em outras áreas endêmicas (GMP/WHO, 2019; RUNGSIHIRUNRAT *et al.*, 2015).

Atualmente, um forte apoio em áreas com fluxo alto de transmissão por *P. falciparum* é a utilização de uma vacina popularmente chamada Mosquirix, elaborada pela empresa GlaxoSmithKline (GSK). Essa vacina tem como alvo a proteína circunsporozoíta do parasita *P. falciparum*, atuando na fase pré-eritrocítica do parasita no hospedeiro. Recentemente, essa vacina começou a ser aplicada em países do continente Africano, após a finalização de todas as fases de testes da mesma. No entanto, dados das fases de teste indicaram uma proteção parcial dessa vacina, cerca de 40%. Porém, bons resultados já foram obtidos na redução de mortalidade em crianças, que são as principais afetadas em regiões endêmicas para o *P. falciparum* (AGNANDJI *et al.*, 2010; RTS, 2015; WHO, 2017).

Para ter noção do controle desses casos de malária e compreender melhor ao redor do mundo o que está acontecendo, dados epidemiológicos são de extrema importância. Por isso, a notificação da malária é de natureza compulsória, sendo exigidas informações como: dados completos dos pacientes, local de provável infecção, intensidade de transmissão, característica dos vetores (quando possível), variabilidade genética e prevalência dos parasitas. Essas informações norteiam futuras pesquisas e ensaios para a elaboração de novas medidas de controle e desenvolvimento de novos fármacos (BARRY, 2005; OWUSU-AGYEI *et al.*, 2009; WHO, 2017).

2.2 Epidemiologia

Segundo a WHO, estima-se que 3,3 bilhões de pessoas correm o risco de se infectar por *Plasmodium* e desenvolver a doença. É estimado que no ano de 2018 ocorreram 228 milhões de casos globalmente, desses 405.000 mil foram a óbito, afetando principalmente crianças menores de 5 anos e grávidas. A maior parte dos casos ocorreram na Região da África (93%), seguida pela Região do Sudeste Asiático (3,4%) e pela Região do Mediterrâneo Oriental (2,1%). De acordo com relatório mais recente da WHO, 106 países e territórios são endêmicos para malária, destes, 43 fazem parte do continente Africano (GMP/WHO, 2019; WHO, 2017).

Todos os países endêmicos para malária estão localizados nos continentes: Africano, Asiático e Americano. A malária ainda tem uma ampla distribuição nesses continentes. No entanto, os números de casos diminuíram desde 2010, onde apresentou cerca de 251 milhões, uma relativa melhora nas medidas de contenção e tratamento podem ter contribuído para isso. Entre 2015 e 2018, apenas 31 países, onde a malária ainda é endêmica, conseguiram reduzir a incidência de casos, porém a WHO já declarou que os planos de redução pré-estabelecidos até 2030 não serão alcançados. Paraguai, Uzbequistão, Argélia e Argentina receberam certificação de eliminação da WHO. Entretanto, são necessárias modificações mais aceleradas para mudarmos o cenário da malária globalmente **Figura 2** (GMP/WHO, 2019; WHO, 2017).

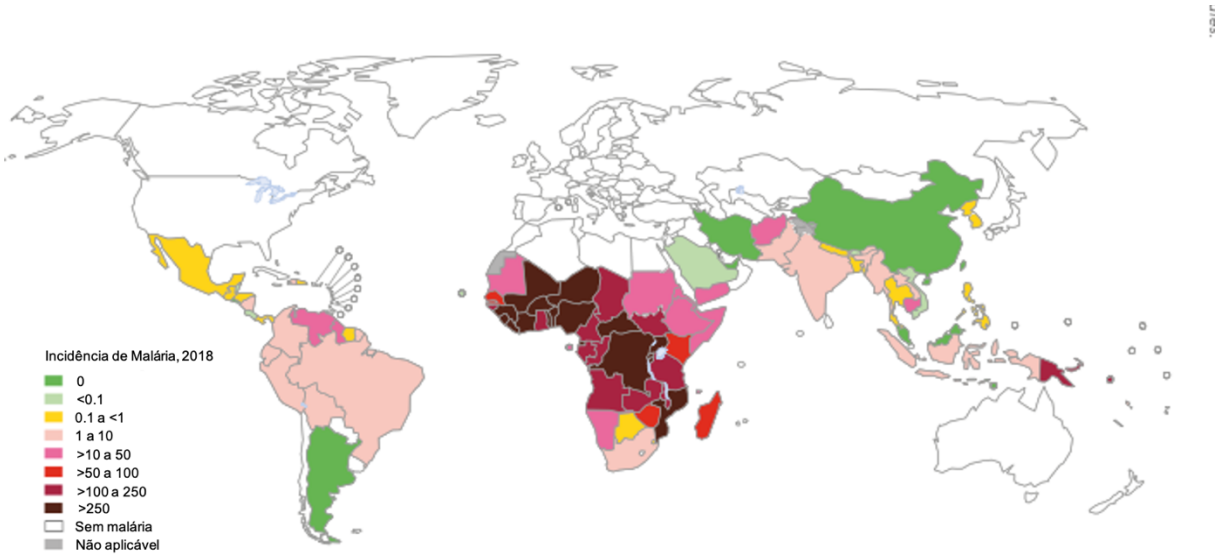

Figura 2: Distribuição Mundial da Malária em 2018 Fonte: (GMP/WHO, 2019)

Estima-se que no continente americano 112 milhões de pessoas estão suscetíveis a malária, em aproximadamente 24 países endêmicos. Ao longo dos anos houve uma diminuição significativa do número de infectados neste continente com 1.2 milhões de casos em 2000 para 427.000 casos que foram diagnosticados em 2013. Sendo o Brasil responsável por 42% das notificações registradas nas américas, dessas 75% correspondem a infecções por *P. vivax* (GMP/WHO, 2019; WHO, 2015).

Atualmente, são reconhecidos seis plasmódios capazes de infectar seres humanos, sendo eles, *P. falciparum, P. knowlesi, P. malarie, P. ovale, P. vivax, P. simium e P. cynomolgy*. No Brasil, a prevalência se restringe ao *P. vivax e P. falciparum* (GRIGG; SNOUNOU, 2017; LEE *et al.*, 2015; OLIVEIRA-FILHO; MARTINELLI, 2009; WHO, 2015). Sendo transmitidos através do mosquito *Anopheles darlingi* fêmea infectado, principal vetor transmissor da malária na região amazônica (BRASIL, 2005a; BRASIL, 2006; LANGHORNE et al., 2008). A malária se restringe em maior parte a essa região, devido as condições climáticas e por dispor de um habitat favorável ao crescimento do *An. darlingi* (OLIVEIRA-FERREIRA *et al.*, 2010; PINA-COSTA *et al.*, 2014).

No Brasil, em particular na região amazônica, o *P. vivax* é responsável por aproximadamente 84% dos casos, enquanto os 16% restantes correspondem a casos de *P. falciparum*. Sendo os casos praticamente restritos à Bacia Amazônica (constituída pelos estados do Amazonas, Acre, Roraima, Amapá, Pará, Tocantins, Rondônia e partes do Mato Grosso e Maranhão). Mesmo em área endêmica, o risco de contrair a malária não é uniforme, sendo 45 municípios foram classificados como de alto risco (alta densidade de vetores/anofelinos), 82 de médio risco (menor densidade de vetores), 370 de baixo risco (baixa transmissão da doença) e áreas Não-Endêmicas (IPA=Zero) correndo nenhum risco da infecção por malária. Em 2019 eles incluíram as áreas com muito baixo risco, segundo estimativas do Índice Parasitário Anual (IPA) (MINISTÉRIO DA SAÚDE, 2020) **(Figura 3)**.

Na Amazônia, o *P. vivax* é responsável pela maioria dos casos e a prevalência de infecção assintomática é muito alta (ANDRADE *et al.*, 2010). Segundo dados do SIVEP-Malária, foram registrados 134.524 casos em 2018. Destes, 53.113 ocorreram no Amazonas, sendo 86% das infecções por *P. vivax* e 14% por *P. falciparum*. A letalidade por malária é considerada baixa nessa região, normalmente no restante do

país chega a ser 128 vezes maior. Muitas vezes nas outras regiões não tem um diagnóstico e tratamento imediato, por conta disso o risco é maior, mas a maior parte desses casos são importados de regiões ou países endêmicos. No geral o pico de malária depende da sazonalidade da região que ocorre entre a mudança de estações mais úmidas para mais quentes (MINISTÉRIO DA SAÚDE, 2020)

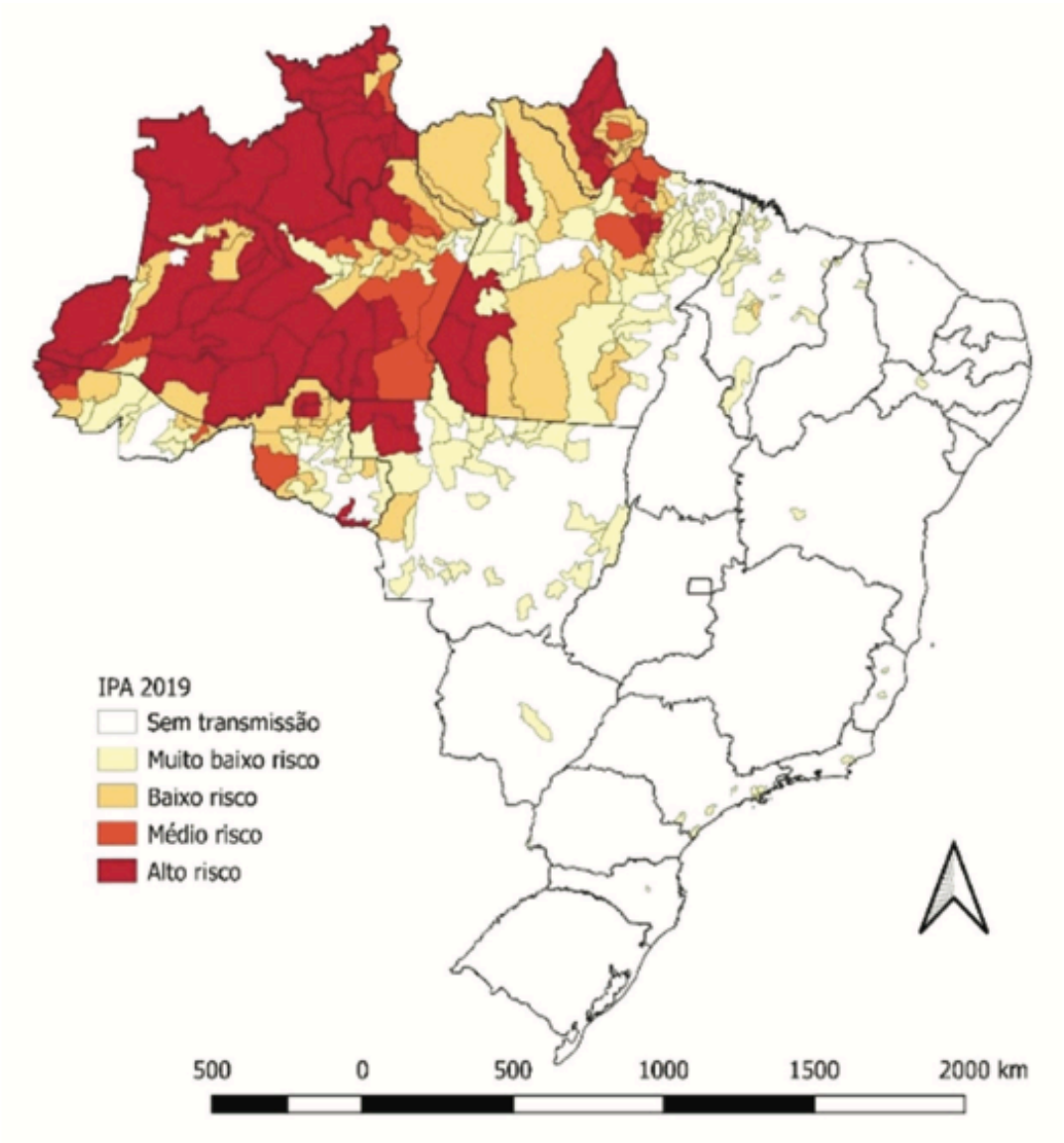

Figura 3: Mapa de risco da malária por município de infecção Fonte: Sivep-Malária, 2020

Embora a maior parte dos casos graves de malária sejam causados por infecções por *P. falciparum*, os casos por *P. vivax* também podem acarretar doença grave. Estudos tem mostrado inclusive na região amazônica associação a malária grave por *P. vivax*. Apesar de infecções por *P. vivax,* a carga parasitária ser menor e casos complicados serem mais raros. No entanto, pode haver uma relação entre

problemas causados por essa espécie, como ruptura do baço, complicação respiratórias ou mesmo anemia grave (ANDRADE *et al.*, 2010; GETHING *et al.*, 2012).

2.3 Ciclo Parasitário

O ciclo de vida do parasita é dividido em duas fases: a fase sexuada, que ocorre no hospedeiro artrópode (*Anopheles darlingi*), e a fase assexuada (que ocorre no hospedeiro vertebrado) iniciada logo após a picada do mosquito, e posterior inoculação dos esporozoítos (PRUDÊNCIO; RODRIGUEZ; MOTA, 2006).

Os vetores estão mais ativos nos horários ao entardecer e ao amanhecer (BRASIL, 2005b). O mosquito *Anopheles* fêmea injeta esporozoítos que através da corrente sanguínea migram para o fígado, em seguida, invadem os hepatócitos e se multiplicam por reprodução assexuada (esquizogonia), originando esquizontes teciduais que amadurecem e liberam milhares de merozoítos hepáticos (KROTOSKI, 1985). Os merozoítos caem na corrente sanguínea, infectam e invadem as hemácias, iniciando a esquizogonia eritrocítica. Nas hemácias, ocorre a maturação do parasita em trofozoíto se transformam em esquizontes, que ao romperem as hemácias liberam merozoítos na corrente sanguínea, que irão invadir novas hemácias podendo dar continuidade ao ciclo ou se diferenciar em gametócitos masculinos e femininos. Estes gametócitos, ao serem ingeridos pelo *Anopheles,* dão continuidade ao ciclo no vetor **(Figura 4)** (COLEMAN *et al.*, 2002; CROMPTON *et al.*, 2014; LANGHORNE *et al.*, 2008).

Em infecções por *P. vivax*, alguns esporozoítos podem originar formas intra-hepáticas conhecidas como hipnozoítos, onde o parasita fica em estado de latência, podendo assim causar episódios de recaídas por um longo tempo (GETHING *et al.*, 2012; KROTOSKI, 1985).

A fase seguinte do ciclo, chamada esporogonia, ocorre no mosquito, quando o *Anopheles darlingi* ingere os gametócitos durante o repasto sanguíneo. Após alguns minutos ocorre a exflagelação de gametócitos masculinos, o que induz a formação de 6 a 8 gametas masculinos ou microgametas, enquanto os gametócitos femininos transformam-se em macrogametas. Uma fusão entre os microgametas e macrogametas gera um estágio móvel chamado oocineto. O oocineto ao penetrar o estômago do mosquito, se transforma em oocisto (onde ocorre a formação dos

esporozoítos). Após a ruptura do oocisto, inúmeros esporozoítos são liberados e estes migram para as glândulas salivares dos mosquitos e estão prontos para infectar novos hospedeiros vertebrados **(Figura 4)** (MATUSCHEWSKI, 2006).

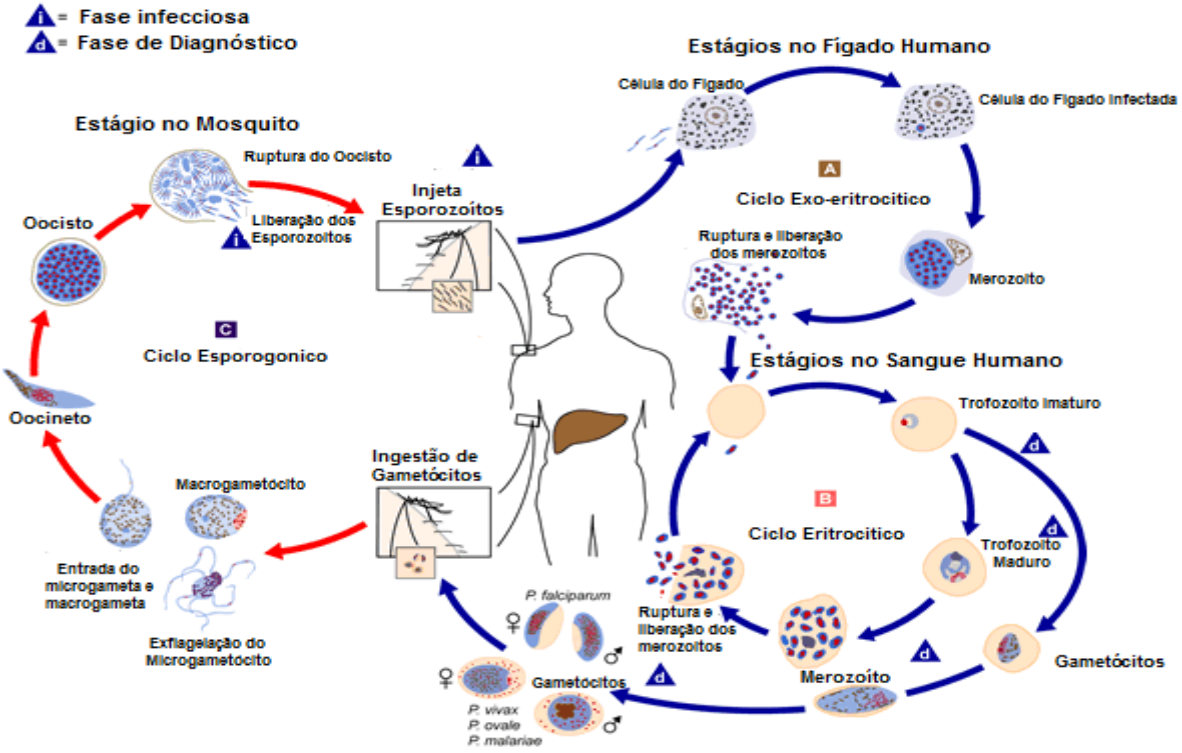

Figura 4. Ciclo de vida do *Plasmodium sp.* Fonte: CDC
Depois que o mosquito fêmea realiza seu repasto sanguíneo os esporozoítos são conduzidos pela corrente sanguínea até o fígado, onde infectam os hepatócitos e se desenvolvem em esquizontes, liberando aproximadamente 30.000 merozoítos na corrente sanguínea. Durante esse processo os esporozoítos de *P.vivax* podem se diferenciar em hipnozoítas, fase dormente do parasita presente em hepatócitos. Os merozoítos invadem hemácias (RBC) e então ocorre a diferenciação em anel, trofozoíto e esquizonte. Após essa etapa o ciclo se perpetua seguindo essa ordem de estágios, em determinados momentos os trofozoítos podem se diferenciar em gametócitos masculinos e femininos. Esses por sua vez são ingeridos pelos mosquitos iniciando a fase sexuada do parasita até a liberação do esporozoíto através das glândulas salivares.

2.4 Diagnóstico e Tratamento da Malária

O diagnóstico diferencial para malária é realizado através do método da gota-espessa, considerado o padrão ouro na área e o único aplicado em regiões endêmicas de difícil acesso. Esse método de diagnóstico requer pessoal treinado e capacitado, além de uma estrutura que seja capaz de disponibilizar a eficácia no diagnóstico. No entanto, não é um teste de alto custo, sendo este, um dos motivos de sua ampla utilização (ALEXANDER *et al.*, 2010; BOWERS *et al.*, 2009; COLEMAN *et al.*, 2002).

Posterior ao diagnóstico realizado por meio do exame de gota espessa, inicia-se o tratamento já preconizado pelo Ministério da Saúde, que consiste em esquemas terapêuticos específicos para cada espécie de *Plasmodium sp.*, utilizando fármacos

como a primaquina (atua principalmente no combate aos hipnozoítos), cloroquina (destrói o parasita na fase eritrocítica), mefloquina (agem aumentando a acidez do parasita, facilitando assim sua eliminação), artesunato (utilizado em pacientes com malária grave por *P. falciparum*) (BRASIL, 2010; KREMSNER; KRISHNA, 2004; ROCHA, 2008).

Outros medicamentos estão sendo desenvolvidos, devido à resistência do parasita aos medicamentos já amplamente comercializados. Destes, a tafenoquina é um grande achado no tratamento de infecções por *P. vivax*. Assim como a primaquina, a tafenoquina é um medicamento ativo contra estágios assexuais do parasita, principalmente contra hipnozoítas responsáveis pela recaída, em infecções por *P. vivax* e *P. ovale*. Essa droga vem sendo desenvolvida para prevenção de recidiva sendo administrada em dose única para infecções por *P. vivax* (GSK, 2017; MILLER et al., 2013a, 2013b). No entanto, esta droga possui um potencial de toxicidade hematológica (anemia hemolítica aguda) em pessoas que possuem deficiência de glicose-6-fosfato desidrogenase (G6PD), enzimopatia relacionada ao cromossomo X, sendo muito encontrada em pessoas que vivem em áreas endêmicas (LLANOS-CUENTAS *et al.*, 2014).

Para a eficácia no tratamento, a utilização correta dos medicamentos é de fundamental importância.

2.5 PAPEL DA RESPOSTA IMUNE INATA NA MALÁRIA

A imunidade inata funciona como a primeira linha de defesa do hospedeiro contra microorganismos patogênicos, pois tem a capacidade de reconhecer padrões moleculares associados a patógenos (PAMPs) e responder rapidamente através do reconhecimento por receptores de reconhecimento padrão (PRRs) (GAZZINELLI *et al.*, 2014; JANEWAY; MEDZHITOV, 2002). Além disso, a imunidade inata orquestra o desenvolvimento de uma resposta imune adaptativa necessária para a proteção do hospedeiro contra uma possível recaída (KAWAI; AKIRA, 2011; ROPERT; FRANKLIN; GAZZINELLI, 2008; TAKEUCHI; AKIRA, 2010).

Durante a fase inicial da infecção por Plasmódio, a ativação de células inatas e inflamação sistêmica levam aos primeiros sinais da infecção. Se não tratada, a malária sintomática pode evoluir para formas mais graves da doença, principalmente em

infecções causadas por *Plamodium falciparum* (GAZZINELLI *et al.*, 2014; GAZZINELLI; DENKERS, 2006).

Os principais eventos envolvidos na fisiopatologia da malária, são: produção e liberação de citocinas pró-inflamatórias; ruptura e remoção de hemácias parasitadas e conhecidamente alteradas por macrófagos esplênicos; e adesão dos eritrócitos infectados aos capilares e vênulas. Todos os três eventos são relacionados a inflamação exacerbada, anemia, acidose metabólica e malária cerebral (CLARK *et al.*, 2006; MILLER, L. H. *et al.*, 2013; SCHOFIELD; GRAU, 2005).

Durante a infecção, os PRRs ativados desempenham um papel indispensável no reconhecimento de PAMPS do *Plasmodium sp*., os quais fazem parte receptores Toll-like (TLRs), receptores semelhantes a NOD (NLRs), receptores RIG-like (RLRs) e lectina do tipo C. Esses receptores estão presentes em células da imunidade inata, tais como, células dendríticas e monócitos/macrófagos (CAMPOS *et al.*, 2001; CHIMMA *et al.*, 2009; CHUA *et al.*, 2013; TAKEDA, 2004). Os TLRs detectam PAMPs na membrana dos endossomas ou na superfície celular, enquanto que os RLRs e NLRs detectam no citosol (BARBALAT *et al.*, 2011; O'NEILL; GOLENBOCK; BOWIE, 2013).

Os PRRs têm a capacidade de ativar uma resposta imunológica a partir do reconhecimento do parasita. É conhecido que âncoras de glicosilfosfatidilinositol (GPI), hemozoína, DNA e RNA derivados do plasmódio fazem parte deste grupo de moléculas reconhecidas, e participam na produção de derivados da resposta imunológica, como citocinas TNF-a e IL-1b (COBAN *et al.*, 2007; DE LEORATTI *et al.*, 2012; GAZZINELLI; DENKERS, 2006; KELLER *et al.*, 2006).

Âncoras de GPI: São encontradas abundantemente em protozoários, produzindo atividade imunoestimulante, sendo de grande importância para a viabilidade parasitária. A estrutura básica das GPIs compreende uma etanolamina substituída com fosfato, uma porção conservada de oligossacarídeo ligada a um grupo fosfatidilinositol. Ele induz respostas celulares, através de receptores TLR1 e TLR2 que reconhece âncoras de GPI contendo três cadeias de ácidos graxos (Heterodímero); em menor grau TLR4 (reconhece Homodímero); TLR2 e TLR6 ativados por âncoras de GPI com duas cadeias de ácidos graxos, via proteína

adaptadora MyD88 (FERGUSON *et al.*, 1999; GAZZINELLI *et al.*, 2014; ROPERT; GAZZINELLI, 2000).

Hemozoína: Também chamada pigmento malárico, é formada a partir da digestão da hemoglobina pelo *Plasmodium sp.* durante seu desenvolvimento intraeritrocítico. Ao digerir a hemoglobina, é liberado o grupo heme tóxico e o parasita forma um dímero cristalino de hematina α, complexada a lipídios e proteínas para detoxificar o produto de seu metabolismo (STEPHENS *et al.*, 2000). A hemozoína é liberada quando ocorre ruptura dos eritrócitos parasitados, podendo atingir altas concentrações na circulação, sendo fagocitada por monócitos e neutrófilos circulantes, bem como por macrófagos presentes nos tecidos (ARESE; SCHWARZER, 1997; DOSTERT *et al.*, 2009; NEWTON; TAYLOR; WHITTEN, 1998; PARROCHE *et al.*, 2007).

Estudos *in vitro* têm demonstrado que na malária, tanto por *P. vivax* como por *P. falciparum,* a GPI e hemozoína via TLR4 podem estimular monócitos e macrófagos a sintetizarem citocinas envolvidas na resposta inata (COBAN *et al.*, 2007; DE LEORATTI *et al.*, 2012; GAZZINELLI; DENKERS, 2006; KELLER *et al.*, 2006). Alguns estudos verificaram também que a hemozoína pode ser reconhecida pelo TLR9, provavelmente complexada ao DNA (COBAN *et al.*, 2005; GRIFFITH *et al.*, 2009; KALANTARI *et al.*, 2014; PARROCHE *et al.*, 2007).

No entanto, a capacidade de TLR reconhecer a hemozoína ainda é controversa. Isso aponta para a possibilidade de que outros receptores podem estar envolvidos neste reconhecimento e consequente resposta imunológica. Neste sentido, estudos observaram que o inflamassoma NLRP3 (que possui o domínio pirínico), composto pelo NLR, adaptador ASC e caspase-1, envolvido na inflamação desencadeada pela hemozoína, é o de maior interesse. Pois o mesmo, atua na produção da citocina IL-1β, relacionada a processos inflamatórios observados durante a infecção por plasmódio (DOSTERT *et al.*, 2009; STRANGWARD *et al.*, 2018; TIEMI SHIO *et al.*, 2009).

DNA do Plasmodium: É um importante ativador da resposta imune inata, não somente na malária, mas também em outras infecções parasitárias (PARROCHE *et al.*, 2007; SHARMA *et al.*, 2011). O genoma do *P. vivax* é caracterizado por aproximadamente 5.500 partes estimuladoras ricas em AT e 2000 partes em CpG (citosina e guanina separadas por um fosfato). Quando chegam aos fagolisossomos

as partes imunoestimuladoras do DNA do plasmódio ativam TLR9, isso acontece muito provavelmente pela degradação do parasita assim que os eritrócitos infectados são fagocitados (GAZZINELLI *et al.*, 2014; PARROCHE *et al.*, 2007; WU, X. *et al.*, 2010).

RNA do Plasmodium: Outro ativador da resposta imune inata é o RNA proveniente do *plasmodium sp*. Estudos *in vivo* demonstraram que camundongos infectados por *P. yoelli* apresentaram maior produção de IFN do tipo I durante o estágio eritrocitário, que tem por base ser dependente da RNA polimerase III. Além disso, o receptor de RNA (TLR7) tem um papel na ativação da resposta imune inata em rato com malária. Nesses estudos o IFN tipo I teve um papel importante no controle da parasitemia (BACCARELLA *et al.*, 2013; LIEHL *et al.*, 2014; MILLER, J. L. *et al.*, 2014; WU, J. *et al.*, 2014).

Os componentes do parasita contribuem direta ou indiretamente para ativar os fatores imunes do hospedeiro **(Figura 5)** (CHUA *et al.*, 2013). Após a invasão dos eritrócitos pelos merozoítos, grandes transformações são induzidas incluindo a expressão de proteínas do parasita na membrana do eritrócito que podem interagir com os PRRs presentes na superfície dos monócitos. Eritrócitos infectados podem levar a ativação do complemento, resultando em deposição do fragmento C3b contribuindo para sua eliminação. Ainda, após a ruptura dos eritrócitos o número de parasitas pode ser reduzido pela ação da fagocitose com opsonização ou sem opsonização (COUPER *et al.*, 2010; SILVER *et al.*, 2010). A ruptura provoca também a liberação de merozoítos que são alvos de anticorpos e ativam monócitos/macrófagos via receptores FcyR e CD36 (CHUA *et al.,* 2013; DASARI *et al.,* 2012).

Os monócitos são fontes inesgotáveis de citocinas durante a infecção por *P. vivax* (DE LEORATTI *et al.*, 2012). É bem descrito, que a diferenciação dos monócitos é extremamente afetada durante a infecção por plasmódio. Os monócitos são divididos em duas subpopulações, os monócitos clássicos $CD14^{+}CD16^{-}$ e os não clássicos (intermediários ou inflamatórios) $CD14^{+}CD16^{+}$ (ANTONELLI *et al.*, 2014).

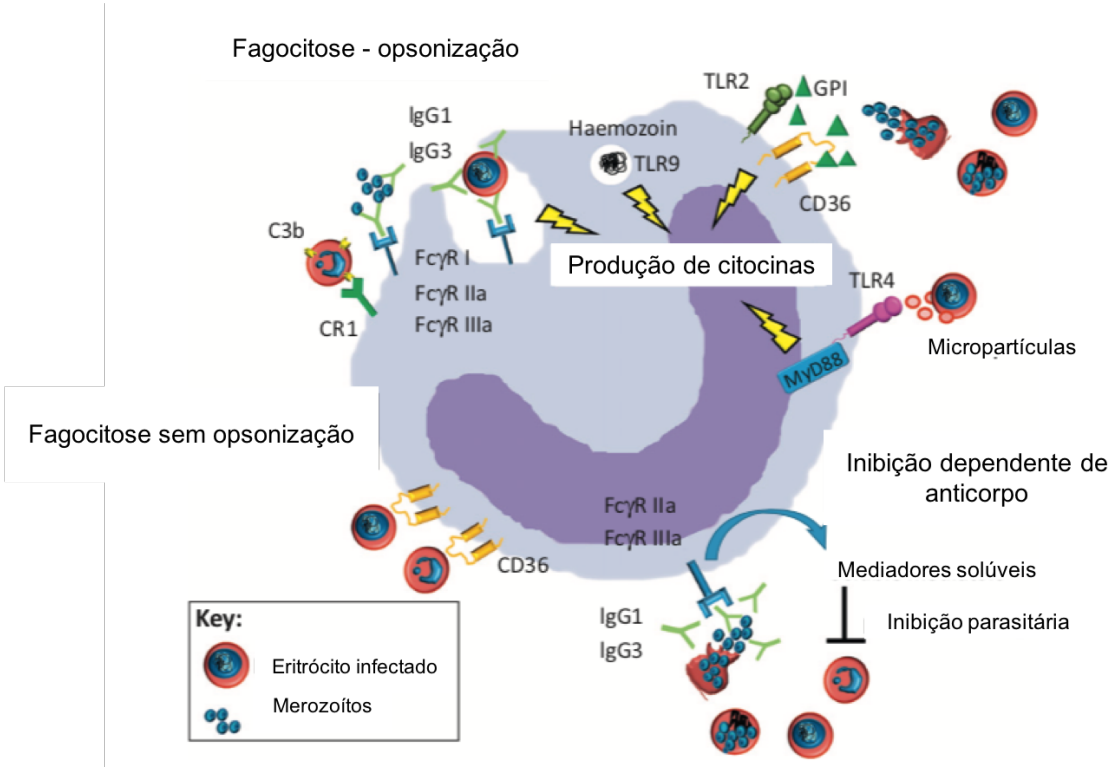

Figura 5: Ativação dos Monócitos/Macrófagos por diferentes PAMPs ligados ao parasita Fonte: Adaptado de (CHUA *et al.*, 2013).
Alguns dos mecanismos utilizados por Monócitos/Macrofágos para o reconhecimento e posterior proteção contra a malária. Essas células podem reduzir o número de parasitas através da fagocitose de eritrócitos inteiros infectados, podendo ser através de opsonização ou não. Os eritrócitos infectados opsonizados pelo sistema complemento podem ser fagocitados através do CR1. A fagocitose opsonica através de receptores Fcγ (FcγR) induz a produção de citocinas, em contraste com a fagocitose não opsonica mediada por receptores CD36. Alternativamente, outros componentes associados ao parasita, incluindo o glicofosfatidilinositol (GPI), micropartículas e hemozoína, também podem ativar as células via CD36 e receptores Toll-like (TLRs) para produzir citocinas, que então alimentam respostas inflamatórias que auxiliam na eliminação do parasita. Além disso, monócitos podem interagir com merozoítos opsonizados por anticorpos via FcγRs para promover a produção de mediadores solúveis que podem inibir diretamente o crescimento do parasita em um processo conhecido como inibição celular dependente de anticorpo.

Dentre as citocinas produzidas por células da imunidade inata temos TNF-α, IL-1, IL-6, IL-12, e IFN-, as quais tem funções importantes no controle do crescimento parasitário. Por outro lado, se a ativação desses mediadores inflamatórios for excessiva, pode ser prejudicial e causar condições patológicas para o hospedeiro, como por exemplo, disfunção cerebral (BRANT *et al.*, 2014; CHAVES *et al.*, 2016; GUIMARÃES DA COSTA *et al.*, 2014; STEVENSON; RILEY, 2004).

Estudos realizados em Manaus apresentaram níveis elevados de citocinas como IL-6, IL-10 e IFN-γ em pacientes com *P. vivax* comparados ao grupo de indivíduos saudáveis (GUIMARÃES DA COSTA *et al.*, 2014; OLIVEIRA *et al.*, 2020). Citocinas reguladoras, tais como IL-10 e TGF–β, são necessárias para reduzir o risco de doença grave, contanto que ocorra um equilíbrio com as citocinas inflamatórias, isto contribui para uma resposta eficiente à malária (GONÇALVES *et al.*, 2012).

Em malária, algumas citocinas como IL-12 e IL-18 podem atuar na redução da carga parasitária e na ativação de IFN-γ, normalmente isso ocorre na fase inicial da infecção, onde o IFN-γ pode ainda atuar na progressão da patologia (ANDRADE *et al.*, 2010; DEROOST *et al.*, 2016; TORRE, 2009). O IFN-γ e TNF-α agem amplamente durante nas respostas imunes aos estágios sanguíneos do parasita, atuando em sinergia para otimizar a produção de óxido nítrico, que pode contribuir para a morte do parasita (BUENO *et al.*, 2010a). Estudos mais recentes mostraram que, indivíduos com primeira malária apresentaram resposta diferenciada em relação a indivíduos com infecção prévia, havendo um aumento de proteínas pró-inflamatórias, mesmo que a parasitemia fosse reduzida (OLIVEIRA *et al.*, 2020; TRAN *et al.*, 2016).

No contexto geral, a imunidade inata é extremamente importante para o controle da infecção. No entanto, a mesma não consegue eliminar com eficiência o parasita e acaba contribuindo para uma inflamação sistêmica, evento já bem conhecido durante a malária. Para diminuir os efeitos da inflamação e gerar uma resposta eficaz, o recrutamento de células T (específicas para o antígeno) é necessário para impedir a progressão da infecção (VELAVAN; OJURONGBE, 2011). Definir melhor como componentes do parasita ativam a resposta imune inata e levam a uma ativação da resposta adaptativa é crucial para entender a fisiopatologia da malária.

É notório que as células TCD4^{+} podem se diferenciar em células Tregs CD4^{+}CD25^{high}FoxP3^{+}CD127^{low}, que tem sido associadas a um importante equilíbrio entre o controle da infecção e a prevenção da imunopatologia durante a malária (BUENO *et al.*, 2010b). No entanto, um número elevado dessas células pode contribuir para o aumento da carga parasitária e desenvolvimento da infecção, devido à supressão da resposta imune (TORRES *et al.*, 2014; WALTHER *et al.*, 2005).

2.6 CÉLULAS T REGULADORAS, IDO E AHR NA MALÁRIA

Alguns estudos têm focado no papel das células T reguladoras (Tregs) CD4^{+} durante a regulação das respostas imunes adaptativas em infecções por *Plasmodium sp*. As células Tregs CD4^{+} são divididas em duas populações distintas: que ocorrem naturalmente (nTreg) constituindo aproximadamente 10% das células T, sendo

derivadas do timo, e as células adaptativas ou induzidas (iTreg) que são provenientes da periferia, em resposta a estímulos antigênicos específicos (MILLS, 2004; SAKAGUCHI, Shimon, 2005).

As células Tregs presentes em indivíduos normais, expressam altos níveis de CD25 (atua como receptor de IL-2), baixa expressão de CD127. Possuem também o CTLA-4 (linfócito T citotóxico associado à proteína 4) e o FoxP3 (fator de transcrição caixa forkhead 3), que desempenham um papel importante no controle das respostas imunológicas aos antígenos (HANSEN, D. S.; SCHOFIELD, 2010; SAKAGUCHI, S.; POWRIE, 2007; SCHMETTERER; NEUNKIRCHNER; PICKL, 2012; WING *et al.*, 2017). São ativadas e iniciam suas atividades a partir da exposição às citocinas, sendo os macrófagos os principais responsáveis pela indução da diferenciação dessas células (HANSEN, D. S.; SCHOFIELD, 2010; SEHRAWAT; ROUSE, 2011).

As células Tregs $CD4^{+}Foxp3^{+}$ naturais têm participação importante durante o resultado da infecção por vários patógenos intracelulares, inclusive parasitas. Já foi demonstrado que em infecções por *Plasmodium sp.*, as células Tregs $CD4^{+}Foxp3^{+}$ atuam inibindo as respostas de células T efetoras, linfócitos B, natural killer (NK), monócitos, DCs e neutrófilos durante a infecção. Isto resulta em um controle imunológico inadequado do patógeno e persistência da infecção em baixo nível. Também é de conhecimento, que células Tregs naturais, em sua capacidade de modular células T efetoras, tem implicação importante no aumento no dano tecidual durante uma infecção parasitária (BELKAID; TARBELL, 2009; LEWKOWICZ *et al.*, 2006; MISRA *et al.*, 2004; OLDENHOVE *et al.*, 2009; STEVENSON *et al.*, 2011; TAAMS *et al.*, 2005; TRZONKOWSKI *et al.*, 2004).

As células Tregs tem sido descritas em vários contextos como um importante fator no balanço entre a resposta imune inata e adaptativa. Na malária, essas células estão mais expressas, e uma exacerbação da mesma, pode desempenhar um controle regulador excessivo, o que permite a replicação do parasita e compromete o hospedeiro (ADALID-PERALTA *et al.*, 2011; SAKAGUCHI, S, 2000). Essas células limitam a amplitude da resposta imunológica celular, devido à produção de fatores solúveis, tais como, TGF-β, IL-10 e IL-35, podendo resultar na incapacidade de controlar adequadamente a infecção (BELKAID; TARBELL, 2009; BUENO *et al.*, 2010a; SEHRAWAT; ROUSE, 2011).

Segundo (HISAEDA et al., 2004) a evasão imune do parasita a resposta imunológica pode estar relacionado a ativação de células Tregs. Seu grupo mostrou que camundongos infectados com *Plasmodium sp.* apresentaram um aumento significativo de células Tregs com atividade supressora. De acordo com o estudo, ao realizar a depleção de células Tregs em camundongos infectados por *P. yoelii* os mesmos conseguiram sobreviver a progressão da infecção, o que levou a conclusão de que as células Tregs estão relacionadas a uma supressão imune e um descontrole da infecção na malária.

Em estudo *in vitro* realizado com *P. falciparum* foi observado uma indução de FoxP3 necessário para a produção de TGF-β e IL-10. Neste estudo, as células T apresentaram os fenótipos CTLA-4^{+}, CD127 em níveis baixos e CD39^{+} (SCHOLZEN et al., 2009). Estudos mostraram que em infecções por *P. falciparum* e *P. vivax* houve aumento sistêmico de IL-10 e TGF-β que se correlacionaram com a maior expressão das células Tregs CD4^{+}CD25^{+}FoxP3^{+} (BUENO *et al.*, 2010a; JANGPATARAPONGSA *et al.*, 2008; WALTHER *et al.*, 2005).

Vários estudos têm demonstrado que células Tregs CD4^{+}CD25^{+}FoxP3^{+} estão aumentadas na malária e possuem uma atividade altamente supressora. Em infecções humanas com *P. vivax* e *P. falciparum* houve uma significativa expansão de células Tregs que expressam o CTLA-4 e PD-1 (GONÇALVES et al., 2010; 25, 45 LIZ Fto). Embora a indução de PD-1 em Treg não tenha sido bem descrita, é importante ressaltar que seu papel na função e desenvolvimento dessas células é bem conhecido (COSTA *et al.*, 2018; GONÇALVES-LOPES *et al.*, 2016b; KURUP *et al.*, 2017). Em tese, camundongos deficientes de CTLA-4 apresentaram alta estimulação de TGF-β não sendo capaz de converter as células T CD4^{+}CD25^{+} em Tregs (ADALID-PERALTA *et al.*, 2011; VELAVAN; OJURONGBE, 2011).

As células Tregs CD4^{+}CD25^{+}FoxP3^{+} podem ser induzidas independente da apresentação a MHC de classe II e sim podem ser resultado de interações indiretas que estejam ocorrendo ao longo da infecção por plasmódio. Levando a ponto onde fatores solúveis como, IL-2, IL-10 e TGF-β contribuam para essa indução (SCHOLZEN *et al.*, 2009). Durante a estimulação das células T específicas para controlar a malária, o TGF-β pode regular a expressão de FoxP3 (fator essencial para a indução,

diferenciação, sobrevivência e função de Tregs) levando ao desenvolvimento de Tregs (ADALID-PERALTA *et al.*, 2011; VELAVAN; OJURONGBE, 2011)

Já está claro que as células Tregs estão em maior expressão durante infecções por *P. vivax*, no entanto como essas células podem controlar as funções deletérias durante a malária e o que levou a alta expressão da mesma, ainda não está esclarecido (BUENO *et al.*, 2010a; GONÇALVES-LOPES *et al.*, 2016a; KHO *et al.*, 2015). Um trabalho publicado por (WOODBERRY *et al.*, 2017), mostrou que na infecção por *P. vivax* o aumento de células Tregs está relacionado a atividade da enzima IDO e consequente aumento de KYN. Adicionalmente, nosso estudo mostrou um aumento de Tregs associado ao metabolismo do TRP (DOS SANTOS *et al.*, 2020).

Conhecer melhor o funcionamento da resposta de células Tregs na malária é de fundamental importância para o entendimento da imunopatologia causada pela infecção.

A indoleamina 2,3-dioxigenase (IDO) é uma enzima encontrada no citosol quando ativada, possui um grupo proteico heme (Fe^{2+}) e esta envolvida no catabolismo do TRP (um aminoácido essencial, ou seja, os indivíduos adquirem através da dieta alimentar) em um metabólito imunossupressor chamado KYN, sendo essa atividade de conversão descrita como a principal função da enzima (MUNN, David H. *et al.*, 2004).

Em células de mamíferos, o aminoácido L-triptofano (TRP) é principalmente catabolizado através da chamada via da quinurenina (KYN). O primeiro passo limitante dessa via pode ser catabolizado por 3 enzimas a IDO1, IDO2 e triptofano 2,3-dioxigenase (TDO). A IDO1 apresenta uma maior afinidade com o L-triptofano sendo considerada a mais bem caracterizada dessas enzimas, a IDO2 é um parálogo evolutivo, tendo menor afinidade e uma baixa expressão. Já a TDO é um órtologo funcional que está presente nos tecidos, principalmente fígado, rins e cérebro. IDO2 e TDO possuem alta especificidade tecidual e nível de expressão muito menor que a IDO1, o que restringe a atividade das mesmas (BALL *et al.*, 2007; METZ *et al.*, 2007; PFEFFERKORN, 1984; URADE *et al.*, 1983; YOSHIDA; HAYAISHI, 1978; YUASA *et al.*, 2007).

A IDO não é expressa constitutivamente. A ativação de diversas vias de sinalização imunológicas, induzem fatores de transcrição que se ligam *upstream* a sequencias específicas de nucleotídeos, como os *interferon sequence response-like elements* (ISRE), *palindromic gamma-activated sequences* (GAS), além da via não canônica do NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells), presentes na região promotora da IDO1 que controlam a tradução da versão metabolicamente ativa da enzima (MBONGUE *et al.*, 2015).

Sendo a única enzima capaz de converter TRP em KYN, a mesma contribui para a regulação imune metabólica. Originalmente, a via de degradação do TRP foi descrita como um mecanismo de defesa da imunidade inata do hospedeiro contra a infecção, no entanto a via mostrou-se ativa em vários contextos imunológicos (MUNN, David H.; MELLOR, 2013).

O TRP é um metabólito essencial proveniente da dieta, metabolizado em duas vias comuns, a via da serotonina e da KYN. A via da quinurenina (Via-KYN) é responsável pelo metabolismo de aproximadamente 95% do TRP livre e compreende a conversão do TRP. A IDO1 converte o TRP em KYN clivando a ligação dupla 2,3 do anel indol enquanto um oxigênio molecular (0_2) se funde na molécula não selada. Sendo assim, se converte em N-formilquinurenina que se transforma rápida e espontaneamente em L-Kyn. Essa via tem como produto final o NAD^+, uma coenzima que tem um papel importante na produção de energia para a célula **(Figura 6)** (FALLARINO; GROHMANN; PUCCETTI, 2012; ROUTY *et al.*, 2016; SAITO; HEYES, 1996).

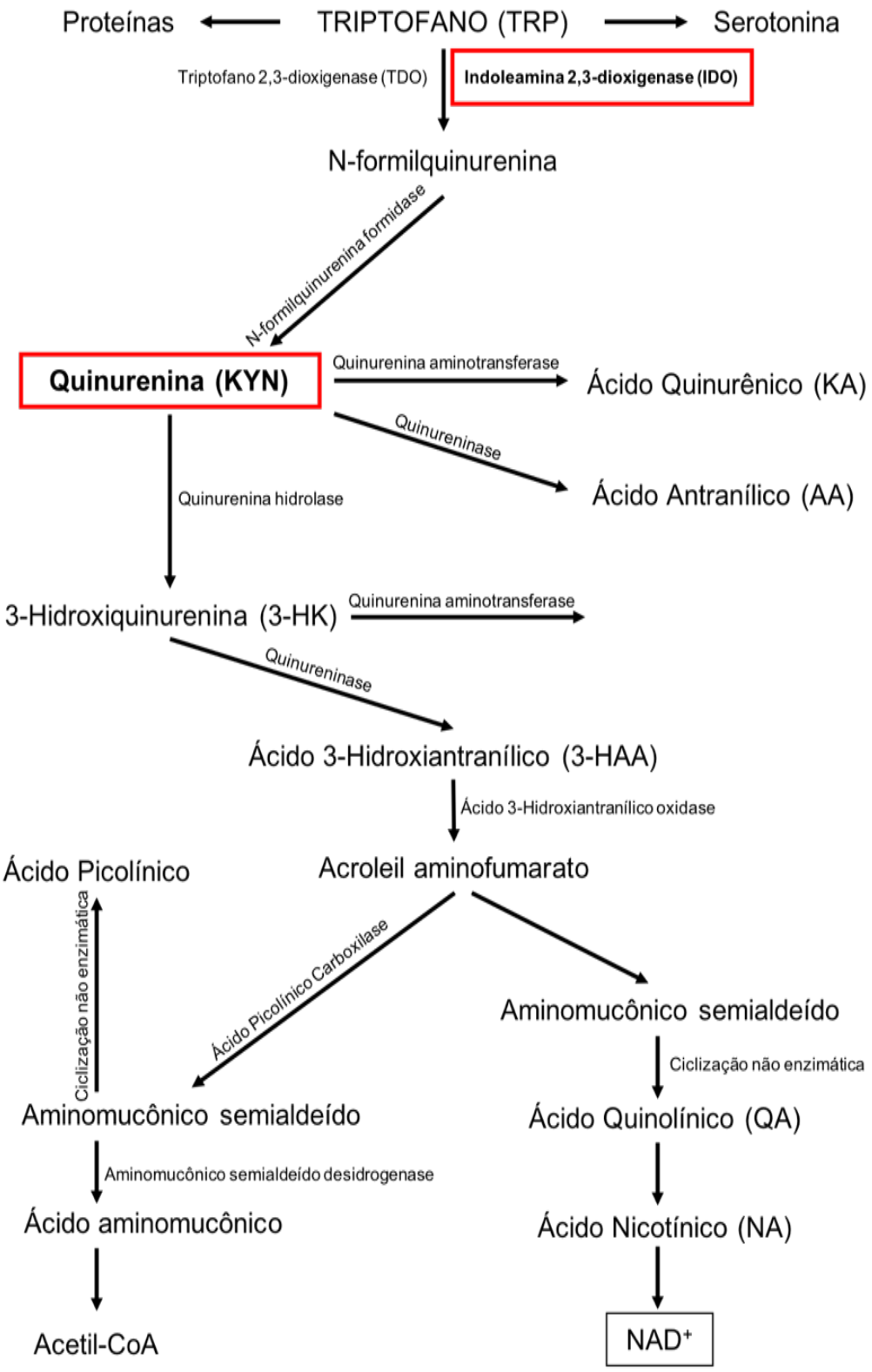

Figura 6: Vias de degradação do Triptofano (adaptado de (ROUTY *et al.*, 2016)**)**
Via da KYN esquematizada. É estimado que maior parte do TRP que não desencadeia para síntese de proteína, é utilizado pela Via da Quinurenina, aproximadamente 95%, a outra parte é convertida em serotonina. As enzimas que desencadeiam a conversão são TDO e IDO. Outros metabólitos do Triptofano são mostrados, até o produto final que é o NAD^+.

A IDO1 no princípio foi descrita como uma molécula de defesa natural do hospedeiro contra infecções patogênicas, porém com o decorrer dos estudos essa enzima foi identificada como um imunorregulador essencial (ADAMS *et al.*, 2004; FRUMENTO *et al.*, 2002; PANTOJA *et al.*, 2000; PFEFFERKORN, 1984; TERNESS *et al.*, 2002). Essa enzima com funções reguladoras inibe respostas de células T e promove tolerância imunológica (MBONGUE *et al.*, 2015). Durante as infecções por Malária (MIU *et al.*, 2009) e Leishmaiose (MAKALA *et al.*, 2011)**,** a atividade da enzima na resposta adaptativa pode facilitar a persistência do parasita e a gravidade da doença (VALLEJO *et al.*, 2018).

Subconjuntos específicos de células como, células dendríticas (DCs), macrófagos e monócitos imaturos expressam níveis elevados de IDO1 em resposta a estímulos inflamatórios como IFN-γ ou indução através da ligação das moléculas co-estimuladoras CD80 e CD86, também conhecidas como B7-1 e B7-2, respectivamente. Suspeita-se que o IFN-γ proveniente da ativação das células T possa ser o sinal para a indução da IDO. Na malária por *P. vivax*, o IFN-γ se apresenta em níveis elevados, sendo que pacientes primo-infectados despontam níveis ainda mais elevados do que pacientes com mais de uma infecção. Esses dados foram correlacionados ao aumento da KYN (CHIMMA *et al.*, 2009; HWU *et al.*, 2000; MOON *et al.*, 2015; OLIVEIRA *et al.*, 2020; PETROFF *et al.*, 2003; POTULA *et al.*, 2005).

A ativação da enzima leva a uma diminuição na concentração de TRP e consequente supressão da ativação, proliferação e diferenciação de células T efetoras, isso já é bem descrito em câncer e HIV. Em infecção parasitária por *Leishmania major*, houve supressão de células T devido à expressão induzida da enzima IDO, em camundongos (MAKALA *et al.*, 2011). Já na infecção por malária, com camundongos infectados por *P. yoelii* e *P. berguei* foi verificado um aumento na produção da enzima IDO que pode ser explicado pelos altos níveis de IFN-γ produzidos por células T $CD4^+$. Foi mostrado ainda, que a inibição da IDO por 1-metil-Triptofano (1-MT) *in vivo* ou camundongos *knockout* para IDO, protegeu parcialmente os camundongos contra infecção letal e que um hiper-catabolismo do TRP pode suprimir a imunidade e também gerar respostas imunes prejudiciais (HANSEN, A. M. *et al.*, 2004; HANSEN, D. S.; SCHOFIELD, 2010; TETSUTANI *et al.*, 2007).

Como uma possibilidade, o acúmulo de KYN pode atuar em sinergia com limitações locais na disponibilidade de TRP para inibir potencialmente a proliferação e ativação de células imunológicas efetoras, isso já vem sendo demonstrado *in vitro* (CHEN *et al.*, 2008; FALLARINO; GROHMANN; PUCCETTI, 2012). Mecanismos indiretos também podem explicar a atividade da IDO-1. As DCs que expressam IDO1, possuem efeitos imunossupressores amplos, uma vez que, elas suprimem diretamente a proliferação e as funções de linfócitos T citotóxicos, células NK e plasmócitos; além de promoverem a conversão de células T $CD4^+$ inativas em Tregs $CD4^+CD25^+FoxP3^+$ ativadas (BABAN *et al.*, 2005; HWU *et al.*, 2000; MELLOR *et al.*, 2003; MOLANO *et al.*, 2008).

Como ocorre essa ativação de células Tregs, ainda não é bem descrita na malária. Mas sabemos que em outras infecções a expressão elevada de CTLA-4 das células Tregs resulta em um aumento adicional da secreção de IDO1 por células dendriticas. Podendo ainda contribuir juntamente com outros marcadores, como PD-1 e PD-L1 para inibição das funções de células T efetoras (CAMPESATO *et al.*, 2020).

A enzima IDO modifica as respostas imunitárias da seguinte forma: através da produção da KYN, que se liga ao receptor de Aril hidrocarboneto (AhR) e pela diminuição do TRP que aciona as vias de transdução dos sinais de aminoácidos podendo atuar como um sinal regulatório potente por meio de resposta moleculares que levam ao estresse, como a cinase GCN2 e o alvo da rapamicina em mamíferos (mTOR), que respondem à retirada de aminoácidos. Com isso ocorre hiper-ativação de células Tregs, supressão, anergia e morte de células T efetoras **(Figura 7)** (MUNN, D H *et al.*, 1999; MUNN, David H.; MELLOR, 2013; PALLOTTA *et al.*, 2011).

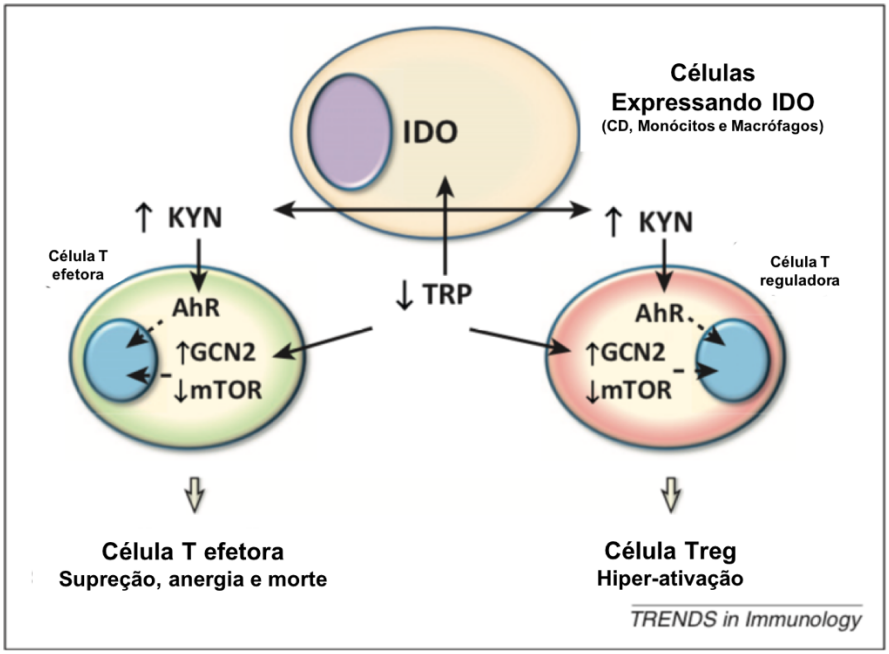

Figura 7. O controle metabólico de células Tregs e T efetoras via IDO Fonte: (MUNN; MELLOR, 2013)

A liberação de KYN e a redução de TRP por células acessórias que expressam IDO, geram sinais através do receptor AhR e sensores de aminoácidos (GCN2, mTOR), respectivamente. Os quais possuem efeitos importantes nas respostas de células T efetoras e T reguladoras a sinais antigênicos e inflamatórios.

O AhR, atua como um fator de transcrição ativado pelo ligante que medeia a toxicidade da dioxina, que há poucos anos, emergiu como um importante fator na regulação das respostas imunes (STEVENS; MEZRICH; BRADFIELD, 2009). Esse receptor pertence à superfamília de proteínas PER-ARNT-SIM (BURBACH; POLAND; BRADFIELD, 1992; EMA *et al.*, 1992). Em seu estado inativo o Ahr reside no citosol como parte de um complexo que inclui outras proteínas, como: a proteína do choque térmico chaperona Hsp90, as proteínas cochaperonas que interagem com o AhR, a fosfoproteína p23 e a proteína quinase C-SRC (JARONEN; QUINTANA, 2014; MA; WHITLOCK, 1997; PERDEW, 1988).

O AhR é altamente conservado, quando ativado por TCDD, ITE, KYN, Laquinimod, FICZ, IL-10 e TGF-β induz uma maior frequência de células Treg FoxP3^{+} através de diferentes mecanismos, incluindo transativação direta e indução de modificações epigenéticas que controlam a transcrição de FoxP3, e também através da modulação de DCs (GOETTEL *et al.*, 2016; KAYE *et al.*, 2016). Em infecções virais o AhR tem propensão para reprimir alguns genes considerados chave para a manutenção do estado antiviral, como CDK1, CDK2 e ciclinas (STEVENS; MEZRICH; BRADFIELD, 2009).

A princípio, uma conexão com o sistema imunológico havia sido previamente reconhecida, baseada principalmente no conhecimento de que a exposição à um ligante exógeno 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) levou à rápida involução do timo. Além disso, era sabido que os animais e seres humanos expostos à TCDD estavam imunossuprimidos. Os efeitos da TCDD em células dendríticas (DCs) e células T têm sido reconhecidos há anos (VORDERSTRASSE; KERKVLIET, 2001). O tratamento *in vitro* (ou *in vivo*) com TCDD leva ao aumento e geração de células T regs por um mecanismo dependente de AhR, que foi identificado pela primeira vez em 2005 (FUNATAKE *et al.*, 2005).

Em 1998, foi relatado que o catabolismo do TRP por IDO1 e outras enzimas eram responsáveis pela prevenção da rejeição fetal alogênica em camundongos, e foi ainda revelado que esta enzima foi gerada por CDs, e de alguma forma aumentou a diferenciação de Tregs (MUNN, D H *et al.*, 1998). O mecanismo pelo qual isso acontecia não estava claro. Porém, havia duas teorias: a privação de TRP poderia contribuir para a diminuição da geração e divisão de células T efetoras, ou certos metabólitos de sua degradação, como a KYN, atuariam através de algum alvo em células T ou outras células, favorecendo a diferenciação em células Tregs (BELLADONNA *et al.*, 2006; MUNN, D H *et al.*, 1998, 1999).

Em um trabalho publicado por (LABADIE; BAO; LUKE, 2018), eles consideraram a hipótese de que a KYN ou um de seus produtos de degradação estava funcionando através do AhR em células T para melhorar a diferenciação e ativação de Tregs. Isso se deve ao fato de que indóis e outros derivados do TRP são ligantes do AhR, tornando a KYN um bom candidato a ligante endógeno. Por fim, eles identificaram que a KYN se liga ao AhR no citosol das células T e aumentam a expressão de Tregs *in vitro*.

A KYN é um ligante do AhR enquanto o TGF-β tem um papel na potencialização do efeito dessa ligação, pois o mesmo pode atuar no aumento da expressão de receptores, silenciando assim a resposta imune (MEZRICH *et al.*, 2010).

Estudos mostram que em câncer o bloqueio do AhR atrasa a progressão de tumores com super expressão de IDO/TDO e sua eficácia é melhorada com o bloqueio de PD-1. Ainda em outro trabalho, autores mostraram que células Knockout para AhR estimuladas com LPS, apresentaram KYN reduzida e inibição da diferenciação de T naive em Treg e que isso foi acompanhado de uma maior frequência de Th17. Chegando à conclusão de que além da diferenciação de células T em Treg o AhR

também é necessário na indução da expressão de IDO (CAMPESATO *et al.*, 2020; NGUYEN *et al.*, 2010). AhR e IDO/TDO estão intimamente interligados, pois o AhR regula a expressão dessas enzimas e a KYN produzida pelo catabolismo do TRP é um agonista do AhR (JARONEN; QUINTANA, 2014).

Em malária um estudo realizado com camundongos mostrou que a depleção de AhR deixa o animal suscetível a infecção levando a lesões renais agudas. Estabelecendo que o AhR tem um papel importante na limitação dos danos ao tecido (LISSNER *et al.*, 2020). Em nosso estudo, mostramos um aumento de AhR em células Tregs de pacientes infectados por *P. vivax* acompanhado de um aumento dessas células. Ao inibir o AhR em cultura de PBMC estimulado com lisado de Pv, houve uma redução de células Tregs e um diminuição de KYN (SANTOS *et al*., 2021; Dados não publicados).

No entanto, essa ligação da KYN ao AhR presente em células T ainda precisa ser completamente elucidado na malária. É bem estabelecido em nosso estudo, que a KYN está elevada na malária e que isso tem uma relação ao aumento de células Tregs.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o papel da atividade da enzima IDO na conversão do TRP em KYN e a influência desse metabólito na maior frequência de células Tregs $CD4^{+}CD25^{+}FoxP3^{+}$ na Malária Vivax.

3.2 Objetivos Específicos

- Quantificar e correlacionar níveis de citocinas, quinurenina e frequência de células Tregs em pacientes infectados por *P. vivax*;
- Investigar o perfil imunofenotípico dos pacientes com malária relacionados células T. Avaliando as diferenças entre pacientes primo-infectados e com mais de uma infecção;
- Determinar o papel da enzima Indoleamina 2,3 dioxigenase (IDO) na produção de quinurenina, frequência de células Tregs e ativação de células T *in vitro* por lisado de eritrócitos infectados por Pv. Avaliando o papel do AhR na plasticidade de células T em Tregs.

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Capítulo 2

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BRIEF DEFINITIVE REPORT

Parasite Immunology WILEY

Kynurenine elevation correlates with T regulatory cells increase in acute *Plasmodium vivax* infection: A pilot study

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Abstract

Background: Disease-tolerance mechanisms limit infection severity by preventing tissue damage; however, the underlying mechanisms in human malaria are still unclear. Tryptophan (TRP), an essential amino acid, is catabolized into tolerogenic metabolites, kynurenines (KYN), by indoleamine 2,3-dioxygenase 1 (IDO1), which can induce Foxp3+ T regulatory cells (Tregs). In this study, we evaluated the relationship of these metabolites with Treg-mediated tolerance induction in acute malaria infections.

Methods: We performed a cross-sectional study that evaluated asymptomatic, symptomatic malaria patients and endemic control patient groups. We assessed plasmatic concentration of cytokines by ELISA. Plasmatic TRP and KYN levels were measured by HPLC. Peripheral T regulatory cells were measured and phenotyped by flow cytometry.

Results: The KYN/TRP ratio was significantly elevated in asymptomatic and symptomatic *Plasmodium* infection, compared to healthy controls. Also, Th1 and Th2 cytokines were elevated in the acute phase of malaria disease. IFN-γ increase in acute phase was positively correlated with the KYN/TRP ratio and KYN elevation was positively correlated with the increase of peripheral FoxP3+ T regulatory cells.

Conclusions: Additional studies are needed not only to identify innate mechanisms that increase tryptophan catabolism but also the role of Tregs in controlling malaria-induced pathology and malaria tolerance by the host.

KEYWORDS

inflammation, kynurenine, *Plasmodium*, T regulatory cells and immunosuppression, tryptophan

1 | INTRODUCTION

Malaria remains a public health challenge in the developing world, with the World Health Organization (WHO) estimating 219 million cases of malaria worldwide in 2017.[1] In Brazil, over 85% of the clinical cases are caused by *Plasmodium vivax* and are localized in the Amazon region. *P vivax* infection is characterized by disease relapses after the first infection. A latent form of the parasite, called hypnozoite, can remain dormant and reactivate if not treated appropriately.[2,3] Additionally, the lack of appropriate in vitro culture and

animal models hampers the understanding of the parasite's biology and pathophysiology, which remain poorly understood. Compared to *P falciparum*, *P vivax* disease is relatively milder and has lower rates of complications; hence, the pathogenesis of severe or complicated *P vivax* malaria is rarely assessed. Recent studies have shown both cytoadherence of parasite-infected erythrocytes to endothelial cells, and sequestration of infected red blood cells in deep vasculature in *P vivax* infection.[4] Recent evidence also demonstrates that *P vivax* infection can cause severe infections that might occasionally be associated with mortality and severe disease,[5,6] but the mechanisms producing these effects are poorly understood.

Malaria infection inflicts host pathology by immune activation and increasing several proinflammatory mediators during the acute phase of the disease,[7-10] whereas the host tries to circumvent these deleterious effects by increasing tolerance mechanisms to avoid tissue damage.[10,11] The increase of tolerogenic FoxP3+ T regulatory cell (Treg) has been described in acute murine and human malaria infections.[12-14] Recent studies demonstrated that kynurenines (KYN), products of the tryptophan (TRP) catabolism pathway, induce the generation of Tregs via the activation of the aryl hydrocarbon receptor (AhR).[15,16] Physiologically, tryptophan 2,3 dioxygenase (TDO), constitutively expressed in the liver, also converts TRP to KYN. However, during inflammation, the inducible enzyme indoleamine 2,3 dioxygenase 1 (IDO1), principally expressed in antigen-presenting cells, rapidly degrades TRP, increasing KYN levels.[17] IDO1 increase in CD1c expressing cells was recently described in acute controlled *P vivax* infections and was associated with increased disease tolerance.[18] Moreover, previous in vitro detailed studies with *P falciparum* have demonstrated the bystander activation and increase of Tregs, independent of antigen presentation.[19] The aim of this pilot study was to investigate the cytokines and kynurenines levels in autochthonous malaria infections and to analyse their correlation with the frequency of T regulatory cells in a small endemic cohort.

2 | MATERIALS AND METHODS

2.1 | Ethics and patient samples

The Research Ethics Review Board of the Institute of Biomedical Sciences of the University of São Paulo (954/CEP) and Federal University of Amazonas (CAAE:36936614.3.0000.5020), Brazil, approved this study. Written informed consent was obtained from all study participants. Participants answered a questionnaire on demographics and clinical symptoms, upon which venous blood was collected. Subsequently, blood was centrifuged and plasma was stored in bio-freezer at −80°C until analysis.

2.2 | Study population

Our cross-sectional study comprised of three malaria patient groups and three control groups of subjects exposed to hypoendemic malaria transmission in the Amazon region. Malaria patients were recruited in Remansinho (Amazonas State): (a) asymptomatic carriers of low-density *P vivax* infection and symptomatic malaria patients infected with (b) *P vivax or* (c) *P falciparum*. Control groups were recruited in Manaus (Amazonas State) and were negative for malaria based on thick blood smear microscopy comprised of (a) healthy endemic controls, (b) patients with febrile disease, and (c) endemic patients with obesity or body mass index (BMI) ≥30. The symptomatic malaria patients were recruited from malaria clinics in Remansinho (Amazonas State), and the clinical spectrum of malaria disease ranged from a very mild illness to full-blown paroxysms, but no patient had severe or complicated malaria as previously described.[13] Samples from asymptomatic carriers of *P vivax* infection were collected from subjects participating in an ongoing prospective cohort study of malaria risk factors in the farming settlement of Remansinho (Amazonas) as described earlier.[9] Additionally, endemic healthy control samples were collected in Manaus (Amazonas state) at the Fundação Medicina tropical (FMT-HVD) hospital; individuals self-reported as healthy, without fever and did not take any medication for chronic or acute disease, also these patients tested negative for malaria by thick blood smear. Patients with acute febrile illness with clinical suspicion of dengue fever and negative for malaria comprised febrile disease group. Individuals with obesity or BMI ≥30 had no measurable fever when recruited at the Hospital Universitário Getúlio Vargas (HUGV) (Manaus, Amazonas state, Brazil).[20] On-site malaria diagnosis, based on thick blood smear microscopy, was further confirmed by nested PCR with species-specific primers that target the 18S rRNA genes of *P falciparum* and *P vivax*.

2.3 | Tryptophan and kynurenines quantification

Metabolites were estimated in plasma samples[20] by high-pressure liquid chromatography (HPLC) as previously described. Retention time (Rt) was used to identify metabolites in the chromatogram, and standard curve was constructed by plotting the ratio of peak area (computed by LCsolution software, Shimadzu) of TRP or KYN against known TRP (Sigma-Aldrich) or KYN (Sigma-Aldrich) calibration controls.

2.4 | Cytokines quantification

Plasma samples were tested in duplicate using a sandwich ELISA kit for each cytokine as per manufacturer's instructions. TNF-a, IFN-γ, IL-4 and IL-10 levels were estimated using the OptEIA capture ELISA kits (BD Biosciences), while levels of IL-6 were measured using the DuoSet capture ELISA kit (R&D Systems).

2.5 | Peripheral blood mononuclear cell separation

Peripheral blood mononuclear cell separations (PBMCs) from *P vivax* patients and endemic controls were separated on-site by density

TABLE 1 Study population characteristics

	Febrile disease	BMI ≥ 30 Mallmann et.al. 2017	Endemic healthy	*P* vivax (n = 105)		*P* falciparum						
	(n = 13)	(n = 44)	(n = 15)[A]	Asymptomatic (n = 36)[B]	Symptomatic (n = 69)[C]	Symptomatic (n = 23)[D]	*P* value[AB]	*P* value[AC]	*P* value[AD]	*P* value[BC]	*P* value[BD]	*P* value[CD]
Gender, Female (%)	6 (46%)	16 (36%)	9 (60%)	19 (53%)	26 (38%)	8 (35%)						
Age, Years mean ± SD	42.3 ± 15.9	34.0 ± 6.2	34.8 ± 11.7	25.4 ± 15.2	31.6 ± 14.2	36.4 ± 13.6						
Parasite/uL Median, (IQR)				1.7 (0.455-14.48)	146.2 (32.33 - 567.2)	53.5 (31.68 - 273.1)						
Haematological parameters (Mean ± SD)												
Haematocrit, %			41.8 ± 5.7	42.4 ± 7.1	39.7 ± 5.5	40.1 ± 5.2	.2452	.6624	.7624	**.0386**	.1388	.6820
Haemoglobin, g/dL			15.1 ± 2.3	12.9 ± 1.9	13.0 ± 1.9	13.1 ± 1.6	**.0114**	**.0021**	**.0096**	**.0386**	.9055	.9732
White Blood Cells, X 103/µL			6.7 ± 1.5	7.7 ± 2.1	6.4 ± 2.3	5.2 ± 1.3	.1210	.3169	**.0180**	**.0058**	**<.0001**	.0534
Red Blood Cells, X 106/µL			5.2 ± 0.9	4.7 ± 0.7	4.5 ± 0.6	4.5 ± 0.6	.3143	**.0083**	**.0251**	.1311	.2765	.9767
Platelets, X 103/µL			261.7 ± 72.4	208.3 ± 55.3	161.3 ± 56.2	183.7 ± 53.4	**.0087**	**<.0001**	**.0003**	**.0033**	.3701	.0552
Cytokines pg/mL (Mean ± SD)												
IL-4			0.39 ± 1.5	5.2 ± 1.3	6.1 ± 12.1	4.1 ± 3.5	**<.0001**	**<.0001**	**<.0001**	**.0005**	**.0003**	.1418
IL-6			0.94 ± 1.6	17.4 ± 29.7	63.3 ± 181.1	21.7 ± 30.8	**<.0001**	**<.0001**	**<.0001**	.0995	.0643	.8940
IL-10			0.51 ± 1.8	25.5 ± 54.8	283.9 ± 249.3	130.2 ± 147.9	**.00271**	**<.0001**	**<.0001**	**<.0001**	**.0030**	>.9999
IFN-γ			0.07 ± 0.1	5.9 ± 3.6	40 ± 70.1	35.5 ± 46.1	**<.0013**	**<.0001**	**<.0001**	**<.0007**	**.0202**	>.9999
TNF			0.0 ± 0.0	5.3 ± 5.6	8.3 ± 6.4	13.7 ± 13.9	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**.0068**

Bold values refer $P < .5$ or statistically significant.

Abbreviation: BMI, Body Mass Index.

gradient centrifugation with Ficoll-Paque Plus (GE Healthcare) as described earlier.[13] Briefly, isolated PBMCs were washed twice in Hanks buffer or PBS, resuspended in 90% heat-inactivated foetal bovine serum (FBS, Gibco) and 10% dimethyl sulfoxide (Sigma) and later frozen in liquid nitrogen for shipping and long-term storage. Frozen cells were thawed in a 37°C water bath and resuspended in complete RPMI 1640 medium before flow cytometric analysis (only samples with ~85% viability were used for analysis).

2.6 | T regulatory cell measurement and phenotyping

Thawed PBMC samples were centrifuged and 10^6 viable cells were fixed and permeabilized using the Human FoxP3 buffer set (BD Pharmingen). Next, directly conjugated monoclonal antibodies were added and cells were incubated at 4°C in dark for 30 minutes, subsequently washed twice to remove unbound antibodies. Cells were then suspended in 100 uL of fixation buffer (1% paraformaldehyde in PBS) and analysed on an LSRFortessa flow cytometer (BD Biosciences) using the FACSDiva software (BD Bio- sciences). Live/Dead Fixable Blue Dead Cell Stain for UV excitation (Invitrogen) was used to determine cell viability and 300 000 events in each live gate were collected. Boolean analysis was applied to evaluate coexpression of different molecules by the same cells. Data analysis was carried out using FlowJo software version 8.8.6 (Tree Star).

2.7 | Statistics

Differences in the means were tested, respectively, using either one-way ANOVA, with Tukey's post hoc test, or the Mann-Whitney *U* test or Kruskal-Wallis test, with Dunn's post hoc test, as appropriate. The linear correlation between variables was determined using the Spearmen correlation coefficient. Data were analysed using Prism software version 7.01 (GraphPad).

3 | RESULTS

Control groups and *Plasmodium*-infected patient characteristics, haematological parameters and cytokine levels are described in Table 1. Malaria asymptomatic and symptomatic infection reduced haemoglobin and platelet levels compared to healthy endemic patients (Table 1). Also, symptomatic *P vivax* and *P falciparum* infection decreased RBC count compared to healthy controls (Table 1). A decrease in WBC was observed in symptomatic *P vivax* and *P falciparum* infection compared to asymptomatic *P vivax* infection (Table 1).

Canonical Th1 and Th2 cytokines were elevated in asymptomatic and symptomatic acute malaria infections compared to the healthy endemic controls (Table 1). IL-4, IL-10, IFN-γ and TNF-α were also elevated in symptomatic *P vivax* and *P falciparum* infection compared to asymptomatic *P vivax*-infected individuals (Table 1), whereas only TNF was elevated in *P falciparum* patients compared to *P vivax*-infected cases.

Dietary intake of TRP, an essential amino acid, varies within individuals; hence we used the KYN to TRP (KYN/TRP) ratio as a measurement of IDO1 enzyme activity.[21] Chronic disease like obesity and acute microbial infections increased KYN/TRP (Figure 1A), which was dependent on the inflammation status of the patient.[16,21] We observed a significant increase in the KYN/TRP ratio in *P vivax* and *P falciparum* patients, compared to healthy controls (Figure 1A). Moreover, we also observed an elevated KYN/TRP ratio in both asymptomatic and symptomatic *P vivax* malaria patients (Figure 1A). Both *P vivax* and *P falciparum* infection increased KYN/TRP ratio; however, no significant difference in KYN/TRP ratio was observed between the two *Plasmodium*-infected groups. Symptomatic malaria-infected patients had elevated IFN-γ and IL-10 levels compared to asymptomatic patients and healthy controls (Table 1 and Figure 1B).

Subsequently, we also noted that the KYN/TRP ratio in these vivax patients was positively correlated with the increase in peripherally circulating Foxp3 + CD4+Tregs; in addition, a positive significant correlation was observed between IFN-γ concentration in symptomatic vivax-infected patients and plasma KYN/TRP ratio (Figure 1C). Overall, a non-significant correlation was observed between KYN/TRP ratio and malaria parasite load or plasma cytokines (Table 2).

4 | DISCUSSION

Plasmodium infection inflicts tissue damage, and the host responds via resistance and tolerance mechanisms to avoid it. IFN-γ is pivotal in controlling liver and blood stage parasites, but unrestricted IFN-γ-mediated inflammation can produce deleterious effects, therefore it is actively regulated in tissues.[22] Furthermore, IFN-γ strongly induces the activation of the tryptophan degradation pathway, mediated by IDO1, with both antimicrobial and tolerogenic effects.[17,21] The consequences of the activation of this pathway in chronic non-communicable diseases,[20,21] chronic viral, bacterial and intracellular parasitic infections are well documented.[23,24] In this study, we report an increase in serum IFN-γ that correlated with increased serum kynurenine levels in *P vivax* and *P falciparum* malaria infection. We also observed a positive relation between serum kynurenines levels and the frequency of tolerogenic CD4+ FoxP3+ Tregs.[25]

Blood canonical Th1 and Th2 cytokines were markedly increased in acute *P vivax* and *P falciparum* malaria infections[9,26,27] and exposure or parasite load-dependent strong association with activation of proinflammatory responses and cytokine imbalance was observed.[7,9,10] Murine and human *Plasmodium* infections have demonstrated that early in infection IFN-γ is produced by innate activation of Natural Killer (NK) cells that is subsequently taken over by CD4+ and CD8+ T cells over the course of infection.[22,28,29] Overall, we observed an increase in kynurenines in patients with disease ranging from asymptomatic infection, very mild illness, to full-blown paroxysms. Our results corroborate the increase in KYN/TRP ratio in

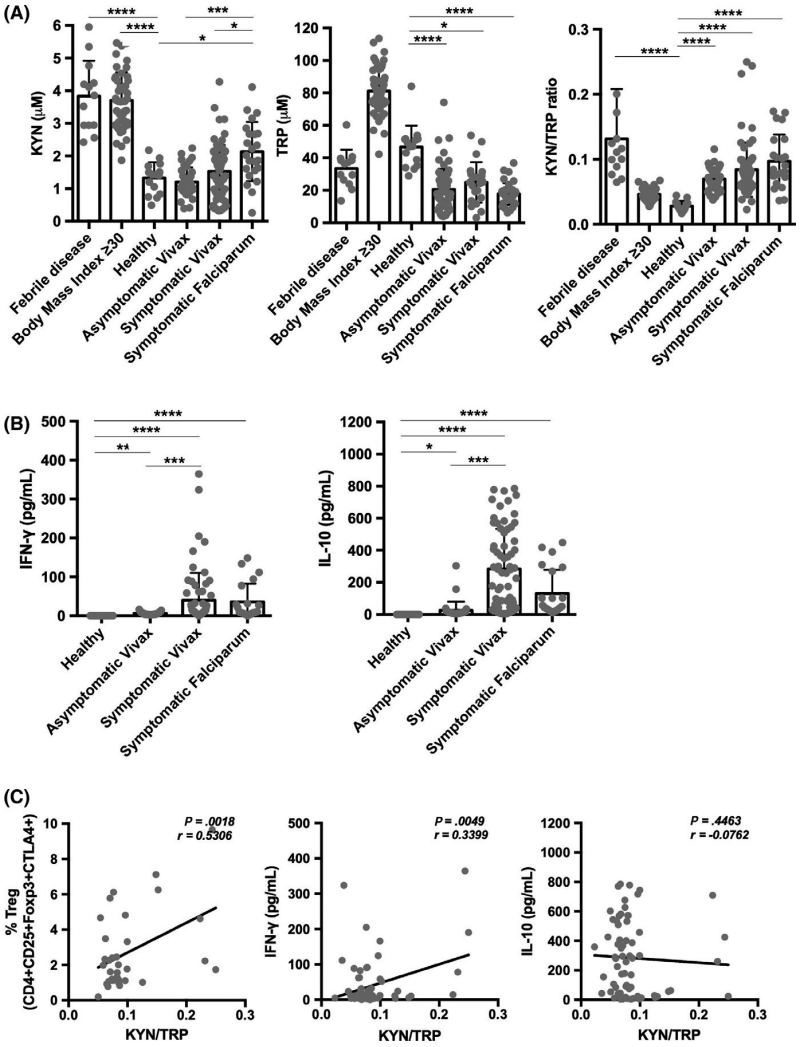

FIGURE 1 *Plasmodium* infection elevates tryptophan metabolism and T regulatory cells. Plasma (A) tryptophan (TRP) and kynurenine (KYN) levels were estimated by HPLC in three control groups comprised of healthy endemic individuals, patients with febrile illness and obesity and three malaria patient groups comprised of asymptomatic *P vivax*-, symptomatic *P vivax*- and symptomatic *P alciparum*-infected individuals before starting antimalarial treatment. (B) Cytokine levels were estimated by ELISA and compared between healthy endemic controls and malaria-infected patient groups. (C) Correlation analysis was performed between circulatory Tregs, IFN-ϒ or IL-10 and KYN/TRP ratio (***P* < .01 ****P* < .001 *****P* < .0001, ANOVA or Spearman correlation)

TABLE 2 Comparison between immunological markers and blood parasite load

	Parasite/μL	
Symptomatic Vivax patients	***r***	***P***
% Tregs (CD4 + CD25+FoxP3 + CTLA4+)	.2235	.2937
IL-10 (pg/mL)	.1578	.4616
IFN-γ (pg/mL)	−.1651	.4407
KYN/TRP ratio		
Asymptomatic Vivax	−.0613	.7224
Symptomatic Vivax	−.0565	.6447
Symptomatic Falciparum	−.1338	.5427

naturally acquired *P vivax* malaria, as demonstrated in human-controlled *P vivax* infections.[18] Also, our observations are consistent with recent studies that characterized serum metabolomics of *Plasmodium*-infected humans and nonhuman primates.[30-32] IFN-γ was shown to be essential for IDO1 induction,[33] and mice genetically deficient for IDO1 were not protected against cerebral malaria[34] in a *P berghei* ANKA (PbA) mouse model. Moreover, treatment of PbA-infected mice with hyperbaric oxygen (HBO) conferred protection against cerebral malaria and improved survival by increasing expression of AhR and decreasing IDO1 expression and KYN metabolites.[35]

The increase of Tregs has been demonstrated in acute human and murine malaria infections.[12-14,36,37] Previously in *P falciparum* infections, it was demonstrated that IL-10 and Tregs cells frequencies are influenced by malaria incidence and by the number of malaria infections.[38,39] Our data also show that the frequency of Tregs increase in acute *P vivax* infection[13,36]; on the contrary, we did not observe positive correlation with parasite load, as previously described.[14] Moreover, in vitro stimulation of PBMCs with *P falciparum* was shown to induce Tregs, independent of direct TCR stimulation but was dependent on IL-10 and TGF-β.[19] Tryptophan-derived

metabolites produced by myeloid cells mediate immune tolerance by inducing apoptosis of activated T cells and by the conversion of naive T cells into Tregs, via the activation of the AhR; tryptophan restriction induces the starvation response in T cells via the activation of the GCN2 kinase.[15,23,40,41] Similarly, an observed increase in KYN in *P vivax* patients might be responsible for this Treg increase observed in our study. Additionally, a recent report showing an increase in PD-1+ Tregs during acute vivax infection,[42] opens up fundamental questions about their ability to control parasite load and reduce pathology in *P vivax* infection.

Although our preliminary results are promising, we also acknowledge some limitations. We determined laboratory parameters retrospectively in a relatively small cross-sectional cohort of healthy individuals and malaria-infected patients from different locations. The exact delay between symptoms onset and malaria diagnosis was not recorded, and we only tested a single blood sample upon inclusion in the study. Longitudinal follow-up measurements of tryptophan metabolism were not available to assess the kinetics during the course of the disease and after parasite clearance. In addition, the cohorts, especially the healthy controls, were not perfectly age-matched. However, the observed KYN/TRP ratio increase in patients might be explained by the acute inflammation that accompanies *Plasmodium* infection, as noted by elevated Th1 and Th2 cytokines, a well-documented phenomenon also observed by others.[13,26,27] We also acknowledge that other concomitant infections can produce similar cytokines and serum kynurenines responses as observed in our cohort, and we cannot exclude other infectious causes for these responses. Further longitudinal cohort studies should address these issues and provide a better understanding of these preliminary observations.

In this study, we observed a serum IFN-γ increase, important for immune activation, and serum kynurenines elevation, that might be induced to avoid host immunopathology; on the other hand, the serum kynurenines increase can also hamper optimal control of parasitemia by increasing tolerance and obstructing an effector T cell response. Different clinical outcomes during malaria infection have been mainly attributed to differences in the host immunity level, and vaccination strategies that explore the boosting of IFN-γ responses might be essential in improving immune response and disease outcome. However, additional studies are needs to not only understand the molecular mechanisms involved in IDO1 and Treg induction, but also their implication in impairing an efficient immune response.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTION

ROS, MUF and PL designed the study, performed statistical analysis and wrote the manuscript. ROS, RMGL, NFL and KKGS collected patient samples, performed experiments and analysed the results.

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Capítulo 3

frontiers in Microbiology

ORIGINAL RESEARCH
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A First *Plasmodium vivax* Natural Infection Induces Increased Activity of the Interferon Gamma-Driven Tryptophan Catabolism Pathway

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The human immune response that controls *Plasmodium* infection in the liver and blood stages of the parasite life cycle is composed by both pro- and anti-inflammatory programs. Pro-inflammatory responses primarily mediated by IFN-γ controls the infection, but also induce tolerogenic mechanisms to limit host damage, including the tryptophan (TRP) catabolism pathway mediated by the enzyme Indoleamine 2,3-Dioxygenase (IDO1), an enzyme that catalyzes the degradation of TRP to kynurenines (KYN). Here we assessed total serum kynurenines and cytokine dynamics in a cohort of natural *Plasmodium vivax* human infection and compared them to those of endemic healthy controls and other febrile diseases. In acute malaria, the absolute free kynurenine (KYN) serum levels and the KYN to TRP (KYN/TRP) ratio were significantly elevated in patients compared to healthy controls. Individuals with a diagnosis of a first malaria episode had higher serum KYN levels than individuals with a previous malaria episode. We observed an inverse relationship between the serum levels of IFN-γ and IL-10 in patients with a first malaria episode compared to those of subjects with previous history of malaria. Kynurenine elevation was positively correlated with serum IFN-γ levels in acute infection, whereas, it was negatively correlated with parasite load and *P. vivax* LDH levels. Overall, the differences observed between infected individuals depended on the number of *Plasmodium* infections. The decrease in the KYN/TRP ratio in malaria-experienced subjects coincided with the onset of anti-*P. vivax* IgG. These results suggest that *P. vivax* infection induces a strong anti-inflammatory program in individuals with first time malaria, which fades with ensuing protective immunity after subsequent episodes. Understanding the tolerance mechanisms involved in the initial exposure would help in defining the balance between protective and pathogenic immune responses necessary to control infection and to improve vaccination strategies.

Keywords: tryptophan, kynurenine, *Plasmodium*, IFN-γ, inflammation, Indoleamine 2,3- Dioxygenase 1

INTRODUCTION

Malaria is a parasitic disease that represents a significant global health problem. *Plasmodium vivax* infects over 20 million people each year (World Health Organization, 2017), and is the most geographically widespread species worldwide (Battle et al., 2019). *P. vivax* infection accounts for more than 85% of the malaria cases in Brazil, and most of the patients are confined to the Amazon region, with isolated cases occurring in other states of the country (Oliveira-ferreira et al., 2010). *P. vivax* is often present in peripheral blood at sub-patent densities and infections include a dormant liver stage invisible to current diagnostic methods, increasing the challenge for its control. Compared to *P. falciparum*, *P. vivax* infection has lower parasitic load, and disease complications are rare, but several recent studies have reinforced the association between severe disease and death in *P. vivax* infections (Poespoprodjo et al., 2009; Alexandre et al., 2012; Mahgoub et al., 2012; Baird, 2013). Overall, *Plasmodium* infections can produce severe forms of the disease, because of an insufficient immune response to control the parasite load or the inability of the host to control inflammation, resulting in immunopathology (Gonçalves et al., 2012; Mendonça and Barral-Netto, 2015), but the mechanisms involved are not fully understood (Cunnington et al., 2013; Crompton et al., 2014).

The innate immune response is pivotal for the initial control of infection and for instructing and directing the ensuing adaptive response. Clinical data from cohorts and experimental evidence from animal models supports the observation that the adaptive immune response, mainly via antibody-mediated anti-plasmodium immunity, restricts the infection and limits pathology (Longley et al., 2016). In addition, the pivotal role of IFN-γ as a central cytokine in controlling *Plasmodium* infection in both the liver and blood stages of the parasite life cycle is well documented (King and Lamb, 2015). Plasmodium infection induces IFN-γ production from a range of innate and adaptive immune cell subsets at different stages of the life cycle, highlighting the roles of both innate and adaptive immunity in controlling the infection. Although *Plasmodium*-specific antibodies are known to play a key role in controlling malarial fever and parasitemia, less is known about cellular and innate immunity to malaria in humans. *Plasmodium*-induced IFN-γ production has been related with clinical immunity to malaria in humans; yet, IFN-γ can induce pathology if not regulated (Andrade et al., 2010; Deroost et al., 2016). Therefore, the mechanisms regulating the balance between immunity and immunopathology during malaria remain unclear. Recent studies have shown that, relative to malaria-experienced individuals, naive individuals had increased activation of pro-inflammatory pathways during primary infection, despite lower parasitemia (Tran et al., 2016), providing evidence for modulation of inflammatory responses during malaria.

The IFN-γ pathway is both an important effector of innate cell-intrinsic immunity, and a key inducer of appropriate subsequent T and B cell adaptive responses. It initiates both potent pro- and anti-inflammatory cell-intrinsic responses in immune cells and in tissue, and their balance and timing can affect the ensuing adaptive mechanisms engaged to control the infection. In addition, IFN-γ strongly induces the activation of the tryptophan degradation pathway, mediated by IDO1, and a large body of evidence indicates that the tryptophan catabolism pathway is involved directly or indirectly in the host response to infection. IDO1, IDO2, and TDO catalyze the first rate-limiting step in this pathway, producing both tryptophan depletion and tryptophan catabolic products collectively known as kynurenines. Both, tryptophan depletion and kynurenines production, can convert naïve T cells into regulatory T cells. In addition, L-kynurenine, the main degradation product, activates the aryl hydrocarbon receptor (AhR), with immunoregulatory effects in both T and myeloid cells (Fallarino et al., 2006; Mellor and Munn, 2008; Grohmann and Puccetti, 2015; Yeung et al., 2015). The consequences of the activation of the tryptophan degradation pathway in chronic viral and bacterial infections, and in intracellular parasite disease, have demonstrated that both, its antimicrobial and tolerogenic effects, can influence the outcome of infection (Munn and Mellor, 2013; Schmidt and Schultze, 2014; Yeung et al., 2015).

We therefore aimed to explore the dynamics of the potent IFN-γ-induced cell intrinsic response mediated by the tryptophan catabolic pathway, assessing its potential role during the development of anti-*P. vivax* malaria immunity in an endemic region. We also aimed to explore its potential role for immunomonitoring disease dynamics in the context of natural infection, where multiple exposures to *P. vivax* are prevalent and define natural protection to the parasite.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Universidade Federal do Amazonas (UFAM). All the study participants provided an informed consent prior to enrollment.

Study Design

All study participants were recruited at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD; Manaus, Amazonas state) from March 2015 to February 2016. We analyzed the immune response to natural *P. vivax* infection by measuring different hematological and immune parameters (serum cytokines, tryptophan, and kynurenines levels) in an endemic region.

A cross-sectional study included three study groups: malaria cases (*P. vivax*-diagnosed patients, with either a first-time malaria episode or more than one episode, $n = 81$), patients with other febrile diseases (non-malaria disease, $n = 21$), and healthy malaria-negative endemic controls ($n = 34$) (**Figure 1**). The clinical spectrum of malaria in the participants ranged from a very mild illness to full-blown paroxysms, and none of the patients had severe or complicated disease. Subjects with a primary malaria episode were defined as subjects with a self-reported history of no previous malaria and seronegative for MSP-1_{19} *P. vivax* antigen. Subjects with previous malaria episodes were identified as subjects that self-reported previous malaria and were seropositive for IgG MSP-1_{19}; last malaria

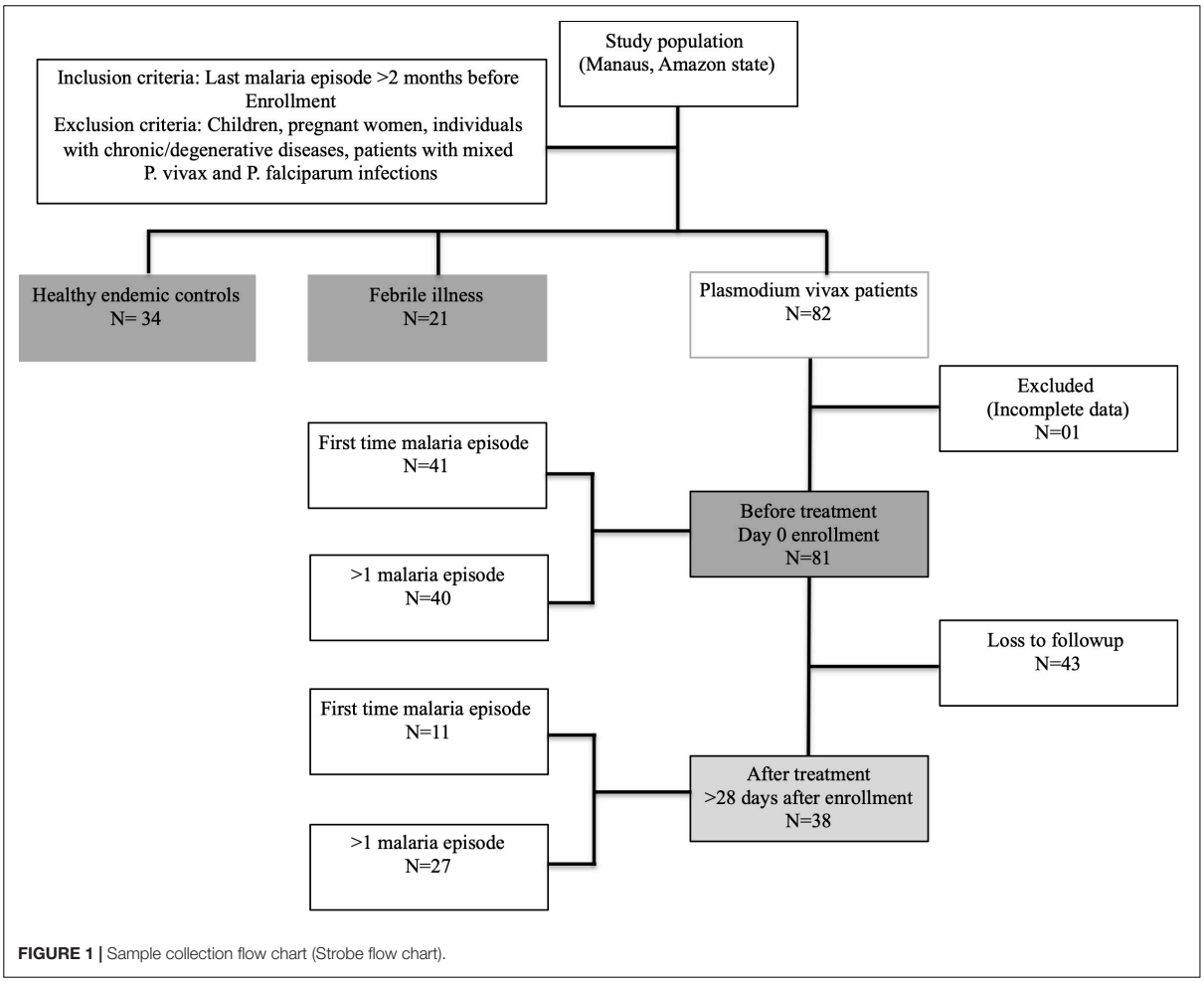

FIGURE 1 | Sample collection flow chart (Strobe flow chart).

episode was at least 2 months before inclusion in the study. One malaria patient was excluded from the analyses due to missing information. Febrile disease patients were confirmed as negative for *P. vivax* infection by thick blood smear microscopy. Healthy endemic controls had negative *P. vivax* diagnosis by thick blood smear microscopy and were also seronegative for malaria, identified by detection of IgM and IgG antibodies against MSP-1_{19} antigen. Individuals with chronic/degenerative diseases, pregnant women or subjects <18 years old were not enrolled in this study.

The study was exploratory and the sample size was not pre-defined by effect size, but limited by voluntary participation and enrollment. Previous malaria history and the number of days with fever and other symptoms before diagnosis were assessed by self-reported medical history. The degree of pre-existing immunity was assessed by the presence of serum anti-*P. vivax* antibodies.

A follow-up assessment was performed to evaluate the dynamics of the hematological and immune response parameters in *P. vivax*-positive patients after successful anti-malaria treatment and clearance of blood-stage parasites, as recommended by the Brazilian Ministry of Health. However, only 47% of the recruited patients returned for followup after anti-malaria treatment [before treatment (BRx, n = 81), and after treatment (ARx, n = 38), respectively]. After treatment, all patients were malaria-negative, confirmed by thick blood smear microscopy.

Peripheral Blood Samples

Peripheral blood samples were obtained from 136 participants. A total of 5 mL of blood was drawn from each participant (using EDTA tubes, BD Vacutainer) and divided into two parts: one part was used for hematological analysis and a second part was used for blood plasma analysis. Whole blood samples were employed for assessing hematological parameters using an automated hematological analyzer (Sysmex KX-21 N®): Hematocrit, Hemoglobin, White blood cells, Red blood cells, Mean corpuscular volume, Mean corpuscular hemoglobin,

Mean corpuscular hemoglobin concentration, Mean platelet volume, Platelets.

Tryptophan and Kynurenine Quantification

After separation from whole blood, plasma samples (200 uL) were treated with 8% Perchloric acid, to precipitate proteins and extract soluble metabolites. After extraction, the aqueous phase was analyzed by High Performance Liquid Chromatography (HPLC) as previously described (Mallmann et al., 2018). For the standard curve, a serial dilution was performed with TRP and KYN (TRP/KYN μM/L): 100/10; 50/5; 25/2.5; 12.5/1.25; 6.25/0.625; 3.125/0.325. HPLC flow rate was 1.0 mL min^{-1} and 20 μL of clear sample was then injected into the HPLC system using an autosampler. TRP was identified at 278 nm and KYN at 360 nm by UV detection (Zhang et al., 2009). Retention time (Rt) was used to identify metabolites in the chromatogram and standard curve was constructed by plotting the ratio of peak area (computed by LCsolution software, Shimadzu) of TRP or KYN (y) against known TRP or KYN concentration (x), respectively. The linearity of the standard curves was confirmed using regression variance analysis and significance of correlation coefficient (r) checked using Student's t-test. KYN/TRP ratio was calculated using the formula KYN concentration (μM/L)/TRP concentration (μM/L).

Cytokines Quantification

Plasma cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A) levels were quantified using the CBA Th1, Th2, and Th17 kit (Cytometric Bead Array, BD Biosciences), following the manufacturer's instructions. Samples were analyzed on a FACSCanto II (Becton, Dickinson and Company, San Jose, CA, United States) flow cytometer and data analyzed by FCAP ArrayTM software (V3.0.1). The mean fluorescence intensity (MFI) of each bead cluster was determined and forth logistic regression applied to build the standard curves. Cytokine concentrations for each sample were then extrapolated from the standard curves and data was expressed as pg/mL for each plasmatic cytokine.

Lactate Dehydrogenase (LDH) ELISA

Plasma samples were analyzed by an *in-house* sandwich ELISA to detect *P. vivax* LDH (PvLDH) in untreated malaria patients as previously described (Sousa et al., 2014). Briefly, ELISA plates were coated with polyclonal anti-PvLDH rabbit antibodies, next patient plasma samples were added to capture cell-free PvLDH. Captured antigen was then identified using a mouse PvLDH-specific primay antibody and a HRP-conjugated goat anti-mouse IgG secondary antibody. Absorbance cut-offs were calculated to determine reactive samples using the mean optical density readings from negative sample plus twice the standard deviation of negative samples.

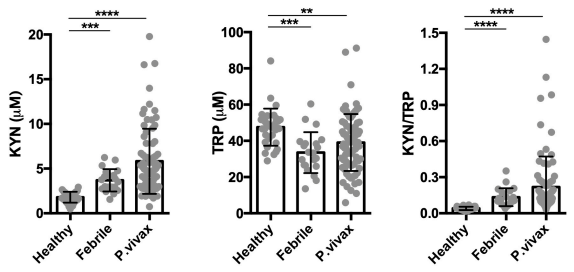

FIGURE 2 | The tryptophan degradation pathway is activated in acute infection. Kynurenine (KYN), tryptophan (TRP) levels were quantified in healthy endemic control group (n = 34), non-malarial febrile patients (n = 21) and vivax infected patients before commencing anti-malarial treatment (n = 81) by HPLC. (**P < 0.01, ***P < 0.001, ****P < 0.0001, ANOVA test with Tukey's *post hoc* test).

Plasmodium vivax Serology

Plasma samples were analyzed by indirect ELISA to detect *P. vivax*-specific total IgG and IgM antibodies using the 19-kDa C-terminal region of the Merozoite Surface Protein-1 of P. vivax ($MSP\text{-}1_{19}$), as previously described (Soares and Rodrigues, 2002). Briefly, recombinant antigen was coated on an ELISA plate, and after blocking, patient plasma samples were added and incubated. Subsequently, HRP-conjugated anti-human IgG or IgM secondary antibodies were added, and optical density readings recorded. For each sample, the reactivity index (RI: absorbance/cut-off) was calculated.

Statistics

Differences in means and medians were tested, respectively, using either one-way ANOVA, with Tukey's *post hoc* test, or the Mann-Whitney U test or Kruskal-Wallis test, with Dunn's *post hoc* test, with correction for multiple comparisons as appropriate. The correlation between variables was determined using the Pearson's correlation coefficient. Data was analyzed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, United States) and Rstudio version 1.1.4 with R version 3.5.0 and RPQ, HNP and Tidyverse packages.

RESULTS

Demographic Description of the Study Groups

A total of one hundred and thirty-six individuals were recruited for this study. One patient was excluded (because of missing information), and out of eighty-one malaria patients, forty-three patients were lost to follow-up after treatment (**Figure 1**). **Table 1** and **Supplementary Table S1** summarize the demographic data and hematological results of the study groups: (1) healthy endemic controls, (2) individuals with non-malarial febrile illness, (3) *P. vivax* malaria patients (enrolled on Day 0 before administering anti-malarial treatment), and (4) *P. vivax* malaria patients followed-up after successful anti-malarial treatment.

TABLE 1 | Patient clinical and demographic details.

Demographic characteristics		Healthy endemic	Febrile patients	Plasmodium vivax		p-value^{AB}	p-value^{BC}	p-value^{AC}	p-value^{CD}
		(n = 34)^{A}	(n = 21)^{B}	Before malaria treatment (n = 81)$^{\#C}$	After malaria treatment (n = 38)^{D}				
Gender	Female (%)	12 (35%)	10 (48%)	22 (27%)	6 (15%)				
Age, mean	Years ± SD	34.7 ± 11.7	40.3 ± 14.6	38.7 ± 10.8	39.9 ± 10.6				
Number of malaria episodes	First malaria			41 (51%)	11 (31%)				
	>1 malaria			40 (49%)	27 (69%)				
Days with fever (symptoms)§	<6 days		11 (73%)	52 (69%)	24 (65%)				
	>6 days		4 (27%)	23 (31%)	13 (35%)				
Hematological parameters (mean ± SD)									
Hematocrit (%)		45.9 ± 5.0	40.5 ± 2.8	41.9 ± 6.7	44.9 ± 3.3	0.0006	>0.9999	0.0002	<0.0005
Hemoglobin (g/dL)		16.4 ± 12.2	13.9 ± 0.9	13.8 ± 2.3	13.8 ± 1.3	>0.9999	>0.9999	0.1852	>0.9999
White blood cells ($\times 10^3/\mu L$)		6.4 ± 1.1	5.7 ± 1.4	4.5 ± 1.8	5.9 ± 1.4	0.0627	0.3913	<0.0001	<0.0001
Red blood cells ($\times 10^6/\mu L$)		5.2 ± 6.4	4.2 ± 1.3	5.1 ± 8.7	4.9 ± 5.1	0.0005	0.0038	0.6103	>0.9999
Mean corpuscular volume (fL)		89.3 ± 4.5	89.4 ± 5.2	87.2 ± 3.4	89.3 ± 3.2	>0.9999	0.2684	0.0398	0.0081
Mean corpuscular hemoglobin (pg)		27.6 ± 1.4	30.7 ± 2.1	27.9 ± 2.4	27.8 ± 1.4	0.0004	<0.0001	>0.9999	>0.9999
Mean corpuscular hemoglobin concentration (g/dL)		31.1 ± 0.9	34.3 ± 1.1	31.6 ± 1.6	31.1 ± 0.8	<0.0001	<0.0001	0.2776	0.8746
Mean platelet volume (fL)		9.3 ± 0.9	8.5 ± 0.8	10.5 ± 1.1	9.8 ± 1.1	0.6782	<0.0001	0.0011	0.1037
Platelet ($\times 10^3/\mu L$)		256 ± 71.6	246 ± 62	110 ± 57.3	235 ± 56.9	>0.9999	<0.0001	0.0001	0.0001

Before malaria treatment (BRx): Day 0 on enrolment; After malaria treatment (ARx):>28 days after treatment; >1 malaria episodes, Median episode number = 3 (IQR: 2-5.75)]; $^{\#}$Median Parasitemia (Parasites/mm^3) = 2158 (IQR, 537.6 - 4185); §Missing patient data.

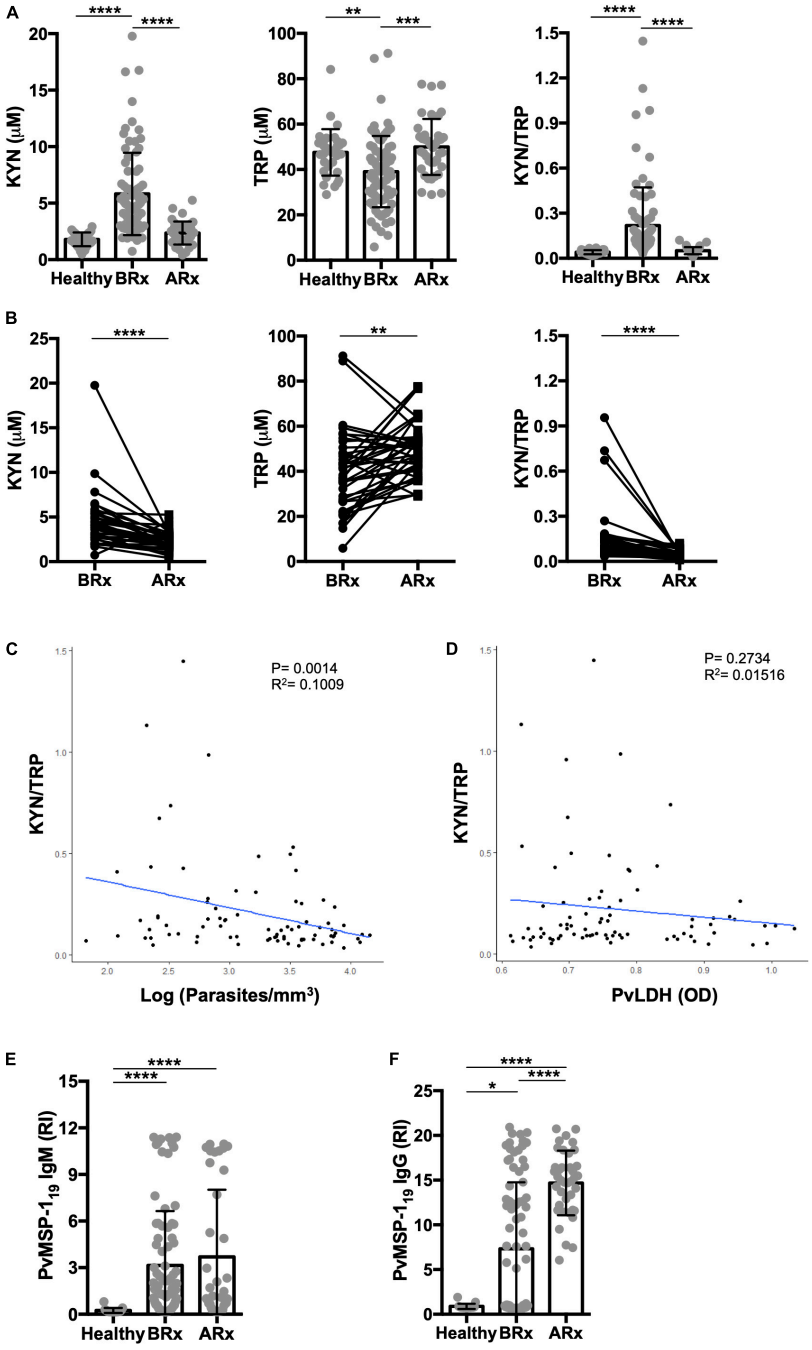

FIGURE 3 | Successful antimalarial treatment reverts kynurenine levels. **(A)** Serum kynurenine (KYN) and tryptophan (TRP) levels were quantified by HPLC in a healthy endemic control group ($n = 34$), and in *P. vivax*-infected patients on day 0 of enrollment (before starting anti-malarial treatment, BRx, $n = 81$), and >28 days after successful malaria treatment (ARx, $n = 38$). **(B)** Paired sample analysis before and after vivax malaria treatment ($n = 38$). Correlation analysis between KYN/TRP ratio and **(C)** parasitemia levels, quantified by thick blood film, or **(D)** vivax lactate dehydrogenase (PvLDH), measured by ELISA. *P.*vivax MSP-1_{19} antigen specific IgM **(E)** and IgG **(F)** antibodies were measured by enzyme-linked immunosorbent assay (ELISA) to confirm sero-conversion (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ANOVA test with Tukey's *post hoc* test, student's paired *t*-test and Pearson's correlation).

TABLE 2 | Blood cytokine levels in *Plasmodium vivax* infected patients.

Cytokines pg/mL (mean ± SD)	Healthy endemic	*Plasmodium vivax*		Number of infections, patients before anti-malarial treatment		*p*-value^{AB}	*p*-value^{BC}	*p*-value^{AC}	*p*-value^{DE}
	(n = 34)^{A}	Before malaria treatment (n = 81)$^{\#B}$	After malaria treatment (n = 38)^{C}	First malaria episode (n = 41)^{D}	>1 malaria episode§ (n = 40)^{E}				
IL-2	0.1 ± 0.1	0.1 ± 0.4	0.1 ± 0.1	0.1 ± 0.25	0.1 ± 0.47	0.3303	0.0890	>0.9999	0.5689
IL-4	0.4 ± 1.5	1.1 ± 4.3	0.1 ± 0.1	0.3 ± 0.78	1.7 ± 6.1	0.0056	< 0.0001	>0.9999	0.6878
IL-6	0.9 ± 1.6	104.2 ± 321.2	1.1 ± 3.2	59.1 ± 133.8	150.4 ± 434.8	< 0.0001	< 0.0001	>0.9999	0.3808
IL-10	0.4 ± 1.4	304.3 ± 855.1	0.3 ± 0.8	198.3 ± 698.2	413.0 ± 988.3	< 0.0001	< 0.0001	>0.9999	0.6206
IL-17	2.2 ± 5.5	23.1 ± 44.1	44.1 ± 58.4	24.3 ± 48.1	21.9 ± 40.2	0.0259	0.0102	< 0.0001	0.9107
IFN-β	0.1 ± 0.1	282.4 ± 849.4	0.0 ± 0.0	533.6 ± 1153	24.2 ± 42.4	< 0.0001	< 0.0001	>0.9999	0.0003
TNF-α	0.3 ± 1.3	0.42 ± 1.7	0.0 ± 0.0	0.1 ± 0.3	0.8 ± 2.3	0.7658	0.0561	>0.9999	0.0201

Before malaria treatment (BRx): Day 0 on enrollment; After malaria treatment (ARx): >28 days after treatment; § > 1 malaria episodes, Median episode number = 3 (IQR: 2-5.75); #Median Parasitemia (Parasites/mm^3) = 2158 (IQR, 537.6 - 4185).

Median (Inter quartile range, IQR) parasite load/mm^3, estimated by thick blood smear, was 2158 (537.6—4185) in *P. vivax* malaria patients, and the mean parasite load was not different between patients with a first-time malaria episode, and individuals with previous malaria history [>1 malaria episode, median episode number = 3 (IQR: 2-5.75)] (**Figure 3B**).

Natural *P. vivax* Infection Induces Hematological Changes, a Complex Serum Cytokine Response, and an Increased Activity of the Tryptophan Degradation Pathway

In this cross-sectional study we evaluated healthy subjects (n = 34), *P. vivax*-malaria patients (n = 81), and patients with other febrile diseases (n = 21). The hematological parameters and serum cytokines levels of these groups are summarized in **Tables 1** and **2**. We observed that malaria induced significant hematological changes that comprised a reduction in hematocrit, leukocyte, and platelet levels (**Supplementary Tables S1, S2** and **Supplementary Figure S1**), as well as a mixed cytokine profile that included the concomitant elevation of canonical Th1, Th2, and Th17 cytokines, with a distinct elevation in serum IFN-γ and IL-10 compared to healthy controls (**Table 2**). These results indicate that acute *P. vivax* infection induces a significant decrease in multiple circulating blood cell lineages, together with a distinct pro- and anti-inflammatory serum cytokine response.

We assessed the activity of the tryptophan catabolism pathway by measuring kynurenines, tryptophan, and the KYN/TRP ratio in sera, a marker for overall tryptophan catabolism by the kynurenine pathway (Widner et al., 1997; Yeung et al., 2015). We observed a significant increase in both serum KYN levels and in the KYN/TRP ratio in acute disease at recruitment, before starting anti-malaria treatment (**Figure 2**). We also observed that other febrile diseases also induce an increase in the serum KYN and the KYN/TRP ratio compared to healthy controls. These results indicate that the induction of the tryptophan degradation pathway is a non-specific host response to infecting pathogens, as observed by others (Schmidt and Schultze, 2014; Yeung et al., 2015).

Next, we evaluated the dynamics of hematologic parameters, serum cytokines, and the tryptophan catabolism pathway in the *P. vivax*-malaria patients before and after anti-malarial chemotherapy in a subgroup that returned for evaluation. At follow up, all patients cleared the parasite after treatment (n = 38). All measured hematological parameters returned to levels comparable to those of the healthy controls (**Table 1**). In a similar fashion, serum cytokine levels returned to levels comparable to those of the healthy controls with the exception of IL-17 (**Table 2**). We also observed that the tryptophan degradation pathway activity (measured by the KYN/TRP ratio) returned to levels comparable to those of the control group (**Figure 3**).

These results show that natural *P. vivax infection* induces a reduction in circulating blood cells, a marked mixed cytokine response, and an increase in the production of tryptophan degradation products that return to healthy control levels after

treatment, as seen by others and in experimental infections (Schmidt and Schultze, 2014; Yeung et al., 2015).

A Single, First Malaria Episode Induces Higher Tryptophan Degradation Pathway Activity Compared to Multiple Infection Episodes

Numerous studies have shown that multiple malaria episodes can induce parasite tolerance by the host. Therefore, we next compared the relationship between the number of malaria episodes and the KYN/TRP ratio, and observed that it was elevated in subjects with a "primary" or first malaria, compared to individuals with previous malaria episodes (>1 malaria episode; **Figure 4A** and **Supplementary Figure 2A**). Moreover, no differences were observed in parasite load and *P. vivax* LDH levels of individuals with a first malaria-infection compared to the ones of individuals with a previous malaria exposure (**Figures 4B,C**). Interestingly, the increased elevation in the KYN/TRP ratio observed in first *Plasmodium* infection negatively correlated with parasite load, while it was independent of parasite load in patients that had a previous infection (**Figures 4D,E**). In addition, we observed that the IgM antibody levels were similar between first malaria patients compared to patients with a previous malaria episode (**Figure 4F**). As expected, first malaria-infected individuals were IgG-negative for MSP-1_{19}, whereas individuals with previous malaria history had circulating antibodies (**Figure 4G**). We hypothesized that the number of febrile days, a proxy for systemic inflammation, would affect the activity of the tryptophan degradation pathway, however, no significant difference in kynurenine levels was observed between the patient groups when stratified by number of days with fever before anti-malaria treatment (**Figure 5** and **Supplementary Figure S2B**). Additionally, we did not observe any relationship between blood parasite stages and KYN/TRP ratio (**Supplementary Figure S3**).

Although all cytokines were elevated in individuals with malaria compared to healthy controls, only IFN-γ and TNF-α were significantly elevated in patients with a first malaria episode compared to individuals with multiple episodes (**Table 2**). IL-10 was elevated in individuals with multiple infections compared to ones with first time malaria; however, the elevation was not significant (**Table 2**). As anticipated, elevated IFN-γ levels were associated with an increase in the KYN/TRP ratio, whereas IL-10 was negatively correlated (**Supplementary Figures S1C,D**). Only individuals with a first malaria episode demonstrated significant positive correlation with IFN-γ and KYN/TRP ratio (**Figure 6**).

These results suggest that a first episode of *P. vivax* malaria produces a distinct host response with a significant induction of the tryptophan degradation pathway, together with a higher pro-inflammatory cytokine response, which correlates positively with serum IFN-γ levels and negatively with parasitemia. In contrast, multiple infections induce a lower activity of the tryptophan degradation pathway, which is independent of parasitemia and coincides with the onset of the production of specific anti-*P. vivax* antibodies.

DISCUSSION

Interferon-gamma is secreted by both innate and adaptive immune cells and is essential for controlling both liver and blood stages of the parasite (King and Lamb, 2015). In addition, IFN-γ drives tryptophan catabolism by inducing the production and enzymatic activity of IDO1. The catabolites -kynurenines- mediate immune tolerance and interfere with pathogen clearance (Mellor and Munn, 2008; Schmidt and Schultze, 2014). *Plasmodium* parasites can synthesize and acquire tryptophan; therefore it is assumed that the activation of the tryptophan degradation pathway only modulates the host response (Liu et al., 2006). In this study, we observed a marked elevation of IFN-γ with a concomitant induction of the tryptophan degradation pathway measured in the serum of naive individuals with a first documented malaria episode, compared to patients with previous malaria. Interestingly, the kynurenine elevation observed in patients with a first *P. vivax* infection was negatively correlated with blood parasite load. We also observed a lack of correlation between parasitemia and level of exposure, a phenomenon described by others (Gonçalves et al., 2014). This may indicate that the potent induction of IFN-γ could be controlling parasite load and simultaneously activating the tryptophan degradation pathway via IDO1 in malaria-naïve individuals, and that in non-naïve individuals in which adaptive immunity is active, the overall response shifts toward lower IFN-γ levels and anti-malaria antibodies, and is independent of parasite load. The shift to antibody-mediated responses and lower pro-inflammatory cytokines is a well-documented phenomenon in the establishment of parasite tolerance and control (Gonçalves et al., 2012; Chaves et al., 2016; Deroost et al., 2016; Longley et al., 2016; Pires et al., 2018).

Myeloid and antigen-presenting cells, as well as parenchymal cells are important for KYN production (Mellor and Munn, 2004). Tryptophan-derived metabolites produced by these cells mediate immune tolerance by inducing apoptosis of activated T cells and by the conversion of naive T cells into T regulatory cells (Tregs), via the activation of the aryl hydrocarbon receptor; tryptophan restriction induces the starvation response in T cells via the activation of the GCN2 kinase (Terness et al., 2002; Mellor and Munn, 2008; Mezrich et al., 2010; Nguyen et al., 2010). Our observations are consistent with recent studies that characterized serum metabolomics of *Plasmodium*-infected humans and non-human primates, and controlled human malaria trials (Woodberry et al., 2017; Vallejo et al., 2018). A recent study that used a controlled human *P. vivax* infection model showed an elevation of the plasmatic KYN/TRP ratio and an increase in activated regulatory T cells (Woodberry et al., 2017), and our findings corroborate these results. Interestingly, we observed a negative correlation between the KYN/TRP ratio and parasitemia, suggesting that the innate immune response is activated at a low blood parasitemia level, compared to what is observed the clinical experimental setting (Woodberry et al., 2017). Our data from a natural infection cohort also corroborates recent published data showing an increased activity of the tryptophan degradation pathway in subjects with no previous history of malaria disease compared to subjects with history of

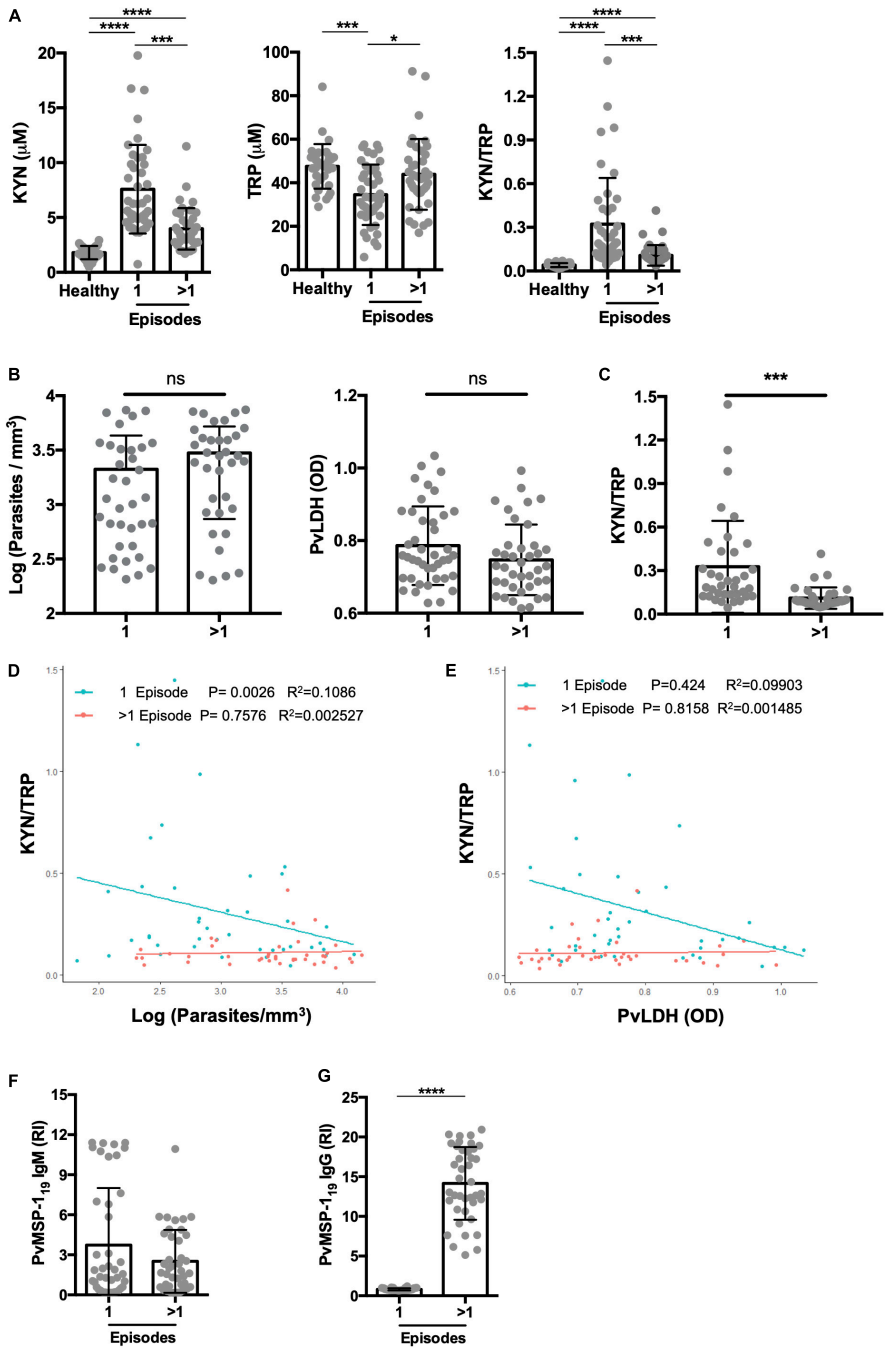

FIGURE 4 | Primary malaria significantly elevates kynurenine levels. **(A)** Kynurenine (KYN) and tryptophan (TRP) levels were quantified by HPLC in healthy endemic control group, malaria-naïve or first malaria episode (1 episode), and individuals with previous malaria history (>1 *P. vivax* malaria episode), before malaria treatment. Individuals with a first malaria (n = 35) and previous malaria exposure (n = 35) were paired by panel **(B)** blood parasite levels or *P.vivax* LDH levels and **(C)** KYN/TRP ratio was compared. Correlation analysis between **(D)** parasites levels quantified by thick blood film or **(E)** *P.vivax* LDH measured by ELISA and KYN/TRP ratio, grouped by patients with a first episode (1 episode, green) or >1 previous malaria episode (>1 episode, red). **(F,G)** *P.vivax* serology was performed using MSP-1_{19} antigen to confirm malaria exposure. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, student's t-test, ANOVA test with Tukey's *post hoc* test and Pearson's correlation).

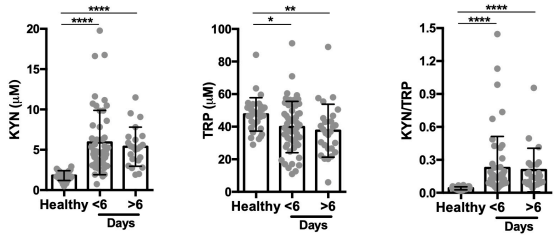

FIGURE 5 | Kynurenine levels are independent of the number of febrile days before treatment. Kynurenine (KYN) and tryptophan (TRP) levels were quantified by HPLC in the healthy control group (n = 34) and the *P. vivax*-infected patients, before malaria treatment (n = 75; six patients failed to respond number of febrile days on inclusion). Data stratified by number of days with fever as self-reported by patients. (*P < 0.05, **P < 0.01, ****P < 0.0001, ANOVA test with Tukey's *post hoc* test).

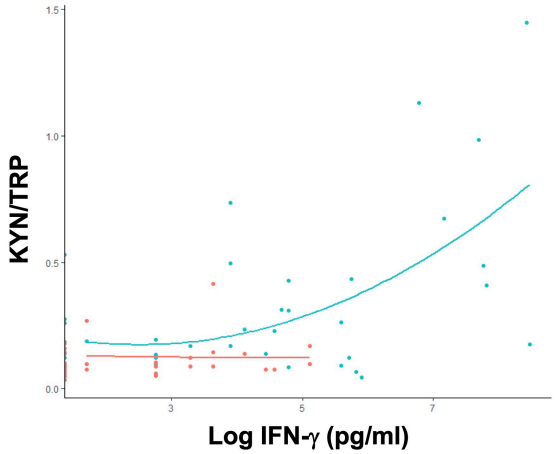

FIGURE 6 | IFN-γ positively modulates tryptophan catabolism in primary *Plasmodium* infection. Correlation between plasma KYN/TRP ratio and IFN-γ levels in untreated *P. vivax* malaria patients, grouped by the number of infections. Single (1 episode, green; n = 41) or multiple malaria (>1 episode, red; n = 40) (**Supplementary Table S2**).

previous exposure (Gardinassi et al., 2018; Cordy et al., 2019). We recently observed that acute *Plasmodium* infection induced an IFN-γ-driven increment in serum kynurenines that correlated with an elevation in the frequency of circulating FoxP3$^+$ T regulatory cells in a hypo-endemic Amazon region (Dos Santos et al., 2020). Our exploratory results grant a more detailed prospective analysis of the relationship between innate immunity, parasitemia, and the balance of pro and anti-inflammatory pathways in human malaria and its dynamics during multiple episodic infections. Of note, we also observed that the tryptophan degradation pathway activity was increased in non-malaria disease as well, suggesting that this pathway is activated by broad non-specific host responses to acute inflammation (Cordy et al., 2019). Previous studies that evaluated the role of the tryptophan degradation pathway in malaria have consistently shown that the activity of IDO1 is deleterious, and that it is associated with the development of disease complications (cerebral malaria, severe disease, hypotension, among others) (Sanni et al., 1998; Hansen et al., 2000; Tetsutani et al., 2007; Wang et al., 2010; Woodberry et al., 2017). Although it is clear that IDO1 plays a significant role in the pathogenesis of the disease and its complications, the bulk of the data is either from *in vitro* cell studies *or in vivo* mouse models. Accordingly, more human data are needed to clearly establish the relevance of these findings.

Our understanding of how, in the context of moderate to high exposure to parasite and host components in the bloodstream of malaria-infected subjects, the majority of clinical cases lack overt clinical disease and complications is still limited (Sinton, 1938; Crompton et al., 2014). In addition to evolutionary human adaptation to malaria parasites that confer host resistance (Júnior et al., 2010; Piel et al., 2013; Vale, 2018; Wang et al., 2018), multiple mechanisms have been proposed to explain malaria tolerance. In this study, we observed that IFN-γ and serum kynurenines increase to both control the infection and to avoid host immunopathology. Our observations in a natural infection cohort suggest that the potent activation of the tryptophan degradation pathway might be part of a host response that uses inflammation to minimize infection intensity while balancing resistance and tolerance (Sears et al., 2011; Ayres and Schneider, 2012). Accordingly, recent studies have demonstrated the role of IDO1 in the induction of host tolerance to inflammation (Bessede et al., 2014; Mondanelli et al., 2017). Our data proposes that a potent IFN-γ-induced IDO1 response might provide protection to naive hosts via parasite tolerance in the context of sub-optimal adaptive immunity, while subsequent tolerated exposures improve adaptive responses via increased antigen exposure and ensuing adaptive immunity, independent of parasite load.

Different clinical outcomes during *Plasmodium* infections have been mainly attributed to differences in host immune responses and parasite load (Garver et al., 2014). These factors, together with pre-existing immunity, either natural or vaccine-induced, can modify disease progression and its complications. Vaccination strategies that explore boosting IFN-γ responses might be essential in improving disease outcome, but their effects in human immune activation function are still poorly defined. In this regard, our observations may be relevant for anti-malaria vaccination strategies, since improving IFN-γ responses via vaccination will increase the activity of the tryptophan degradation pathway. In a recent tuberculosis vaccine trial that failed to show protection, baseline IDO activity negatively correlated with vaccine-specific IFN-γ responses, suggesting that IDO1 activity may impair the generation of T cell memory responses (Tanner et al., 2014). Our study was limited by several factors, including a relatively low number of participants, and the lack of a baseline metabolic assessment before the infecting episodes. We also observed a ∼50% of participant dropout in the study follow-up, limiting the interpretation of our follow-up preliminary findings. We also acknowledge that the measurement of the TRP/KYN ratio is a crude approximation (or proxy) of

the activity of the inducible tryptophan degradation pathway, since multiple factors, including diet and other concomitant infections can have an effect on serum TRP or KYN levels (Yeung et al., 2015). Our exploratory results grant a more detailed assessment of the tryptophan degradation pathway in a natural infection setting.

CONCLUSION

We observed that in the setting of natural infection, a first *P. vivax* infection produces an increased activity of the IFN-γ-driven tryptophan catabolism pathway, an effect that fades after subsequent exposures. Since IFN-γ-induced tryptophan degradation is a potent and conserved host response, exploring malaria disease tolerance mechanisms induced via tryptophan-derived catabolites can be important for understanding the balance between protective and pathogenic immune responses to the parasite, and may help further vaccination strategies and in the design and interpretation of controlled human infection studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Universidade Federal do Amazonas (UFAM). The patients or participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS, ML, and PL contributed conception and study design. RS, MC, and SL collected and analyzed the patient samples. LO, PN, IS, and FK provided reagents and performed malaria serology. EL assisted with HPLC analysis. AC provided reagents and cytokine analysis. RS, FC, CG, and PL analyzed the data and prepared the figures. RS, CG, and PL wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00400/full#supplementary-material

FIGURE S1 | Correlation between platelets, leukocytes cytokines and KYN/TRP ratio. Correlation between **(A)** platelets, **(B)** leukocytes, **(C,D)** cytokines and KYN/TRP ratio in malaria-infected patients before treatment (n = 81) was evaluated (Pearson's correlation).

FIGURE S2 | The tryptophan degradation pathway is elevated in acute vivax infection, decreases with subsequent malaria episodes, and is independent of the number of days with fever before treatment. Kynurenine (KYN) and tryptophan (TRP) levels were quantified in healthy control group (n = 34) and vivax infected patients before malaria treatment by HPLC. Data was then stratified by panel **(A)** number of malaria episodes or **(B)** number of days with fever before enrollment as self-reported by patients. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, Kruskal-Wallis test with Dunn's *post hoc* test).

FIGURE S3 | Vivax malaria blood stage compared with KYN/TRP ratio. At least hundred parasites or fifty microscopic fields were counted to estimate percentage of parasite blood stages as observed in thick smear stained with Hematoxylin and Eosin. Each column represents one vivax positive patient.

TABLE S1 | Hematological characteristic of patients with *Plasmodium vivax* infection.

TABLE S2 | Estimates of parameters based on gamma regression model for KYN/TRP ratio and Interferon-gamma.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Capítulo 4

MyD88-IDO1-AhR axis increases T regulatory cells in vivax malaria infection

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Abstract

Malaria remains a major public health problem in Brazil where *Plasmodium vivax* (Pv) is the predominant species. An inappropriate immune response to parasite infection is one of the primary drivers of malaria pathogenesis. Indoleamine 2,3-Dioxygenase (IDO) catabolizes tryptophan (TRP) an essential amino acid into an immunosuppressive metabolite kynurenine (KYN), which binds to the Aromatic hydrocarbon Receptor (AhR) promoting differentiation of naive $CD4^+$ T cells into (Tregs). In acute vivax malaria infection KYN/TRP ratio and Tregs were elevated, which were positively correlated. Also, IFN-γ and KYN/TRP ratio was higher in patients with first malaria episode compared to patients with previous malaria history. *In vitro* stimulation of PBMCs with Pv-infected erythrocyte (Pv-iE) lysate increased IDO1 expression in $CD14^+$ cells, KYN/TRP ratio and pro-inflammatory cytokines. Furthermore, in presence of IDO inhibitor a decrease in KYN/TRP ratio and Treg cell frequencies was observed upon iPv-RBC lysate stimulation. In addition, inhibition of MyD88 decreased KYN/TRP ratio, IDO1 enzyme expression. Inhibition of AhR with CH-223191 also decreased Treg frequencies and $CD4^+$ T cell proliferation. These data provide new insight into the mechanisms of the innate activation-induced tolerogenic phenotype in vivax infection, which can help the better understanding of processes involved in induction and resolution of chronic inflammation and tolerance.

Keywords: tryptophan, kynurenine, Plasmodium, Tregs, AhR, Indoleamine 2,3-Dioxygenase

Introduction

Plasmodium vivax is a less widely studied form of the malaria parasite that infects over 20 million people each year. Vivax is the predominant species in Brazil accounting for more than 80% of the malaria infections. Most of these episodes are confined to the Amazon region, with isolated cases occurring in other states of the country (1, 2). Additionally, dormant liver stage hypnozoites that can be activated several weeks or months or even years after primary infection possess serious challenge in eliminating vivax malaria (3). In vivax malaria infections the parasitic load is the lowest and complicated cases are rare. Several recent studies have reinforced the association between severe disease and death in vivax infections (4, 5). *Plasmodium* infection can produce a severe form of the disease, because of an insufficient immune response to eliminate the parasite or the inability of the host to control inflammation, resulting in immunopathology.

The human immune response that controls *Plasmodium* infection in the liver and blood stages of the parasite life cycle is composed by both pro- and anti-inflammatory programs. Enzyme Indoleamine 2,3-Dioxygenase (IDO) catabolizes tryptophan (TRP) an essential amino acid into an immunosuppressive metabolite kynurenine (KYN), which binds to the aromatic hydrocarbon receptor (AhR) promoting differentiation of naive $CD4^+$ T cells into regulatory $CD4^+FOXP3^+$ T cells (Tregs) and effector T cell suppression, anergy and death (6, 7). In malaria Tregs frequencies have been shown to increase in human and mouse infections, which have been shown to delay parasite clearance and decrease effector T cell function(8, 9). Tregs play an important role in maintaining a delicate balance between immunopathology and parasite clearance. Hence, in this study we investigated relationship between innate immune activation, tryptophan catabolism and regulatory T cells in human malaria infection.

Materials and Methods

Ethics Statement

This study was approved by the Universidade Federal do Amazonas (UFAM). All the study participants provided an informed consent prior to enrollment.

Patients samples

A cross-sectional study to characterize the immune response of patients with malaria (infected with *P. vivax*). The samples were collected at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD; located in Manaus, Amazonas state) from June/2015 to January/2018. The selection of patients occurred after the diagnosis of Malaria. To confirm the species, a revision of thick blood smear microscopy was performed, with differential parasitemia count (sexual and asexual stages) in 200 leukocytes.

Two groups were included in the study: patients positive for *P. vivax* (Pv) with first malaria (1) or more (>1) malaria episodes (n=23) and healthy endemic controls with a negative result of the thick blood smear for malaria (n=11). The signs and symptoms ranged from very mild to more advanced, however none of the patients presented severity of the disease. Primoinfected individuals were those who obtained a positive result at the time of inclusion and declared that they had not previously presented malaria (n=14). Regarding individuals with more episodes (n=9), they were classified according to the previous malaria report (being considered from 2 cases of malaria).

Blood cell count

About 10ml of venous blood were collected in EDTA tubes (BD Vacutainer). Initially, a complete blood count of the blood samples was performed using an automated hematological analyzer (Sysmex KX-21 ⓃR), acquiring parameters such as: hematocrit, hemoglobin, white blood cells, red blood cells, average corpuscular volume, average corpuscular hemoglobin, average corpuscular hemoglobin, platelet volume and Platelets. Then, plasma and PBMCs were separated from these samples, being frozen at -80ºC.

ELISA - Lactate Dehydrogenase (LDH)

Plasma samples from malaria-positive patients were analyzed by sandwich ELISA to detect *P. vivax* LDH (PvLDH), according to the author (Sousa et al., 2014). In summary, the ELISA plates were coated with polyclonal rabbit anti-PvLDH antibodies, subsequently the patients' plasma samples were added to detect free PvLDH from the cells accompanied by the addition. Soon after, the captured antigen was identified using a specific primary anti-mouse antibody and a secondary anti-mouse IgG antibody conjugated with fluorescence. Optical density of the negative samples and twice the standard deviation were used to calculate the cutt-offs to determine the reactive samples

PBMC separation and freezing

Blood samples from the endemic control group (without complaints of malaria in the last 30 days) and individuals infected with *P.vivax* (Pv), were initially subjected to separation of mononuclear cells from peripheral blood (PBMC), through isolation by gradient of density using Ficoll Plaque-Plus reagent (GE lifescience). The blood was

diluted in PBS-1X (1:1) and transferred to conical tubes containing Ficoll (2:1). The tubes were centrifuged for 35 minutes (3 acceleration, 0 deceleration, 450 RCF at 25ºC). After centrifugation, the haze containing PBMC was removed and these cells were washed with PBS-1X. The cell pellet was resuspended and samples from all individuals were frozen (cryopreservation tubes, 90% SFB + 10% DMSO) and stored in liquid nitrogen for further analysis.

***P. vivax*-infected red cell lysate separation and preparation (iPV-RBC)**

Blood was collected from 40 patients on alternate days, who had a mature stage of the parasite (trophozoite and schizont) in the blood, visualized on the thick blood smear. First the blood was washed 3 times with RPMI medium, then it was applied to a column with cellulose, to separate the red blood cells from the leukocytes and platelets. After filtration, the red cell pellet was washed 3 times with RPMI, then resuspended in RPMI medium and added to a tube containing 45% percoll (45% percoll, 45% filtered H2O and 10% RPMI 10X). They were centrifuged for 15 min (0 acceleration, 0 deceleration, 1500 RPM at 25ºC) to separate iPV-RBC. After centrifugation, the parasite-containing haze was removed and transferred to another tube. The pellet was washed with RPMI 3 times. To define the percentage of infected erythrocytes, a thick blood smear was performed from the pellet, where 500 erythrocytes were counted. Then the total number of erythrocytes was divided by the amount of parasitized erythrocytes, generating the percentage of iPV-RBC. In the preparation of the infected erythrocyte extract, a pool was made with the iPV-RBC of 5 to 10 patients in each microtube. This pool was then frozen at -80ºC. The microtubes containing the iPV-RBC were thawed and frozen again, this procedure was repeated three more times for total lysis of the red cells.

PBMC culture with iPV-RBC lysate

Initially, the PBMC was separated, then the cells were cultured in RPMI 1640 (Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Life Technologies, 24 and 96 well plates, for a maximum of 144 hours in an incubator at 37ºC with 5% CO_2. Proportions of two iPV-RBCs per PBMC (2:1) were used. The same concentration of uninfected erythrocyte lysate (uRBC) was used as a negative control. We used the indirect inhibitor of the IDO enzyme, 1-Methyl-D-tryptophan (1-MT) (Ref: 452483; Lot: MKBQ3449V; SIGMA-ALDRICH) in the concentration of 1mM, and Methanol was used as a negative control. For the blocking of the MyD88 adapter protein, the inhibitor peptide was synthesized that mimics the protein portion preventing homodimerization and interfering with its interaction at the MyD88-TIR domain (DRQIKIWFQNRRMKWKKRDVLPGT) and how to control the peptide (DRQIKIWFQNRRMKWKK) (GenOne, Rio de Janeiro, Brazil). To evaluate the AhR KYN binding receptor on T cells, we used an antagonist Aril hydrocarbon receptor (AhR) inhibitor CH-223191 at a concentration of 10mM, as a negative control, DMSO was used.

Cytokine quantification by CBA

The cytokine dosage of the patient's plasma samples, and cell culture supernatant was performed using the CBA technique (Cytometric Bead Array) by flow cytometry using the BD™ Human T_H1, T_H2 e T_H17 Kit, following the guidelines provided in the manufacturer. The quantified cytokines were: IL-2, IL-4, IL-6, IL-10, IL-17, TNF and IFN-γ. These samples were then passed on the FACSCanto II flow cytometers (Becton, Dickinson and Company, San Jose, CA, USA) of the Leônidas and Maria Deane

Institute (ILMD) - Fiocruz Amazônia and FACSVerse flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA) from the Instituto René Rachou (IRR) - Fiocruz Minas Gerais, where the PE fluorescence intensity of each complex reveals the concentration in pg/mL of each cytokine. To calculate the concentrations in pg/mL the FCAP Array™ software (V3.0.1) was used.

Identification and quantification of Tryptophan (TRP) and kynurenine (KYN) by HPLC

The patient plasma and cell culture supernatant (200μl) were subjected to treatment with 8% perchloric acid, for protein precipitation and the extraction of TRP and KYN, analyzed by High Performance Liquid Chromatography (HPLC) as previously described (Mallmann et al., 2018; Santos et al., 2020).

Cellular immunophenotyping by flow cytometry

Cultured and stimulated PBMCs were fixed and marked with monoclonal antibodies conjugated to fluorochromes (Supplementary Table 1). Then immunophenotypic characterization was performed, using flow cytometry. The following antibodies were used for the Treg panel in patient samples: CD4 (BV510), CD25 (BV605), FoxP3 (AF647), CD45-RA (PerCP-Cy5.5), CTLA-4 (PE-CF594), AhR (FITC), Ki67 (PE-Cy7). In this study, markings were performed for IDO1 and Tregs for cell culture using markers: CD4 (APC-H7), CD25 (BB515), FoxP3 (PE-Cy7), Ki-67 (PE), CTLA-4 (APC), CD45-RA (PerCP-Cy5.5), for the Treg panel; CD14 (PE), HLA-DR (APC), IDO1 (PerCP-eFluor 710) for the IDO1 panel. First the patient samples were thawed (cryogenic tubes were placed in the water bath for 25 seconds), cell cultures were removed in 72h and 144h. The cells were initially incubated with surface antibodies for

30 minutes. Then they were fixed, permeabilized and stained for intracellular markers for 45 minutes. For the readings, flow cytometers FACSCanto II (Becton, Dickinson and Company, San Jose, CA, USA) from Instituto Leônidas and Maria Deane (ILMD) - Fiocruz Amazônia and cytometer LSRFortessa (Becton, Dickinson and Company, San Jose, CA, USA) of the Instituto René Rachou (FIOCRUZ-MG). Data were analyzed using FlowJo™ 10.0.

RNA extraction

For the RNA extraction, the RNeasy Mini Kit (Qiagen) was used, following the supplier's protocol and to a final volume of 30 µl per sample. Then, the RNA yield for each sample was measured through Nanodrop. In order to prevent genomic DNA contamination, an additional step of residual DNA digestion was performed with DNase I (Ambion, 2U/ul) at 37ºC for 30 minutes, with further inactivation of the enzyme by incubating the samples at 75ºC for 10 minutes after adding EDTA 5 mM to preserve RNA integrity. In cDNA synthesis, once the total RNA samples were completely DNA-free, the following *in vitro* cDNA synthesis employed the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) supplemented with additional $MgCl_2$. The qPCR cycles were performed following the manufacturer's instruction. For quantitative PCR (qPCR) in order to measure the gene expression of IDO1 and IDO2, qPCR was carried out in QuantStudio 6 Flex Real-Time PCR System. GAPDH, ACTB and B2M were chosen as normalizing genes for this experiment. In short, on a 96-well plate, 1 ul of cDNA sample was added to each well containing qPCR mix, to a total of three replicates per sample. The qPCR mix was prepared with 5 ul of Power SYBR Green Master Mix (ThermoFisher), 0.2 ul of each primer (forward and reverse) and 3.6 ul of DEPC-treated water per reaction. Additionally, the plates also contained three negative

control replicates for each gene analyzed. The primers were obtained with Origene and are described on the **Supplementary table 2**.

Statistical Analysis

Statistical analyzes were performed using the GraphPadPrism software version 8 (GraphPad Software, La Jolla, CA, United States). Our data were presented in a non-normal distribution and, therefore, non-parametric tests, ANOVA or Test t Kruskal-Wallis or Mann-Whitney were performed. For the correlation, the variables were determined using Pearson's correlation coefficient. For all tests, p values <0.05 were considered. In the flow cytometry analysis, the FlowJo program (version 10.0) was used.

Results

Demographic and Hematological characteristics

Table 1 summarizes the demographic and hematological data of endemic healthy controls (n=11) and acute *Plasmodium vivax* (Pv) patients (n=23). Patients with malaria infection were divided into two groups, first malaria infection (1 episode, n=14) or with previous plasmodium infection (>1 episode, n=9) as self-reported by study participants. There was a predominance of males in both the study groups and the mean age of study participants was 38 years. The median parasitic load estimated by thick blood smear was 3083/mm3 (IQR, 533-2556) in Pv patients. Individuals with previous malaria infection or episodes had twice the number of parasites/mm3 compared to the first-time malaria patients. We observed that malaria infection induces significant hematological changes that include a reduction in peripheral leukocytes and platelets (Table 1).

Malaria infection elevates Tregs cells and AhR in patients

We assessed the activity of the tryptophan catabolism pathway by measuring kynurenines, tryptophan, and the KYN/TRP ratio in plasma, a marker for overall tryptophan catabolism of the kynurenine pathway. We observed a significant increase in both serum KYN levels and in the KYN/TRP ratio in acute disease at recruitment, before starting anti-malaria treatment (Figure 1A).

Next, we investigate if there are any differences between the *P. vivax*-infected group and endemic healthy controls or Pv patients with previous malaria episode (Episode >1) and patients with first-malaria infection (Episode 1) with respect to T cell regulation and the levels of Treg cell populations. Peripheral blood mononuclear cells (PBMCs) were analyzed directly without stimulation (ex vivo), we observed no differences in

frequency of $CD4^+$ and $CD4^+CD25^+FoxP3^-$ cell subsets between endemic controls and acute malaria patients (Figure 1B and 1C). However, a significant increase of regulatory $CD4^+CD25^+Foxp3^+$ T cells was observed in acute malaria infection (Figure 1D). Additionally, we observed an increase in expression of Ki-67 a proliferation marker on $CD4^+$, $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+$ T lymphocytes in acute disease (Figure 1B-D). An increase in intracellular AhR expressing lymphocyte subsets was observed in acute malaria patients. However, no differences between control and disease patients, on the expression of CD45-RA a naïve T cell marker was observed in the T cell subsets (Figure 1B-D). A positive correlation between classical regulatory $CD4^+CD25^+Foxp3^+$ T cells and KYN/TRP ratio was observed in acute Pv infections, accompanied by an overall increase in plasma IL-6, IFN-γ and IL-10 cytokines (Figure 1G and 1H).

A further analysis of Pv-infected individuals on the basis of the number of malaria episodes, we observed an elevated KYN/TRP ratio in first-time malaria infection or episode (Figure 2A). $CD4^+$ cell percentages did not differ between the two malaria subgroups; however, we observed an increase in $CD4^+$ T cell proliferation in the first-episode malaria group (Figure 2B). Overall expression of Ki67 in non-classical Tregs was similar patient malaria patient group. Expression of AhR and CTLA-4 in non-classical $CD4^+CD25^+FoxP3^-$ Treg significant increase in first-time malaria individuals (Figure 2C). Interestingly, a significant elevation in percentage of peripheral $CD4^+CD25^+FoxP3^+$ Treg cells was observed in first-time malaria infected patients (Figure 2D). Individuals with >1 malaria episode had higher levels of parasitemia and PvLDH, compared to patients with first-time malaria. Patients with first malaria infection

had a significant increase in IFN-γ, while patients with previous malaria infection had an increase in IL-6 and IL-10 (Figure 2G and Table 2).

In vitro Plasmodium vivax stimulation of PBMCs increases tryptophan catabolism to kynurenine

We evaluated whether *P. vivax* antigens can induce kynurenine production in vitro, peripheral blood mononuclear cells (PBMCs) from six healthy malaria-naive donors were stimulated with RPMI1640+10% FCS or negative control, uninfected red blood cells (uRBCs), or *P. vivax* infected RBC lysate (iPv-RBC).

Initially, we stimulated PBMCs with iPv-RBC to define the ideal parasite to PBMC ratio, and time for assessing the KYN/TRP ratio. An 2:1 ratio (iPV-RBC:PBMC) showed a better result than the other ratio with an increase in KYN/TRP ratios (Supplementary Figure 1A). Also, we observed a gradual increase in KYN/TRP ratio, compared to the negative control or uRBC groups (Supplementary figure 1B). At 3- and 6-days post stimulation of iPV-RBCs, KYN/TRP ratio was elevated in cell culture supernatant (Figure 3A). We observed at day 3 a decrease in %$CD14^+$HLA-DR^+IDO1^+ and increase in %$CD14^-$HLA-DR^+IDO1^+ cell population in iPv-RBC stimulated PBMCs compared to the controls. Whereas a significant increase in IDO1 MFI was observed in $CD14^+$HLA-DR^+ and $CD14^-$HLA-DR^+ cell population upon iPv-RBC stimulation. iPv-RBC stimulation significantly increased IDO1 gene expression on day 3 post stimulation that reduced on day 6. Whereas IDO2 gene expression was not significantly altered upon stimulation. To evaluate role of innate activation via pattern recognition receptors, we stimulated PBMC in presence of MyD88 inhibitory peptide or control peptide. Upon MyD88 inhibition of iPv-RBC stimulated PBMCs, we found a decrease in KYN/TRP

ratio on day 3 and 6. Also, we observed an overall decrease in IDO1 MFI upon MyD88 inhibition.

IDO-AhR is essential for T regulatory cell expansion

Next, we evaluate effect of in vitro stimulation of PBMCs with iPv-RBCs and, the interaction between the T cell compartment and kynurenines. *In vitro* stimulation of PBMCs by iPv-RBCs increased pro-inflammatory cytokines on day 3 and 6 (Figure 5A). We also found a significant increase in $CD4^+CD25^+FoxP3^+$ Tregs 6 days post stimulation with iPv-RBC compared to RPMI1640+10%FCS or uRBC (Figure 5B and C). Elevated Tregs also had an increase in proliferation marker Ki-67 and activation marker CTLA-4, and a decrease in CD45-RA a naïve T cell marker (Figure 5C). In addition, a significant increase in $CD4^+CD25^+Foxp3^-$ cells upon iPv-RBC stimulation was observed, accompanied by an increase of Ki-67 and CTLA-4 markers compared to the uRBC group (Figure 5D). Similarly, an increase in $CD4^+Ki\text{-}67^+$ and $CD4^+CTLA4^+$ T cells was observed in iPv-RBC compared to uRBC stimulation.

To characterize the activity of the IDO enzyme and its influence on the responses of KYN and Treg cells, we added to the cell culture a competitive inhibitor of the IDO enzyme, 1-Methyl-D-tryptophan (1-MT). By inhibiting IDO, there was a significant reduction in KYN in iPV-RBC stimulated cells compared to the control group (Figure 6A). Also, enzyme inhibition significantly reduced levels of cytokines IL-4, TNF and IFN-y, showing a clear reduction in 6 days post stimulation. Likewise, we observed a reduction in the populations of $CD4^+CD25^+FoxP3^-$ T cells and $CD4^+CD25^+FoxP3^+$ Tregs by inhibiting IDO, which was accompanied by reduction in the proliferation (Ki-67) and regulatory signaling (CTLA-4) of these cells at 6 days. However, inhibition of IDO did not influence the naive $CD4^+$ T cells expressing CD45-RA (Figure 6D and 6E).

The analysis also observed an reduction in $CD4^+$ T cells expressing Ki-67 and CTLA-4 markers.

We next tested the hypothesis that iPV-RBC induced increase of KYN mediates expansion of Tregs cells via the aryl hydrocarbon receptor (AhR). Therefore, we used an AhR antagonist inhibitor (CH-223191) to evaluate this response. We observed that by inhibiting AhR there was a reduction of KYN/TRP ratio in culture with iPV-RBC that was significant in 6 days (Figure 7A). In general, AhR block significantly increased expression of several pro-inflammatory cytokines IL-2, IL-4, IL-17, TNF and IFN-γ at 3- and 6-days post stimulation. Whereas we observed a reduction in IL-10 (Figure 7B). Interestingly, the frequency of $CD4^+CD25^+FoxP3^+$ Treg cells was reduced by inhibiting AhR, although there were no differences in Ki-67 and CTLA-4 expression on Tregs (Figure 7C). Our results showed an increase in $CD4^+CD25^+FoxP3^-$ T cells by inhibiting AhR, accompanied by a reduction in proliferation (Ki-67) at 6 days and a decrease in CD45-RA at 3 days (Figure 7D). We observed an increase in $CD4^+$ T cells in 6 days, contrary to expectations the proliferation (Ki-67) of these cells was reduced, we also identified a reduction in CTLA-4 signaling and a reduction in naive cells in3 days (Figure 7C). In summary, innate immune activation via MyD88 induces kynurenine pathway and increases Treg frequencies in Pv infection via AhR.

Discussion

Physiopathology of the disease is driven by changes in the host metabolism and immune response. T regulatory cells increase has been described in human and mice malaria infection (8), however mechanisms that lead to its increase and role of host metabolism is not completely understood in malaria infection. In this study, we observed an increase in $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) in acute vivax infection, and a positive correlation between Tregs and kynurenine a product of tryptophan catabolism. Moreover, the increase in Tregs and kynurenine was significantly higher in primary or first-time malaria infections compared to patients with previous malaria exposure. *In vitro* stimulation of healthy donor PBMCs with iPV-RBC we observed a direct activation of innate immune response via MyD88 increased tryptophan catabolism and elevated kynurenine levels via the IDO enzyme. This increase of kynurenine metabolites increased the tolerogenic Treg cell frequencies by direct activation of AhR. Overall, our data provide new insight into the mechanisms of the innate activation-induced tolerogenic phenotype in vivax infection, which can help the better understanding of the processes involved in induction and resolution of chronic inflammation and tolerance.

Myeloid and antigen-presenting cells, as well as parenchymal cells are important for KYN production (6). Tryptophan-derived metabolites produced by these cells mediate immune tolerance by inducing apoptosis of activated T cells and by the conversion of naive T cells into T regulatory cells (Tregs), via the activation of the AhR; tryptophan restriction induces the starvation response in T cells via the activation of the GCN2 kinase (7, 10-12). A recent study that used a controlled human *P. vivax* infection model showed an elevation of the plasmatic KYN/TRP ratio and an increase in activated Treg

cells (13). A positive correlation between Tregs and KYN/TRP ratio observed was observed in this study. These results corroborate with previous results from another Brazilian cohort (14). Also, a negative correlation between the KYN/TRP ratio and parasitemia suggests that the innate immune response is activated at a low blood parasitemia level, compare to what is observed the clinical experimental setting (13, 15). Our results confirm previous findings that Tregs increase in acute human vivax infections (16-18). Increase in Treg was higher in first-time vivax infections compared to individuals with previous malaria exposure, moreover this increase did not correlate with parasite load. Also, we did not observe positive correlation with parasite load as previously describe in vivax patients (16). But we observed an increase in AhR expressing Tregs in acute vivax infection.

I*n vitro* stimulation of PBMCs with *P. vivax* antigens increased proinflammatory cytokines, increased KYN/TRP ratio and induced Tregs (19, 20). MyD88 inhibition reduced KYN/TRP ratio and IDO1 enzyme expression. These results are in concordance that TLRs signalling via MyD88 is important for innate immune activation during malaria infection, (21) and might play a role in increasing IDO expression. Previously, *P. falciparum* was shown to induce Tregs *in vitro* independent of direct TCR stimulation but was dependent on IL-10 and TGF-β (20). Additionally, in other systems KYN binding to AhR was shown to convert T naive cells into Tregs (7). On the other hand, memory T cells were shown to convert into Treg cells upon stimulation with *P. falciparum* (19). We observed a decrease in CD45-RA positive cells upon vivax antigen stimulation. However, CD45-RA positive cell data was not consistent when IDO enzyme or AhR was blocked. Hence, further experiments are need to understand the role of naïve and memory cell compartment in induction of Treg cells upon vivax stimulation. Nevertheless, IDO and AhR inhibitors in PBMC stimulated with vivax

antigens efficiently reduced KYN/TRP ratio and Treg cell frequencies. Inhibition of IDO enzyme also reduced IFN-gamma levels, whereas inhibition of AhR elevated IFN-gamma levels. IFN-gamma has been shown to play an important role in IDO enzyme induction and tryptophan catabolism (15, 22). We believe that in addition to innate activation, AhR might also play role in the regulation of IDO enzyme in myeloid cells in vivax stimulated PBMCs (23, 24). Thus, blocking of AhR in vivax stimulated cells reduced KYN/TRP ratio even in the presence of high levels of IFN-gamma.

Overall, IDO blocking in vivo and AhR knockout mice experiments demonstrate an essential role of these molecules in reducing controlling parasitemia (25-28). Although it is clear that IDO and AhR plays a significant role in the pathogenesis of the disease and its complications, the bulk of the data is either from *in vitro* cell studies *or in vivo* mouse models. Additionally, more human data are needed to clearly establish the relevance of these findings. A further analysis of endogenous AhR ligands in necessary to understand its role in the clinical spectrum of malaria (27, 29, 30). Additionally, a recent report on increase in PD-1 Tregs during acute vivax infection (31) opens up fundamental question about their ability to control parasite load and reduce pathology in vivax infection. In summary, accelerated metabolism of TRP into KYN during acute phase of vivax infection promotes an immunosuppressive environment with an induction of Tregs. However, additional studies are needs to understand not only the molecular mechanisms involved in innate immune activation, AhR-ligand interaction and Treg induction but also its implication in impairing an efficient immune response.

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Figure Legends

Figure 1: Positive correlation between peripheral $CD4^+CD25^+FoxP3^+$ regulatory T cells and kynurenine in patients acutely infected with *P. vivax*

Plasma **(A)** kynurenine (KYN) and tryptophan (TRP) levels were quantified by HPLC in an endemic control group (n=11) and symptomatic patients with *P. vivax* infection confirmed by light microscopy (n=23). Characterization of (B) $CD4^+$, (C) $CD4^+CD25^+FoxP3^-$ and (D) $CD4^+CD25^+FoxP3^+$ lymphocyte population in the peripheral blood of endemic controls and patients acutely infected with *P. vivax*. **(E)** Blood parasite levels estimated by blood smear and **(F)** *P. vivax* LDH measured by sandwich ELISA. **(G)** Correlation between the KYN/TRP ratio and $CD4^+CD25^+FoxP3^+$ Tregs cells. **(H)** Plasma cytokines were measured in endemic controls and *P. vivax* patients by cytometric bead assay. Asterisks (*) alongside cytokines denoted the p value for comparison between control and the *P. vivax* patients. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). The data are shown as mean ± SD. Student's *t*-test and Pearson's correlation.

Figure 2: First-time malaria significantly elevates plasma kynurenine and peripheral $CD4^+CD25^+FoxP3^+$ regulatory T cells.

Plasma **(A)** kynurenine (KYN) and tryptophan (TRP) levels were quantified by HPLC and compared between patients with first-time malaria or with first episode (n=14) and those with previous history of (n=9) *P. vivax*-infection (>1 episode). Characterization of **(B)** $CD4^+$, **(C)** $CD4^+CD25^+FoxP3^-$ and **(D)** $CD4^+CD25^+FoxP3^+$ lymphocyte population in the peripheral blood of endemic controls and patients acutely infected with *P. vivax*. **(E)** Blood parasite levels estimated by blood smear and **(F)** plasma *P. vivax* LDH

measured by sandwich ELISA. **(G)** Plasma cytokines were measured by cytometric bead assay and compared between patients with first malaria episode or individuals with more than one malaria episode. Map is based on the mean, asterisks (*) alongside the cytokines denoted the p value for comparison between the two study groups. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). The data are shown as mean ± SD. Student's *t*-test and Pearson's correlation.

Figure 3: In vitro stimulation of healthy donor PBMC with Plasmodium vivax-infected erythrocytes (iPV-RBC) lysate elevates IDO expression.

In vitro healthy malaria naïve donor PBMCs were stimulated with freeze thaw lysate of *Plasmodium vivax*-infected erythrocytes lysate (iPv-RBC) in a 1:2 ratio. **(A)** Cell culture supernatants were used to quantify TRP and KYN levels by HPLC, 72 hours and 144 hours post stimulation. **(B and C)** Cells were stained 72hour post stimulation for CD14 and HLA-DR expression, permeabilized, and then stained for intracellular IDO1 protein. Stained cell was analyzed by flowcytometry, data is represented as percentage or mean fluorescence intensity (MFI). Alternatively, **(D)** IDO1 and **(E)** IDO2 gene expression was assessed by RT-qPCR. The data are shown as mean + SD of one representative donor PBMC stimulated in quadruplicate. Experiment was performed with five independent malaria naïve donors. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and not significant - NS. Two-way ANOVA test with Tukey's post hoc test for multiple comparisons or Student's *t*-test was used to calculate p value.

Figure 4: MyD88 adaptor protein driven innate activation increases tryptophan catabolism upon Vivax stimulation

In vitro healthy malaria naïve donor PBMCs were stimulated with freeze thaw lysate of *Plasmodium vivax*-infected erythrocytes lysate (iPv-RBC) in a 1:2 ratio. **(A)** Cell culture supernatants were used to quantify TRP and KYN levels by HPLC, 72 hours and 144 hours post stimulation. **(B and C)** Cells were stained 72-hours post stimulation in presence of control peptide or inhibitory peptide (26 amino acid) that blocks MyD88 signaling by inhibiting its homodimerization for CD14 and HLA-DR expression, permeabilized, and then stained for intracellular IDO1 protein. Stained cell was analyzed by flowcytometry, data is represented as percentage or mean fluorescence intensity (MFI). The data are shown as mean + SD of one representative donor PBMC stimulated in quadruplicate. Experiment was performed with three independent malaria naïve donors. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and not significant - NS. Two-way ANOVA test with Tukey's post-hoc test or Kruskal-Wallis test followed by post-hoc Dunn's multiple comparisons test was used to calculate the p value.

Figure 5: Treg cells are induced and activated by Plasmodium vivax-infected erythrocytes lysate (iPv-RBC)

Healthy malaria naïve donor PBMCs were cultured for up to 6 days or 144 hours treated with RPMI1640 with 10% growth medium as negative control, uninfected red blood cells (uRBCs), or iPv-RBC (1 PBMC: 2 parasite ratios, equivalent to $2x10^6$ parasites/mL). Cells were stained for T cell markers and the percentage of $CD4^+$, $CD4^+CD25^+FoxP3^-$ and $CD4^+CD25^+FoxP3^+$ cells were determined ex vivo, after 3 and 6 days of culture. **(A)** Cytokines in supernatant of *in vitro* stimulated PBMCs were measured by cytokine bead assay. Heatmap was plotted using the means for each

cytokine and stimulation. **(B)** Representative dot plot shows the $CD4^+CD25^+FoxP3^+$ cell population. **(C)** $CD4^+CD25^+FoxP3^+$, **(D)** $CD4^+CD25^+FoxP3^-$ and **(E)** $CD4^+$ cell population with proliferation, activation and memory markers. Two-way ANOVA test with Tukey's post-hoc test or Mann-Whitney test was used to calculate the p value. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, not significant - ns.

Figure 6: IDO enzyme is essential for generation of $CD4^+CD25^+FoxP3^+$ Regulatory T cells

Healthy malaria naïve donor PBMCs were cultured for up to 6 days or 144 hours stimulated with uninfected red blood cells (uRBCs) or iPv-RBC (1 PBMC: 2 parasite ratios, equivalent to 2 × 106 parasites/mL), in presence of methanol (MET,vehicle) or 1-Methyl Tryptophan (1-MT) a potent and specific inhibitor of Indoleamine 2,3-dioxygenase (IDO) enzymatic activity. Cells were stained for T cell markers and the percentage of $CD4^+CD25^+FoxP3^+$, $CD4^+CD25^+FoxP3^-$ and $CD4^+$ cells were determined ex vivo, after 3 and 6 days of culture. **(A)** Kynurenine levels quantified by HPLC in supernatants. **(B)** Cytokines in supernatant of *in vitro* stimulated PBMCs were measured by cytokine bead assay. Heatmap was plotted using the means for each cytokine and stimulation. **(C)** $CD4^+CD25^+FOXP3^+$, **(D)** $CD4^+CD25^+FOXP3^-$ and **(E)** $CD4^+$ cell population with proliferation, activation and memory markers. Two-way ANOVA test with Tukey's post-hoc test was used to calculate the p value. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, not significant - ns.

Figure 7: *P. vivax* mediates kynurenine increase and generation of FoxP3$^+$ Tregs in an AHR-dependent manner.

Healthy malaria naïve donor PBMCs were cultured for up to 6 days or 144 hours stimulated with uninfected red blood cells (uRBCs) or iPv-RBC (1 PBMC: 2 parasite ratios, equivalent to 2x10^6 parasites/mL), in presence of DMSO (vehicle) or CH-223191 a potent and specific aryl hydrocarbon receptor (AhR) antagonist. Cells were stained for T cell markers and the percentage of CD4$^+$CD25$^+$FoxP3$^+$, CD4$^+$CD25$^+$FoxP3$^-$ and CD4+ cells were determined ex vivo, after 3 and 6 days of culture. **(A)** Kynurenine and tryptophan levels quantified by HPLC in supernatants. **(B)** Cytokines in supernatant of *in vitro* stimulated PBMCs were measured by cytokine bead assay. Heatmap was plotted using the means for each cytokine and stimulation. **(C)** CD4$^+$CD25$^+$FOXP3$^+$, **(D)** CD4$^+$CD25$^+$FoxP3$^-$ and **(E)** CD4$^+$ cell population with proliferation, activation and memory markers. Two-way ANOVA test with Tukey's post-hoc test was used to calculate the p value. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, not significant - ns.

Supplementary figure 1: In vitro stimulation of PBMC with iPv-RBC

In vitro culture experiment of PBMC only with and stimulated with uninfected erythrocyte lysate (uRBC) or with Pv-infected erythrocyte lysate. **(A)** Kynurenine (KYN) and Tryptophan (TRP) levels in culture supernatant for different concentrations of parasites (PBMC:iPV-RBC) in the ratios 1:0, 1:1, 1:2 and 2:1 **(B)** and quantify at different cell cultivation time 12, 24, 72 and 144 hours by HPLC. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, not significant - NS). ANOVA test.

Table 1: Patient clinical and demographic details

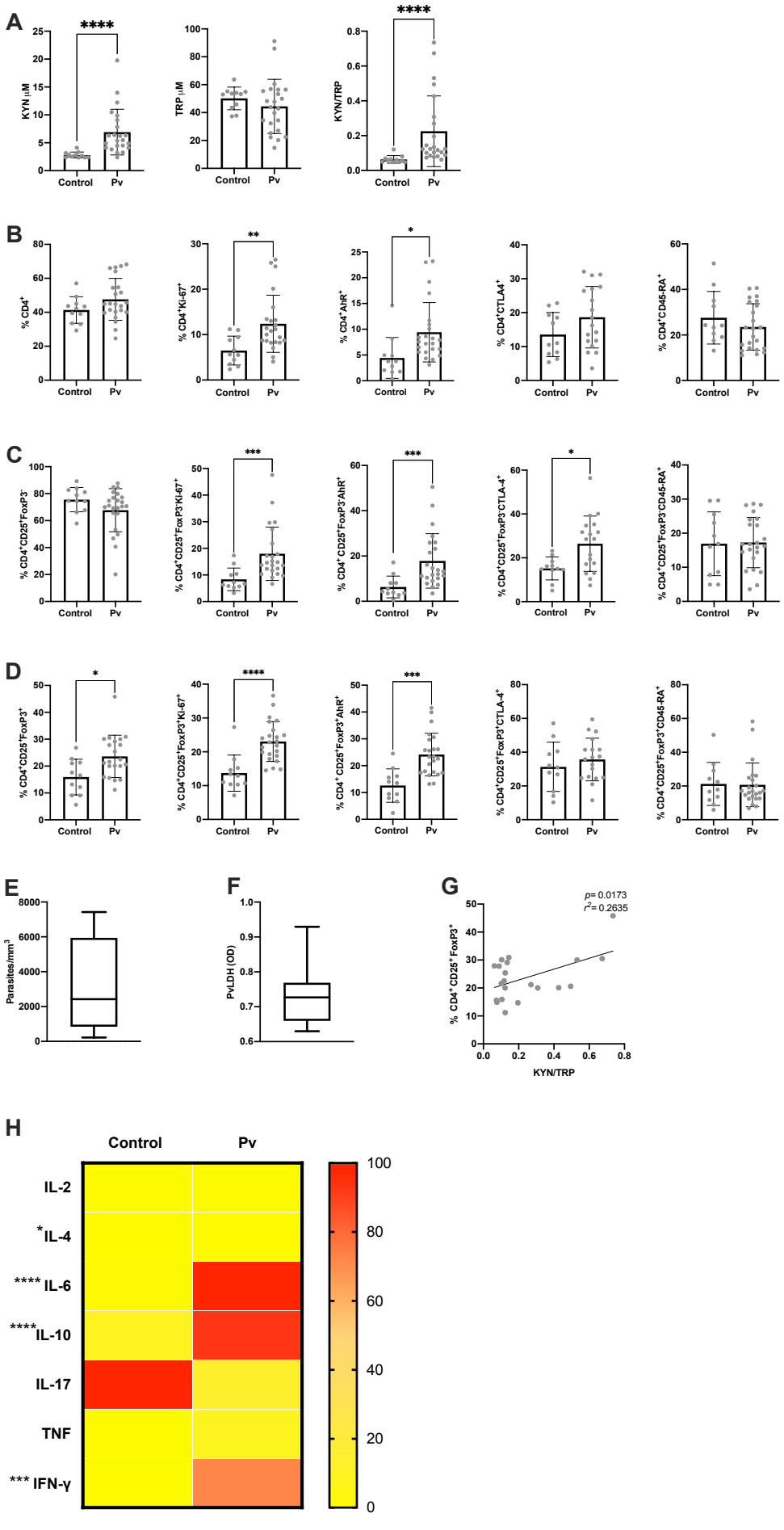
Demographic Characteristics		Endemic Control	*P. vivax* (n=23)			*p value*[AB]	*p value*[AC]	*p value*[AD]	*p value*[CD]
				Episodes					
		(n=11)[A]	Total (n=23)[B]	1 (n=14)[C]	>1 (n=9)[D]				
Gender	Female (%)	2 (18,2%)	7 (30,43%)	5 (35,7%)	2 (22,2%)				
Age, mean	(Years ± SD)	38.8 ± 12.2	38,6 ± 12.2	38.8 ± 12.2	38.9 ± 12.2				
Parasitemia, median	(Parasites/mm3. IQR)		3083 (533 - 2556)	2432 (598.8 - 2240)	4096 (937,9 – 2814)				
Haematological parameters (mean ± SD)									
Haematocrit (%)		43.3 ± 3.3	43.6 ± 5.5	41.2 ± 3.1	42.2 ± 8.2	>0.9999	0.5810	>0.9999	>0.9999
Haemoglobin (g/dL)		13.3 ± 1.2	13.2 ± 1.8	13.2 ± 1.1	13.2 ± 2.6	>0.9999	>0.9999	>0.9999	>0.9999
White blood cells (x 10^3/µL)		6.1 ± 1.4	4.5 ± 1.3	3.9 ± 1.1	5.2 ± 1.2	**0.0257**	**0.0014**	0.9073	0.0798
Red blood cells (x 10^6/µL)		4.8 ± 4.9	4.8 ± 6.5	4.7 ± 3.6	4.8 ± 9.6	>0.9999	>0.9999	>0.9999	>0.9999
Mean corpuscular volume (fL)		89.3 ± 4.5	85.7 ± 2.6	85.8 ± 2.6	90.8 ± 5.7	**0.0375**	**0.0228**	>0.9999	**0.0322**
Mean corpuscular haemoglobin (pg)		27.6 ± 1.4	28.4 ± 2.2	28.2 ± 2.4	28.9 ± 1.9	0.7062	0.9390	0.2369	>0.9999
Mean corpuscular haemoglobin concentration (g/dL)		31.1 ± 0.9	32.1 ± 1.8	32.2 ± 1.6	31.8 ± 0.8	0.0691	**0.0449**	0.2465	>0.9999
Mean platelet volume (fL)		9.3 ± 0.9	10.6 ± 1.4	10.9 ± 1.4	9.9 ± 1.1	0.0724	**0.0162**	0.8766	0.6997
Platelet (x 10^3/µL)		229 ± 48.6	96 ± 4.5	91 ± 41.1	105 ± 50.8	**<0.0001**	**<0.0001**	**0.0029**	>0.9999

1: First malaria; >1: malaria episodes

Table 2: Blood cytokine levels in vivax infected patients

Cytokines pg/mL (mean ± SD)	Endemic Control	*P. vivax*			*p value*^{AB}	*p value*^{AC}	*p value*^{AD}	*p value*^{CD}
			Episodes					
	(n=11)^{A}	Total (n=23)^{B}	1 (n=14)^{C}	>1 (n=9)^{D}				
IL-2	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.4	0.1 ± 0.2	0.0694	0.7813	0.3991	>0.9999
IL-4	0.1 ± 0.2	0.8 ± 1.1	0.8 ± 1.2	0.8 ± 1.1	**0.0255**	0.1416	0.1761	>0.9999
IL-6	0.4 ± 0.3	126.9 ± 444.4	22.2 ± 27.9	289.7 ± 702.1	**<0.0001**	**0.0004**	**0.0007**	>0.9999
IL-10	0.1 ± 0.1	118.7 ± 286.3	33.6 ± 23.7	251.3 ± 438.3	**<0.0001**	**0.0020**	**<0.0001**	0.9871
IL-17	51.5 ± 69.3	17.9 ± 24.1	18.5 ± 21.9	16.9 ± 28.5	0.1433	>0.9999	0.2978	>0.9999
TNF	0.0 ± 0.0	0.48 ± 2.1	0.0 ± 0.0	1.2 ± 3.3	0.5349	>0.9999	0.3565	0.3042
IFN-γ	0.0 ± 0.0	91.5 ± 264.2	187.9 ± 389.0	25.1 ± 30.7	**0.0002**	**0.0022**	0.0724	**0.0173**

1: First malaria; >1: malaria episodes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TRP µM
KYN/TRP
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Pv
B
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% CD4+AhR+
% CD4+CTLA4+
% CD4+CD45-RA+
C
% CD4+CD25+FoxP3-
% CD4+CD25+FoxP3-Ki-67+
% CD4+ CD25+FoxP3-AhR+
% CD4+CD25+FoxP3-CTLA-4+
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% CD4+CD25+FoxP3+CTLA-4+
% CD4+CD25+FoxP3+CD45-RA+
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Parasites/mm3
F
PvLDH (OD)
G
p= 0.0173
r2= 0.2635
% CD4+ CD25+ FoxP3+
KYN/TRP
H
Control
Pv
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*IL-4
**** IL-6
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IL-17
TNF
*** IFN-γ
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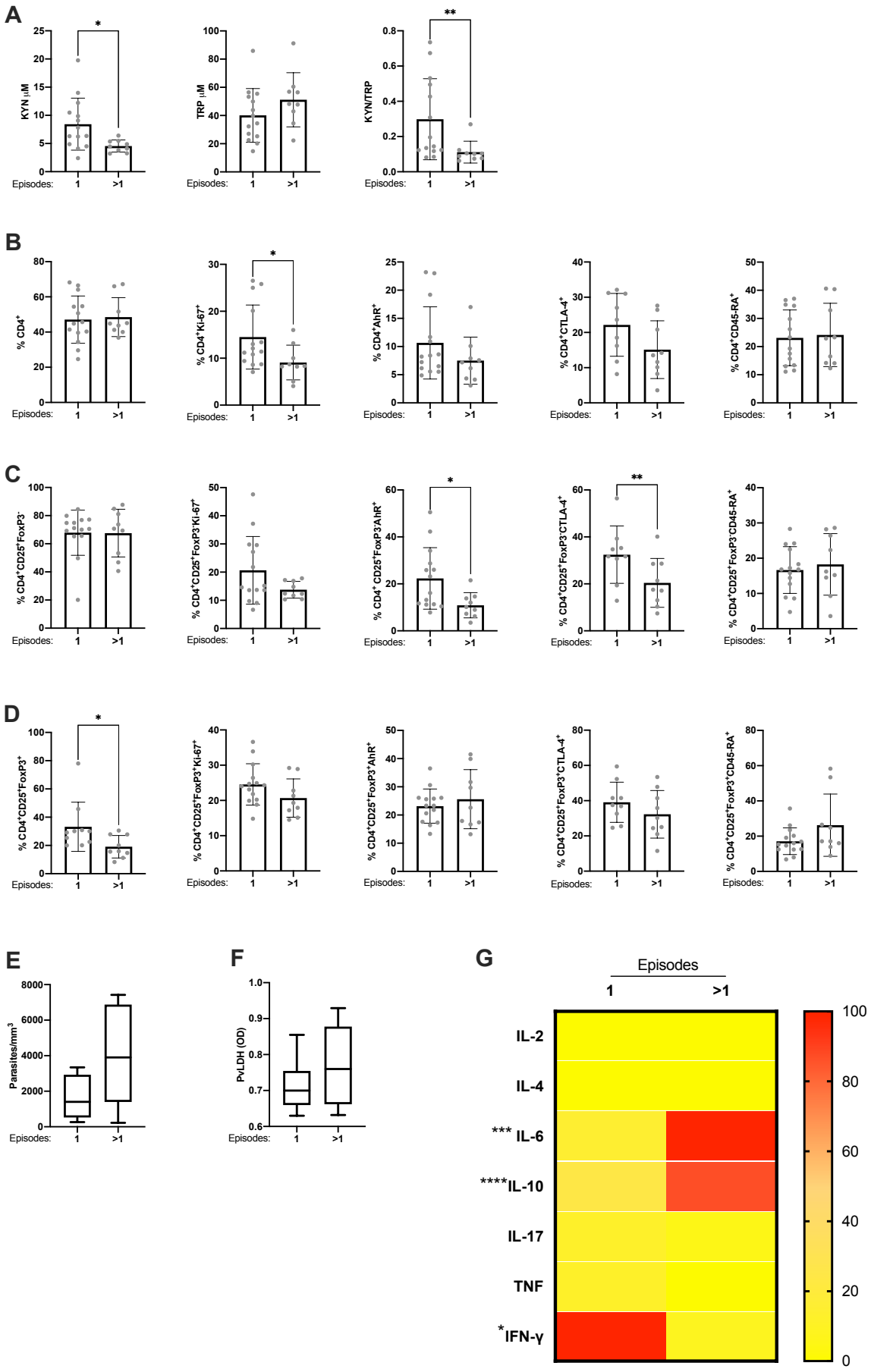
80
60
40
20
0

Figure 1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TRP µM
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C
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Episodes
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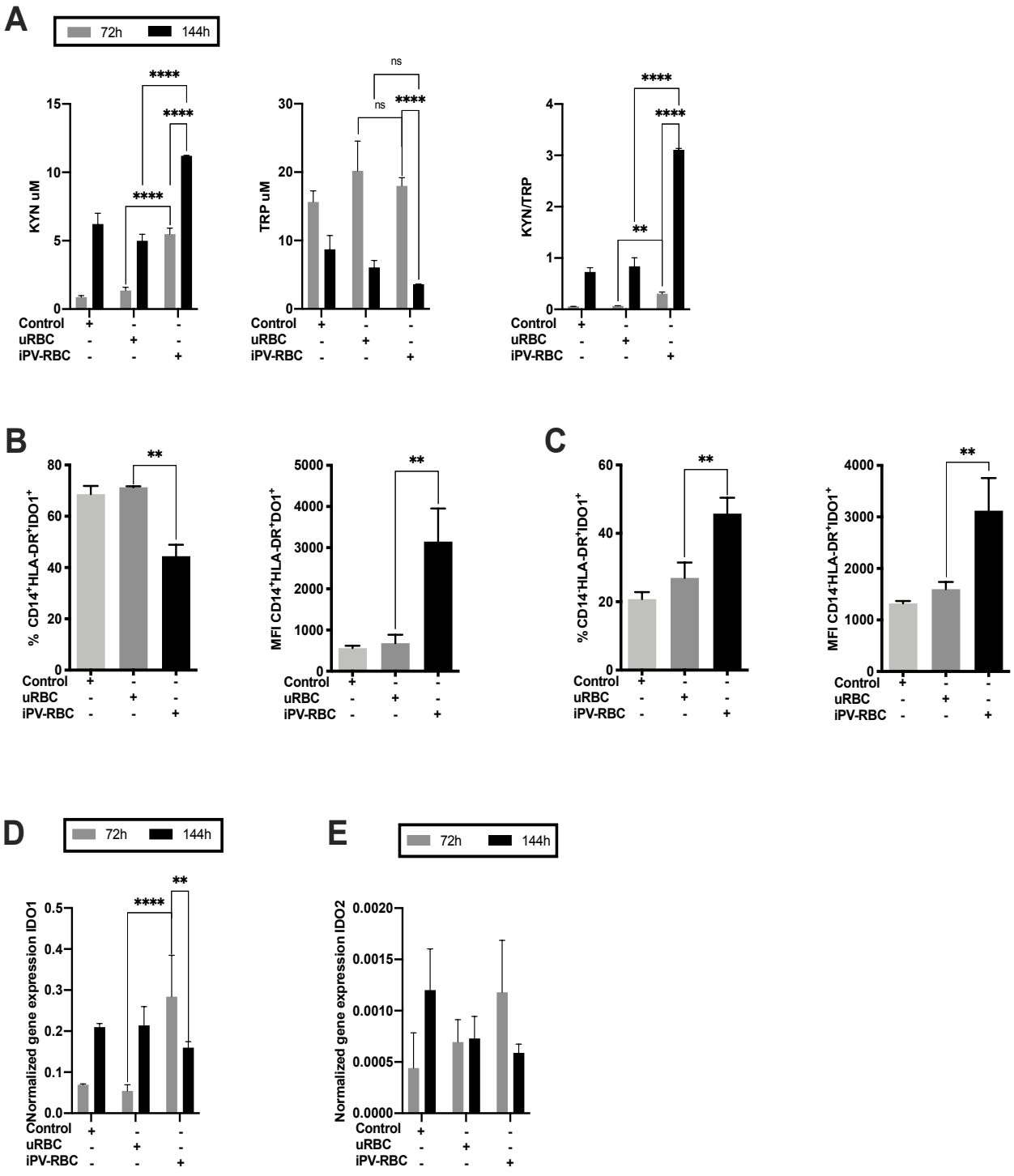
Figure 2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MFI CD14+HLA-DR+DO1+
C
% CD14+HLA-DR+IDO1+
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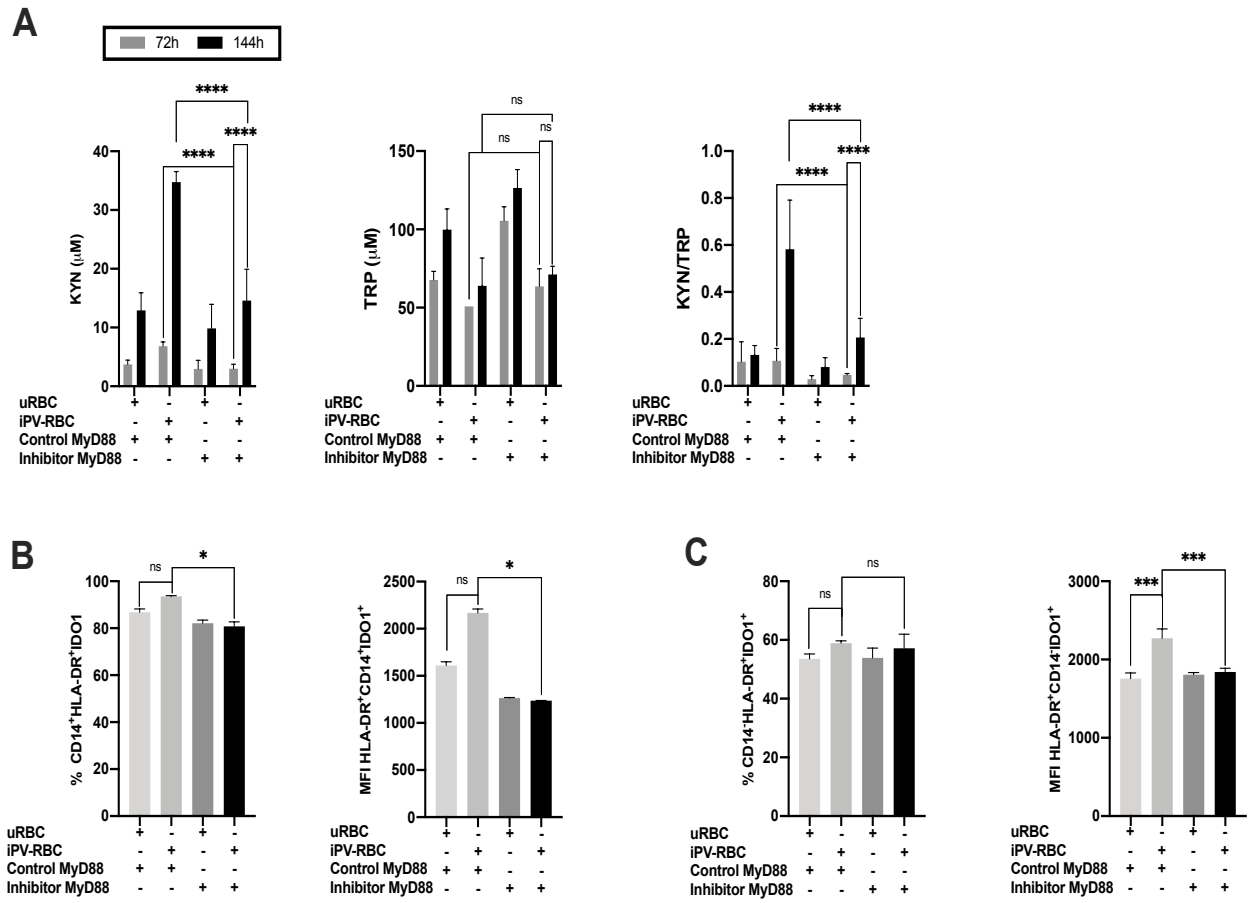
D
Normalized gene expression IDO1
E
Normalized gene expression IDO2

Figure 3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144h
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TRP (μM)
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uRBC
iPV-RBC
Control MyD88
Inhibitor MyD88
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MFI HLA-DR+CD14+IDO1+
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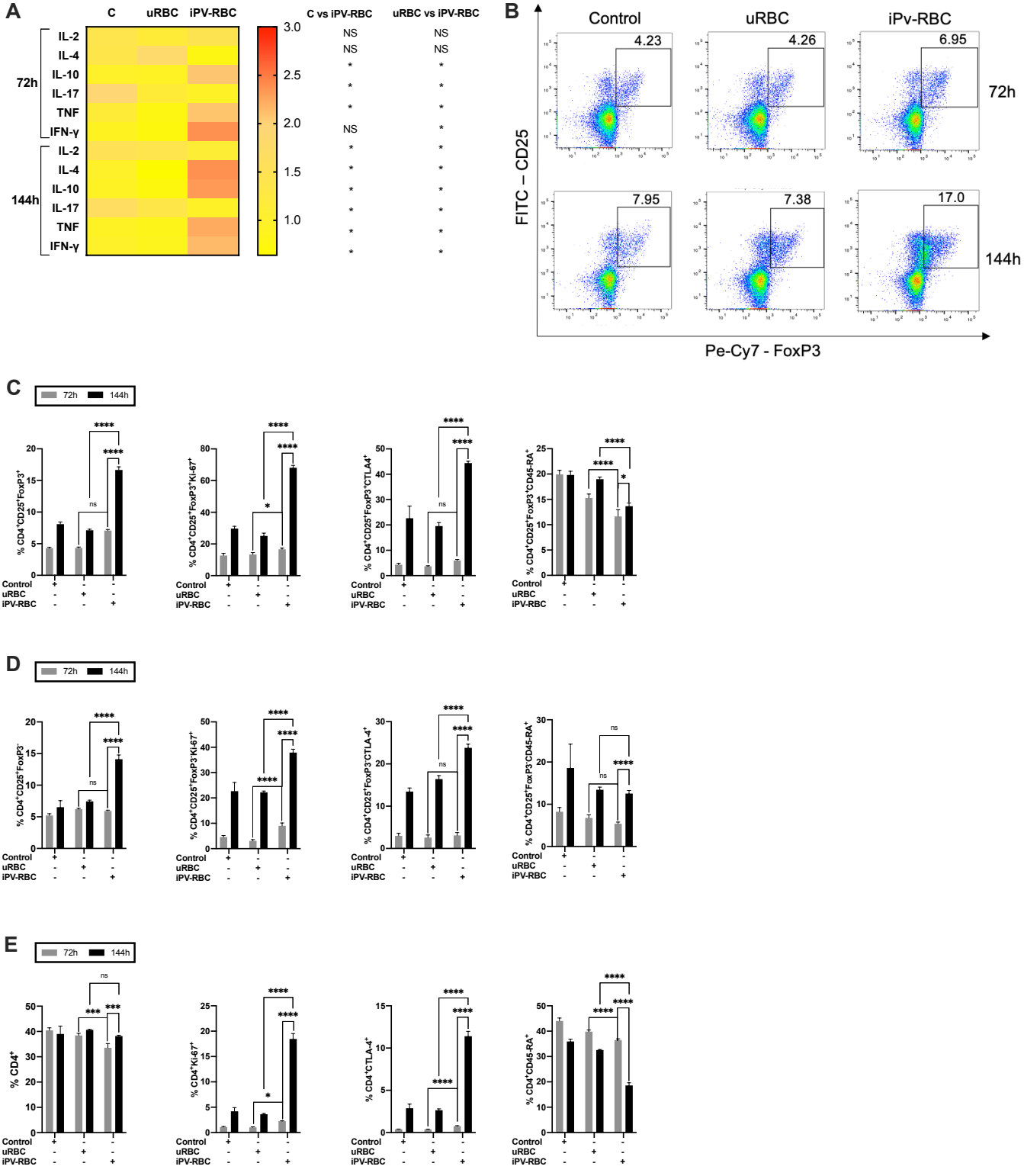
MFI HLA-DR+CD14+IDO1+
ns
*
***
****

Figure 4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144h
IL-2
IL-4
IL-10
IL-17
TNF
IFN-γ
3.0
2.5
2.0
1.5
1.0
NS
B
Control
uRBC
iPv-RBC
4.23
4.26
6.95
7.95
7.38
17.0
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Pe-Cy7 - FoxP3
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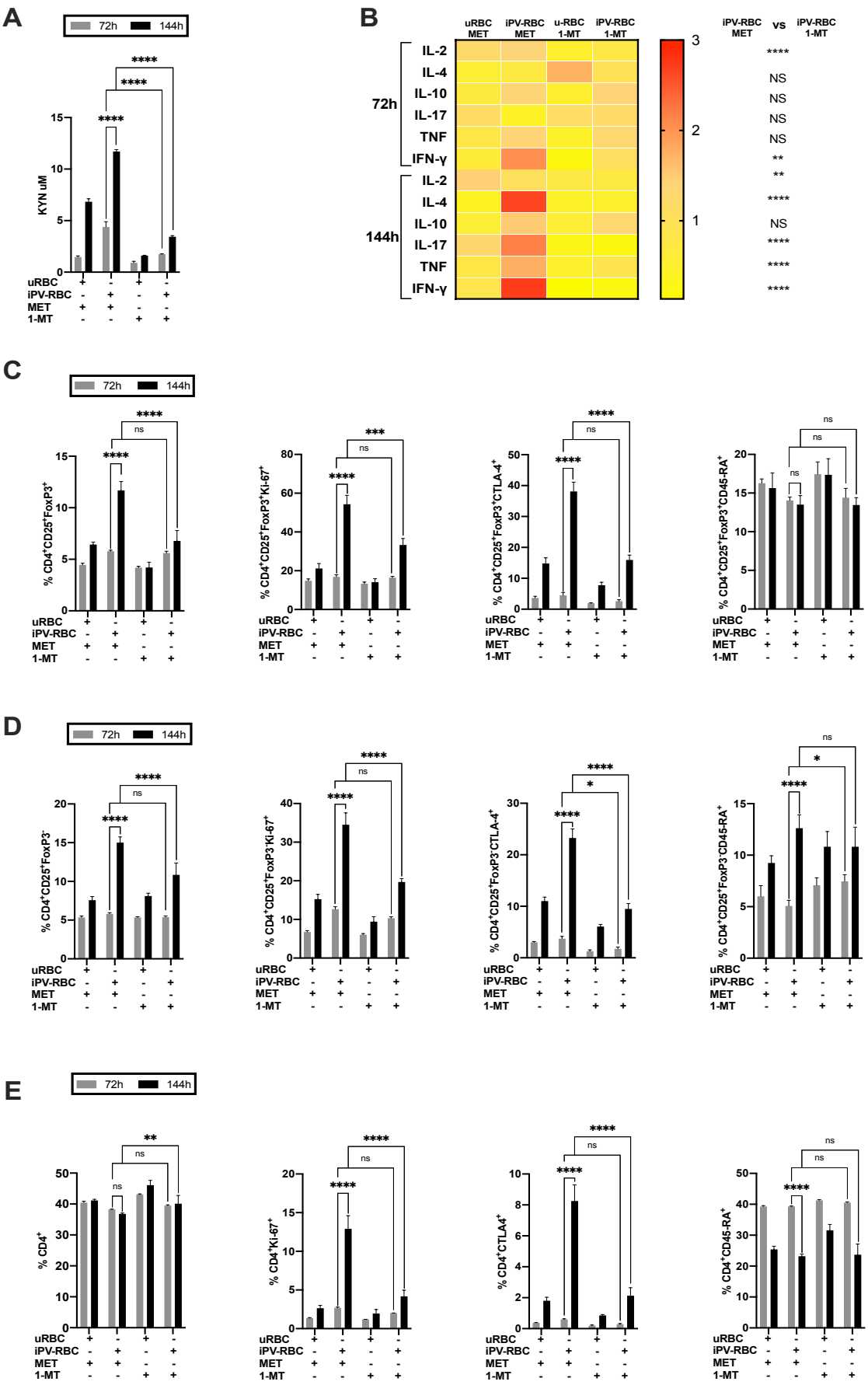
D
E
72h
144h
Control
uRBC
iPV-RBC

Figure 5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iPV-RBC 1-MT
72h
144h
IL-2
IL-4
IL-10
IL-17
TNF
IFN-γ
iPV-RBC MET vs iPV-RBC 1-MT
C
% CD4+CD25+FoxP3+
% CD4+CD25+FoxP3+Ki-67+
% CD4+CD25+FoxP3+CTLA-4+
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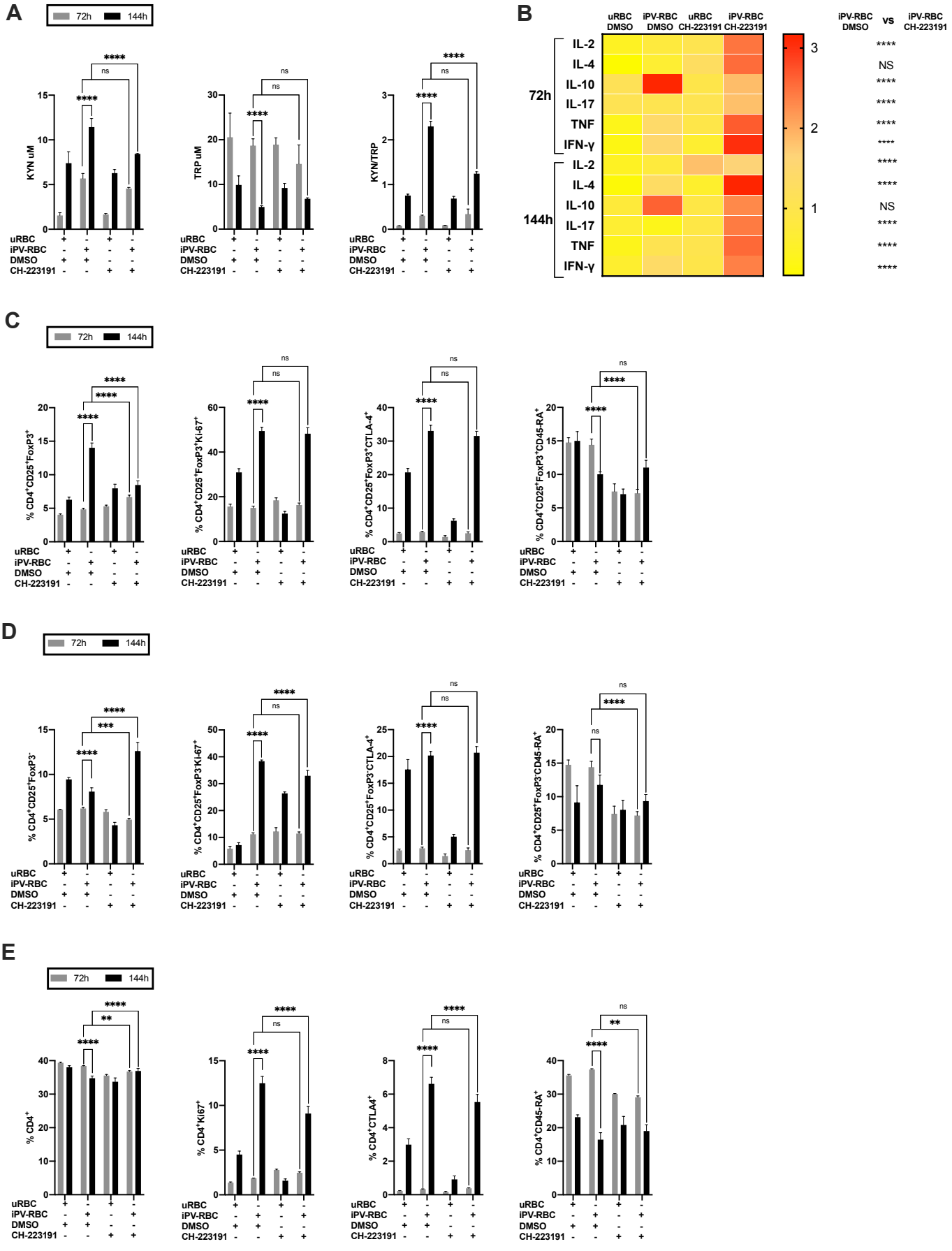
% CD4+CTLA4+
% CD4+CD45-RA+

Figure 6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uRBC CH-223191
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72h
144h
IL-2
IL-4
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IL-17
TNF
IFN-γ
iPV-RBC DMSO vs iPV-RBC CH-223191
C
% CD4+CD25+FoxP3+
% CD4+CD25+FoxP3+KI-67+
% CD4+CD25+FoxP3+CTLA-4+
% CD4+CD25+FoxP3+CD45-RA+
D
% CD4+CD25+FoxP3-
% CD4+CD25+FoxP3-KI-67+
% CD4+CD25+FoxP3-CTLA-4+
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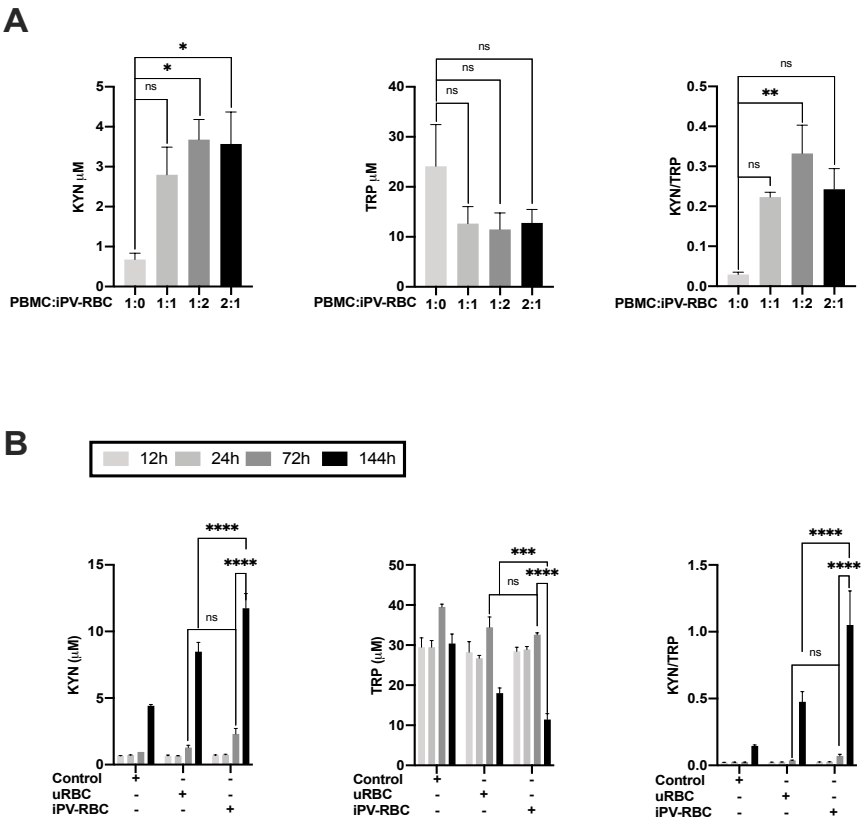
% CD4+CD45-RA+

Figure 7



Supplementary Figure 1

Supplementary Table 1: Flow cytometry markers

Panel	Antibody	Fluorochrome	Catalog	Clone	Mark
Treg (Patients)	CD4	BV 510	317444	OKT4	BioLegend
	CD25	BV 605	302632	BC96	BioLegend
	FoxP3	AF647	560045	259D/C7	BD
	CD45RA	PerCP-Cy5.5	563429	HI100	BD
	CTLA4	PE-CF594	562742	BNI3	BD
	AhR	Primario	14-9854-82	FF3399	eBioscience
	Secundário	FITC	-	-	BD
	Ki67	Pe-Cy7	12-5699-42	B56	BD
IDO1 (*in vitro*)	CD14	PE	555398	M5E2	BD
	HLA-DR	APC	557344	G46-6	BD
	IDO1	PerCP-eFluor 710	46-9477-42	eyedio	eBioscience
Treg (*in vitro*)	CD4	APC-H7	560158	RPA-T4	BD
	CD25	BB515	564467	2A3	BD
	FoxP3	PE-Cy7	25-4777-42	236A/E7	eBioscience
	Ki-67	PE	350504	Ki-67	BioLegend
	CTLA-4	APC	555855	BNI3	BD
	CD45-RA	PerCP-Cy5.5	563429	HI100	BD

Supplementary Table 2: Primers used in qPCR

Target	Forward	Tm (ºC)	Reverse	Tm (ºC)
IDO1	GCCTGATCTCATAGAGTCTGGC	56.7	TGCATCCCAGAACTAGACGTGC	56.7
IDO2	GTTATGTCTGGCAGGAAGGAGAG	57.1	GTCCAGTTCGTCAGCACCAAGT	56.7
GAPDH	GTCTCCTCTGACTTCAACAGCG	56.7	ACCACCCTGTTGCTGTAGCCAA	56.7
ACTB	CACCATTGGCAATGAGCGGTTC	56.7	AGGTCTTTGCGGATGTCCACGT	56.7
B2M	CCACTGAAAAAGATGAGTATGCCT	54	CCAATCCAAATGCGGCATCTTCA	55.3

Anexo 1 – Artigo Publicado em parceria com Dr. Fábio Costa

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Inhibition of hypoxia-associated response and kynurenine production in response to hyperbaric oxygen as mechanisms involved in protection against experimental cerebral malaria

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ABSTRACT: Cerebral malaria (CM) is a multifactorial syndrome involving an exacerbated proinflammatory status, endothelial cell activation, coagulopathy, hypoxia, and accumulation of leukocytes and parasites in the brain microvasculature. Despite significant improvements in malaria control, 15% of mortality is still observed in CM cases, and 25% of survivors develop neurologic sequelae for life—even after appropriate antimalarial therapy. A treatment that ameliorates CM clinical signs, resulting in complete healing, is urgently needed. Previously, we showed a hyperbaric oxygen (HBO)-protective effect against experimental CM. Here, we provide molecular evidence that HBO targets brain endothelial cells by decreasing their activation and inhibits parasite and leukocyte accumulation, thus improving cerebral microcirculatory blood flow. HBO treatment increased the expression of aryl hydrocarbon receptor over hypoxia-inducible factor 1-α (HIF-1α), an oxygen-sensitive cytosolic receptor, along with decreased indoleamine 2,3-dioxygenase 1 expression and kynurenine levels. Moreover, ablation of HIF-1α expression in endothelial cells in mice conferred protection against CM and improved survival. We propose that HBO should be pursued as an adjunctive therapy in CM patients to prolong survival and diminish deleterious proinflammatory reaction. Furthermore, our data support the use of HBO in therapeutic strategies to improve outcomes of non-CM disorders affecting the brain.—Bastos, M. F., Kayano, A. C. A. V., Silva-Filho, J. L., Dos-Santos, J. C. K., Judice, C., Blanco, Y. C., Shryock, N., Sercundes, M. K., Ortolan, L. S., Francelin, C., Leite, J. A., Oliveira, R., Elias, R. M., Câmara, N. O. S., Lopes, S. C. P., Albrecht, L., Farias, A. S., Vicente, C. P., Werneck, C. C., Giorgio, S., Verinaud, L., Epiphanio, S., Marinho, C. R. F.,

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; ATA, atmosphere pressure; BBB, blood-brain barrier; CM, cerebral malaria; CSF, cerebrospinal fluid; C_t, cycle threshold; ECM, experimental cerebral malaria; EPCR, endothelial protein C receptor; *fl/fl*, flox/flox; HBO, hyperbaric oxygen; HIF-1α, hypoxia-inducible factor 1-α; Hmox1, heme oxygenase-1 mRNA; HO-1, heme oxygenase-1; HPRT, hypoxanthine-guanine phosphoribosyltransferase; ICAM-1, intercellular adhesion molecule-1; IDO-1, indoleamine 2,3-dioxygenase 1; iEs, infected erythrocytes; Itga/b1/b2, integrin α1/β1/β2; KA, kynurenine acid; KP, kynurenine pathway; Kyn, kynurenine; LFA-1, lymphocyte function-associated antigen-1; NI, noninfected; PbA, *Plasmodium berghei* ANKA; p.i., postinfection; QA, quinolinic acid; qRT-PCR, quantitative RT-PCR; Trp, tryptophan

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KEY WORDS: brain · aryl-hydrocarbon receptor · tryptophan metabolism · endothelial cell activation

Among malaria complications, cerebral malaria (CM) is associated with high mortality and morbidity rates, mainly in children in sub-Saharan Africa, and accounts for almost one-half of a million deaths per year (1). Although a tremendous effort has been made toward controlling CM mortality, including numerous clinical trials of different adjunctive therapies, 18% of African children and 30% of Southeast Asian adults with CM still die. Moreover, nearly 25% of survivors develop neurologic sequelae and cognitive impairments, even after optimal antimalarial treatment (2–5).

Uncontrolled production of proinflammatory cytokines, coagulopathy, endothelial activation, and oxidative stress is often associated with poor CM outcomes (6–9). CM pathologic features also include vascular occlusion, ischemia, brain edema and hemorrhages, neuronal and endothelial damage, blood-brain barrier (BBB) dysfunction, convulsions, coma, and death (7, 10). Although the host immune response plays a pivotal role in CM pathogenesis, congestion of deep microvasculature is reported in autopsies of CM patients, as well as endothelial activation and hypoxia, which correlate to BBB breakdown in CM patients (11–14). In mice, lethal infection with *Plasmodium berghei* ANKA (PbA) is a murine model widely used for experimental CM (ECM) that shares several pathogenesis components of human CM, mostly pediatric CM (10, 15).

Administration of pressurized oxygen, also referred as hyperbaric oxygen (HBO) therapy, has been successfully used in humans in a variety of disorders, such as refractory wounds, radiation injury, and decompression sickness (16). This therapy is also able to suppress transiently the inflammatory process of ischemic trauma and improve blood flow, thus ameliorating brain disorders, such as stroke (17). We have previously demonstrated that HBO therapy, in conditions suitable for human use, protects mice against the lethal disease induced by PbA infection (18). However, the molecular mechanisms involved in the neuroprotective effect of HBO during ECM remained unknown. Here, we evaluate the mechanisms of action of HBO treatment on leukocyte and parasite accumulation in the microvasculature and hence, on cerebral blood flow. Furthermore, we assess its molecular mechanisms triggered on the brain by evaluating the expression of proteins and genes encoding adhesion molecules, hypoxia-associated response, and generation of metabolites derived from the tryptophan (Trp) metabolism.

MATERIALS AND METHODS

Ethics statement

All experiments and procedures were approved by the University of Campinas Committee for Ethics in Animal Research (1366-1 and 2200-1) and by Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee (2013-0144). The protocols adhered to the guidelines of the Brazilian National Council for the Control of Animal Experimentation and to the *Guide for the Care and Use of Laboratory Animals* [National Institutes of Health (NIH), Bethesda, MD, USA].

Mice and murine parasites

Female C57BL/6 mice (7–10 wk old) were purchased from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science, University of Campinas, and maintained in our specific pathogen-free animal facility. Hypoxia-inducible factor 1-α flox/flox (HIF-1$\alpha^{fl/fl}$) mice were provided by Dr. George Deepe (University of Cincinnati, Cincinnati, OH, USA) (19) and crossed with Tie2cre to generate endothelial cell conditional knockout mice for HIF-1α (Tie2cre HIF-1$\alpha^{fl/fl}$).

A cloned line of PbA was kindly provided by Dr. Laurent Rénia (Singapore Immunology Network, Agency for Science, Technology and Research, Singapore). The blood-stage forms of PbA parasites were stored in liquid nitrogen after *in vivo* passages in C57BL/6 mice, as described in refs. 15 and 18. Mice were infected intraperitoneally with 10^6 infected erythrocytes (iEs), and parasitemia and CM neurologic signs were monitored daily. ECM was defined by the presence of at least 2 of the following clinical signs of neurologic involvement: ataxia, limb paralysis, poor righting reflex, seizures, rollover, and coma.

Administration of HBO in infected mice

HBO treatment in mice was conducted as described in Blanco *et al*. (18). In brief, groups of 8–10 PbA-infected mice were exposed for 1 h daily to 100% oxygen at a pressure of 3.0 atmospheres (ATA) in a hyperbaric animal research chamber (Model HB 1300B; Sechrist, Anaheim, CA, USA) from d 0 until the day the assay was performed. The chamber was pressurized and decompressed at a rate of 0.5 ATA/min, as previously described (18). Infected mice in the control group (nonexposed) were left in an ventilated room (normal oxygen tension and normal local atmospheric pressure, ~0.98 ATA).

Real-time quantitative RT-PCR

Gene expression levels and parasite load in mice brains were assessed by real-time quantitative RT-PCR (qRT-PCR). On d 6–7 postinfection (p.i.), when mice showed signs of ECM, PbA-infected mice were perfused intracardially with PBS using a peristaltic pump (Harvard Apparatus, Cambridge, MA, USA) to remove circulating iEs and leukocytes. Extraction of total RNA from mice brains was performed with the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Total RNA samples (1 μg) were reverse transcribed using the oligo(dT) primer from the High Capacity cDNA Reversion Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression of intercellular adhesion molecule-1 (*Icam1*); heme oxygenase-1 (HO-1) mRNA (*Hmox1*); endothelial protein C receptor (*Epcr*); *tissue factor*; lymphocyte function-associated antigen-1 (*Lfa1*); *perforin*; indoleamine 2,3-dioxygenase 1 (*Ido1*); integrin α1 (*Itga*), β1 (*Itgb1*), and β2 (*Itgb2*); and 18S was analyzed by qRT-PCR, using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Transcripts of *Il12a*, *Il12b*, aryl hydrocarbon receptor

(*Ahr*), and *Hmox1* were also evaluated by qRT-PCR (Qiagen). The oligonucleotides used are described in Supplemental Table S1. The median cycle threshold (C_t) value and $2^{-\Delta\Delta Ct}$ method were used for relative quantification analysis, and all C_t values were normalized to the hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA expression level. Results were expressed as means, and SD of biologic triplicates is shown compared with noninfected (NI) mice.

Immunoblotting

Mice brain protein crude extracts were homogenized in extraction buffer [EDTA, pH 7.4, 0.01 M; Tris-HCl, pH 7.4, 0.1 mM; sodium pyrophosphate, 10 mM; sodium fluoride, 100 mM; sodium orthovanadate, 10 mM; Triton, 1%; and protease inhibitor cocktail, 1% (MilliporeSigma, St. Louis, MO, USA)]. Protein concentration was determined by the Bradford method. Protein crude extract (100 μg) was incubated at 95°C for 5 min with 1 vol Laemmli sample and submitted to electrophoretic separation on an 8% SDS-PAGE. The proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad) and incubated for 1 h with blocking solution [5% dried milk (Nestlé, Vevey, Switzerland), 50 mM PBS, pH 7.4, 150 mm NaCl, 0.1% PBS-Tween]. The transferred proteins were incubated with mAb against AhR and β-actin (all purchased from Santa Cruz Biotechnology, Dallas, TX, USA) for 12 h at 4°C and diluted at 1:1000 in a PBS-Tween solution. After washing with PBS-Tween, blots were incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse secondary antibody (Zymax; Zymed Laboratories, San Francisco, CA, USA), diluted at 1:10,000. The immunoreactive blots were detected by autoradiography on Kodak film GBX2 with SuperSignal West Pico Chemiluminescence Kit (Thermo Fisher Scientific). The optical density of immunoreactive bands was determined by digital optical densitometry (Scion Image Software, v.4.0.3.2, Scion, Frederick, MD, USA) and the values expressed in relation to the α-tubulin used as internal control.

Craniotomy and intravital microscopy

Intravital microscopy of brain microvasculature was performed, as described in Lacerda-Queiroz *et al.*(20), with slight modifications. In brief, on d 5 p.i., groups of 4–6 PbA-infected mice or NI animals, exposed or not to HBO, were anesthetized with ketamine (100 mg/kg) and xylazine (16 mg/kg) and maintained at 37°C using a heating pad. A skull opening of 3–4 mm diameter was made in the left parietal lobe using a surgical drill (Beltec, Araraquara, Brazil). Dura mater and arachnoid were lifted away from the skull to expose the pia mater blood vessels. To observe leukocyte/endothelium interactions, leukocytes were fluorescently labeled by intravenous administration of rhodamine 6G (0.3 mg/kg; MilliporeSigma) and observed using an Intravital Microscope (Axio ImagerA2; Carl Zeiss, Jena, Germany) with a ×10 objective and a 590 nm emission filter, coupled with a camera (AxioCam) to record images. The images obtained were analyzed using ImageJ software (NIH), and adhered leukocytes were counted. Adhered leukocytes were considered as those cells that remained attached for 30 s or more onto the endothelium. The counting of adherent leukocytes was expressed as cells attached to the endothelium in a 100-μm length of the vessel. Velocity (pixel/ms) of rhodamine 6G-labeled cells was determined using manual tracking plugin (ImageJ) in 3–4 movies for each condition. After the procedure, all animals were euthanized with a high dose of ketamine and xylazine.

Tissue processing

The presence of HIF-positive cells in the brain was considered evidence of tissue hypoxia. For this at a comparable time point, all mice were euthanized in an experiment when susceptible mice exhibited clinical signs of CM. PbA-infected mice demonstrated signs of CM at d 6–7 p.i., and most of these mice had entered the terminal phase of murine CM. On the day of euthanasia, groups of 4–6 PbA-infected mice or NI animals, exposed or not to HBO, were anesthetized with ketamine (100 mg/kg) and xylazine (16 mg/kg) and were perfused intracardially with phosphate-buffered 4% paraformaldehyde for 20 min. The brain was removed quickly, split sagittally, and immersion fixed in phosphate-buffered 4% paraformaldehyde for 2 h at room temperature before transfer to 70% ethanol. Tissues were processed for paraffin inclusion, which included immersion baths in ethanol-xylol-paraffin gradients. After inclusion, sections of 5 μm were obtained for immunohistochemical analysis.

Immunohistochemical analysis

The presence of HIF-1α-positive cells in the brain of all mice groups was detected by an immunohistochemical stain. Brain sections from midbrain were deparaffinized with xylene and hydrated in an ethanol gradient before staining. Directly after, incubation was performed with 1% H_2O_2 to block endogenous peroxidase activity (10 min). Henceforth, all procedures were done according to an ImmunoCruz rabbit ABC Staining System (sc-2018; Santa Cruz Biotechnology) datasheet. In brief, all sections were incubated with goat serum (MilliporeSigma) to avoid secondary antibody nonspecific binding for 1 h at room temperature and then incubated with specific primary antibodies to mouse HIF-1α (sc-10790; Santa Cruz Biotechnology) for 16 h at 4°C. After washing with saline phosphate buffer, sections were overlaid for 1 h with the biotin-conjugated secondary antibody at room temperature. This was followed by incubation with AB Enzyme reagent to amplification of the signal reaction. Bound antibodies were detected by reactivity with 3,3′-diaminobenzidine plus H_2O_2. After tap-water washing, the slides were counterstained by Harris Hematoxylin and mounted with Entellan. For immunohistochemical controls, primary antibodies were omitted from the staining procedure and were negative for any reactivity. Quantification of the immunostaining was done with ImageJ software. All slides were blinded and assessed using digital images. In total, 1000 cells were counted for each specimen using an ocular grid. The percentage of HIF-1α-positive cells was determined by a single observer (C.F.), blinded to the animal status, and defined as follows: percent of HIF-1α-positive cells = HIF-1α-positive cells/total cells.

Endothelial cell flow cytometry

Brains were removed from wild-type or Tie2cre HIF-1$\alpha^{fl/fl}$-infected mice (d 6 p.i.). Brains were digested with collagenase I for 30 min at 37°C. Cells (1–5 million) were incubated with FcBlock (BD Biosciences, San Jose, CA, USA), along with anti-F4/80 or anti-CD11b (Brilliant Violet 510), rat IgG1 isotope control or anti-CD31 (peridinin chlorophyll protein complex-cyanine 5.5), anti-CD54 (FITC), anti-CD142 (FITC), and anti-EPCR (phycoerythrin) for 30 min at 4°C in FACS buffer. Cells were then washed, resuspended in FACS buffer, and acquired using a BD LSR Fortessa cytometer (500,000 events/sample). Expression profiles in $CD31^+CD11b^-$ (endothelial cells) for ICAM-1 (CD54), tissue factor (CD142), and EPCR were then determined by the mean fluorescence intensity using FlowJo software (Ashland, OR, USA).

Total free heme quantification

Infected animals were anesthetized with ketamine (100 mg/kg) and xylazine (16 mg/kg), and blood samples were collected on d 6 p.i. from the vena cava using EDTA as an anticoagulant.

Thus, plasma was obtained after centrifugation at 1000 *g* for 15 min at 4°C for total free heme assessment. Total heme was quantified using a chromogenic assay, according to the manufacturer's instructions (QuantiChrom Heme Assay Kit; Bioassay Systems, Hayward, CA, USA).

Simultaneous Trp and kynurenine quantification

Trp and kynurenine (Kyn) concentrations in mouse serum were measured simultaneously by reverse-phase HPLC, as described in ref. 21, with some modifications. The HPLC system (Shimadzu, Kyoto, Japan) was coupled with an analytical octadecylsilyl C18 column of 15 cm, 4.6 μ, and a photodiode array detector (Shimadzu; collecting UV–visible spectra from 190 to 800 nm, which can provide chromatograms at the desired wavelength in this range). The mobile phase consisted a mixture of 15 mM sodium acetate buffer, with 27 ml acetonitrile adjusted to a final pH of 4 with acetic acid. Perchloric acid (final concentration 8%) was added to 200 μl mouse serum sample, homogenized by vortexing for 1 min, and centrifuged at 10,000 rpm at 4°C for 10 min. A clear supernatant was then injected into the HPLC system using an autosampler. In addition, Trp and Kyn HPLC grade reagents were purchased from MilliporeSigma, and stock solutions were prepared in mobile-phase buffer. For the standard curve, serial dilutions were used for Trp and Kyn (Trp/Kyn micromolars): 100/10, 50/5, 25/2.5, 12.5/1.25, 6.25/0.625, 3.125/0.325. The flow rate was 1.0 ml/min, and the volume per sample was 20 μl. Trp and Kyn were detected at 278 and 360 nm, respectively (22). Retention time was used to identify metabolites in the chromatogram, and standard curve was constructed by plotting the ratio of peak area (computed by LCsolution software; Shimadzu) of Trp or Kyn (*y* axis) against known Trp or Kyn concentration (*x* axis), respectively. The linearity of the standard curves was confirmed using regression variance analysis and significance of correlation coefficient, checked using Student's *t* test. A derived equation was used to quantify unknown concentrations in the mice samples. In addition, the intra-assay (reproducibility) variability was determined by analyzing samples in triplicate and the interassay (repeatability) variability by testing samples, 3 and 6 d in triplicate after the first analysis. The concentration compared in inter- and intra-assay had no statistically significant difference.

Statistical analysis

Statistical significance was determined using 1-way ANOVA or Student's *t* test for parametric data. Kruskal-Wallis and *post hoc* or Mann-Whitney *U* tests were used for nonparametric data. Mantel-Cox test was used for comparison of survival curves. All statistical analyses were performed using BioEstat v.5.0 (CNPq, Brasília, Brazil) and Prism v.5.0 (GraphPad Software, La Jolla, CA, USA). Values were considered significant when $P < 0.05$.

RESULTS

HBO treatment decreases parasite and leukocyte accumulation in the brain of mice during PbA infection

We have shown that HBO protects mice against ECM by reducing expression of proinflammatory cytokines and sequestration of T cells in the brain of PbA-infected mice, which in turn, prevent BBB disruption and delay CM-specific neurologic signs (18). In addition to sequestration of $CD8^+$ T cells in the CNS, the concomitant presence of parasitized erythrocytes is thought to be crucial for CM development (23). Thus, we asked whether pressurized oxygen would modulate the parasite load in the brain. To this end, qRT-PCR for PbA 18S rRNA was performed on material extracted from the brains of PbA-infected mice untreated (PbA) and HBO treated (PbA-HBO), either before or after intracardiac perfusion. There was no significant difference in parasite 18S rRNA in the brain of infected animals before intracardiac perfusion (**Fig. 1*A***). However, parasite 18S rRNA was significantly lower ($P = 0.0079$) in the brain of PbA-HBO mice compared with PbA mice after intracardiac perfusion at d 6 p.i. (Fig. 1*B*).

Cerebral blood flow impairment by adherent leukocytes and parasites is a common feature in CM (6–8). Therefore, we also assessed whether HBO would affect leukocyte interaction with brain endothelium by intravital microscopy of PbA-infected mice. Representative intracranial photomicrographs for each tested group are shown (Fig. 1*C, D*), and movies of NI animals—PbA-infected mice, exposed or not to HBO—were recorded at real-time conditions (Supplemental Movies S1–3). In sharp contrast to PbA mice, exposure to HBO significantly inhibited leukocyte adhesion to the brain microvasculature ($P < 0.0001$; Fig. 1*C*) and significantly improved microcirculation velocity (Fig. 1*D*).

HBO treatment also resulted in reduction of mRNA expression of *Lfa1* and perforin, by 50 and 70%, respectively, in the brain of PbA mice (Fig. 1*E, F*). As these molecules are closely related to adhesion and function of effector leukocytes cells, these observations support our hypothesis that HBO treatment decreases leukocyte adhesion in the brain microvasculature.

To address whether decreased leukocyte adhesion to brain endothelial cells in PbA-HBO mice was a result of decreased activation of leukocytes from the periphery, we analyzed mRNA expression of different integrins—*itga1*, *itgb1*, and *itgb2* (components of the LFA-1 protein complex)—molecules expressed in activated leukocytes. Surprisingly, in total peripheral leukocytes (peripheral blood or spleen derived), mRNA expression of all integrins analyzed was similar in PbA-HBO and PbA control mice (**Fig. 2**). Furthermore, only mRNA expression of *itga1* in leukocytes from blood and *itgb1* in leukocytes from spleen was increased after HBO treatment (Fig. 2). Taken together, these observations indicate that the protective effect of HBO treatment during ECM is not related to a direct immunosuppressive effect on immune cells. Instead, our data point to the fact that HBO may be targeting activation of the brain endothelial cells during ECM.

HBO treatment decreases activation of brain endothelial cells during PbA infection

It is known that brain endothelial cell activation is one of the hallmarks of ECM development (23–27), and our

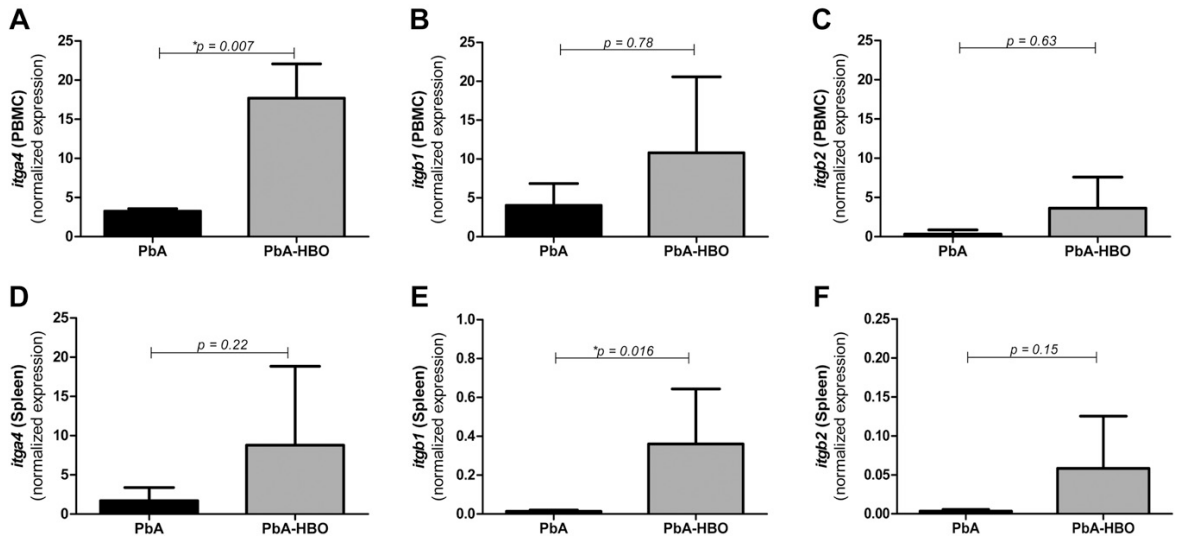

Figure 2. Genetic expression of integrins in peripheral blood- or spleen-derived leukocytes. Groups of 5–7 animals infected with 10^6 PbA iEs (PbA group) were daily exposed, or not, to HBO (PbA-HBO) conditions (100% O_2, 3 ATA, 1 h). On d 6–7 p.i., when PbA mice showed signs of ECM, leukocytes were isolated from the peripheral blood [peripheral blood mononuclear cell (PBMC)] (*A–C*) or from the spleen and *itga4*: ITGA4 (*D*); *itgb1*: ITGB1 (*E*); and *itgb2*: ITGB2 (*F*) levels were assessed by qRT-PCR. Results were normalized to HPRT, and means and SD of biologic triplicates are shown compared with NI mice. $^*P < 0.05$.

HBO treatment decreases cerebral hypoxia induced by PbA infection

It has been demonstrated in postmortem studies in CM patients and murine models of CM that some of the deleterious consequences of leukocyte and parasite accumulation in the activated endothelium of the host brain vessels are vascular obstructions and impairment of tissue perfusion (28). Impairments of cerebral microcirculatory blood flow caused an ischemic process leading to cerebral hypoxia (10, 28, 29), which was associated with endothelial activation and BBB disruption, resulting in an increased permeability, subsequent edema, and tissue damage (29–31). Therefore, hypoxia is also a key event in development of acute cerebral disease (29, 30). Importantly, during the hypoxia-associated response, the transcription factor HIF-1α is rapidly upregulated, and its transcriptional program is activated in different cell types, including endothelial cells (32, 33). Thus, because we observed a lower parasite and leukocyte accumulation, as well as lower endothelial cell activation in the brain microvasculature after HBO treatment, we investigated whether exposure to HBO also reduced the hypoxic response in the brains of PbA mice. As shown in **Fig. 4*A***, few areas and cells are HIF-1α$^+$ in NI mice. However, the levels of HIF-1α$^+$ cells were significantly increased in PbA mice when compared with PbA-HBO animals (Fig. 4*A*). To regulate gene expression, HIF-1α requires its binding partner, the Ahr nuclear translocator (ARNT). ARNT is also required by the AhR, a crucial regulator that mediates many of the responses to toxic environmental chemicals (34). It has been shown that HIF-1α and AhR compete for binding to ARNT, thus establishing a crosstalk between hypoxia- and AhR-induced gene-expression profiles (34). Moreover, HIF-1α induction promotes AhR ubiquitination and proteasomal degradation (35). Accordingly, here we observed a lower AhR protein expression in the brains of PbA mice, whereas HBO treatment prevented downregulation of AhR expression (Fig. 4*B*).

It has been shown that HIF-1α transcriptional activity results in upregulation of HO-1 expression (36, 37). In addition, HO-1 is induced by the release of free heme in the plasma as a result of parasite growth (38, 39). We assessed *Hmox1* expression to verify whether ECM-induced tissue hypoxia increases transcriptional activity of HIF-1α and if HBO inhibits this process. Accordingly, Fig. 4*C* shows upregulation of *Hmox1* expression in the brain of PbA-infected mice, whereas *Hmox1* was significantly reduced (2-fold; $P < 0.0001$) in the brain of PbA-HBO mice at d 6–7 p.i. This set of results shows that HBO treatment resulted in reduction of hypoxia-associated HIF-1α transcriptional activity in the brains of PbA-infected mice.

However, the observation that HBO strikingly reduces expression of *Hmox1* and modestly reduces expression of HIF-1α raises the possibility that HBO could downregulate HO-1 expression by another mechanism. HO-1 is also induced by free heme (40). Here and previously (18), we showed that HBO therapy resulted in a significantly reduced parasite burden in the brain of PbA-infected mice, which could decrease the release of free heme into the plasma. Accordingly, here, we observed that administration of pressurized oxygen in PbA-infected mice significantly reduced total heme levels on d 5 and 6 p.i., when ECM clinical signs begin to appear (Fig. 4*D*). Thus, HBO treatment downregulates HO-1 expression in brains of infected mice by both reducing hypoxia and by slowing parasite growth and the release of free heme into the plasma. Taken together, these results show that the

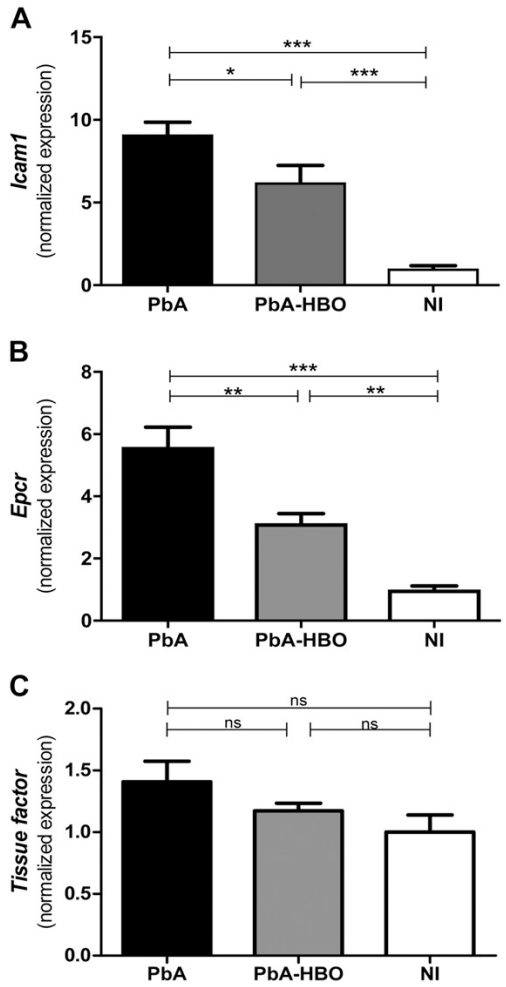

Figure 3. HBO reduces endothelial activation in the brain of infected animals. Total brain mRNA from PbA-infected mice or PbA mice exposed to HBO (PbA-HBO) were submitted to qRT-PCR for *Icam1*(*A*), *Epcr* (*B*), and *tissue factor* (*C*) genes. Results were normalized to HPRT, and means ± SD of biologic triplicates are shown compared with NI mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA test).

hypoxia-triggered response during PbA infection is inhibited by HBO treatment.

Lack of HIF-1α expression in endothelial cells is protective against ECM

Our results indicate that endothelial cells are an important target of HBO-induced protective molecular mechanisms during ECM, probably by downregulation of the proinflammatory hypoxic response (10, 29). Thus, to address whether during ECM, HBO inhibits the hypoxia-triggered response in brain endothelial cells through inhibition of HIF-1α-dependent transcriptional activity, we created a Tie2cre HIF-1α$^{fl/fl}$ mouse strain that lacked HIF-1α in endothelial cells (19). As *Ahr* mRNA and *Hmox1* expression is regulated by HIF-1α-induced transcriptional activity, we tested whether HIF-1α deletion in brain endothelial cells would affect expression of both genes during infection with PbA. Similar to PbA-HBO mice, **Fig. 5*A*** shows that *Ahr* gene expression was significantly higher, whereas *Hmox1* expression was lower in the absence of endothelial cell HIF-1α (Fig. 5*B*). These results indicate that the PbA-induced hypoxic response, represented by the decrease of *AhR* expression and increase of *Hmox1* expression, was abrogated in the absence of HIF-1α in the brain endothelial cells. Furthermore, in the brains of PbA-infected Tie2cre HIF-1α$^{fl/fl}$ mice, we found lower mRNA levels of the proinflammatory cytokine IL-12, also a target of HIF-1α transcriptional activity and associated with murine CM pathogenesis (41) (Fig. 5*C, D*). Although we did not observe changes in ICAM-1 and EPCR protein expression (data now shown), we verified lower tissue factor (CD142) expression in brain-derived endothelial cells from the Tie2cre HIF-1α$^{fl/fl}$ gene during PbA infection (Fig. 5*E*). These observations indicate that brain endothelial cell activation is, in part, induced by the HIF-1α-mediated proinflammatory hypoxic response during ECM, as previously described in hypoxic-ischemic conditions in Kaur *et al*. 30. Consistent with our hypothesis that the ECM-protective activity of HBO occurs by dampening an HIF-1α-mediated hypoxic proinflammatory response, Tie2cre HIF-1α$^{fl/fl}$ mice were resistant to development of ECM and showed a survival rate similar to that of PbA-HBO mice (Fig. 5*F*) (18).

HBO treatment inhibits Kyn pathway of Trp metabolism

Our results show that the balance between the response to hypoxia mediated by HIF-1α and AhR expression might play a critical role during CM pathogenesis.

Several endogenous ligands have been identified to bind, activate, and decrease AhR expression following ligand binding, for instance, Kyn, a product of the IDO-1-dependent Trp metabolism through the Kyn pathway (KP) (34, 42, 43). IDO-1, which catalyzes the initial and rate-limiting step of this pathway, is upregulated by IFN-γ in the cerebral microvascular endothelium in *Plasmodium*-infected mice with CM (44). Kyn levels, the first breakdown product of the KP, increase in the plasma and accumulate in the cerebral blood vessels of mice infected with PbA, reaching concentrations in the high micromolar to low millimolar range (45). In addition, a decrease of Trp levels and an increase in the Kyn:Trp ratio have been described in several pathologies associated with chronic immune activation, including malaria, implicating activation of the KP in CM pathogenesis (42, 44, 46). In this regard, because HBO seems to restore the balance toward increased AhR expression, as observed in physiologic conditions, we evaluated if HBO treatment could also affect the expression of some key components of the KP. We verified that *Ido1* mRNA expression is upregulated in the brain of PbA-infected mice. Kyn levels and the Kyn:Trp ratio in the serum of PbA mice were increased, whereas Trp levels did not change (**Fig. 6**). Importantly, HBO treatment

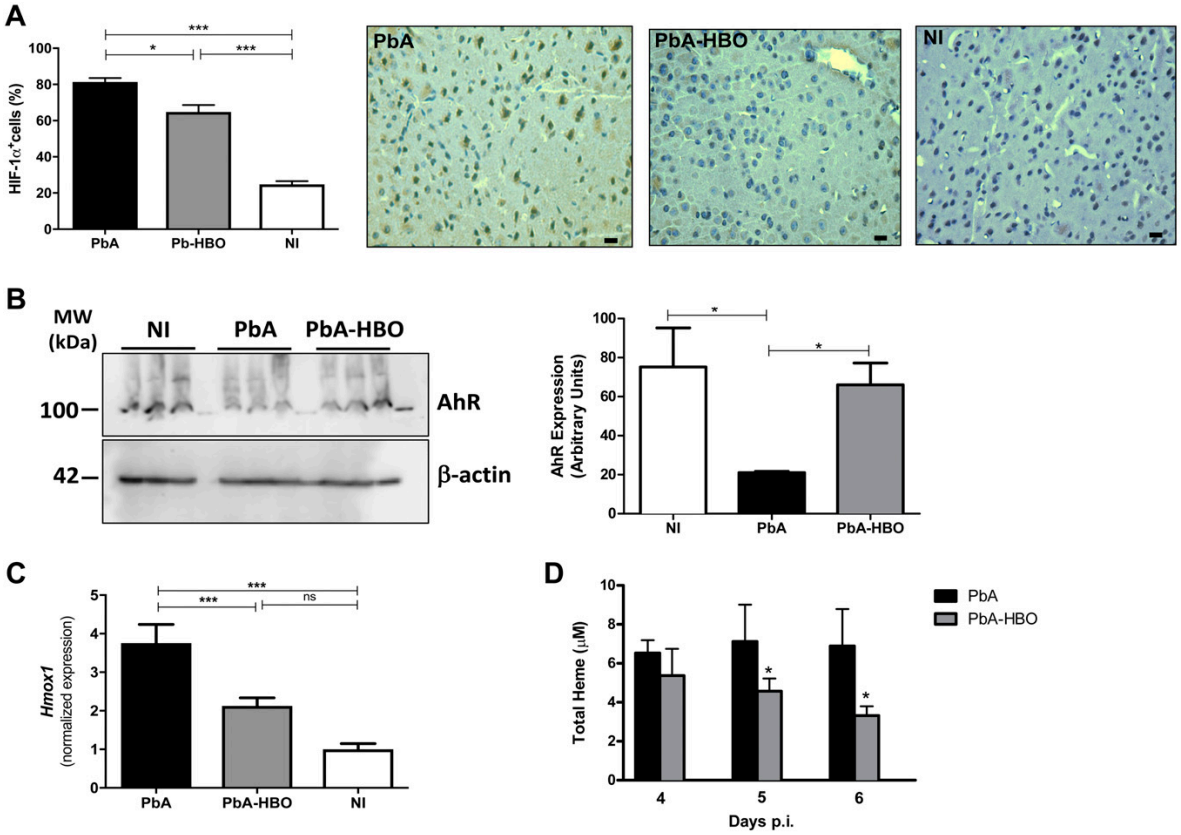

Figure 4. HBO reduces hypoxia in the brain of infected animals. *A*) Representative images of HIF-1α$^+$ cells in mice brains of NI, PbA, and PbA-HBO are shown. Values represent means ± SD of 6 mice per group and are representative of 2 independent experiments with similar results. *$P < 0.05$, ***$P < 0.001$ (ANOVA test). *B*) On d 6–7 p.i., mice brains were collected and AhR expression assessed by Western blot. Values represent means ± SD of 6 mice per group and are representative of 2 independent experiments with similar results. *$P < 0.05$ (ANOVA test). *C*) *Hmox1* brain expression levels from PbA-infected mice or PbA mice exposed to HBO (PbA-HBO) were determined by qRT-PCR. Results were normalized to HPRT, and means and SD of biologic triplicates are shown compared with NI mice. ***$P < 0.001$ (ANOVA test). *D*) Quantification of free heme was determined, at d 4–6 in the plasma of infected mice exposed, or not, to HBO. The results represent the average of 6–8 animals per group ± SD. *$P < 0.05$ (ANOVA test).

inhibited upregulation of *Ido1* expression and reduced Kyn production and the Kyn:Trp ratio in the infected mice (Fig. 6), indicating that HBO inhibits PbA-induced Kyn production and probably the generation of downstream neurotoxic metabolites (41, 42).

DISCUSSION

Previously, we demonstrated a neuroprotective effect of HBO during ECM (18). In the present study, our goal was to provide an understanding of the protective molecular mechanisms of pressurized oxygen in PbA-infected animals. Our results here show that HBO treatment reduced parasite and leukocyte accumulation in the brain by targeting the activation of brain microvascular endothelial cells. In addition, HBO treatment prevented an hypoxia-mediated proinflammatory response, as demonstrated by decreased HIF-1α expression and transcriptional activity, whereas stabilizing AhR expression in the brain of PbA mice. The biologic significance of such effects was confirmed in mice lacking HIF-1α in endothelial cells (Tie2cre HIF-1α$^{fl/fl}$), which were resistant to ECM.

It is important to consider which aspects of this model fit better with human CM. It has been demonstrated in the murine model that parasite accumulation in the brain microvasculature activates endothelial cells through release of inflammatory ligands, such as glycosylphosphatidylinositol anchors and hemozoin crystals bound to parasite DNA (31). In turn, activated brain endothelial cells respond to these stimuli by upregulating adhesion receptors and molecules involved in antigen presentation and secreting chemokines and cytokines. Consequently, leukocytes and platelets are recruited and activated, feeding a local proinflammatory cycle by promoting more endothelial activation, leukocyte/platelet sequestration, and parasite accumulation (24, 47). Moreover, activated CD8$^+$ T cells migrate toward the chemokine gradient to the brain, and LFA-1 promotes their adhesion to endothelial ICAM-1. Furthermore, locally secreted proinflammatory cytokines stimulate brain endothelial cells to phagocytose and cross present

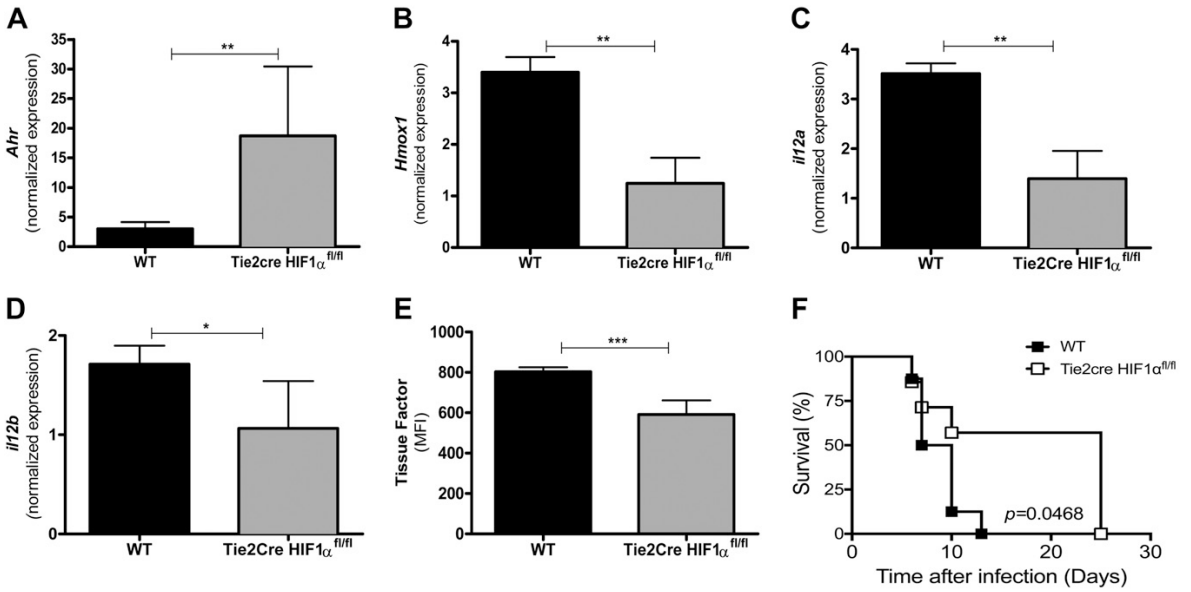

Figure 5. Lack of HIF-1α expression in endothelial cells is protective against ECM. *A–D*) Groups of 10–15 wild-type (WT) or Tie2cre HIF-1α$^{fl/fl}$ mice lacking HIF-1α in endothelial cells were infected with 10^6 PbA-iEs. On d 6–7 p.i., *Ahr* (*A*), *Hmox* (*B*), *il12a* (*C*), and *il12b* (*D*) levels were assessed by qRT-PCR. As controls, brains of NI mice were used. Values were expressed as the means of specific *Ahr*-, *Hmox*-, *il12a*-, or *il12b*-normalized expression of 10 mice ± SD. $^{**}P < 0.01$, $^{*}P < 0.05$ (ANOVA test). *E*) Tissue factor protein expression was determined by the mean fluorescence intensity (MFI) in $CD31^+CD11b^-$ (endothelial cells). The results represent the average of 10 animals per group ± SD. $^{***}P < 0.001$ (ANOVA test). *F*) Survival of the indicated PbA-infected mouse strains.

parasite-derived epitopes to $CD8^+$ T cells. In turn, $CD8^+$ T cells secrete perforin and granzymes, which disrupt the BBB (24, 47). In regard to human CM, a study conducted with >100 isolates collected from *P. falciparum*-infected individuals demonstrated an association between endothelial cell activation and CM (14). Moreover, different studies show that ICAM-1 and EPCR are upregulated in the brain endothelium of CM patients and are implicated in parasite accumulation (13, 14, 47). Importantly, in a systematic postmortem study of the brains of Malawian children with CM, few $CD8^+$ T cells were observed intravascularly in distended capillaries (48). This is similar to mice infected with PbA, where the relatively small numbers of sequestered $CD8^+$ T cells are diffcult to observe by

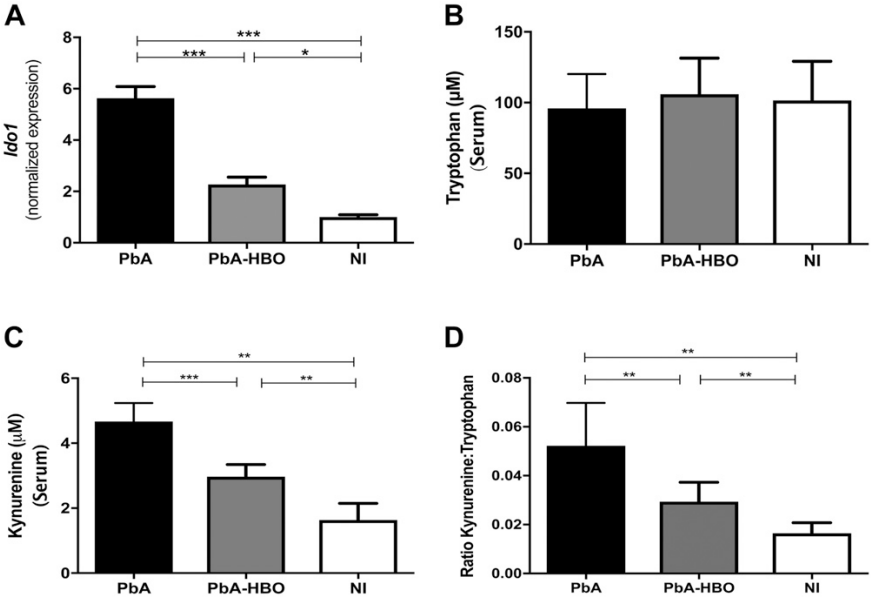

Figure 6. HBO treatment inhibits the KP of the Trp metabolism. *A*) *Ido1* mRNA brain expression levels from PbA iEs or exposed to HBO (PbA-HBO) mice groups were determined by qRT-PCR. The NI mice group was used as control. $^{*}P < 0.05$, $^{***}P < 0.001$ (ANOVA test). *B–D*) Concentration of Trp (*B*), Kyn (μm) (*C*), and Kyn:Trp (*D*) ratio were determined in the serum of the different mice groups. Values represent means ± SD of 7 mice per group and are representative of 2 independent experiments with similar results. $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA test).

histology (49), which do not exclude the possibility that $CD8^+$ T cells play an important role in human CM pathogenesis. In this context, we have shown that HBO treatment reduces the expression of proinflammatory cytokines and sequestration of $CD4^+$ and $CD8^+$ T cells in the brain of PbA mice, which in turn, prevent BBB disruption and delay CM-specific neurologic signs (18). Here, we also verified that HBO significantly reduces parasite and leukocyte accumulation in the brain microvasculature by inhibiting endothelial cell activation, which improved microcirculation velocity. Interestingly, even though leukocyte activation is not modulated by pressurized oxygen, as demonstrated by the expression of *ItgaI*, *Itgab1*, and *Itgab2* in cells isolated from the peripheral blood or the spleen, expression of LFA-1 and perforin in the brain of PbA-HBO mice was significantly decreased compared with control mice. These data suggest that HBO treatment did not target immune cells, but instead, it may target brain endothelial cells affecting their capacity to promote leukocyte sequestration.

It has also been demonstrated in postmortem studies in CM patients and murine models of CM that parasite and leukocyte accumulation, associated to endothelial cell activation, increases vascular resistance and promotes obstructions, thereby contributing to impaired cerebral perfusion during CM. These impairments of cerebral microcirculatory blood flow are thought to cause ischemia and cerebral hypoxia. The microvascular hemodynamics and oxygenation are drastically compromised during ECM, resulting in lower O_2 delivery and O_2 extraction by the brain tissue (28, 29). To maintain normal brain function and cell survival, oxygen-sensing mechanisms operate at the cellular level in response to hypoxia. In particular, the transcription factor HIF-1α plays a central role in hypoxia sensing. In the brain, HIF-1α expression is induced by hypoxia in neurons, astrocytes, ependymal cells, and endothelial cells (33, 34). Under normal conditions, the oxygen-regulated HIF-1α subunit is rapidly degraded *via* prolyl hydroxylation that targets its degradation in the proteasome. Hypoxia inhibits prolyl hydroxylase activity, resulting in HIF-1α subunit stabilization in the cytoplasm and translocation to the nucleus, where it dimerizes with ARNT to form HIF-1. HIF-1 then binds to hypoxia-responsive elements in promotor regions of target genes involved in cellular adaptation to hypoxic stress and induces their expression (33, 34). However, HIF-1α signaling could be deleterious in some circumstances by inducing expression of proapoptotic proteins of the B cell lymphoma 2 family, leading to cell death (33, 34). Hypoxia could also induce BBB dysfunction through increased tyrosine phosphorylation and redistribution of tight junction proteins away from cell borders and VEGF secretion (31). Conversely, inhibition of HIF-1 improves barrier function in hypoxic cells, indicating that temporal suppression of HIF-1 activity may be essential to preserve barrier function during injury (31). In addition, we cannot rule out that other factors, including cytokines, such as IFN-γ, IL-1β, or TNF and NF-κB, may play a more direct role in inducing HIF-1α, regardless of the oxygen levels (41, 50). In turn, hypoxia also triggers a proinflammatory response that can activate endothelial cells (30). Furthermore, the biologic relevance of such HBO effects, particularly those targeting endothelial cells, was unequivocally demonstrated in PbA-infected mice lacking HIF-1α expression in endothelial cells (Tie2cre HIF-$1\alpha^{fl/fl}$), which showed a comparable level of survival improvement and protection against CM with that observed in PbA-HBO mice. Thus, reduction of the proinflammatory hypoxic response, resulting from HBO treatment, may explain the protective effects observed in PbA-HBO mice (18).

It is known that there is a crosstalk between hypoxia and AhR signaling pathways, as both require ARNT as a binding partner (33, 34). In addition, HIF-1α activity promotes AhR ubiquitination and proteasomal degradation (35). This interaction has been described in human cerebral microvascular endothelial cells, indicating that it could play a role in cellular responses after reduced oxygen availability at the BBB (51). In this regard, AhR plays a protective role during ECM. There is a significant downregulation of AhR expression in the brain during PbA infection, and AhR-deficient mice are highly susceptible to PbA infection (52). These mice display increased parasitemia, earlier mortality, enhanced leukocyte sequestration, and increased inflammation in the brain (52). Thus, AhR-protective effects seem to involve control of parasite replication and induction of immune responses required for host resistance, probably *via* activation of suppressor of cytokine signaling 1 and 3 pathways (52). Accordingly, here, we observed reduced AhR expression in brain endothelial cells of PbA-infected mice, whereas HBO treatment prevented downregulation of AhR expression. Together, our data show an inverse correlation of HIF-1α and AhR expression during ECM, indicating that the predominant orchestration of HIF-1α or AhR signaling pathways in the brain could determine a better or worse malaria outcome.

Significant alterations of the Trp metabolism have been described in different pathologic conditions related to immune activation and inflammation, such as infections, including malaria, autoimmune syndromes, various types of cancer, cardiovascular disease, and neurodegenerative processes (44, 46). A decrease of Trp concentration, along with an elevated Kyn:Trp ratio in plasma, cerebrospinal fluid (CSF), and other body fluids, is common in these conditions (44, 46). In addition, evidence shows that the IDO-dependent Trp degradation through the KP is involved in CM pathogenesis (42, 44, 46). Quinolinic acid (QA) levels, a metabolite downstream of the KP and associated with glutamate receptor-mediated excitotoxicity and impairment of BBB integrity, are elevated during CM (44). Furthermore, the concentration of Kyn acid (KA), an upstream metabolite of KP, thought to be neuroprotective, is decreased (44). These findings suggest that the Trp metabolism, through the KP, could be relevant to the hyperexcitability observed in murine and human CM. Accordingly, with the findings in murine models, Kenyan children with CM had a 14-fold greater concentration of QA in their CSF compared with an age-matched control set (44). Elevated QA was also found in CSF of Malawian

children with CM associated with a clinical history of convulsions (44). In Vietnamese adults, the QA level in the CSF and QA:KA ratio was significantly elevated, although this might have been a consequence of renal failure in these patients (44). At the cellular level, endothelial cells at the BBB and pericytes constitutively express components of the KP and synthesize Kyn and KA, which are secreted basolaterally (46, 53). The secreted Kyn can be further metabolized by perivascular macrophages and microglia with synthesis of QA (53). This could explain why KP activation at the BBB could result in local neurotoxicity, pointing to a mechanism whereby a systemic inflammatory signal can be transduced across an intact BBB to cause local neurotoxicity. However, despite the fact that the KP is highly active in the periphery, only Trp, Kyn, and 3-hydroxy-*l*-Kyn can be transported through the BBB to serve as substrates for production of neurotoxic metabolites in the CNS (54). Thus, another potential molecular mechanism of protection induced by HBO treatment during CM could be a result of the overall decrease in the availability of Kyn in the periphery and CNS that might subsequently affect the generation of neurotoxic metabolites derived from Trp degradation through the KP. Future in-depth studies are needed to ascertain the impact of HBO treatment on Trp catabolism in the CNS.

In summary, our data show, for the first time, the neuroprotective molecular pathways induced by HBO treatment during ECM. These insights support its potential as a supportive therapy in association with conventional treatment to improve poor CM outcomes. In addition, our data provide a molecular basis for the use of HBO in therapeutic strategies to improve treatment outcomes of other disorders affecting the brain. FJ

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AUTHOR CONTRIBUTIONS

M. F. Bastos, A. C. A. V. Kayano, N. Shryock, J. Aliberti, and F. T. M. Costa designed research; M. F. Bastos, A. C. A. V. Kayano, Y.C. Blanco, C. Judice, J. L. Silva-Filho, J. C. K. Dos-Santos, N. Shryock, M. K. Sercundes, L. S. Ortolan, C. Francelin, J. A. Leite, R. M. Elias, R. Oliveira, N. O. S. Câmara, S. C. P. Lopes, L. Albrecht, A. S. Farias, and C. P. Vicente analyzed data; R. M. Elias, N. O. S. Câmara, C. C. Werneck, S. Giorgio, L. Verinaud, S. Epiphanio, C. R. F. Marinho, P. Lalwani, R. Amino, J. Aliberti, and F. T. M. Costa performed research; J. L. Silva-Filho, M. F. Bastos, A. C. A. V. Kayano, J. C. K. Dos-Santos and C. Judice wrote the paper; C.C. Werneck, S. Giorgio, L. Verinaud, S. Epiphanio, C. R. F. Marinho, P. Lalwani, R. Amino, J. Aliberti and F. T. M. Costa contributed new reagents or analytic tools; M. F. Bastos, A. C. A. V. Kayano, Y.C. Blanco, J. C. K. Dos-Santos, C. Judice, J. A. Leite, M. K. Sercundes, L. S. Ortolan, C. Francelin, R. M. Elias, N. O. S. Câmara, N. Shryock, J. Aliberti, and F. T. M. Costa performed and recorded experiments; and N. Shryock and J. Aliberti performed all experiments with Tie2cre HIF-1a$^{fl/fl}$ mice.

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Anexo 2 – Questionário

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Instituto Leônidas e Maria Deane

QUESTIONÁRIO

ID paciente:_____

Nome:

_____

1. **Endereço: _____ CEP: _____**
2. **Bairro/ Comunidade: _____**
   Cidade:_____
3. **Há quanto tempo você mora nesta área? _____**
4. **Idade: _____** **Gênero/Sexo:** ☐ Masculino ☐ Feminino
5. **Qual seu Grupo Sanguíneo: _____** **RH:** ☐ Positivo ☐ Negativo
6. **Como você classifica a cor de sua pele/raça?**
   ☐ Branco ☐ Negro ☐ Pardo ☐ Amarelo ☐ Indígena
   ☐ Negro x índio ☐ Branco x índio ☐ Branco x negro
7. **Qual o seu nível de escolaridade?**
   ☐ Analfabeto ☐ Ensino fundamental completo
   ☐ Alfabetizado ☐ Ensino médio completo
   ☐ 1ª a 4ª série do ensino fundamental ☐ Superior incompleto
   ☐ 5ª a 8ª série do ensino fundamental ☐ Superior completo
   ☐ 1ª a 3ª série do ensino médio ☐ Não sabe
8. **Renda Familiar:** ☐ até 1 salário ☐ 2 salários ☐ 3 salários ☐ 4 salários ☐ 5 ou mais salários
9. **Você trabalha?** ☐ Sim ☐ Não
10. **Nos últimos 12 meses, qual foi sua principal ocupação?** [Entrevistador: com a ajuda do entrevistado, classifique a ocupação no grupo ocupacional mais adequado]
    ☐ Não tinha. Estavas desempregado (a)
    ☐ Altos funcionários do governo, dirigentes, gerentes ou altos funcionários de empresa
    ☐ Profissionais de nível superior
    ☐ Profissionais das artes
    ☐ Profissionais ou técnicos de nível médio
    ☐ Trabalhadores de serviços administrativos

☐ Trabalhadores da prestação de serviços e comerciários

☐ Trabalhadores de serviços domésticos

☐ Trabalhadores agropecuários, florestais de caça e pesca

☐ Trabalhadores manuais (produção de bens e serviços industriais)

☐ Trabalhadores manuais da construção civil

☐ Trabalhadores manuais de reparação e manutenção

☐ Membros das forças armadas, policiais e bombeiros militares

☐ Ocupações mal especificadas do trabalho informal (ambulante, manobrista, guardador de carro...)

☐ Autônomo: _____

☐ _____

11. **Você já teve Malária?** ☐ Sim ☐ Não **Se sim, quantos episódios?** _____
12. **Quando foi a última malária?** ☐ <2 meses ☐ 2-6 meses ☐ 6-12 meses ☐ >1 ano
13. **Já apresentou alguns dos sintomas abaixo durante algum episódio da malária?**
    ☐ Pele Amarelada ☐ Urina escura ☐ Não
14. **Tempo da doença atual? _____**
15. **Já está em uso de antimalárico?** ☐ Sim ☐ Não
    Se sim, qual antimalárico? _____
16. **Você tem: Diabetes, Hipertensão, Lúpus, Câncer ou outra doença?** ☐ Sim ☐ Não
    _____ **Quanto tempo? _____**
17. **Está tomando algum outro medicamento?** ☐ Sim ☐ Não
    Se sim, qual outro medicamento? _____
18. **Você já teve Dengue?** ☐ Sim ☐ Não _____
    Se sim, Quando? ☐ <2 meses ☐ 2-6 meses ☐ 6-12 meses ☐ >1 ano
19. **Você já tomou vacina contra Febre Amarela?** ☐ Sim ☐ Não ☐ Não sabe

Informações adicionais:

_____

_____

Análise Laboratorial

20. **Malaria**

    Gota espessa: ☐ Positivo ☐ Negativo **Fase do plasmódios:**_____

    Plasmodium Spp. ☐ Vivax ☐ Falciparum ☐ Ovale ☐ Malariae

    Parasitemia: _____

21. **Dengue:** ☐ IgM ☐ NS1 ☐ PCR **Dengue tipo:** ☐1 ☐ 2 ☐ 3 ☐ 4
22. **Outros**_____
    _____

23. **Leucócitos totais:** _____
24. **Eritrócitos:** _____
25. **Plaquetas:**_____
26. **Hemoglobina:** _____
27. **Neutrófilos:**_____
28. **Eosinófilos:**_____
29. **Basófilos:**_____
30. **Temperatura Corporal:** _____

Anexo 3 – Termo de Consentimento

UFAM

Universidade Federal do Amazonas, Faculdade de Ciências Farmacêuticas

Termo de Consentimento live e Esclaredcido (TCLE)

Você está sendo convidado para participar de uma pesquisa chamada: "**Avaliação da resposta Imune em paciente com Malária-Dengue co-infecção**", realizada por pesquisadores da Universidade Federal do Amazonas (UFAM).

A Malária e a Dengue são um crescente problema de saúde pública no Brasil e, em especial na região Amazônica, estão associadas ao aumento da morbidade e mortalidade. A influência da co-infecção na resposta imunológica de doenças não está clara, sendo assim, o objetivo deste projeto é tanto obter informações e dados para diferencia-las clinicamente e biologicamente das infecções sozinhas, assim como, determinar os mecanismos e a via de sinalização em células e identificar biomarcadores em pacientes co-infectatos para Malária e Dengue, e compará-los às infecções sozinhas. Estes estudos terão impacto no conhecimento básico desses patógenos humanos e, também, serão primordiais para a criação de novas terapias e vacinas eficazes contra doenças transmissíveis por artrópodes.

A sua participação nesta pesquisa consiste em permitir a coleta de 60mL de sangue da veia, em três momentos: dia 0 (quando apresentam sintomas e incluídos no estudo), dias 8-10 e dias 25-28 com o uso de agulha e seringa descartáveis, além de responder a um questionário com dados epidemiológicos. O seu sangue será analisado nos laboratórios da UFAM e poderá ser guardado para futuras pesquisas, se você permitir. Os exames realizados no seu sangue servem para detecção de infecções, marcadores da resposta imunológica e genéticos do patógeno e do hospedeiro. O FMT dará a você o resultado dos testes para dengue e malária .

Os riscos relacionados com sua participação são a dor causada pela picada da agulha e um pequeno sangramento que pode acontecer após a coleta de sangue. Para amenizar estes possíveis desconfortos a coleta de sangue será realizada por pessoal treinado e experiente na coleta de sangue e com a adoção rigorosa dos procedimentos padrão de biossegurança para a coleta de sangue. O único benefício relacionado com a sua participação é o de nos ajudar a conhecer essa doença no seu município e as situações de risco para sua transmissão, além do conhecimento gerado concorrem para tal desfecho.

A sua participação neste estudo é voluntária, não implicando em qualquer custo para você. A qualquer momento você pode desistir de participar e isso não trará nenhum prejuízo em sua relação com o pesquisador ou com a UFAM. Todas as informações coletadas serão mantidas confidencialmente. Os seus dados serão armazenados em um computador e seu nome não aparecerá em nenhuma publicação, apresentação ou documento.

Solicitamos sua autorização para armazenamento de suas amostras de sangue por dez anos, e se o Sr(a) autorizar ela poderá ser utilizado em estudos posteriores desde que autorizado pelo Comitê de Ética em Pesquisa da Universidade Federal do Amazonas ou, caso necessário, a Comissão Nacional de Ética em Pesquisa e pelo responsável por esta pesquisa atual.

Por isso, pedimos que o Sr(a). se manifeste abaixo sobre o armazenamento e uso da sua amostra de sangue em estudos posteriores:

[ ] Não, a minha amostra não deverá ser armazenada.

[ ] Sim, concordo que a minha amostra seja armazenada e utilizada em estudos posteriores.

Se concordar com o armazenamento da amostra de sangue, pedimos que se manifeste abaixo sobre a necessidade de ser consultado para cada nova pesquisa com a sua amostra e seus dados:

[ ] Não quero ser consultado, mesmo sabendo que assim não terei os possíveis benefícios dos resultados do novo projeto de pesquisa.

[ ] Sim, exijo ser consultado para autorizar o uso de minha amostra no novo projeto de pesquisa.

Também em caso de concordar com o armazenamento da sua amostra de sangue, e não exigir ser consultado em estudos posteriores, é possível ainda que sua identidade seja desvinculada dos seus dados e da amostra de sangue, sendo substituídos por códigos, o que aumentaria a segurança de seu anonimato. Porém, a decisão de desvincular a sua identidade da amostra significa que em todos os estudos posteriores onde a sua amostra será utilizada não será possível relacionar os resultados à sua pessoa e assim o Sr.(a) não teria os possíveis benefícios dos resultados do novo projeto de pesquisa. Um benefício de sua participação neste estudo é a contribuição para o conhecimento científico na área da saúde e doenças infecciosas.

Assim, pedimos que se manifeste sobre a desvinculação de sua identidade da amostra de sangue e dados:

[ ] Não, a minha identidade não pode ser desvinculada da amostra e dados

[ ] Sim, concordo que minha identidade seja desvinculada da amostra e dados, mesmo sabendo que assim não terei os possíveis benefícios dos resultados do novo projeto de pesquisa.

Nome _____

Endereço _____

E-mail _____

Celular/ telefone _____

O Sr(a) receberá uma via deste termo, que será assinado pelo Sr(a) e o pesquisador responsável. Logo abaixo, você tem o nome, telefone e o endereço do pesquisador principal e CEP/UFAM que poderá tirar suas dúvidas sobre o projeto e sua participação quando você desejar.

Declaro que entendi os objetivos, riscos e benefícios de minha participação na pesquisa e concordo em participar.

_____, ____ de _____ de _____

Assinatura do Pesquisador responsável

_____, ____ de _____ de _____

Assinatura do Participante

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Assinatura alternativa
(Impressão do dedo polegar)