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"Desenvolvimento de Drogas Experimentais e Imunopatogênese na Malária"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Imunologia.

Orientador: Prof. Dr. Fabio Trindade Maranhão Costa

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Lista de Abreviaturas

ACT	Tratamento combinado à base de artemisinina
	Artemisinin Combined Treatment
Akt	Proteína quinase B
ALI	Dano Pulmonar Agudo
	Acute Lung Injury
ARDS	Síndrome Respiratória de Desconforto Agudo
	Acute Respiratory Dystress Syndrome
ARM	Artemether
ARS	Artesunato
ART	Artemisinina
BSA	Albumina bovina
	Bovine Serum Albumine
CD36	Cluster de Diferenciação 36
CEEA	Comitê de Ética em Experimentação Animal
CEMIB	Biotério Central da Unicamp
CHO	Células de Ovário de Hamster Chinês
	Chinese Hamster Ovarium
CPM	Contagens por minuto
CQ	Cloroquina
CS	Proteína do Circumsporozoíta
CSA	Condroitin-Sulfato A
DHA	Dihydroartemisinina
DMSO	Dimethil Sulfóxido
DO	Densidade Óptica
ECGS	Suplemento de Crescimento de Célula Endotelial
	Endothelial Cell Growth Suplement

EI	Eritrócito Infectado
ELISA	Ensaio Imuno-enzimático
	Enzyme-Linked Immunoabsorbent Assay
ERO	Espécie Reativa de Oxigênio
FMT-HVD	Fundação de Medicina Tropical Heitor Vieira Dourado
GMAP	Plano de Ação Global em Malária
	Global Malaria Action Plan
GPARC	Plano Global de Contenção de Resistência à Artemisinina
	Global Plan for Artemisinin Resistance Containment
HLEC	Célula endotelial pulmonar humana
	Human Lung Endothelial Cell
HPLC	Cromatografia Líquida de Alta Eficiência
	High Performance/Pressure Liquide Chromatography
i.p.	Intraperitoneal
IC_{50}	Concentração inibitória 50%
	Inhibition concentration 50%
ICAM-1	Molécula de Adesão Intercelular 1
	Intra-Cellular Adhesion Molecule 1
IFN	Interferon
ILMD	Instituto Leônidas e Maria Deane
LDH	Lactato Desidrogenase
LPS	Lipopolissacarídeo
mAb	Anticorpo monoclonal
	Monoclonal Antibody
MC	Malária cerebral
MQ	Mefloquina
NCI	Instituto Nacional do Câncer
	National Cancer Institute
NFκB	Fator nuclear κB
NP-violaceína	Violaceína encapsulada em PLGA

NT	Nanotubos de carbono
OMS	Organização Mundial de Saúde
PAM	Malária associada à gravidez *Pregnancy associated malaria*
PBS	Tampão fosfato *Phosphate Buffer Saline*
PcchAJ	*Plasmodium chabaudi chabaudi* AJ
PcchAS	*Plasmodium chabaudi chabaudi* AS
PCL	Poli ε- caprolactona
Pf-EI	Eritrócitos Infectados por *P. falciparum*
PfEMP-1	Proteína 1 da Membrana do Eritrócito Infectado por *P. falciparum*
PLGA	Co-polímero de ácido lático e ácido glicólico
PNCM	Programa Nacional de Controle da Malária
Pv-EI	Eritrócitos Infectados por *P. vivax*
QN	Quinino
Rb	Retinoblastoma
RBM	*Roll Back Malaria*
SIVEP	Sistema de Informação em Vigilância Epidemiológica
SP	Sulfadoxina- Pirimetamina
SUSAM	Secretaria do Estado da Saúde do Amazonas
TCLE	Termo de Consentimento Livre e Esclarecido
TNF	Fator de Necrose Tumoral *Tumor Necrose Factor*
TNFR1	Receptor de Fator de Necrose Tumoral 1 *Tumor Necrose Factor Receptor 1*
TRAP	Proteína adesiva relacionada a trombospondina

Lista de figuras

Resumo

Este trabalho de tese está dividido em dois capítulos. Abaixo descreveremos brevemente os achados de cada um deles.

O primeiro capítulo entitulado ***Desenvolvimento de Drogas Experimentais*** trata da atividade antimalárica de um composto natural, a violaceína e suas formulações geradas com o auxílio da nanotecnologia. Neste sentido, a violaceína foi eficiente em eliminar *in vitro* formas sanguíneas de *P. falciparum* 3D7 e *P. falciparum* S20, sendo este último isolado resistente à cloroquina. Quando testada *in vitro* em isolados frescos de *P. vivax,* a violaceína não foi capaz de inibir o desenvolvimento parasitário. No entanto, acreditamos que novos ensaios são necessários para confirmar este dado, uma vez que o IC_{50} (concentração inibitória 50%) observado para cloroquina apresentou-se estranhamente elevado. Os experimentos *in vivo* utilizando camundongos infectados com *Plasmodium chabaudi chabaudi* AS revelaram uma potente atividade desta droga, inclusive quando o tratamento foi iniciado após o estabelecimento de infecção patente nos animais. Quando administrada em animais infectados com uma cepa murina letal (*Plasmodium chabaudi chabaudi* AJ), a violaceína protegeu 80% dos animais. Como a violaceína é um composto insolúvel em água, novas formulações com essa molécula foram feitas visando sua solubilidade e também potencializar sua atividade antimalárica. Neste sentido, tanto a violaceína encapsulada com co-polímero de ácido láctico e ácido glicólico quanto as formulações de violaceína com três diferentes nanotubos de carbono também foram capazes de inibir o crescimento parasitário *in vitro* contra *P. falciparum*, no entanto esta atividade não foi potencializada. Em síntese, este capítulo nos mostra que a violaceína apresenta capacidade de

inibir o crescimento parasitário de *Plasmodium* sp., no entanto as suas formulações não foram capazes de potencializar a atividade antimalárica.

O segundo capítulo entitulado ***Imunopatogênese na malária vivax*** trata da capacidade adesiva de eritrócitos infectados por *P. vivax* (Pv-EI) a diferentes receptores endoteliais e da capacidade deste parasita em formar rosetas. Uma vez que há uma baixa proporção de formas maduras principalmente esquizontes na circulação periférica de pacientes infectados por *P. vivax*, estabelecemos uma técnica de cultivo *in vitro* para obtenção destas formas maduras e investigamos a capacidade adesiva destas formas do parasita. Neste sentido, observamos que Pv-EI pós-amadurecimento apresentam maior capacidade adesiva em HLEC (células endoteliais de pulmão humano) que parasitas do mesmo isolado antes do amadurecimento. Quando avaliado os receptores envolvidos antes e após o amadurecimento, ensaios com células transfectadas ou uso de inibidores específicos, revelou a participação de ICAM-1 (molécula de adesão intercelular 1) e CD36 (cluster de diferenciação 36) na citoadesão deste parasita enquanto a participação de CSA (Condroitin Sulfato A) permanece a ser elucidada. Ao passo que ICAM-1 parece estar envolvida na citoadesão de todos os estágios do parasitas, o CD36 parece ser estritamente envolvido na citoadesão de formas maduras, essencialmente esquizontes. Além disso, a citoadesão de Pv-EI em HLEC foi potencializada na presença de soro do próprio paciente, sugerindo a existência no soro dos infectados de mediadores adesivos. Quando investigada a formação de rosetas por Pv-EI verificamos que a porcentagem de rosetas foi maior nas formas amadurecidas e também foi potencializada na presença de soro autólogo. Os dados aqui apresentados abrem perspectivas para novos estudos visando compreender o fenômeno patológico envolvido na malária vivax grave, incluindo a participação de mediadores plasmáticos e a identificação dos receptores celulares e dos ligantes parasitários envolvidos.

Abstract

This thesis was divided into two chapters, and below we briefly describe the findings of each one.

The first chapter entitled ***Experimental Drug Development*** investigates the antimalarial activity of a natural compound, violacein, and its formulations developed by nanotechnology. Violacein effectly killed in vitro blood stages of *P. falciparum* 3D7 and *P. falciparum* S20, a chloroquine resistant strain. When tested in vitro against *P. vivax* fresh isolates, violacein did not inhibit parasite development. However, further experiments are needed to confirm this finding, as chloroquine IC_{50} (inhibition concentratios 50%) was strangely high. The in vivo experiments using mice infected with *Plasmodium chabaudi chabaudi* AS revealed a potent activity of this drug, even when treatment was initiated after the establishment of patent infection in animals. When administered to lethal strain (*Plasmodium chabaudi chabaudi* AJ) infected mice, violacein protected 80% of animals. As violacein is insoluble in water, new formulations were developed to enhance its solubility and also its antimalarial activity. However, violacein encapsulated with PLGA (poly(lactic-co-glycolic acid)) and violacein formulations with three different carbon nanotubes did not enhanced violacein activity in vitro against *P. falciparum.* In summary, this chapter shows that violacein has the capacity to inhibit *Plasmodium* sp. growth; still their formulations were not able to potentiate violacein antimalarial activity.

The second chapter entitled ***Immunopathogenesis of vivax malaria*** investigates the adhesive capacity of *P. vivax* infected erythrocytes (Pv-IE) in different endothelial cell receptors and the ability of this parasite to form rosettes. Since there are a low proportion of mature forms,

especially schizonts, in peripheral circulation of malaria vivax patients, we established an in vitro technique to obtain these mature forms and investigate their adhesive capacity in endothelial cells. Here we showed that post-maturation Pv-IE presented more adhesive capacity in HLEC (human lung endothelial cells) than the same isolate before maturation. The search for receptors involved in cytoadhesion before and after maturation, using transfected cells or specific inhibitors, showed that ICAM-1 (intracellular adhesion molecule 1) and CD36 (cluster of differentiation 36) were involved in Pv-IE cytoadhesion whereas CSA (chondroitin sulfate A) involvement remains to be elucidated. While ICAM-1 seems to be involved in all stages of Pv-IE cytoadhesion, CD36 seems to be strictly involved in mature forms cytoadhesion, essentially schizonts. Furthermore, Pv-IE cytoadhesion in HLEC was enhanced in patient's serum presence, suggesting the existence of adhesive mediators in Pv-IE infected serum. Rosette formation by Pv-IE was also higher in mature forms and rosette formation was enhanced in autologous serum presence. These data collectively open new perspectives to study the pathological phenomenon involved in severe vivax malaria, including the involvement of plasma mediators and identification of cell receptors and parasite ligands involved in Pv-IE cytoadhesion.

Sumário

Anexos

On cytoadhesion of Plasmodium vivax: raison d'être?

Costa FT, **Lopes SC**, Ferrer M, Leite JA, Martin-Jaular L, Bernabeu M, Nogueira PA, Mourão MP, Fernandez-Becerra C, Lacerda MV, del Portillo H. Mem Inst Oswaldo Cruz. 2011 Aug;106 Suppl 1:79-84.

In vitro and in vivo assessment of the anti-malarial activity of Caesalpinia pluviosa.

Kayano AC, **Lopes SC**, Bueno FG, Cabral EC, Souza-Neiras WC, Yamauchi LM, Foglio MA, Eberlin MN, Mello JC, Costa FT. Malar J. 2011 May 2;10:112.

On the cytoadhesion of Plasmodium vivax-infected erythrocytes.

Carvalho BO, **Lopes SC**, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, Mamoni R, Leite JA, Rodrigues MM, Soares IS, Oliveira TR, Wunderlich G, Lacerda MV, Del Portillo HA, Araújo MO, Russell B, Suwanarusk R, Snounou G, Rénia L, Costa FT. J Infect Dis. 2010 Aug 15;202(4):638-47.

Violacein extracted from Chromobacterium violaceum inhibits Plasmodium growth in vitro and in vivo.

Lopes SC, Blanco YC, Justo GZ, Nogueira PA, Rodrigues FL, Goelnitz U, Wunderlich G, Facchini G, Brocchi M, Duran N, Costa FT. Antimicrob Agents Chemother. 2009 May;53(5):2149-52.

Regulatory T cell induction during Plasmodium chabaudi infection modifies the clinical course of experimental autoimmune encephalomyelitis.
Farias AS, Talaisys RL, Blanco YC, **Lopes SC**, Longhini AL, Pradella F, Santos LM, Costa FT. PLoS One. 2011 Mar 25;6(3):e17849.

Immunogenic properties of a recombinant fusion protein containing the C-terminal 19 kDa of Plasmodium falciparum merozoite surface protein-1 and the innate immunity agonist FliC flagellin of Salmonella typhimurium.
Bargieri DY, Leite JA, **Lopes SC**, Sbrogio-Almeida ME, Braga CJ, Ferreira LC, Soares IS, Costa FT, Rodrigues MM. Vaccine. 2010 Apr 1;28(16):2818-26.

Hyperbaric oxygen prevents early death caused by experimental cerebral malaria.
Blanco YC, Farias AS, Goelnitz U, **Lopes SC**, Arrais-Silva WW, Carvalho BO, Amino R, Wunderlich G, Santos LM, Giorgio S, Costa FT. PLoS One. 2008 Sep 4;3(9):e3126.

Apresentação

A malária é uma doença infecciosa parasitária causada por protozoários pertencentes ao filo Apicomplexa, à família Plasmodiidae e ao gênero *Plasmodium*. Dezenas de espécies de *Plasmodium* são capazes de infectar uma ampla faixa de espécies animais, como répteis, aves e mamíferos, entretanto somente quatro espécies são reconhecidas como parasitas naturais do homem: *Plasmodium falciparum*, *P. vivax*, *P. malariae* e *P. ovale*. Recentemente, observou-se um aumento nos relatos de infecções maláricas em humanos nas quais o agente etiológico é *P. knowlesi*, parasita que normalmente circula entre símios (Sing et al., 2004). Contudo, essa espécie ainda apresenta um caráter de zoonose, diferente das outras quatro espécies que infectam o homem.

Apesar de haver peculiaridades de cada espécie de Plasmodium, o ciclo biológico é bem similar entre as espécies que infectam o homem.

1. Ciclo Biológico

Os plasmódios apresentam um ciclo de vida complexo (Figura 1) com o envolvimento de dois hospedeiros, um hospedeiro intermediário vertebrado e um hospedeiro definitivo invertebrado.

Resumidamente, o ciclo de vida dos plasmódios inicia-se com a picada do mosquito fêmea do gênero *Anopheles* sp., que durante o repasto sanguíneo, inocula formas esporozoítas no hospedeiro vertebrado. Recentemente, observou-se que a maioria dos esporozoítos são retidos na pele por minutos ou horas antes de atingir o fluxo sanguíneo e ainda que alguns deles migram ativa ou passivamente para os linfonodos (Amino et al., 2006). Após invadirem os hepatócitos,

apenas as formas que atingem a corrente sanguínea são capazes de se desenvolverem (Amino et al., 2007).

Nos hepatócitos, os parasitos se desenvolvem em esquizontes que irão originar milhares de merozoítos. Ainda no fígado, *P. vivax* e *P. ovale* evoluem para uma forma latente conhecida como hipnozoíta que, posteriormente, por mecanismos ainda desconhecidos podem dar início a um novo ciclo sanguíneo, sendo responsável pela recaída da doença. O ciclo hepático dura entre 6 a 15 dias, e corresponde ao período de incubação da doença.

Os merozoítos liberados pelos hepatócitos nos sinusóides hepáticos, inicialmente em pequenas vesículas chamadas merossomos (Sturm et al., 2006), irão rapidamente invadir os eritrócitos, desenvolvendo-se em trofozoítos, que através de através da divisão nuclear, darão origem aos esquizontes. Os esquizontes se rompem, culminando com a ruptura dos eritrócitos infectados, e então há liberação de merozoítos na corrente sanguínea, que rapidamente invadem novos eritrócitos, repetindo o ciclo assexuado. O ciclo eritrócito se repete a cada 48h nas espécies *P. falciparum*, *P. vivax* e *P. ovale* e a cada 72h em *P. malariae*. Esse sincronismo é o que causa o cenário de febres cíclicas em infecções estabelecidas.

Alguns merozoítos diferenciam-se em formas sexuais masculinas e femininas (gametócitos), que são ingeridas pelo mosquito durante o repasto sanguíneo. A fusão dos gametas forma o zigoto, que se desenvolve em um oocineto. Este penetra na parede do intestino do inseto, formando um oocisto entre o epitélio intestinal e a lâmina basal. Então, muitos esporozoítos são formados assexuadamente dentro do oocisto, e são liberados ao amadurecer. Esses esporozoítos migram para a glândula salivar do mosquito, onde podem ser transmitidos para o hospedeiro intermediário durante o repasto sanguíneo, dando continuidade ao ciclo.

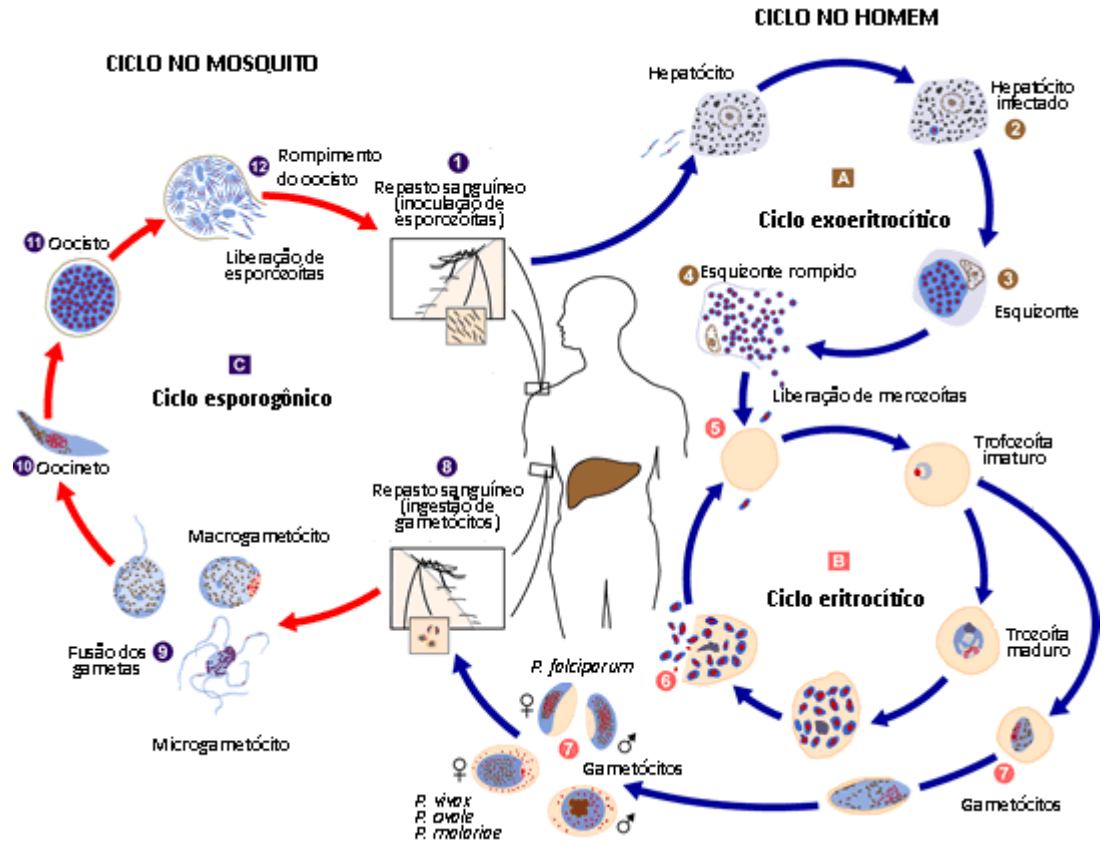

Figura 1. Ciclo de vida de Plasmodium sp. O ciclo da malaria envolve dois hospedeiros. Durante o repasto sanguíneo, o mosquito *Anopheles* fêmea infectado inocula esporozoítas no hospedeiro humano ❶. Os esporozoítas infectam as células do fígado ❷ e se desenvolvem em esquizontes ❸, que se rompem liberando merozoítas ❹. Após esta primeira replicação no fígado (Ciclo exoeritrocítico A), o parasita se multiplica assexuadamente no eritrócito (Ciclo eritrocítico B). Os merozoítas liberados infectam os eritrócitos. ❺. Os trofozoítas amadurecem em esquizontes, que se rompem culminando com a liberação de mais merozoítas ❻. Alguns parasitas se diferenciam em estágios sexuados (gametócitos) ❼. Os gametócitos, macho (microgametócitos) e fêmea (macrogametócito), são ingeridos pelo mosquito *Anopheles* durante o repasto sanguíneo ❽.A multiplicação do parasita no mosquito é conhecida como Ciclo Esporogônico C. No estômago do mosquito o microgameta penetra o macrogameta gerando o zigoto ❾. O zigoto se torna móvel e alongado (oocineto) ❿, invade a parede do intestine do mosquito e se desenvolve em oocisto ⓫. O oocisto cresce e se rompe, liberando esporozoítas ⓬, que migram para a glândula salivar do msoquito. A inoculação de esporozoítas ❶ em um novo hospedeiro humano perpetua o ciclo da malária. Fonte:.http://www.cdc.gov/malaria/about/biology/index.htm

2. A doença Malária

A malária na sua forma mais frequente, não grave, traduz-se clinicamente em uma síndrome febril aguda indiferenciada. O paroxismo febril é decorrente da ruptura de eritrócitos parasitados (Figura 1, item 6), já que nesta etapa ocorre a liberação na circulação sanguínea de antígenos constituintes do parasito e os formados em consequência de seu metabolismo, que causam uma intensa ativação do sistema imune (de Souza & Riley, 2002).

Outra manifestação clínica observada em indivíduos infectados é o quadro de anemia, que pode ser decorrente de um somatório de eventos responsáveis pelo seu desenvolvimento, entre eles o processo de destruição de eritrócitos durante a liberação do parasito, o aumento da eritrofagocitose esplênica e a deficiência do mecanismo de eritropoiese (Chang & Stevenson, 2004).

A malária grave, por ser responsável por grande número de óbitos em áreas tropicais, tem sido o alvo de grande parte dos estudos em malária. Alguns fatores estão envolvidos com a virulência dos plasmódios, como a capacidade de multiplicação do parasita, a capacidade do parasita em citoaderir ao endotélio capilar e tecido placentário, a imunidade do hospedeiro (Good et al., 2005), além de questões geográficas e sociais (Miller et al., 2002).

As infecções por *P. falciparum* levam a mais quadros de complicação e, portanto maior número de óbitos (WHO, 2011). Essa maior patogenicidade da malária falciparum é atribuída à capacidade de eritrócitos infectados por *P. falciparum* citoaderirem na microvasculatura de diferentes órgãos como cérebro, pulmões e placenta (MacPherson et al., 1985; Rowe et al., 1995; Newbold et al., 1999; Roberts et al., 2000; Pain et al., 2001). Nesse sentido, em 1990 a Organização Mundial de Saúde (OMS) estabeleceu os sinais e sintomas clínicos que caracterizam

a malária grave por *P. falciparum* (WHO, 2000). São consideradas formas graves da doença malária cerebral (MC), anemia grave, insuficiência renal aguda, edema pulmonar, hipoglicemia, colapso circulatório e acidose metabólica (WHO, 2000).

Apesar de ainda serem desconhecidos os mecanismos fisiopatogênicos, casos de malária vivax grave, inclusive com óbitos, têm sido relatados em várias partes do mundo (Price et al., 2007; Kochar et al., 2005; Kochar et al., 2007; Kochar et al., 2009; Barcus et al., 2007; Baird et al., 2007; Baird et al., 2009; Tjitra et al., 2008; Genton et al., 2008; Tanwar et al., 2011), inclusive na Amazônia brasileira (Lomar et al., 2005; Alexandre et al., 2010; Andrade et al., 2010; Lacerda et al., 2012). Nesse sentido trabalho recente de nosso grupo constatou que eritrócitos infectados por *P. vivax* são capazes de citoaderir *ex vivo* em diferentes receptores de células endoteliais e em criocortes de tecido placentário (Carvalho et al., 2010).

3. Situação da malária no Brasil e mundo

A malária é a doença parasitária mais comum, causadora de mortalidade e morbidade em países localizados em regiões tropicais e subtropicais, e continua sendo o maior desafio em saúde pública em todo o mundo (WHO, 2011). A doença é endêmica em mais de cem países (Figura 2), e cerca de 40% da população do mundo permanece sob o risco de infecção por malária (WHO, 2011).

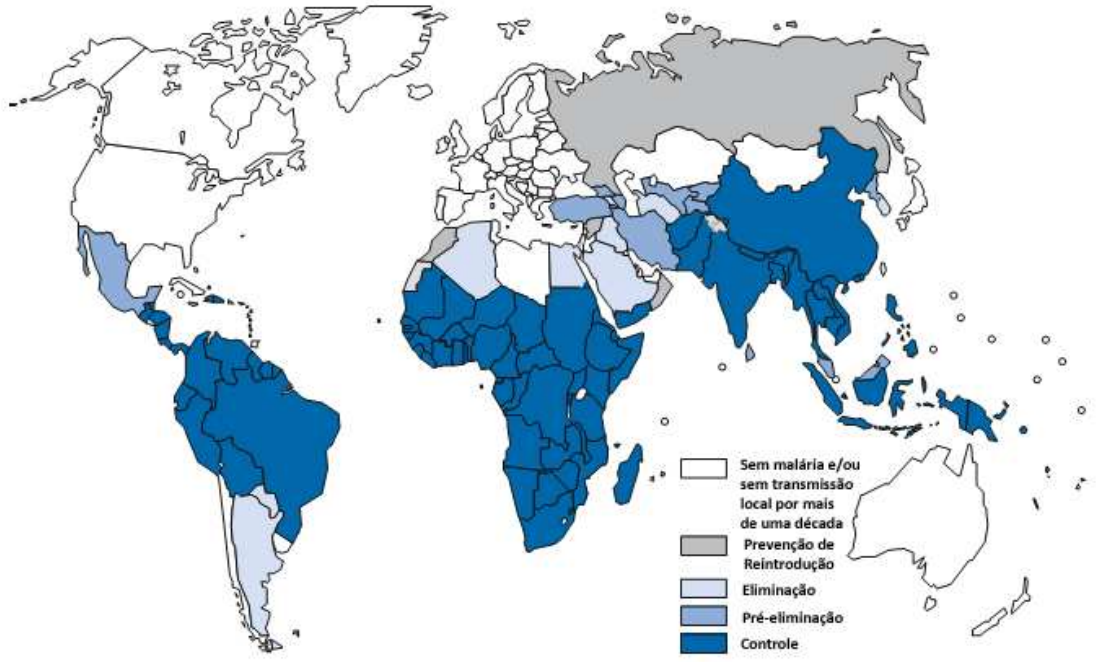

Figura 2: Mapa de distribuição global de malária em 2008. Adapatado de: WHO, 2008..

Em 2010, foram estimados 216 milhões de novos casos de malária, e cerca de 660 mil mortes por esta infecção (WHO, 2011). Aproximadamente 81% do total de casos (174 milhões) e 91% das mortes ocorreram na África (WHO, 2011), em parte devido à maior incidência de malária falciparum nessa população. Embora, a maioria dos casos ocorra na África, a doença está amplamente distribuída na América Latina, sudeste asiático e Oceania, como podemos ver na Figura 2.

Enquanto *P. falciparum* predomina no continente africano, *P. vivax* é a espécie mais prevalente nas outras áreas onde a doença é endêmica, como por exemplo, sul e sudeste da Ásia, Pacífico Ocidental, leste do Mediterrâneo, América Central e América do Sul (Bassat & Alonso, 2011).

Na América do Sul e América Central, em 2010, foram mais de 700 mil casos registrados, sendo o *P. vivax* responsável por mais de 70% dos casos de malária nessa região. O Brasil é responsável por mais de 50% das infecções maláricas nas Américas, e em 2009 foram registrados 306 mil casos sendo 83,7% por *P. vivax* (Oliveira-Ferreira et al., 2010). A malária no Brasil ocorre quase que exclusivamente (99,8%) na região da Amazônia Legal, que inclui os estados do Amazonas, Pará, Acre, Roraima, Rondônia, Amapá, Mato Grosso, Tocantins e Maranhão (Figura 3).

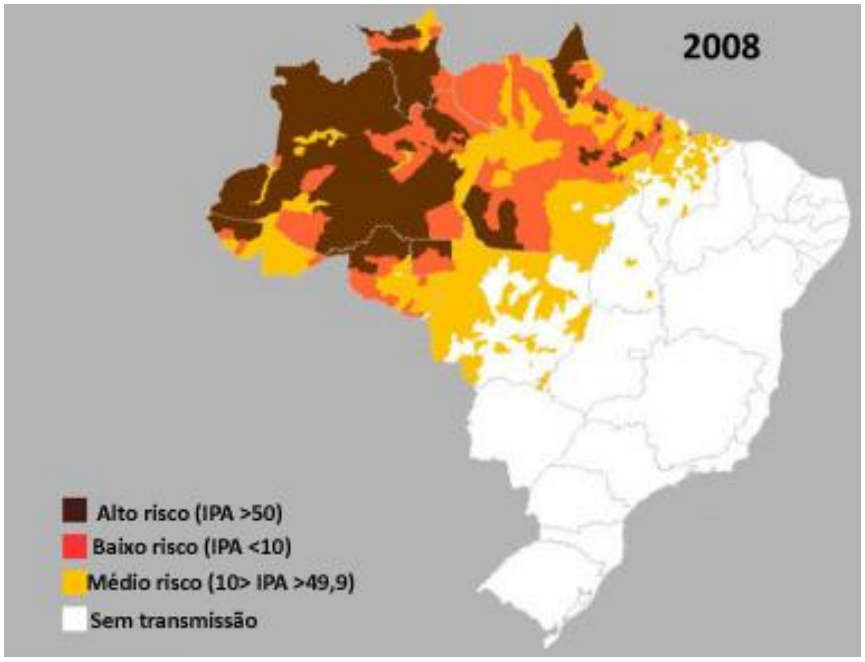

Figura 3: Mapa de distribuição da malária no Brasil em 2008. IPA: Indíce Parasitário Anual. Adaptado de Oliveira-Ferreira et al., 2010.

Desde a década de 60, diversas campanhas para erradicação da malária vêm sendo seguidas mundialmente, mais recente o Plano de Ação Global em Malária (GMAP) coordenado pela OMS em parceria com *Roll Back Malaria* (RBM) vem apresentando resultados satisfatórios. Nesse

sentido, 43 de 99 países em área de transmissão reduziram em 50% ou mais o número de casos entre os anos 2000 e 2010 (WHO, 2011). No Brasil, o Programa Nacional de Controle de Malária (PNCM) também apresentou resultados bastante promissores, com a redução de 50% dos casos de malária em 47 municípios que juntos eram responsáveis por 70% dos casos no país (Oliveira-Ferreira et al., 2010). Entretanto, globalmente os números ainda estão abaixo do esperado (redução de 17% na incidência e 26% na mortalidade entre os anos 2000 e 2010) e novos financiamentos, melhorias em prevenção, diagnóstico e tratamento são necessários para a idealizada eliminação da malária.

Capítulo 1

Desenvolvimento de drogas experimentais

Introdução

Antimaláricos e Resistência

O quinino (QN), extraído da raiz da árvore chinchona, foi o primeiro agente antimalárico e foi utilizado no tratamento de malária desde 1632 (Talisuna et al., 2004). Até o século 19 era o único antimalárico conhecido, mas após a Primeira Guerra Mundial, foram produzidos alguns derivados de QN, como a primaquina (PQ) e a cloroquina (CQ).

CQ é um 4-aminoquinoleína derivada de quinino sintetizada pela primeira vez em 1934 (Coatney, 1963) e desde então foi a droga antimalárica mais amplamente usada. Historicamente, foi a droga de escolha para o tratamento de malária não-grave ou malária não complicada por muitos anos, entretanto a resistência do parasita à CQ reduziu drasticamente sua utilidade (WHO, 2011).

Acredita-se que a resistência à CQ tenha emergido independentemente em quatro focos (Talisuna et al., 2004). O primeiro foco de resistência à CQ por *P. falciparum* foi identificado na divisa entre Tailândia e Cambódia em 1957 (Harinasuta et al., 1965). Posteriormente, em 1960 dois focos foram identificados na América do Sul, um na Venezuela e outro na Colômbia (Moore & Cowman, 1994). Em 1976, o quarto foco de resistência foi reportado na Papua Nova Guiné (Talisuna et al., 2004). A resistência à CQ na África foi encontrada em 1978 no Quênia (Fogh et al., 1979) e na Tanzânia (Campbell et al., 1979). Hoje a resistência à CQ está presente em praticamente todos os países onde há infecção por *P. falciparum* como podemos ver na Figura 4.

Em 1973, a Tailândia foi o primeiro país a trocar o tratamento de primeira escolha para malária falciparum com CQ, pela combinação Sulfadoxina-Pirimetanina (SP), seguido por países da Ásia e América do Sul (Talisuna etal., 2004). Na África, somente em 1988 o tratamento com CQ foi substituído por SP.

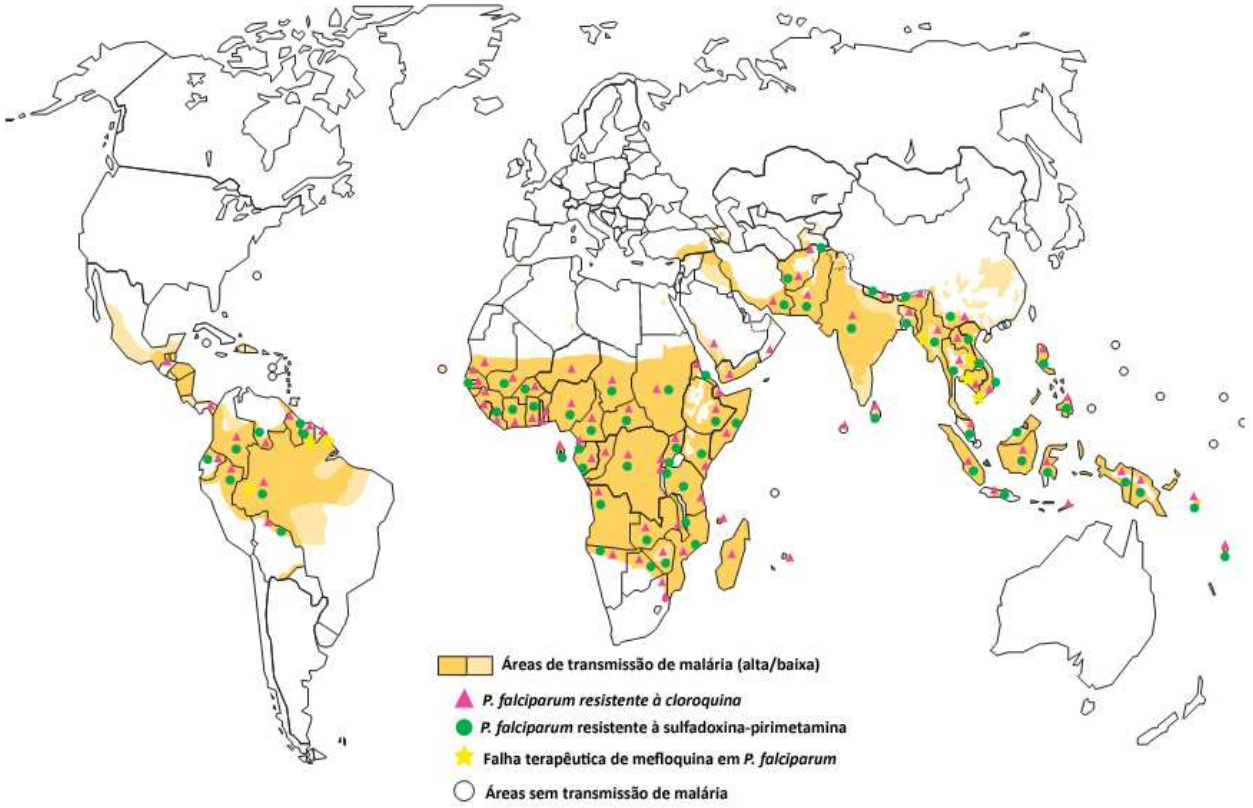

Figura 4: Mapa de distribuição global de resistência aos antimaláricos por *P. falciparum* em 2004. Adaptado de WHO, 2005..

A resistência de *P. falciparum* à SP emergiu quase que simultaneamente à sua introdução, por exemplo, na Tailândia esse tratamento foi introduzido em 1967 e no mesmo ano já houve relato de resistência (Wernsdorfer & Payne, 1991). Isso pode ser devido ao pequeno número de mutações genéticas envolvidas (em comparação à CQ) e também a elevada meia-vida da droga (Foote & Cowman, 2004). Em 1980, depois destas duas drogas (CQ e SP) terem sido consideradas ineficientes, os pacientes com malária *falciparum* passaram a ser tratados com QN. No entanto, a resistência ao QN foi constatada logo após a sua introdução e em 1981 foi iniciado

um tratamento combinado com QN e tetraciclina (Trigg & Kondrachine, 1998). Todavia, um dos problemas associados a esta combinação de drogas era a necessidade de longos períodos de tratamento para a completa eliminação do parasita dificultando o monitoramento do tratamento. Sendo assim, em 1984, foi introduzido um regime de tratamento à base de mefloquina (MQ) administrada em dose única. Entretanto, nos anos 90, casos de resistência à MQ começaram a ser constatadas na Tailândia, principalmente nas áreas de fronteira com o Camboja e o Vietnãm (Nosten et al., 2000).

A resistência à CQ por *P. vivax*, a segunda espécie de *Plasmodium* mais prevalente, surgiu tardiamente, com os primeiros casos descritos em 1989 na Papua Nova Guiné (Rieckmann et al., 1989) e ainda hoje não é tão disseminada como em *P. falciparum* (Figura 5). Estudos posteriores avaliando os índices de resistência à cloroquina em *P. vivax* mostram claramente que a Indonésia Oriental apresenta o problema da resistência mais avançado, com somente 50% de sucesso terapêutico em média (Baird et al., 1995; Baird et al., 1997a; Baird et al, 1997b; Ratcliff et al., 2007; Sumawinata et al., 2003; Sutanto et al., 2009). Já em outros países asiáticos, como Tailândia (Congpuong et al., 2002; Krudsood et al., 2007; Tasanor et al., 2006; Vijaykadga et al., 2004), Índia (Nandy et al., 2003; Valecha et al., 2006), Paquistão (Leslie et al., 2007), Afeganistão (Kolaczinski et al., 2007) e Azerbaijão (Valibayov et al., 2003) a resistência é baixa (menor que 5%) ou mesmo ausente. Pesquisas na América do Sul mostraram evidências que vão desde ausência a um baixo nível de resistência (5%) (Castillo et al., 2002; Ruebush et al., 2003), no entanto um estudo com apenas 27 pacientes encontrou 11% de resistência (Soto et al., 2001). Um recente estudo realizado em Manaus, Brasil com 109 pacientes encontrou 10% de falha terapêutica, mesmo tendo estes pacientes níveis adequados de cloroquina no plasma no momento da parasitemia recorrente (de Santana Filho, 2007).

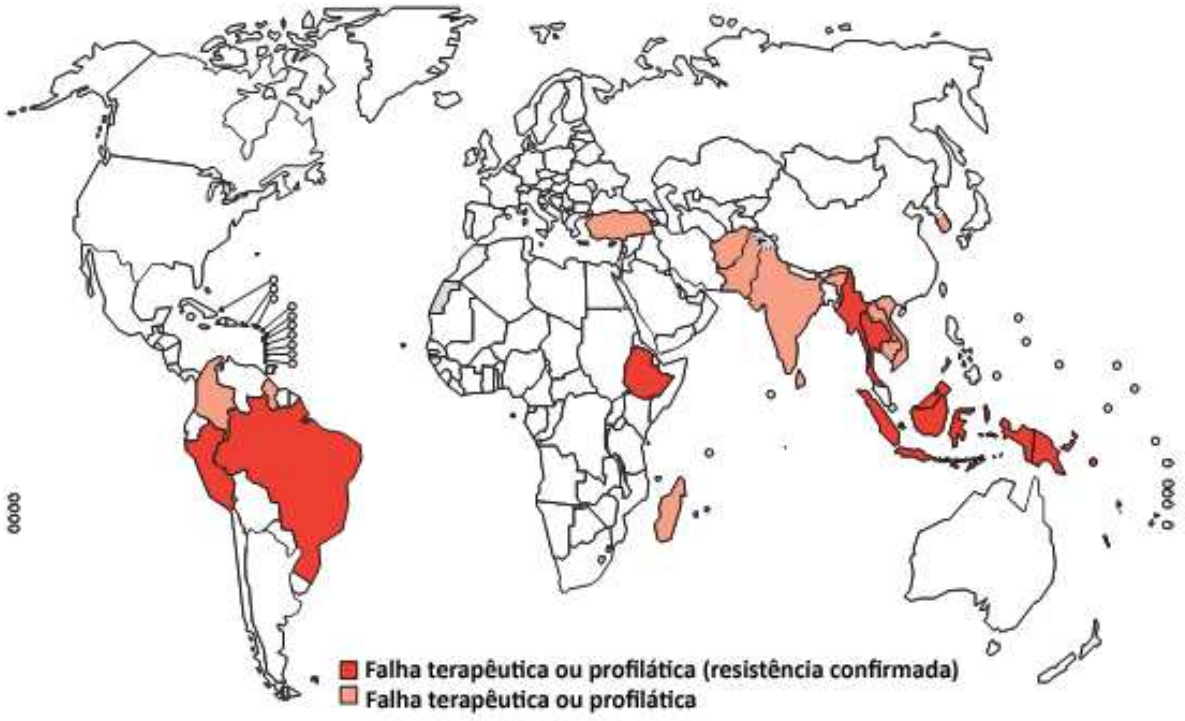

Figura 5: Mapa de distribuição global de resistência a cloroquina por *P. vivax* em 2010. Adapatado de WHO, 2010.

O tratamento da malária vivax na maioria dos países ainda se dá através da combinação CQ e PQ (WHO, 2011). Entretanto, em países do sudeste asiático como Papua Nova Guiné e Indonésia, onde a resistência de *P. vivax* à CQ é alta, o tratamento da malária vivax é à base de artemisininas (ART). Alguns autores defendem o uso do tratamento combinado à base de artemisininas (ACT) na malária vivax em todos os países onde haja possibilidade de infecções mistas com *P. falciparum* (Price et al., 2011).

Com elevados índices de resistência a todos os antimaláricos no mercado, desde 2001 a OMS recomenda o uso do ACT como primeira linha de tratamento para malária falciparum nos países onde há resistência aos outros antimaláricos (WHO, 2011).

Na década de 70 foi descoberto o potencial antimalárico da ART, usada extensamente na Medicina Tradicional Chinesa (White, 2008). A sua descoberta foi rapidamente seguida de sua identificação química, como peróxido sesquiterpeno, e do desenvolvimento de métodos para a produção de seus derivados: artesunato (ARS), artemether (ARM) e dihydroartemisinina (DHA). Como antimalárico, os derivados de ART apresentam a habilidade de rapidamente diminuir o número de formas sanguíneas, além de um amplo espectro de ação sendo eficaz contra formas extremamente jovens e também contra trofozoítos maduros, além de sua ação gametocida (Kumar & Zheng, 1990). A tolerabilidade dessas drogas é excelente também (White & Olliaro, 1998). A única barreira no uso dos derivados de ART é sua meia vida curta, em geral entre 1 e 3 horas (Olliaro & Taylor, 2003). Assim, clinicamente a monoterapia com esses derivados só é curativa quando administrada por 7 dias e a recrudescência é comum (Stepniewska et al., 2010). Sendo assim, os derivados de ART foram combinados com drogas de meia vida mais longas, e assim os tratamentos puderam ser reduzidos, normalmente para 3 dias, e também proteger contra a emergência de resistência (Wells et al., 2009).

Em 2010, dos 87 países em áreas de infecção malárica por *P. falciparum*, 84 já adotaram o ACT (Figura 6) (WHO, 2011). Ainda, alguns países, como Indonésia e Papua Nova Guiné, já o utilizam também no tratamento da malária vivax (WHO, 2011). Hoje temos cinco combinações no mercado (ARM-Lumefantrina, ARS-MQ, DHA-Piperaquine, ARS-Amodiaquina e ARS-SP), e algumas outras em etapas de ensaios clínicos.

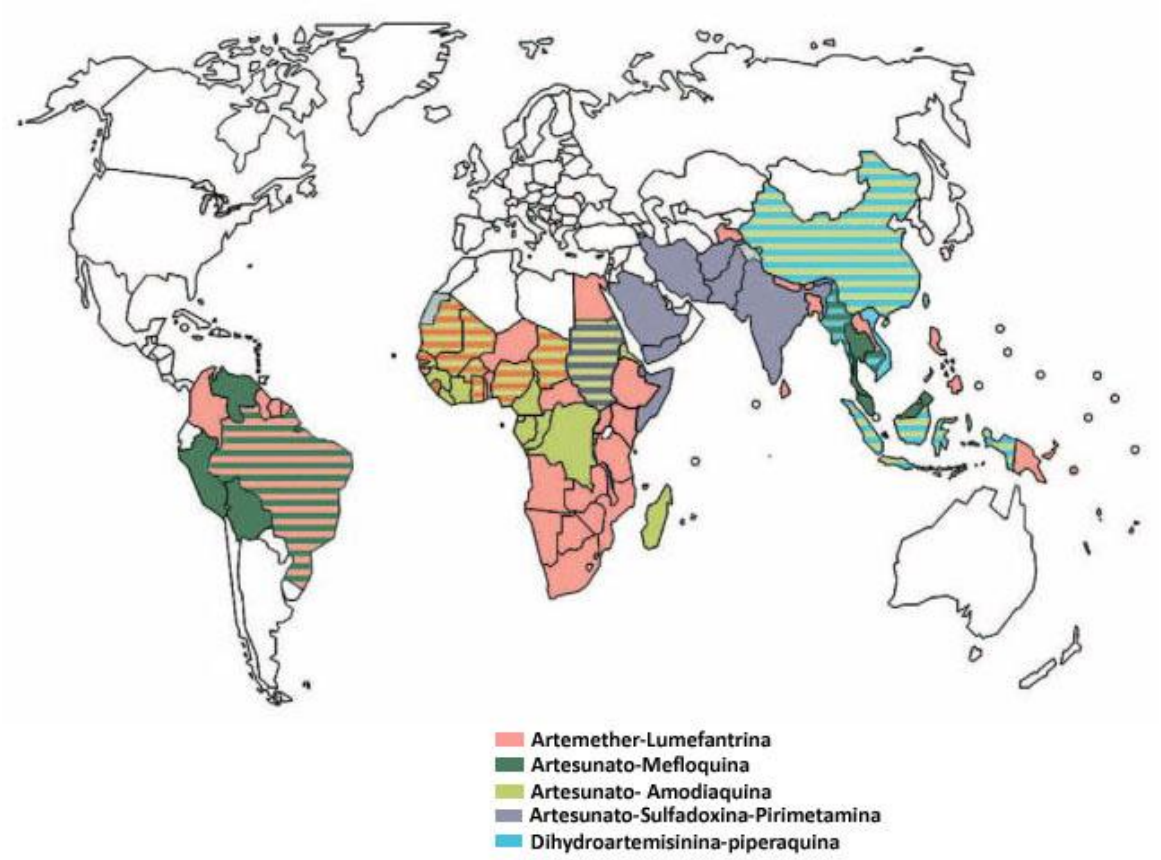

Figura 6: Atual distribuição global do tratamento de primeira linha com ACT para malária falciparum não complicada. Adaptado de O'Brien et al., 2011.

Apesar do uso terapêutico de formulações contendo ART ser recomendado pela OMS, vem sendo reportados atrasos no *clearence* parasitário e também casos de falha terapêutica por essa droga em regiões da Tailândia, Cambódia, Myanmar e Vietnãm (WHO, 2010).

As primeiras indicações de falha terapêutica com ACT se deram no início dos anos 2000 em regiões da divisa Tailândia- Cambódia (WHO, 2010; Vijaykadga et al., 2006; Denis et al., 2006). No entanto não ficou claro se essa falha devia-se à resistência ao derivado de ART, à droga em combinação, ou ainda devido a fatores farmacológicos ou do indivíduo. Em 2008, Noedl et al.

(2008) identificou dois pacientes, no oeste do Cambódia, com evidências claras de resistência ao ARS. Esses pacientes apresentaram rescrudescência parasitária após sete dias de monoterapia com ARS, mesmo em concentrações plasmáticas adequadas da droga (Noedl et al., 2008). Outro estudo, que dá suporte à emergência da resistência ao ARS, mostrou um atraso no *clearence* parasitário em pacientes de Paillin (oeste do Cambódia) em relação aos pacientes de Wang Pha (nordeste da Tailândia) sob tratamento de ARS e também da combinação ARS-MQ (Dondorp et al., 2009). Em 2008, para conter a emergência da resistência ao ARS nessa região do Cambódia, a OMS mudou o tratamento de primeira linha para a combinação DHA-piperaquine (WHO, 2011). Entretanto, após a implantação do novo tratamento houve um aumento na porcentagem de pacientes com parasitemia positiva no dia 3 (8% em 2008, 27% em 2010), indicando queda na eficácia do tratamento devido à pressão seletiva da droga (Yadav et al., 2011; WHO, 2011b).

Os fatores associados à emergência da resistência ao ARS no oeste do Cambódia incluem o uso de sua monoterapia por 30 anos, o uso de drogas fora do padrão e/ou o uso de doses abaixo da curativa, e possivelmente um *background* genético desses parasitas que favorece ao surgimento de parasitas multiresistentes (Dondorp et al., 2010).

Em outros países da região, como Tailândia, Myanmar e Vietnãm, estudos já mostram que em algumas áreas mais de 10% dos pacientes tratados com ACT tem parasitemia positiva no dia 3 pós-tratamento (Figura 7) (WHO, 2011b). Na Tailândia, nas províncias de Tak e Ranong, a falha terapêutica já é maior que 10% em estudos de seguimento por 42 dias (WHO, 2011b).

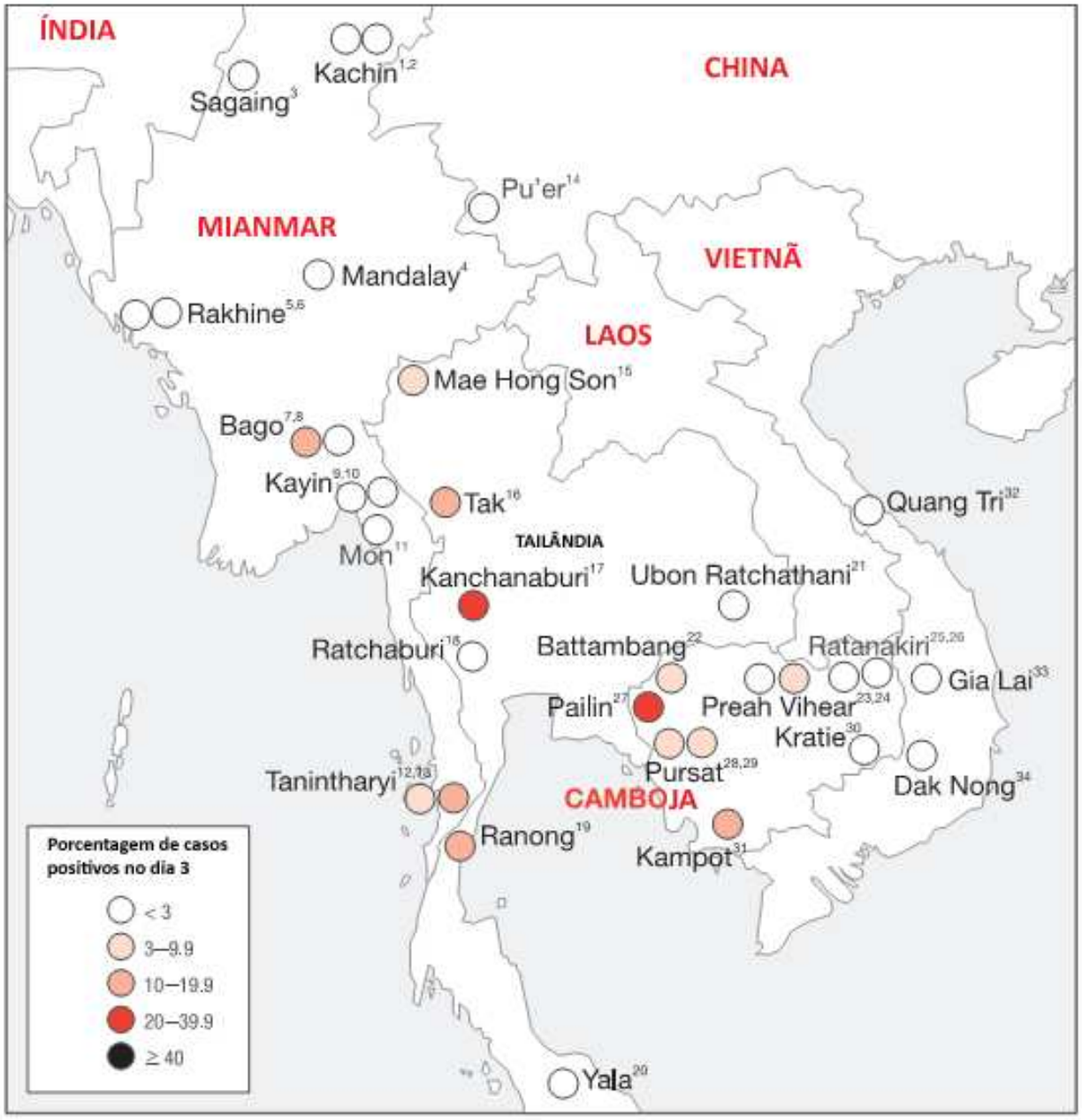

Figura 7. Porcentagem de pacientes de *P. falciparum* com parasitemia positiva no dia 3 pós-tratamento com derivados de ART. Adaptado de WHO, 2010.

Apesar do atraso na resposta ao ACT em algumas áreas da sub-região de Mekong, ACT continuam sendo o tratamento mais eficaz para malária falciparum não complicada; e a maioria

dos pacientes com resposta atrasada são curados se a droga combinada for eficaz (O'Brien et al., 2011). No entanto, a OMS está preocupada com a crescente evidência de resistência, e em resposta aos novos dados, projetos de contenção da resistência aos ACTs estão sendo iniciados, como o GPARC (*Global Plan for Artemisin Resistance Containment*).

Uma vez que a resistência às drogas tem sido implicada na propagação da malária para novas áreas e re-emergência de malária em áreas onde a doença havia sido erradicada e também teve um significativo papel na ocorrência e gravidade de epidemias em algumas partes do mundo, é de grande importância o planejamento de novas formas de controle da malária. E mais devido à emergência de resistência a todos os antimaláricos no mercado, inclusive aos derivados de ART, mesmo que em menor proporção, é urgente o desenvolvimento de novos compostos antimaláricos que apresentem novas estruturas químicas e também mecanismos de ação distintos das drogas já disponíveis (White et al., 1999).

As drogas antimaláricas disponíveis tem como alvo diferentes estágios durante o ciclo do *Plasmodium*, entretanto a maioria delas agem nos estágios intraeritrocíticos (Olliaro, 2001). O mecanismo de ação da maioria dos antimaláricos em uso ainda não foi completamente elucidado. A seguir descreveremos o que até então sabe-se a respeito do mecanismo de ação deles.

Alguns dos antimaláricos disponíveis, como as sulfonamidas e pirimetaminas, agem inibindo enzimas da via do folato, e assim há uma diminuição da síntese de pirimidinas e portanto redução na replicação do DNA, formação de serina e metionina (Olliaro, 2001). Já a atovaquona, utilizada tanto como no tratamento quanto na prevenção da malária, age essencialmente nas funções mitocondriais interferindo com a cadeia de transporte de elétrons e também no potencial de membrana mitocondrial (Srivastava et al., 1997). Os antimaláricos mais comuns, como cloroquina, quinino e mefloquina, pertencem à classe das quinolinas. Vários mecanismos de ação

já foram propostos para as quinolinas, no entanto a hipótese mais aceita é que essas drogas agem primariamente na detoxificação do grupamente heme liberado durante o metabolismo parasitário (Olliaro et all, 2001). O mecanismo de ação dos derivados de artemisinina, drogas de escolha no tratamento antimalárico em infecções por *P. falciparum*, ainda não foi completamente elucidado, a hipótese prevalente é de que a clivagem do peróxido intacto pela protoporfirina IX geraria radicais que poderão alquilar biomoléculas levando a morte do parasita (Olliaro et al., 2001).

1. Violaceína

A violaceína (Figura 8) é o principal pigmento violeta produzido pela bactéria *Chromobacterium violaceum*. A primeira descrição documentada sobre a bactéria e seu pigmento foi feita pelo italiano Curzio Bergonzini em 1881, porém a fórmula química e a estrutura exata da violaceína só foram determinadas entre 1958-1960 (Ballantine et al., 1960). A violaceína é um derivado indólico caracterizado como 3(1,2- dihidro-5-(5-hidroxi-1H-indol-3-il)-2-oxo-3H-pirrol-3-ilideno)-1,3-dihidro-2H-indol-2-ona.

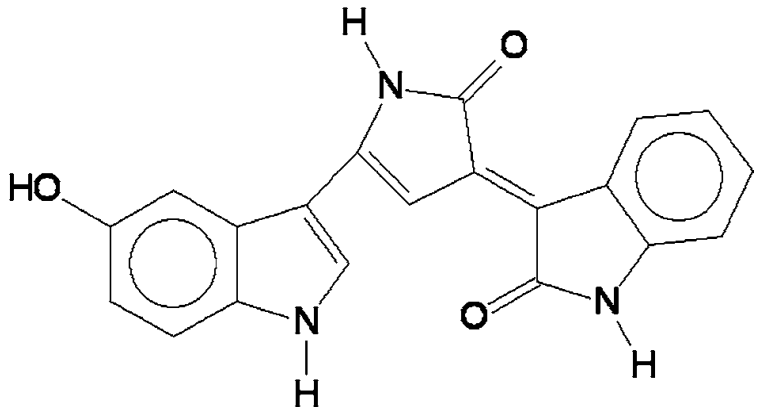

Figura 8: **Estrutura química da violaceína.** Adaptado de Lopes et al., 2009.

A *Chromobacterium violaceum* é uma bactéria Gram-negativa pertencente à família Neisseriaceae e à classe das β-proteobactérias, é anaeróbia facultativa em forma de bastonetes (Duran & Menck, 2001). Trata-se de uma bactéria saprófita amplamente distribuída, podendo ser encontrada em águas e solos de regiões tropicais e subtropicais do Brasil onde constitui um componente minoritário da microbiota total. Seu genoma foi sequenciado por um Consórcio Brasileiro em 2003, e revelou diversas vias alternativas para a geração de energia, complexos e extensivos sistemas de adaptação ao estresse, amplo uso de *quorum sensing* a fim de aumentar a versatilidade e adaptabilidade do organismo (Brazilian National Genome Project, 2003).

A partir do estabelecimento da cepa CCT 3496 da *Chromobacterium violaceum*, a produção, extração e purificação do pigmento foram otimizadas e estudos de sua biossíntese, bem como reações de bio-transformação foram realizadas (Rettori & Duran, 1998; Bromberg & Duran, 2001; Duran, 1997; Bromberg, 2000).

Diversos trabalhos atribuíram à violaceína diferentes atividades biológicas tais como bacteriostática (Lichstein & Van De Sand, 1946; Duran et al., 1983), antiviral (Andrighetti-Frohner et al., 2003; Andrighetti-Frohner et al., 2006), antioxidante (Rettori & Duran, 1998), antimicobacteriana (De Souza et al., 1999), fungicida (Shirata et al., 2000; Barreto et al., 2008), antiulcerogênica (Duran et al., 2003), antitumoral (Melo et al., 2003; Saraiva et al., 2004; de Carvalho et al., 2006; Kodach et al., 2006) e também contra protozoários patogênicos (Duran & Menck, 2001; Leon et al., 2001; Duran et al., 1989; Haun et al., 1992).

Seu potencial antibiótico foi sugerido ao notar-se que nas lesões na pele causadas por essa bactéria, nenhum outro organismo contaminante era encontrado. Um estudo com 51 linhagens de bactérias, totalizando 21 espécies, mostrou que a violaceína apresenta um notável efeito inibitório

no crescimento de bactérias Gram positivas, mas pequeno em Gram negativas (Lichstein & Van De Sand, 1946). Posteriormente foi demonstrada atividade antibacteriana *in vitro* em ambos os grupos (Durán et al., 1983), apesar de mais reduzida no grupo das Gram negativas.

Diversos trabalhos mostraram o potencial citotóxico da violaceína e na Tabela 1 apresentamos um resumo da citotoxicidade mostrando o tipo celular e a IC_{50} (concentração inibitória responsável por 50% de morte celular) encontrada (Duran et al., 2007). Em ensaios de citotoxicidade utilizando fibroblastos V79 de pulmão de hamster chinês, a violaceína demonstrou um potencial citotóxico com valores de IC_{50} de 5-12 μM e essa citotoxicidade deve-se a apoptose e não necrose (Melo et al., 2003; Haun et al., 1992; Melo et al., 2000). A citotoxicidade da violaceína também foi avaliada em linfócitos humanos normais e o ensaio de MTT mostrou IC_{50} maior que 10 μM. Apesar dessa toxicidade a linfócitos normais, a violaceína foi capaz de inibir o crescimento de células leucêmicas em concentrações bem abaixo (IC_{50}< 1 μM) da encontrada em para linfócitos normais (Bromberg et al., 2005). Entretanto, em algumas linhagens celulares, como FRhK-4 (células embrionárias de rim de macaco Rhesus) e Vero (células epiteliais de rim de macaco), concentrações baixas de violaceína (IC_{50}< 1,5 μM) foram capazes de provocar dano no DNA (Andrighetti-Frohner et al., 2006).

Tabela 1. Citotoxicidade da violaceína frente a vários tipos celulares. Adaptada e modificada de Duran et al., 2007.

Célula	IC_{50} ou GI_{50}* (μM)	Referência
V79	1,7-4,1 (5,0-12)	Haun et al., 1992; Melo et al., 2000.
Linfócito humano	>3,4 (>10)	Bromberg et al., 2005.
HL60	0,27 (0,8)	Melo et al., 2003; Ferreira et al., 2005.
MOLT-4	0,01 (0,03)*	Melo et al., 2000.
NCI-H460	0,01 (0,03)*	Melo et al., 2000.
KM12	0,02 (0,06)*	Melo et al., 2000.
92.1	0,95 (2,78)	Saraiva et al., 2004.
COM-1	1,27 (3,69)	Saraiva et al., 2004.
HT29	>3,4 (>10)	de Carvalho et al., 2006.
Caco-2	0,68 (2,0)	de Carvalho et al., 2006; Kodach et al., 2006.
SW480	0,68 (2,0)	Kodach et al., 2006.
DLD1	0,51 (1,5)	Kodach et al., 2006.
HCT116	0,51 (1,5)	Kodach et al., 2006.
RENCA	0,2 (0,6)	Justo et al., 2005.
EAT	1,7 (5,0)	Justo et al., 2005; Bromberg et al., 2010.
FRhK-4	1,08 (3,14)	Andrighetti-Frohner et al., 2003.
VERO	1,02 (2,96)	Andrighetti-Frohner et al., 2003.
MA104	1,22 (3,55)	Andrighetti-Frohner et al., 2003.
Hep2	1,18 (3,42)	Andrighetti-Frohner et al., 2003.
EOMA	0,17 (0,5)	Justo et al., 2005.

A maioria dos estudos de atividade biológica da violaceína tem seu foco na sua atividade antitumoral e sua habilidade de induzir apoptose nessas células, assim como as vias moleculares para atingir esse efeito. Nesse sentido, o primeiro trabalho demonstrando a atividade antitumoral da violaceína utilizou um painel de células tumorais provenientes do NCI (*National Cancer Institute*) e obteve boas taxas de inibição do crescimento das células nas linhagens MOLT-4 (células leucêmicas), NCI-H460 (células de câncer de pulmão) e KM12 (células de câncer de cólon) (Melo et al., 2000). Com base nesses resultados, diversos trabalhos vêm investigando não só a atividade citotóxica da violaceína em diferentes linhagens tumorais (como células de câncer

de cólon Caco-2, DLD-1, SW480 e HCT116; células leucêmicas HL60; Tumor ascítico de *Ehrlich*; fibrosarcoma 2237; células de melanoma B16F10, 92.1 e OCM-1), mas também buscando o mecanismo de ação nessas diferentes linhagens (de Carvalho et al., 2006; Kodach et al., 2006; Ferreira et al., 2004; Justo et al., 2005; Bromberg et al., 2010).

A fim de melhor investigar o mecanismo envolvido na citotoxicidade da violaceína em células leucêmicas, estudo conduzido em células de leucemia mielóide humana (HL60) demonstrou que o composto é capaz de mediar a apoptose via ativação específica do receptor do fator de necrose tumoral 1 (TNFR1), e que sua citotoxicidade é precedida pela ativação da caspase-8, da transcrição de NFκB (fator nuclear κB) e da ativação da p38 MAP quinase (Ferreira et al., 2004).

Além dos estudos em células leucêmicas, o mecanismo de ação da violaceína também foi investigado em linhagens de células de câncer de cólon (de Carvalho et al., 2006; Kodach et al., 2006). Neste sentido, de Carvalho et al.(2006) demonstraram que o estresse oxidativo induzido pela violaceína é um dos mediadores dos níveis de apoptose em células Caco-2 do cólon intestinal. Entretanto, a violaceína foi incapaz de aumentar os níveis de EROs (Espécies reativas de oxigênio) em outra linhagem de câncer de cólon (HT29), sugerindo a existência de mecanismos célula-específico do composto (de Carvalho et al., 2006).

Nas células HCT116, o tratamento com a violaceína causou bloqueio no ciclo celular em G1, devido ao aumento nos níveis das proteínas p27 e p21, diminuição nos níveis da ciclina D1 e bloqueio da fosforilação da proteína Rb (Retinoblastoma). Além disso, a violaceína levou à inibição de Akt (proteína quinase B) com subseqüente ativação da via apoptótica (caspases) e inibição da sinalização por NF-κB (Kodach et al., 2006).

O papel das caspases na apoptose mediada pela violaceína também foi demonstrado no carcinoma murino renal (RENCA) e no tumor de *Ehrlich* (Justo et al., 2005). Além da habilidade da violaceína em inibir o crescimento do tumor de *Ehrlich in vitro*, seu potencial também foi avaliado *in vivo*. Os estudos *in vivo* mostraram que tratamentos com 0.1 e 1 mg/kg diários por via i.p. foram capazes de reduzir o tamanho do tumor nos animais e mais aumentou a sobrevida nesses animais (Justo et al., 2005; Bromberg et al., 2010), sendo esse o primeiro trabalho a mostrar a atividade antitumoral *in vivo* da violaceína.

Efeitos moderados da violaceína também foram observados contra protozoários patogênicos. Em ensaios para medir sua atividade contra *Trypanosoma cruzi* a violaceína apresentou ação similar ao *Nifurtimox*, um importante quimioterápico utilizado no tratamento da doença de Chagas (Duran & Menck, 2001; Duran et al., 1989). No entanto, seu potencial quimioterápico *in vivo* foi extremamente baixo (Haun et al., 1992).

Além de atividade tripanossomicida, estudos de Leon et al. (Leon et al., 2001) mostraram que a violaceína também apresenta atividade contra promastigotas de *Leishmania amazonensis*, apesar de apresentar-se dez vezes menos ativa que a pentamidina, uma das drogas mais utilizadas no tratamento de leishmanioses. Além disto, neste estudo (Leon et al., 2001) não foi avaliado o potencial deste composto na célula hospedeira (macrófagos) alvo do parasita,e nem nos modelos murinos das leishmanioses.

A violaceína apresenta uma baixa solubilidade em água, característica essa que faz com que alguns procedimentos experimentais não sejam viáveis, devido à citotoxicidade do solvente em que ela é diluída como, por exemplo, DMSO (Dimetil Sulfóxido). Assim, uma estratégia que está sendo explorada é sintetizar derivados de violaceína com grupos glicosídicos (Duran et al., 2007)

ou a sua biotransformação por enzimas oxidativas (Bromberg & Duran, 2001) permitindo maior solubilidade e atividade biológica.

Tentativas de aumentar a solubilidade e a atividade biológica através da complexação da violaceína com β-ciclodextrina também têm sido relatadas (Duran et al., 2003; Duran et al., 2007). As ciclodextrinas apresentam uma cavidade interna com caráter hidrofóbico (apolar) o que faz com que compostos orgânicos com baixa solubilidade possam interagir com esta cavidade aumentando sua solubilidade. A este respeito, a capacidade de violaceína em evitar a ulceração gástrica foi potencializada pela complexação com β-ciclodextrina em diferentes proporções molares (Duran et al., 2003).

O uso de polímeros biocompatíveis e biodegradáveis em formulações contribui para o aumento da eficácia terapêutica e redução dos efeitos colaterais de drogas. Estudos de citotoxicidade realizados em células HL60 têm demonstrado que a incorporação de violaceína em microesferas de PCL (poli-ε-caprolactona) potencializa a sua atividade citotóxica em comparação à violaceína livre (Duran et al., 2007).

A citotoxicidade da violaceína encapsulada em PLGA (co-polímero de ácido lático e glicólico) foi estudada em diferentes linhagens celulares tumorais. Neste sentido, uma correlação entre a citotoxicidade e o tempo de exposição foi observada em células HL60 tratadas com a violaceína encapsulada com PLGA (NP-violaceína), sugerindo que a violaceína foi liberada de forma dependente do tempo. No entanto, a formulação da violaceína em PLGA reduziu seu efeito citotóxico em HL60 (Melo et al., 2009).

Diante do potencial biológico da violaceína e seus derivados aqui apresentado, este trabalho visa em avaliar o potencial antimalárico da violaceína livre e também de suas formulações.

Objetivos

1. Objetivo Geral

Este projeto visa avaliar a capacidade quimioterápica deste composto, livre e formulado, no combate de formas sangüíneas de *Plasmodium* sp. *in vivo* e *in vitro*.

2. Objetivos específicos

2.1. Avaliação do potencial antiplasmodial da violaceína livre *in vitro*, em culturas de *P. falciparum.*

2.2. Avaliação do potencial antiplasmodial da violaceína livre *in vivo* em camundongos infectados com *P. chabaudi chabaudi*, levando-se em consideração: (i) a parasitemia, (iii) a taxa de mortalidade.

2.3. Avaliação do potencial antiplasmodial da violaceína livre em isolados de *P. vivax* coletados de pacientes infectados provenientes de Manaus, AM.

2.4. Avaliação do potencial antiplasmodial da NP-violaceína *in vitro* contra estágios sanguíneos de *P. falciparum* e *in vivo* contra estágios sanguíneo de *P. chabaudi chabaudi.*

2.5. Avaliação do potencial antiplasmodial da violaceína funcionalizada com diferentes nanotubos de carbono (NTs) *in vitro* contra estágios sanguíneos de *P. falciparum.*

Métodos

1. Violaceína

1.1 Purificação da violaceína

A violaceína (3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3ilydene) -1,3-dihydro-2H-indol-2-one) foi isolada a partir de *Chromobacterium violaceum* (CCT3496) como descrito previamente (Rettori & Duran, 1998). Através da purificação com clorofórmio, a parede celular e os resíduos da membrana foram extraídos, e usando éter dietil e posteriormente etanol, foi obtido um extrato contendo a violaceína. Após a evaporação do etanol, a violaceína foi purificada por cristalização (metanol/água) seguida de HPLC (Cromatografia Líquida de Alta Eficiência). DMSO foi utilizado para dissolver a violaceína devido sua pobre solubilidade em água. Todo esse procedimento de obtenção e purificação da violaceína foi realizado em colaboração com o grupo do Prof. Dr. Nelson Duran (Instituto de Química, Unicamp). Como a violaceína é um produto derivado de bactéria, avaliamos se havia resíduos de lipopolissacarídeo (LPS) presente nas amostras purificadas da violaceína. Para isso, utilizou-se o kit Limulus Amebocyte Lysate QCL-1000® (Cambrex) e os procedimentos foram feitos de acordo com o protocolo do produto. Não foi detectada a presença de LPS em nenhum os lotes utilizados.

1.2 Encapsulação da violaceína em PLGA

Devido a pobre solubilidade da violaceína, nós preparamos NP-violaceína segundo o método de nanoprecipitação (Cui et al., 2006). Resumidamente, violaceína e PLGA foram dissolvidos em acetona na concentração de 0,63% e 0,05%, respectivamente. Essa solução foi adicionada em

uma fase aquosa contendo 0,06% (peso/volume) de surfactante Pluronic F68 e PVA (solução aquosa saturada de álcool polivinílico). A mistura foi mecanicamente agitada por 5 minutos e evaporada por rotação até atingir o volume de 5 ml de suspensão. O encapsulamento da violaceína foi feito pelos grupos do Prof. Nelson Durán (Departamento de Química Biológica, Instituto de Química, UNICAMP) e do Prof. Marcelo Brocchi (Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, UNICAMP).

1.3 Funcionalização da Violaceína com Nanotubos de Carbono

NTs consistem exclusivamente de átomos de carbono dispostos em uma série de anéis benzeno laminados em uma estrutura tubular. Este novo nanomaterial pertence à família dos fulerenos, a terceira forma alotrópica de carbono, juntamente com grafite e diamante. NTs podem ser classificados em duas categorias gerais, com base na sua estrutura: paredes simples (*single-walled*), que são constituídos por uma única camada cilíndrica de grafeno; e paredes mútiplas (*multi-walled*), que contêm várias folhas de grafeno concêntricas. Existe um interesse crescente em explorar todas estas propriedades que os NTs possuem para aplicações diversas, inclusive como sistemas de distribuição para uma variedade de agentes diagnósticos ou terapêuticos (Martin & Kohli, 2003).

Neste sentido, utilizamos três diferentes nanotubos de carbono, dois deles com paredes simples*:* SW e NT-1 e um com paredes múltiplas: NTC-2. Os NTs foram gentilmente cedidos pelo grupo do Prof. Helder José Ceragioli (Faculdade de Engenharia Elétrica e Computação-Unicamp).

Para a funcionalização da violaceína com os NTs, a mesma quantidade em massa dos dois compostos (violaceína e nanotubos de carbono NT-1, SW e NTC-2) foi reunida em um mesmo tubo de 1,5 ml, seguido de sonicação por 30 minutos. Para a violaceína a quantidade utilizada foi a IC_{50} pré-determinada para *P. falciparum* 3D7 e para os NTs foi utilizada essa mesma quantidade em massa. Nos ensaios antimaláricos foi utilizada a violaceína livre e os NTs puros na mesma concentração da funcionalização para controle.

2. Atividade antimalárica *in vitro* contra formas sanguíneas de *P. falciparum*

2.1 Cultivo

P. falciparum 3D7 e *P. falciparum* S20 foram cultivados em meio RPMI completo (RPMI 1640, Sigma, USA), suplementado com 10% de plasma humano decomplementado através do método de *candle jar* (Trager & Jensen, 1976). O hematócrito final foi ajustado a 4% com eritrócitos sadios O^+ fornecidos pelo banco de sangue da UNICAMP.

2.2 Sincronização

Para alguns ensaios *in vitro,* os parasitas foram sincronizados em Sorbitol como descrito em (Fernandez, 2004). Resumidamente, o pellet de eritrócitos infectados (EI) com a parasitemia maior que 5%, sendo a maioria das formas no estágio de anel, foi ressuspenso em uma solução 5% de Sorbitol (J.T.Baker, USA). Após 5 minutos em temperatura ambiente, o pellet foi lavado

duas vezes em meio completo e a cultura foi retomada. O ensaio foi repetido pelo menos uma vez para assegurar a sincronização do parasita.

2.3 Atividade antimalárica da violaceína

2.3.1 Por incorporação de [^{3}H] Hipoxantina

A atividade da droga foi determinada como descrito por Schmidt (2004), sendo que o crescimento parasitário foi medido através da incorporação da [^{3}H] Hipoxantina (GE Healthcare), essencial na replicação parasitária. Os ensaios foram feitos em placas de cultura de 96 poços com o volume final de 200 μL sendo 100 μL de meio RPMI completo suplementado com 10% de plasma humano com diferentes concentrações da droga testada (violaceína) ou sem droga (controle) e 100 μL de suspensão de Eritrócitos infectados por *P. falciparum* (Pf-EI) (2% hematócrito final e 1% parasitemia). Como a violaceína está dissolvida em DMSO, na concentração máxima de 0.25%, essa quantidade de DMSO (Sigma, USA) foi adicionada ao meio completo nos respectivos controles. No caso dos ensaios com a violaceína em PLGA, não foi utilizado DMSO para diluí-la, portanto nos poços controle colocamos somente meio completo.

Após 30 horas de incubação a 37°C em *candle jar*, 50 μL de meio completo contendo 10 μCi [^{3}H] Hipoxantina foi adicionado a cada poço. Após 18 horas a 37°C em *candle jar*, os eritrócitos foram lisados com o auxílio de *Cell Harvester* semi-automático (Skatron) em filtros de papel (Millipore). Após secos, os filtros foram revelados em um líquido de cintilação (2,5 Diphenyl oxazole- Sigma, USA - diluído em Toluol - Merck). A leitura da radiotividade (contagens por

minuto- CPM) se deu em um cintilador Beta (Beckman 56000TA). Também se incubou eritrócitos sadios nas mesmas condições anteriores e a radioatividade foi determinada e usada como branco. Cada concentração da droga foi testada em triplicata, e pelo menos três ensaios diferentes foram feitos utilizando-se diferentes amostras das drogas. O crescimento parasitário foi comparado com o controle (ausência de droga), representando 100% de crescimento parasitário. A inibição do crescimento parasitário foi calculada de acordo com a fórmula: % Inibição = 100 – [(CPM amostra – CPM Eritrócitos sadios / CPM Controle – CPM Eritrócitos sadios) x 100]. A IC_{50} foi calculada através de um gráfico do Log da dose *versus* inibição (expresso em porcentagem em relação ao controle).

Para os experimentos envolvendo parasitas sincronizados, realizou-se somente uma incubação a 37°C por 18 horas na presença de 10 μCi [^{3}H] Hipoxantina.

2.3.2 Por citometria de fluxo

Por impossibilidade de se utilizar o cintilador Beta para análise da atividade antimalárica nos ensaios com a violaceína associada aos nanotubos de carbono utilizamos marcação com Acridina Orange e leitura em citômetro de fluxo BD FACSCanto II.

Nesses ensaios após 48h de cultivo em meio completo, na presença ou não de violaceína e/ou NTs, acrescentou-se 20 μL de Solução de Acridine Orange 200 μM (Invitrogen) em cada poço e após 5 minutos de incubação procedeu-se a leitura no citômetro. Como esse corante tem afinidade por ácido nucléico, somente os eritrócitos infectados são marcados e assim é possível determinar a parasitemia em cada poço. A fim de eliminar o *background* de fluorescência pela presença do corante *per se*, eritrócitos sadios foram incubados com a mesma concentração de

Acridine Orange. A inibição do crescimento parasitário foi calculada de acordo com a fórmula: % Inibição = 100 – [(Parasitemia amostra – *background* Eritrócitos Sadios / Parasitemia Controle – *background* Eritrócitos sadios) x 100].

3. Atividade antimalárica *in vivo* contra formas sanguíneas de *P. chabaudi*

3.1 Animais e parasita

Foram utilizados camundongos isogênicos da linhagem C57BL/6 de 7 a 10 semanas de vida provenientes do Centro de Bioterismo (CEMIB) - UNICAMP. Os animais foram mantidos em condições *Germ-Free* e todos os procedimentos foram analisados de acordo com o comitê de ética em experimentação animal desta instituição (CEEA/UNICAMP) (protocolo n° 1436-1).

Duas linhagens de *Plasmodium* foram testadas: *P. chabaudi chabaudi* AS (PcchAS) e *P. chabaudi chabaudi* AJ (PcchAJ), cedidos gentilmente, respectivamente, pelos professores Dr. Hernando Del Portillo (Departamento de Parasitologia, ICB, USP, São Paulo, SP, Brasil) e Dra. Maria Regina d'Imperio (Departamento de Imunologia, ICB, USP, São Paulo, SP, Brasil). As formas sanguíneas dos dois parasitas foram coletadas de animais doadores (C57BL/6) previamente infectados com 100 μL de pellet de eritrócitos infectados mantidos em criotubos a -80°C; de acordo com o protocolo descrito por (Engwerda et al., 2005). Os animais foram infectados por via intraperitoneal (i.p.) com 10^6 EI.

3.2 Toxicidade

A toxicidade da violaceína foi avaliada através da análise diária de sobrevida, densidade de eritrócitos e peso de 5 camundongos C57BL/6 injetados i.p. com a maior dose de violaceína (7,5 mg/kg) durante 11 dias consecutivos, ou apenas com solução salina (controle). A densidade de eritrócitos foi determinada diariamente, através da retirada de 10 uL de sangue da cauda do animal, diluição em PBS com heparina e contagem em Câmara de Neubauer. O valor diário encontrado para cada animal foi dividido pelo valor desse mesmo animal no dia 0 da infecção, obtendo-se assim a densidade relativa (Número de eritrócitos por mL de animal num determinado dia pós-infecção /Número de eritrócitos por mL do mesmo animal no dia 0). A massa corpórea foi calculada de acordo com a fórmula: [(peso do animal em determinado dia de tratamento / peso do animal no dia anterior ao tratamento) x 100].

3.3 Análise da Parasitemia

A parasitemia foi determinada diariamente por meio de esfregaços de sangue obtido da cauda do animal a partir do dia 3 pós-infecção até a morte do animal ou a cronificação da doença na qual é observada uma baixa quantidade de parasitas nos esfregaços (menor que 1%). Os esfregaços foram fixados e corados através do kit Panótico Rápido (Laborclin). A porcentagem de parasitemia foi determinada calculando-se o número de EI em 1.000 eritrócitos.

3.4 Atividade antimalárica

3.4.1 PcchAS (Tratamento a partir do dia 0)

Para a análise da atividade antimalárica da violaceína *in vivo*, grupos de 7 a 10 camundongos foram infectados, i.p., com 10^6 EI por PcchAS. Os camundongos foram tratados diariamente com 100 µL de diferentes doses de droga (7,5; 3,75; 0,75 e 0,075 mg/kg de violaceína ou salina) durante 11 dias consecutivos (dia 0-10 pós-infecção) por via i.p.. A primeira administração da droga foi efetuada 1 hora após a infecção, as administrações sucessivas ocorreram 24 horas após a anterior. O DMSO, usado para solubilizar a droga não ultrapassou 1.25%, sendo que o grupo controle recebeu essa mesma concentração diluída em salina. Nesse tratamento acompanhou-se a parasitemia até dia 22 pós-infecção a fim de avaliar se haveria recrudescência parasitária, uma vez que o tratamento se encerra no dia 10 pós-infecção.

3.4.2 PcchAS (Tratamento a partir do dia 5)

Para verificar se o tratamento teria eficácia se iniciasse após o estabelecimento da infecção, ou seja, já com a infecção patente, camundongos infectados com 10^6 EI por PcchAS foram tratados diariamente a partir do dia 5 pós-infecção (5-10) com 100 µL de 7,5 mg/kg de violaceína ou DMSO 1,25% em salina.

3.4.3 PcchAJ (Tratamento a partir do dia 0)

A fim de avaliar se a violaceína teria efeito protetor na mortalidade, camundongos foram infectados com 10^6 EI por PcchAJ, uma cepa letal. Esses animais foram tratados diariamente com 100 μL de 7,5 mg/kg de violaceína ou DMSO 1,25% em salina durante 11 dias consecutivos (dia 0-10 pós infecção) e foram monitorados diariamente quanto a sobrevivência.

4. Atividade antimalárica contra formas sanguíneas de *P. vivax*

Os estudos de novas drogas contra *P. vivax* esbarram na dificuldade em se cultivar esse parasita que infecta preferencialmente senão exclusivamente, reticulócitos. Até hoje a metodologia utilizada para o cultivo de *P. vivax* só permite seu cultivo por um curto período de tempo (Chotivanich et al., 2001; Udomsangpetch et al., 2008). Por esta razão, os ensaios conduzidos em *P. vivax* foram realizados em área endêmica de malária (Manaus-AM) em parceria com a Fundação de Medicina Tropical Heitor Vieira Dourado (FMT-HVD) e Instituto Leônidas e Maria Deane (ILMD- Fiocruz) a fim de trabalhar com isolados frescos de pacientes positivos para malária vivax e que ainda não deram início ao tratamento.

4.1 Procedimentos com paciente

A unidade de saúde de seleção de pacientes foi a FMT-HVD, situada em Manaus- AM, unidade de referência da Secretaria do Estado da Saúde do Amazonas (SUSAM) para o atendimento de pacientes com doenças infecciosas.

Os indivíduos que obedeceram aos critérios de inclusão foram informados e convidados a participar do projeto. Os pacientes que aceitaram participar do estudo fizeram a leitura e concordância do termo de consentimento livre e esclarecido (TCLE- em Anexo). Posteriormente foi preenchido um Questionário (Anexo) e coletados cerca de 10 ml de sangue por punção venosa (um tubo com EDTA). Após o preenchimento do questionário e coleta de sangue, o paciente recebeu atendimento médico e tratamento antimalárico apropriado dando seguimento a rotina ambulatorial da FMT-HVD.

4.1.1 Critérios de inclusão

- Pacientes com mais de 18 anos.
- Pacientes com diagnóstico microscópico positivo para malária vivax com parasitemia maior ou igual a uma cruz (1- parasito/campo).

4.1.2 Critérios de não inclusão.

- Pacientes com diagnóstico microscópico para malária por *P. falciparum*, ou co-infecção vivax e falciparum (malária mista).
- Mulheres com história de gestação atual.
- Paciente na vigência de tratamento antimalárico.

4.1.3 Critério de exclusão **a posteriori**

- Paciente com diagnóstico molecular para *P. falciparum* ou infecção mista

4.2 Cultivo de *P. vivax*

Inicialmente para a contagem apurada da parasitemia no tempo zero, um esfregaço sanguíneo da amostra foi efetuado. O sangue coletado foi lavado três vezes em meio Waymouth (Sigma, USA) com bicarbonato para a retirada de leucócito e células do sistema fagocitário. O sangue então foi diluído 25 vezes em Meio RPMI-Waymouth (1:1) suplementado com 20% de Plasma AB^+ decomplementado (hematócrito 4%). Então, 100 µl dessa solução de hematócrito 4% foram colocados em cada poço da placa na presença de 100 µl de diferentes concentrações de violaceína (4000; 2000; 1000; 500; 250; 125 e 62,5 ηM), cloroquina (240; 120; 60; 30; 15; 7,5 e 3,75 ηM), artesunato (60; 30; 15; 7,5; 3,75; 1,88; 0,94 ηM) ou 100 µl de meio (controle). Todas as condições foram testadas em quadruplicata. Após 48h de cultivo em *candle jar* foram feitos esfregaços de um poço controle e a placa foi armazenada a -20°C.

4.3 Atividade antimalárica

Primariamente, a placa foi congelada e descongelada cinco vezes para hemolisar os eritrócitos e liberar os antígenos. O ensaio de ELISA (*Enzyme-Linked Immunoabsorbent Assay*) para o antígeno LDH (Lactato Desidrogenase) foi feito como descrito anteriormente para *P. falciparum* (Druilhe et al., 2001), com algumas modificações (Druilhe et al., 2007). Brevemente, 100 µl do lisado de cada poço foi transferido para a placa de 96 poços previamente marcada com o anticorpo de captura contra a proteína LDH de *P. vivax*, e as placas foram incubadas por 1h a 37°C. Também foi colocado PBS (tampão fosfato) em um poço para a leitura do branco. Após

cinco lavagens com PBS-1% BSA (albumina bovina), o anticorpo secundário biotinilado anti-LDH foi adicionado e as placas incubadas por 1 h a 37°C. Após 5 lavagens com PBS-BSA 1%, as placas foram incubadas com Streptavidina-peroxidase por 30 min a temperatura ambiente. Após oito lavagens com PBS-1% BSA, o substrato da peroxidase 3,3′,5,5′-tetramethylbenzidine foi adicionado. Após 5 minutos a temperatura ambiente a reação foi bloqueada com ácido fosfórico 1M, e a densidade óptica (DO) foi quantificada em espectrofotômetro a 450 ηm. A porcentagem de inibição da droga foi calculada de acordo com a fórmula: % Inibição = 100-[(DO poço com violaceína – DO poço branco/ média DO poço controle – DO poço branco)x100]. A IC_{50} foi calculada através de um gráfico do Log da dose *versus* inibição (expresso em porcentagem em relação ao controle).

5 Análise estatística

A análise estatítica deste trabalho foi efetuada com o auxílio do software Prism™ versão 5.0 (Graphpad, USA). Os testes utilizados para comparação de parasitemia, inibição de parasitemia e densidade de glóbulos vermelhos entre os grupos tratados e não tratados foram *Teste U Mann-Whitney* (comparação de 2 grupos) ou *Kruskal-Wallis- Dunn's post test* (comparação de 3 ou mais grupos). As diferenças entre as taxas de sobrevivência foram analisadas pelo teste de *Log-Rank*.

Resultados e Discussão

Baseado nas propriedades quimioterápicas da violaceína contra células tumorais, bactérias, fungos e protozoários patogênicos ao homem, somadas a urgência na descoberta de novos compostos com atividade antimalárica, esta tese teve como objetivo avaliar a capacidade quimioterápica da violaceína no combate de formas sangüíneas de *Plasmodium* sp. *in vivo* e *in vitro*.

1. Atividade antimalárica *in vitro* contra *P. falciparum*

Devido à elevada citotoxicidade da violaceína, já relatada anteriormente em diversos trabalhos (Tabela 1), nosso primeiro objetivo foi verificar se a violaceína era tóxica aos eritrócitos humanos não infectados, uma vez que o parasita utiliza estas células para completar seu ciclo de vida e sem elas não sobrevive. Assim, após 48h de incubação dos eritrócitos humanos não infectados (hematócrito 4%) a 37ºC com diferentes concentrações de violaceína, observamos uma leve redução na densidade de eritrócitos em concentrações acima de 14.0 μM (P<0.05) (Figura 9). Nota-se, portanto, que a violaceína é menos tóxica aos eritrócitos humanos que aos linfócitos (IC_{50}= 10 μM) (Bromberg et al., 2005) e ao se utilizar doses abaixo de 14.0 μM para verificar se a violaceína é citotóxica ao *Plasmodium*, não teremos nenhuma alteração na densidade eritrocitária e assim podemos acessar a sua atividade antimalárica.

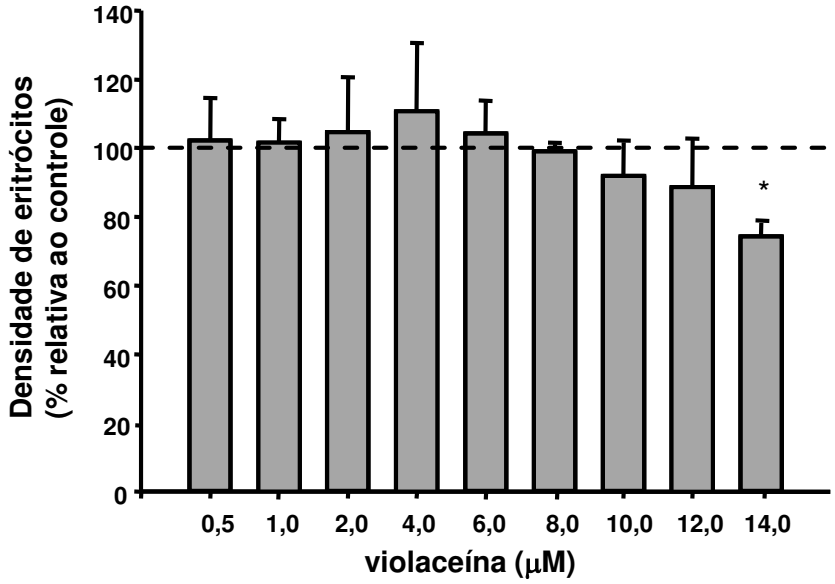

Figura 9. Toxicidade da violaceína em eritrócitos sadios. Os eritrócitos foram quantificados, com auxílio da Câmara de Neubauer, após 48h de incubação com diferentes concentrações de violaceína ou DMSO 0,25% (controle). Os dados estão expressos em porcentagem em relação ao controle e referem-se a média de triplicata ± desvio padrão.
* *P< 0,05* violaceína *versus* controle (*Kruskall-Wallis, Dunn's post test*)

Para os ensaios contra *P. falciparum,* utilizamos doses abaixo de 10 μM. Nesse sentido, *P. falciparum* 3D7 cultivados por 48h na presença de diferentes concentrações de violaceína apresentaram inibição em seu crescimento de maneira dose-dependente (Figura 10) revelando que a violaceína possui atividade antimalárica. A IC_{50} da violaceína para *P. falciparum* 3D7 foi de 825 ηM (média de 3 experimentos independentes utilizando diferentes lotes de violaceína).

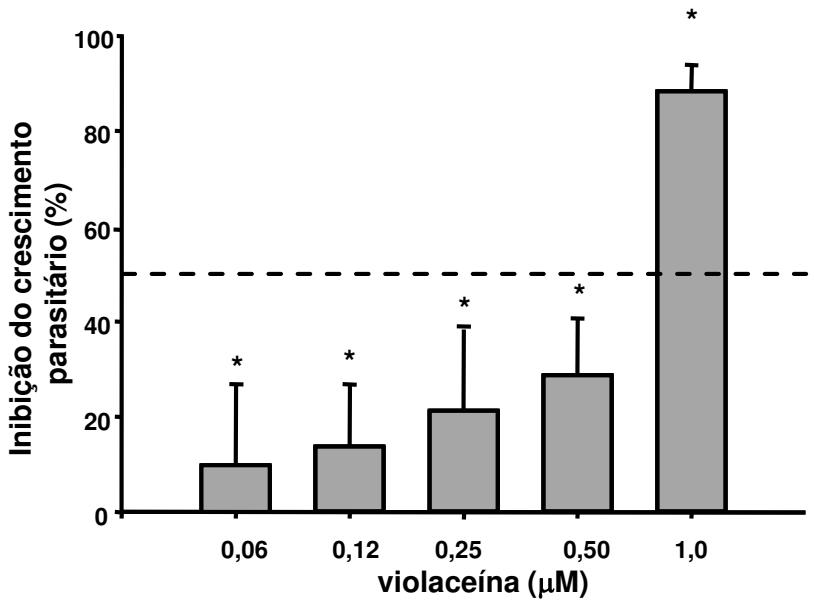

Figura 10. Atividade antimalárica da violaceína em formas sanguíneas de *P. falciparum* 3D7. *P. falciparum* 3D7 (parasitemia 1%, hematócrito 2%) foram incubados em *candle jar* com diferentes concentrações de violaceína ou DMSO 0,25% (controle), a 37°C por 48 horas. A inibição da parasitemia foi calculada através da fórmula: % Inibição = 100 – [(CPM amostra – CPM Eritrócitos sadios / CPM Controle – CPM Eritrócitos sadios) x 100]. Os resultados estão expressos como a média de triplicatas ± desvio padrão.
* *P< 0,05* violaceína *versus* controle (*Kruskall-Wallis, Dunn's post test*)

Como um dos maiores desafios no controle das infecções maláricas é o surgimento de resistência aos antimaláricos disponíveis e existem parasitas resistentes a um medicamento que apresentam resistência cruzada a outros antimaláricos (multi-resistentes) (Nateghpour et al., 1993), verificamos se a violaceína, que é capaz de inibir o crescimento de *P. falciparum* 3D7, é também eficaz no combate a formas sanguíneas de *P. falciparum* S20. Esse parasita é um isolado brasileiro resistente à CQ, droga que foi amplamente usada no tratamento da malária falciparum (Talisuna et al., 2004) e ainda é bastante utilizada no tratamento da malária vivax (WHO, 2011). Como pode ser visto na Figura 11, a violaceína também foi capaz de inibir o crescimento de *P. falciparum* S20 de maneira dose-dependente. Neste sentido, a violaceína aboliu o

desenvolvimento parasitário na concentração de 1 μM, e a IC_{50} da violaceína para esse parasita foi de 625 ηM. Esses dados mostram que a violaceína é eficaz contra um isolado CQ-resistente e sugerem fortemente que essa droga apresenta mecanismo de ação diferente da CQ (Nateghpour et al., 1993).

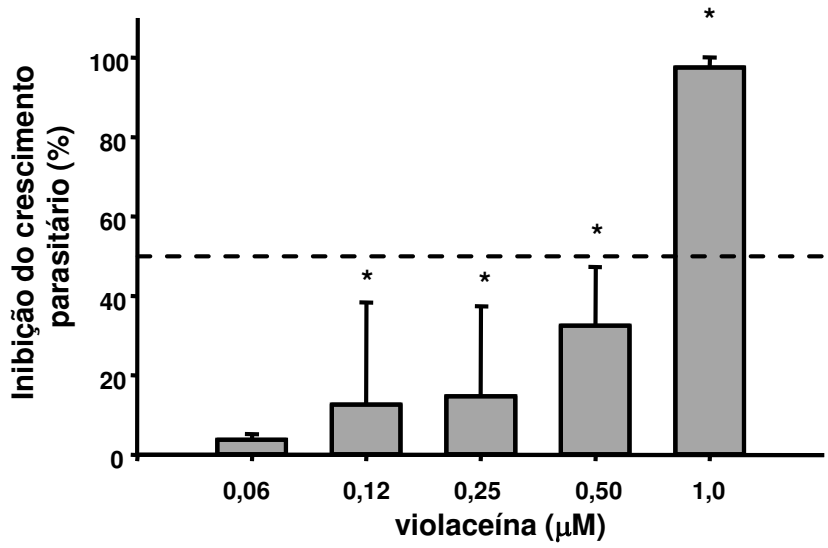

Figura 11. Atividade antimalárica da violaceína em formas sanguíneas de *P. falciparum* S20. *P. falciparum* S20 (parasitemia 1%, hematócrito 2%) foram incubados em *candle jar* com diferentes concentrações de violaceína ou DMSO 0,25% (controle), a 37°C por 48 horas. A inibição da parasitemia foi calculada através da fórmula: % Inibição = 100 – [(CPM amostra – CPM Eritrócitos sadios / CPM Controle – CPM Eritrócitos sadios) x 100]. Os resultados estão expressos como a média de triplicatas ± desvio padrão.
* *P< 0,05* violaceína *versus* controle (teste *Kruskall-Wallis, Dunn's post test*)

Uma vez que o ciclo eritrocitário de *Plasmodium* sp. é bastante complexo, com proteínas sendo expressas diferentemente em cada um dos estágios pelo qual o parasita passa, as drogas antimaláricas também apresentam ação diferencial em cada um dos estágios. A CQ, por exemplo, apresenta atividade somente contra os estágios maduros do parasita (Ashley, 2007) , já os

derivados de ART possuem um espectro de ação bastante grande atuando em todos os estágios sanguíneos de desenvolvimento do parasita (White, 2008). Assim, a fim de verificar se a violaceína tem sua atividade restrita a um estágio parasitário, parasitas sincronizados através do método de Sorbitol (Fernandez, 2004) foram colocados em cultura com diferentes concentrações de violaceína. Neste sentido, incubamos somente formas jovens (anel e trofozoíta jovem, 0-18 h do ciclo) ou então formas maduras (trofozoíto maduro e esquizonte, 24-42 h do ciclo) de *P. falciparum* 3D7 com diferentes concentrações de violaceína. O ensaio teve duração de 18 horas e a incorporação de [^{3}H] Hipoxantina foi medida. Como podemos observar na Figura 12, a violaceína, assim como os derivados de ART, é eficaz contra parasitas em ambos os estágios sanguíneos.

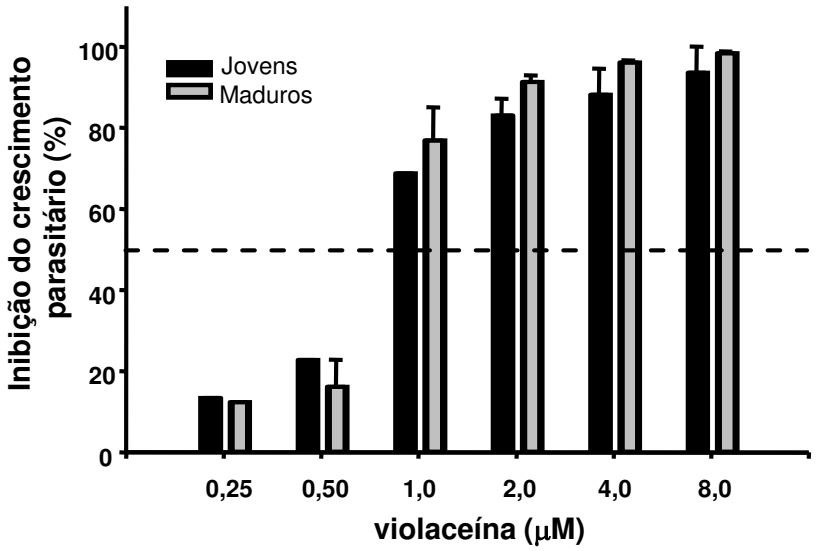

Figura 12. Atividade antimalárica da violaceína em diferentes estágios de desenvolvimento de *P. falciparum* 3D7. *P. falciparum* 3D7 sincronizados no estágio de anel ou trofoítas maduros (parasitemia 1%, hematócrito 2%) foram incubados em *candle jar* com diferentes concentrações de violaceína ou DMSO 0,25% (controle), a 37°C por 18 horas. A inibição da parasitemia foi calculada através da fórmula: % Inibição = 100 – [(CPM amostra – CPM Eritrócitos sadios / CPM Controle – CPM Eritrócitos sadios) x 100]. Os resultados estão expressos como a média de triplicatas ± desvio padrão.

2. Atividade antimalárica *in vivo* contra *P.chabaudi chabaudi*

Uma vez demonstrada sua atividade *in vitro*, investigamos se a atividade antimalárica da violaceína é sustentada *in vivo* em um modelo animal, onde outras características como biodisponibilidade e farmacocinética são levadas em consideração (Landau, 1998).

Antes de avaliar a atividade antimalárica da violaceína *in vivo,* verificamos a tolerabilidade dos animais *naive* a esse composto e acompanhamos não só a sobrevida, mas também a densidade eritrocitária e o peso dos animais ao longo dos 11 dias de tratamento com 7,5 mg/kg de violaceína ou PBS (controle). Nenhum animal sucumbiu ao tratamento com a violaceína, e mais não houve perda de peso (Figura 13A) ou alteração na densidade eritrocitária (Figura 13B) durante o tratamento. Portanto, essa dose de violaceína não se mostrou tóxica aos animais, corroborando com trabalho anterior no qual a violaceína utilizada na dose de 10 mg/kg não foi tóxica aos animais (Justo et al., 2005).

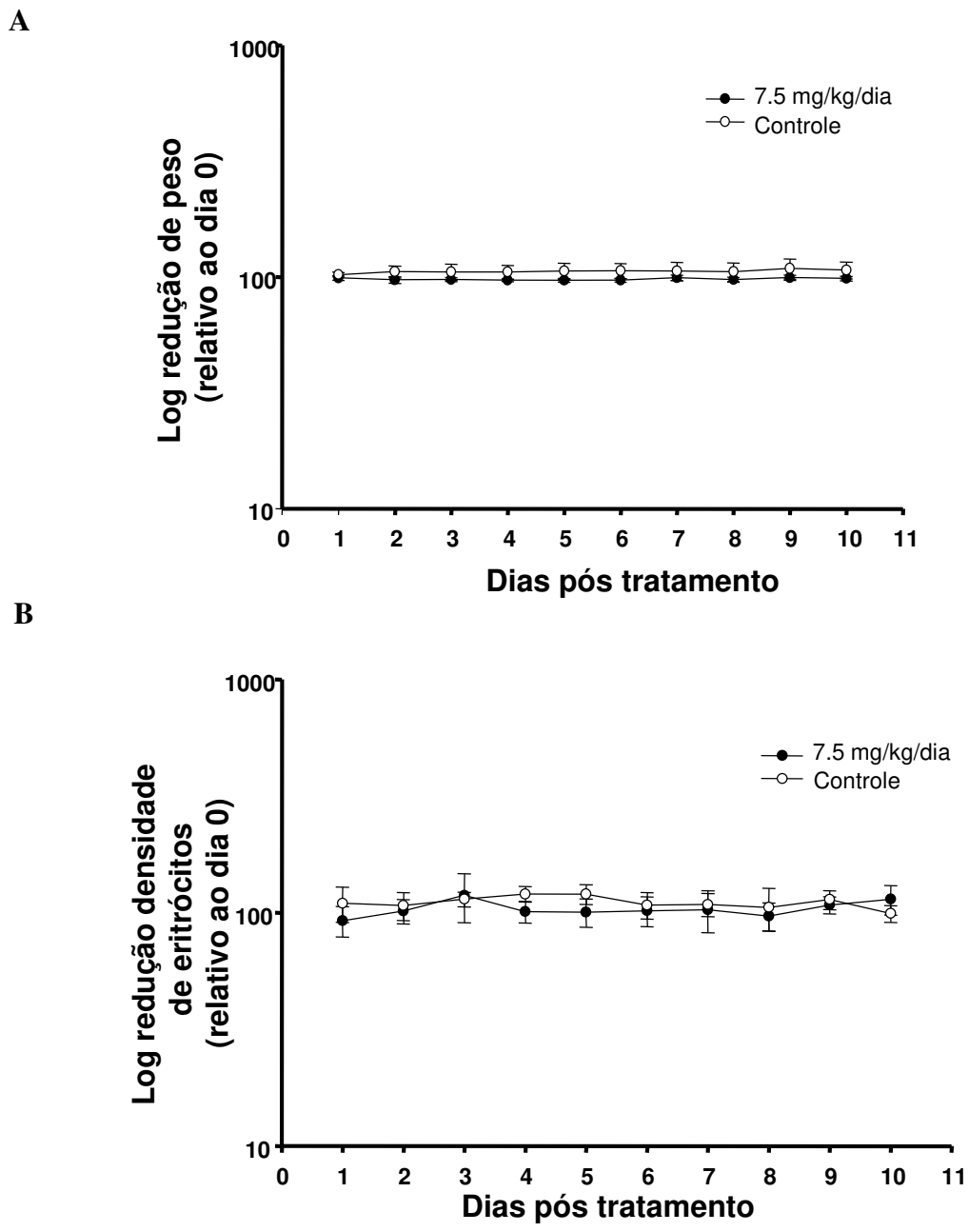

Figura 13: Toxicidade da violaceína em animais naive. Animais naive (n=5/grupo) tratados por 11 dias consecutivos com 7,5 mg/kg de violaceína ou solução salina, foram acompanhados quanto a perda de peso (A) e a densidade de eritrócitos (B). Os dados estão expressos em relação ao mesmo animal antes do tratamento (dia 0).

Como a violaceína não foi tóxica em animais *naive* na dose de 7,5 mg/kg, avaliamos a sua atividade antimalárica *in vivo* em animais infectados por PcchAS. Para isso, esses animais foram tratados diariamente, por 11 dias consecutivos (dia 0-10 pós infecção), com diferentes doses de violaceína. Como podemos observar na Figura 14, os animais do grupo controle apresentaram um pico médio de parasitemia de 37,2% no dia 7 pós-infecção. Semelhantemente ao controle, o grupo tratado com a menor dose (0,075 m/kg) apresentou um elevado pico de parasitemia (38,0%) no dia 7 pós-infecção. Sendo assim a violaceína nessa concentração não reduz a carga parasitária. Já o grupo tratado com 0,75 mg/kg apresentou um pico de parasitemia bem reduzido em relação ao controle, tendo inibido em 39,25% o desenvolvimento parasitário (P<0,01). Os animais tratados com as maiores doses (3,75 mg/kg e 7,5 mg/kg) quase eliminaram por completo o parasita, inibindo em 82,12% ± 4,30 (P<0.001) e 86,90% ± 3,53 (P<0,001) respectivamente.

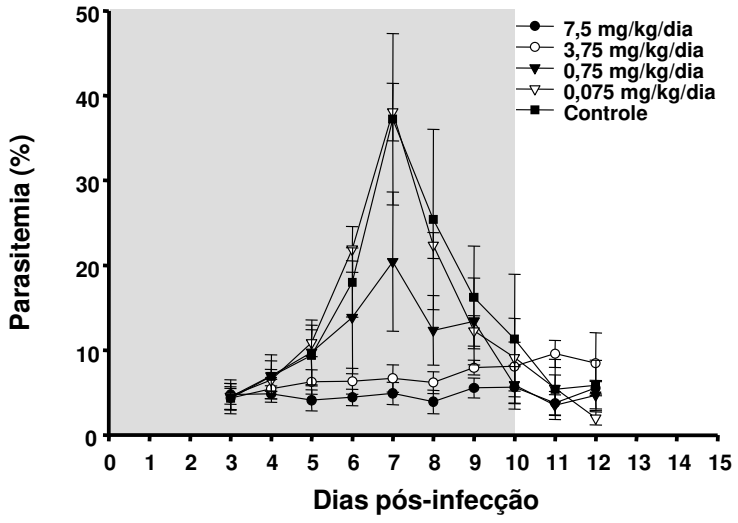

Figura 14: Atividade antimalárica da violaceína em formas sanguíneas de PcchAS. Camundongos C57BL/6 (n=10 para cada grupo) infectados com 10^6 EI de PcchAS foram tratados diariamente (dia 0-10 pós-infecção) via i.p. com diferentes doses de violaceína ou com DMSO 1,25% (controle). A parasitemia foi determinada diariamente e está expressa como média do grupo ± desvio padrão. A caixa cinza representa os dias de tratamento.

Sabe-se que ao retirar-se a pressão da droga, se houver algum parasita restante, o parasita multiplica e ocorre a recrudescência parasitária (Stepniewska & White, 2006). Uma vez que a violaceína não foi capaz de eliminar o parasita e a pressão da droga foi removida no dia 10 pós-infecção, monitoramos a parasitemia dos animais tratados com a maior dose até o dia 22 para verificar se com a retirada da droga, os parasitas restantes se multiplicariam e teríamos um pico de parasitemia tardio. Como podemos observar na Figura 15, nos animais tratados a parasitemia não ultrapassou 5% mesmo após a retirada da droga. Já nos animais não tratados, podemos observar a existência de um segundo pico menos expressivo de 9,1% no dia 16. Esse segundo pico de parasitemia é devido à variação antigênica, que possibilita um escape do sistema imunológico e assim multiplicação parasitária (McLean et al., 1982). Na Figura 15, podemos observar também que a maior dose de violaceína ainda foi capaz de inibir (58,9%) significativamente o segundo pico parasitário ($p < 0,05$, teste U de *Mann Whitney*).

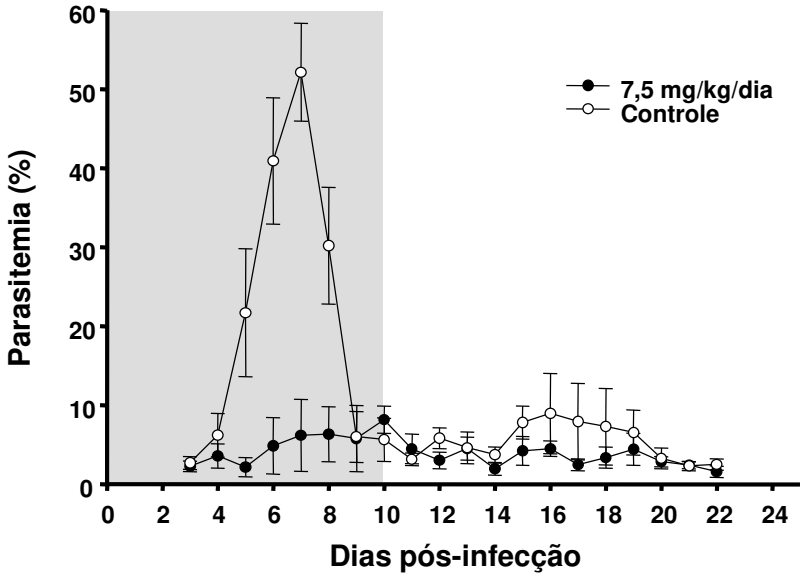

Figura 15: Atividade antimalárica da violaceína na recrudescência parasitária em PcchAS. Camundongos C57BL/6 (n=8 para cada grupo) infectados com 10^6 EI de PcchAS foram tratados diariamente (dia 0-10 pós-infecção) via i.p. com 7,5 mg/Kg de violaceína ou com DMSO 1,25% (controle). A parasitemia foi determinada diariamente até o dia 22 pós-infecção e está expressa como média do grupo ± desvio padrão. A caixa cinza representa os dias de tratamento.

Apesar de a violaceína apresentar demonstrada atividade antimalárica *in vivo* contra formas sanguíneas de PcchAS, o tratamento foi iniciado no mesmo dia que procedemos a infecção, e portanto antes da existência de infecção patente. A fim de verificar se a violaceína tem algum efeito contra o parasita após o estabelecimento da infecção, animais infectados com PcchAS foram tratados com 7,5 mg/kg de violaceína dos dias 5-10 pós infecção. Como mostrado na Figura 16, nos animais controle o pico da infecção se deu no dia 7 pós-infecção e foi de 48% de parasitemia, já nos animais tratados houve uma redução de mais de 50% na parasitemia (23% no dia 7) no dia de pico (P<0,01). Assim, a violaceína foi capaz de inibir consideravelmente a parasitemia quando administrada em esquema de tratamento iniciado durante a infecção patente,

refletindo o período em que pacientes com malária apresentariam sintomas, teriam o diagnóstico e receberiam o tratamento.

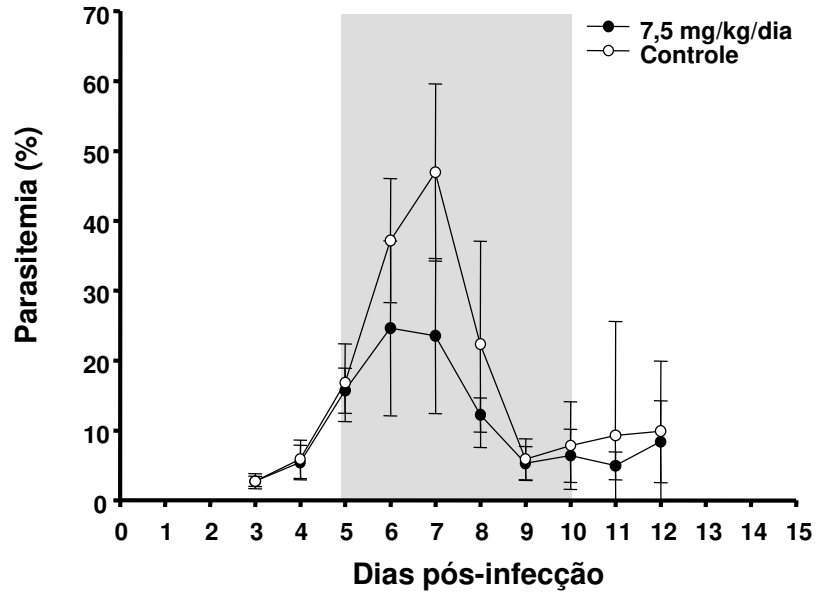

Figura 16. Atividade antimalárica da violaceína em formas sanguíneas de PcchAS após a instalação de infecção patente. Camundongos C57BL/6 (n=8 para cada grupo) infectados com 10^6 EI de PcchAS foram tratados diariamente após a instalação de infecção patente (dia 5-10 pós-infecção) via i.p. com 7,5 mg/Kg de violaceína ou com DMSO 1,25% (controle). A parasitemia foi determinada diariamente e está expressa como média do grupo ± desvio padrão. A caixa cinza representa os dias de tratamento.

A fim de verificar se a violaceína seria eficaz no controle de infecções maláricas mais graves, utilizamos um modelo murino no qual os animais infectados sucumbem à infecção. Neste sentido, animais infectados com a cepa letal PcchAJ foram tratados com 7,5 mg/kg de violaceína por 11 dias consecutivos (0-10 pós infecção) e a taxa de sobrevivência avaliada. Como podemos verificar na Figura 17, 100% dos animais não tratados sucumbiram à infecção enquanto 80% dos animais tratados sobreviveram a ela ($P < 0,001$, teste *Log Rank*). Esses dados revelam que a violaceína apresenta efeito protetor, mesmo ao se utilizar parasita murino bastante agressivo e,

portanto, se extrapolarmos a sua atividade para infecções em humanos, a violaceína poderia ser utilizada nos casos de malária grave.

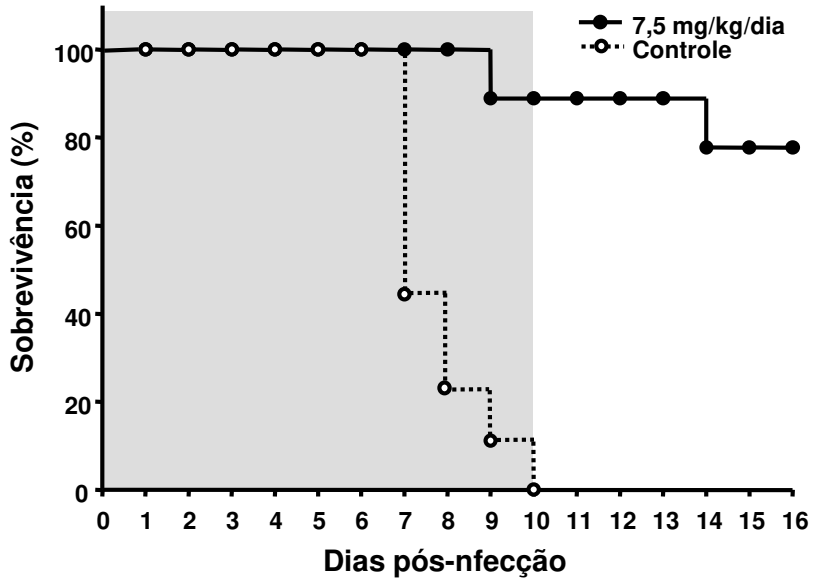

Figura 17. Atividade antimalárica da violaceína em formas sanguíneas de PcchAJ, uma cepa letal. Curva de sobrevivência de camundongos C57BL/6 (n=8 para cada grupo) infectados com 10^6 EI de PcchAJ e tratados diariamente via i.p. (dia 0-10 pós-infecção) com 7,5 mg/Kg de violaceína ou com DMSO 1,25% (controle). A caixa cinza representa os dias de tratamento.

3. Atividade antimalárica da violaceína encapsulada em PLGA

Todos esses resultados somados demonstram a capacidade antimalárica da violaceína e seu potencial como droga de escolha no tratamento da malária. No entanto, a fim de aperfeiçoar a atividade antimalárica da violaceína, nós obtivemos em associação com os grupos do Prof. Marcelo Brocchi (Instituto de Biologia, Departamento de Genética, Evolução e Bioagentes) e Prof. Nelson Durán (Instituto de Química, Departamento de Química Biológica), uma nova

formulação da violaceína baseada em nanopartículas (NP-violaceína) que aumentou sua solubilidade e estabilidade.

Nesse sentido, *P. falciparum* 3D7 cultivados por 48h na presença de diferentes concentrações de NP-violaceína apresentaram inibição similar a violaceína livre (Figura 18), com IC_{50} de 730 ηM, revelando que a encapsulação não alterou a atividade antimalárica da violaceína.

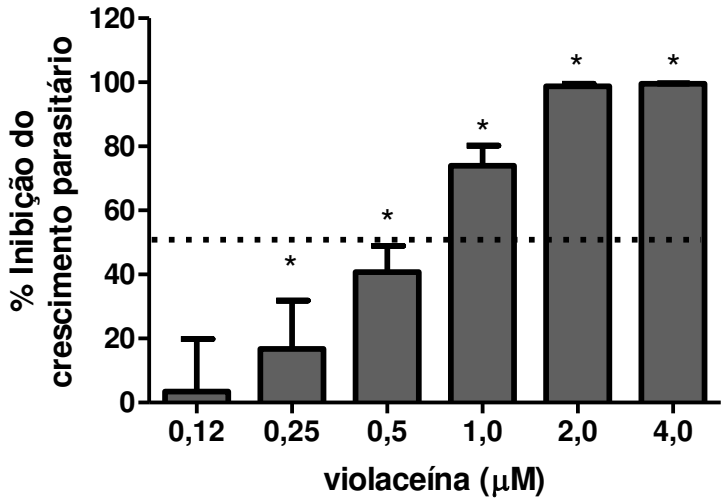

Figura 18: Atividade antimalárica da NP-violaceína *in vitro* contra formas sanguíneas de *P. falciparum* 3D7. *P. falciparum* 3D7 (parasitemia 1%, hematócrito 2%) foram incubados em *candle jar* com diferentes concentrações de NP-violaceína ou meio (controle), a 37°C por 48 horas. A inibição da parasitemia foi calculada através da fórmula: % Inibição = 100 – [(CPM amostra – CPM Eritrócitos sadios / CPM Controle – CPM Eritrócitos sadios) x 100]. Os resultados estão expressos como a média de quadruplicatas ± desvio padrão.

Uma vez que nos ensaios *in vitro*, a NP-violaceína apresentou atividade similar a violaceína livre, verificamos a atividade antimalárica da NP-violaceína *in vivo* frente a formas sanguíneas de PcchAS. Para isso, 5 animais infectados com PcchAS foram tratados com 7,5 mg/kg/dia a partir do dia 0 pós-infecção. Entretanto, no dia 3 pós-infecção e tratamento todos os animais

sucumbiram, mostrando elevada toxicidade da NP-violaceína (Figura 19). Essa elevada toxicidade do composto encapsulado, provavelmente deve-se ao fato da violaceína ser liberada de forma gradual quando encapsulada (Melo et al., 2009; Martins et al., 2010). Neste sentido, estudo *in vitro* da cinética de liberação da NP-violaceína mostrou que a liberação da violaceína das nanopartículas tem uma explosão inicial após três horas de incubação, seguida de uma liberação gradual. Tal comportamento sugere que o sistema tenha violaceína em sua superfície externa ou misturado com a superfície externa da matriz (sendo, portanto, facilmente liberado em incubações com curto espaço de tempo) e um conteúdo interno de droga que é liberado gradualmente, chegando a uma liberação de 80% do teor inicial após 72 horas (Martins et al., 2010).

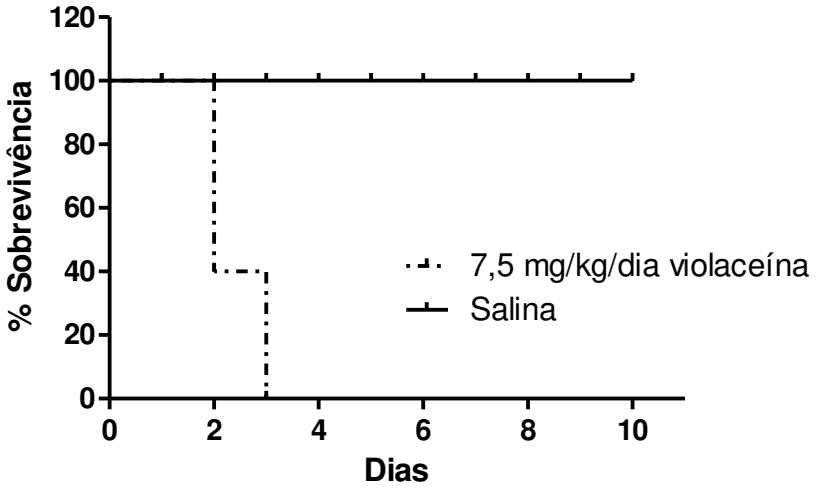

Figura 19: Toxicidade da NP-violaceína em animais infectados com PcchAS. Curva de sobrevivência de camundongos C57BL/6 (n=5 para cada grupo) infectados com 10^6 EI de PcchAS e tratados diariamente i.p.(dia 0-10 pós-infecção) com 7,5 mg/Kg de violaceína ou com solução salina(controle). *P< 0,05* violaceína *versus* controle (Teste *Log Rank)*.

Portanto, antes de avaliar a atividade antimalárica *in vivo* da NP-violaceína é necessário o estudo da farmacocinética desse composto, definindo o tempo de liberação e meia vida da NP-violaceína, assim como concentrações plasmáticas tóxicas desse composto. Diante disso, a atividade antimalárica *in vivo* da NP-violaceína não foi determinada.

4. Atividade antimalárica da violaceína complexada a nanotubos de carbono

Nas últimas duas décadas, uma série de partículas em nanoescala foi desenvolvida para o uso terapêutico. Uma propriedade interessante e muito útil dos NTs é sua capacidade de penetrar nas membranas celulares, proporcionando uma rota de entrega de cargas para o citoplasma e, em muitos casos, para o núcleo das células (Kam et al., 2006). O mecanismo pelo qual isso ocorre não é bem entendido, mas pode ser mediado por endocitose (Kam et al., 2006) ou inserção direta dos nanotubos através de membranas celulares (Pantarotto et al., 2004). Portanto, NTs têm sido estudados para entrega intracelular de proteínas e peptídeos e fármacos (*drug* delivery) (Martin & Kohli, 2003).

Para investigar se a violaceína funcionalizada com diferentes NTs apresenta atividade antimalárica potencializada, devido a um *delivery* mais eficiente, avaliamos a capacidade das três formulações (NT1-Violaceína, NTC2-Violaceína, SW-Violaceína) em inibir o crescimento parasitário de *P. falciparum* 3D7. Como podemos observar na Figura 20, nesse ensaio a violaceína na concentração de 825 ηM (IC_{50} pré-determinada para *P. falciparum* 3D7) foi capaz de inibir em 31% o crescimento parasitário. Já quando avaliada a atividade antimalárica dos três NTs livres (0,28 µg/ml), nenhum deles foi capaz de inibir o crescimento parasitário. A violaceína (825 ηM) funcionalizada com os três diferentes NTs não foi capaz de potencializar a inibição da

parasitemia que foi similar à inibição pela violaceína livre (31%, 35%, 37% e 33% para Violaceína, NT1-Violaceína, NTC2-Violaceína e SW-Violaceína, respectivamente). A não potencialização da atividade da violaceína pela funcionalização com NTs pode ser devido à falha no processo de funcionalização, uma vez que não determinamos a eficácia deste procedimento. Até então não há uma ferramenta adequada para determinar a porcentagem de composto que realmente foi incorporado pelo NT e o quanto de composto e/ou NT permaneceram livres e, portanto a atividade da violaceína funcionalizada com os diferentes NTs pode ter sido similar a da violaceína livre, pois esta poderia estar livre e não incorporada aos NTs.

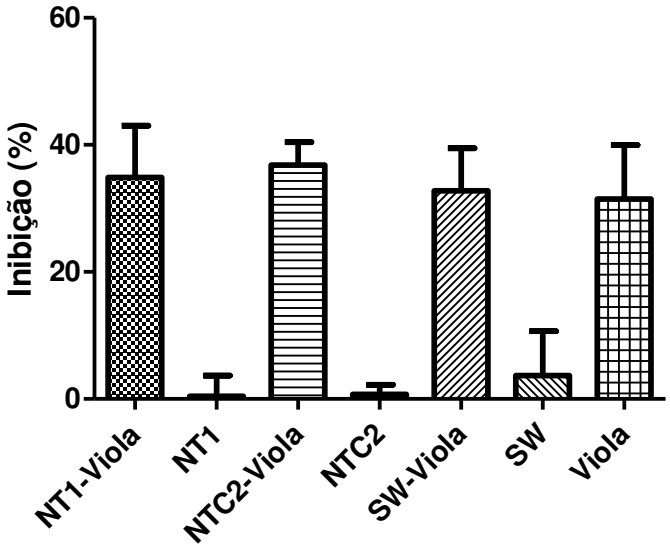

Figura 20: Atividade antimalárica da violaceína funcionalizada com três diferentes NTs *in vitro* contra formas sanguíneas de *P. falciparum* 3D7. *P. falciparum* 3D7 (parasitemia 1%, hematócrito 2%) foram incubados em *candle jar* com a violaceína funcionalizada com três diferentes nanotubos (NT-1, NTC2 e SW), os três nanotubos puros ou a violaceína livre, a 37°C por 48 horas. A inibição da parasitemia foi calculada de acordo com a fórmula % Inibição = 100 – [(Parasitemia amostra – *background* Eritrócitos Sadios / Parasitemia Controle – *background* Eritrócitos sadios) x 100]. Os dados apresentados são a média e desvio padrão de quadruplicata em cada condição.

5. Atividade antimalárica da violaceína *in vitro* contra *P. vivax*

Para investigar a atividade antimalárica da violaceína contra formas sanguíneas de *P. vivax*, foram coletados 13 isolados provenientes de pacientes que procuraram atendimento médico no Ambulatório da FMT em Manaus- Amazonas. Dentre os 13 isolados, somente 9 apresentaram desenvolvimento adequado *in vitro.* O desenvolvimento *in vitro* foi considerado adequado quando a porcentagem de esquizontes após 48h de cultivo em *candle jar,* era maior, similar ou até 50% da parasitemia do mesmo isolado no tempo 0 (Russell et al., 2003). Então, procedemos a avaliação da atividade antimalárica da violaceína através de ELISA nos 9 isolados restantes. O ensaio de ELISA só foi considerado satisfatório quando a leitura da DO do poço controle (sem droga) fosse maior ou igual a 0,5; sendo assim os isolados que obtiveram leitura menor foram descartados. Ao final, somente em 4 dos 13 isolados foi possível avaliar a atividade antimalárica. Como podemos observar na Tabela 2, em 3 dos quatro isolados, nenhuma dose de violaceína testada foi capaz de inibir o desenvolvimento parasitário e, portanto não foi possível fazer o cálculo da IC_{50}. No único isolado que inibiu o desenvolvimento parasitário, a IC_{50} foi de 3241 ηM, bem acima do encontrada para *P. falciparum* 3D7 (825 ηM). Nos mesmos isolados também testamos a atividade da cloroquina e do artesunato. Em todos os isolados a cloroquina inibiu o desenvolvimento parasitário e a IC_{50} média foi de 269,3 ηM (206,5-485,8 ηM), bastante superior a IC_{50} encontrada para *P. falciparum* 3D7 (30,82 ηM). Já o artesunato inibiu o desenvolvimento parasitário em três dos quatro isolados e a IC_{50} média foi de 33,61 ηM (9,5-54,2 ηM) também acima da IC_{50} para *P. falciparum* 3D7 (3,2 ηM).

Tabela 2: IC_{50} (ηM) da violaceína, cloroquina e artesunato encontrada para diferentes isolados de *Plasmodium vivax*.

	IC_{50} (ηM)		
	Violaceína	**Cloroquina**	**Artesunato**
Isolado 1	NI	206,5	37,08
Isolado 4	NI	271,9	54,2
Isolado 6	NI	112,9	9,55
Isolado 11	3241	485,8	NI
P. falciparum 3D7	825	30,82	3,2

NI: Não Inibiu

Esses dados geram bastante dúvida, uma vez que todas as drogas testadas apresentaram IC_{50} bastante superior a IC_{50} para *P. falciparum* e também a encontrada na literatura para *P. vivax* (Russell et al., 2003; Kaewpongsri et al., 2011; Chotivanich et al., 2009). Assim, questionamos a eficácia da nossa metodologia em investigar a atividade antimalárica contra *P. vivax*.

A parasitemia periférica de pacientes com malária vivax geralmente é bastante baixa, e ensaios para medir o desenvolvimento parasitário através da quantificação de pLDH por ELISA tem sua eficácia reduzida quando iniciados com baixa parasitemia (Basco et al., 1995). Segundo Basco et al. (1995), para uma performance ótima do ensaio quando se utiliza isolados frescos é necessário iniciar com parasitemias enre 1 e 2%. E mais, Kaewpongsri et al. (2011) mostraram que a presença de leucócitos durante os ensaios de atividade antimalárica aumentou a IC_{50} de cloroquina e artesunato em relação ao mesmo isolado testado após a remoção de leucócitos. E como em nossos ensaios não utilizamos nenhuma técnica para remoção de leucócitos, podemos ter obtidos valores equivocados de IC_{50} para as drogas testadas. Portanto, é necessário aprimorar a técnica de avaliação da atividade antimalárica em *P. vivax*, testando diferentes metodologias

simultaneamente, como microscópica (Russell et al., 2011; Kaewpongsri et al., 2011), marcação com fluoróforo e contagem em citômetro de fluxo (Malleret et al., 2011) e ELISA (Druilhe et al., 2007) antes de avaliar a ação antimalárica de novos compostos.

Conclusões

Com os dados apresentados neste capítulo, podemos concluir que a violaceína:

1. Não apresentou citotoxicidade *in* vitro sob eritrócitos humanos não infectados em concentrações menores que 12 μM e também não se mostrou tóxica em animais *naive* tratados com 7,5 mg/kg/dia durante 11 dias consecutivos, sendo a toxicidade nos animais avaliada quanto a sobrevida, peso e densidade de eritrócitos.
2. Apresentou atividade antimalárica *in vitro* contras as formas sanguíneas de *P. falciparum* 3D7 e S20, mas não contra formas sanguíneas de *P. vivax.*
3. Apresentou atividade antimalárica *in vivo* contras as formas sanguíneas de *P. chabaudi chabaudi*:
    a. Quando administrada por 11 dias consecutivos a partir do dia 0, foi capaz de inibir o crescimento parasitário nos animais infectados com PcchAS em diferentes doses;
    b. Na maior dose testada (7,5 mg/kg/dia) evitou a recrudescência parasitária, mesmo sem a pressão da droga e reduziu em 80% a mortalidade de animais infectados com a cepa letal PcchAJ;
    c. Foi capaz de inibir o crescimento parasitário quando administrada após o estabelecimento de infecção patente (tratamento entre os dias 5-10 pós-infecção).
4. Quando encapsulada em PLGA apresentou atividade antimalárica *in vitro* contra formas sanguíneas de *P. falciparum* 3D7 similar ao composto livre, mas foi tóxica aos animais infectados com PcchAS quando administrada diariamente na dose de 7,5 mg/kg.
5. Quando funcionalizada com três diferentes formulações de nanotubos de carbono (NT-1, NTC2, SW) não teve atividade antimalárica aumentada em relação ao composto livre.

Capítulo 2

Imunopatogênese na malária vivax

Introdução

1. Malária e Imunopatogênese

Dentre as espécies de *Plasmodium* que infectam o homem, sabe-se que o *P. falciparum* é o mais virulento, sendo responsável por cerca de 90% das mortes por malária (WHO, 2011). *P. falciparum* é conhecido pela sua capacidade em aderir ao endotélio capilar e pós-capilar (processo conhecido como citoaderência), e esta capacidade está associada à patologia da malária grave, como MC, malária associada à gravidez (PAM) e síndrome do desconforto respiratório agudo (ARDS) (MacPherson et al., 1985; Rowe et al., 1995; Newbold et al., 1999; Roberts et al., 2000; Pain et al., 2001).

A citoaderência refere-se a um conjunto de mecanismos no qual os eritrócitos infectados por formas maduras de *P. falciparum* se ligam a receptores expressos na superfície de diferentes células do hospedeiro (Figura 21). Dentre esses mecanismos destacam-se: a sequestração, que corresponde à retirada das formas maduras do parasita da circulação periférica para a microvasculatura, onde aderem às células endoteliais; a formação de rosetas, que é a adesão de eritrócitos infectados a outros não infectados; e a autoaglutinação, que corresponde à adesão entre eritrócitos infectados, podendo esta ser mediada por plaquetas (Handunetti et al., 1992; Ho & White, 1999).

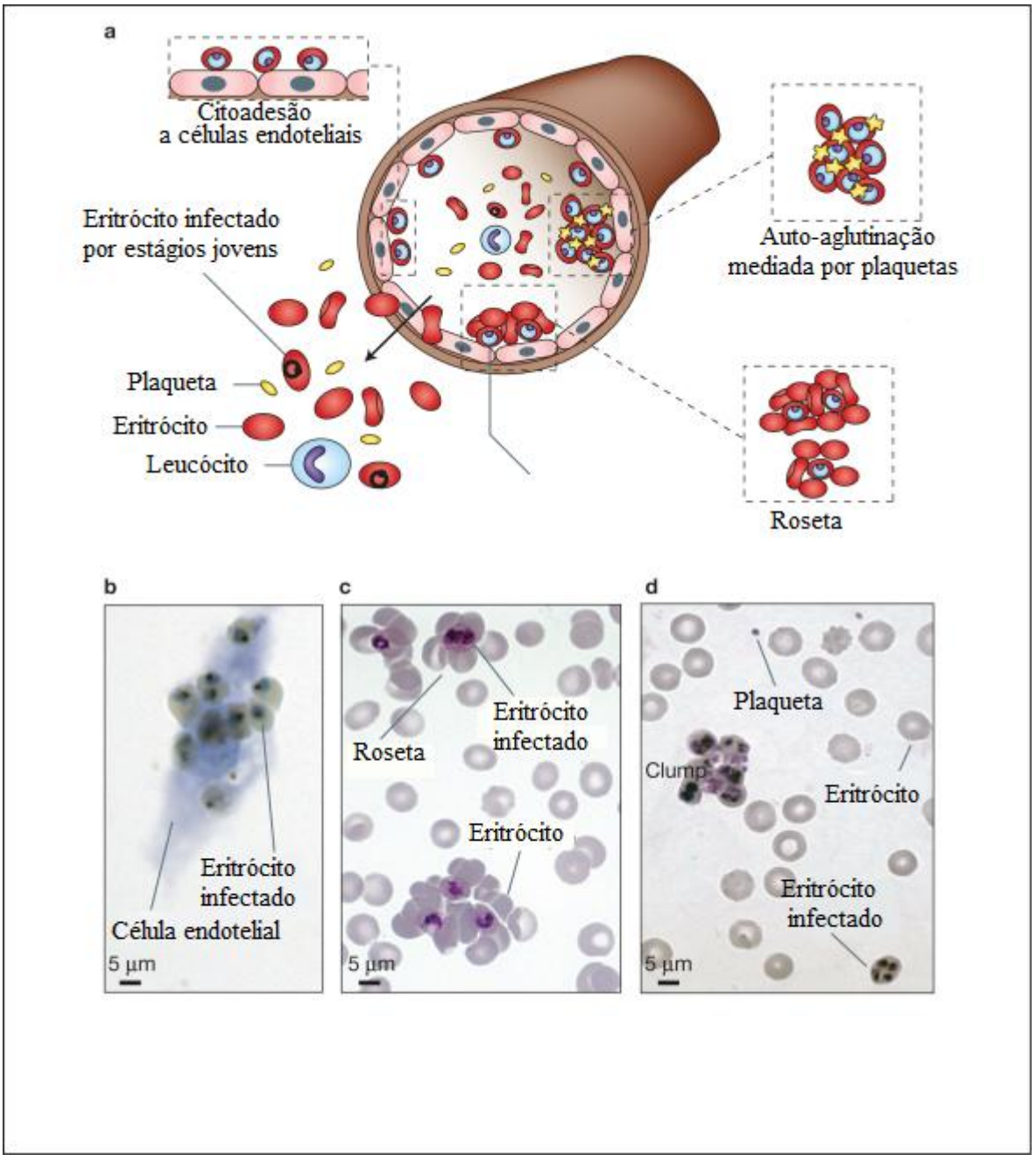

Figura 21: Adesão de eritrócitos infectados por *Plasmodium falciparum* (PF-EIs) a células humanas. Adaptado de Rowe et al., 2009.

Como a citoaderência está relacionada à gravidade da doença, os fatores que medeiam a adesão têm sido o foco de numerosos estudos (Franke-Fayard et al., 2010). Estes estudos resultaram na identificação de proteínas do parasita (ligantes) e do endotélio do hospedeiro (receptores) que estão diretamente envolvidas no processo (Kraemer & Smith, 2006; Sherman et al., 2003; Deitsch & Hviid, 2004; Gamain et al., 2007; Sharma, 1991; Craig & Scherf, 2001; Baruch, 1999).

A infecção do eritrócito por *P. falciparum* acarreta em modificações morfológicas e estruturais da membrana eritrocítica, com o aparecimento de protuberâncias elétrondensas denominadas *knobs*. Estas protuberâncias foram descritas como sendo os locais pelos quais os Pf-EIs aderem a superfícies endoteliais (Sharma, 1991). Os *knobs* são constituídos por diversos polipeptídios, tais como a proteína de membrana 1 do eritrócito infectado por *P. falciparum* (PfEMP-1). Esta proteína é expressa a partir de 60 genes variáveis (*var*) por genoma haplóide do parasita. Devido à recombinação ectópica que ocorre entre regiões teloméricas que contém os genes *var,* o número de alelos é muito grande. Entretanto, apesar desta variabilidade, apenas uma variante antigênica é expressa na superfície dos Pf-EIs de cada vez (Craig & Scherf, 2001). Isto possibilita um constante e eficiente escape da resposta imune antes que sejam formados novos anticorpos que revertam a citoadesão. Por este mecanismo, a PfEMP-1 é capaz de mediar uma ampla gama de interações adesivas a diversos receptores endoteliais, incluindo o CD36 (*Cluster of Differentiation 36*) (Ockenhouse et al., 1989), a ICAM-1 (*Intercelular Adhesion Molecule 1*) (Berendt et al., 1989) e a CSA (*Chondroitin Sulfate A*) (Robert et al., 1995; Rogerson et al., 1995). Muitas dessas moléculas do hospedeiro, que podem agir como receptores de adesão para Pf-EIs, têm sido identificados *in vitro* utilizando células endoteliais, no entanto o papel de cada uma delas na patogênese da malária permanece incerto (Beeson & Brown, 2002).

Acredita-se que a citoadesão de Pf-EI seja importante para a sobrevivência do parasita, uma vez que os eritrócitos modificados pela infecção por *Plasmodium* são reconhecidos como anormais pelo baço e retirados da circulação juntamente com o parasita intracelular (Wyler, 1983). Portanto, ao citoaderir no endotélio, o eritrócito infectado não circula evitando sua depuração pelo baço (Berendt et al., 1990). Além disso, acredita-se que a citoaderência possa aumentar a taxa de reinvasão de eritrócitos, elevando a densidade populacional. Neste sentido, trabalho de Handunnetti et al. (1989) sugere que a formação de rosetas propiciaria um maior contato entre os merozoítos liberados pelos esquizontes e os eritrócitos não infectados, aumentando a eficiência da reinvasão. Outro trabalho sugere que o sequestro de Pf-EI possa garantir um ambiente mais favorável à maturação parasitária (Ho & White, 1999).

A adesão de Pf-EIs às células endoteliais pode ocorrer na microvasculatura de vários órgãos e tecidos, como coração, pulmão, cérebro, rins, fígado, tecido adiposo e placenta (Rowe et al., 2009; Baruch et al., 1999). Diversos fatores estão envolvidos na citoadesão de *P. falciparum* ao endotélio, entre eles podemos destacar os elevados níveis de citocinas circulantes, como TNF-α, que propicia um aumento na quantidade de receptores presentes no endotélio devido sua ativação via NF-κB e as plaquetas que aderem à parte da proteína multimérica Von Willebrand Factor (VWF) presente no endotélio ativado, e podem aumentar a citoaderência de *P. falciparum* agindo como ponte entre as células endoteliais e os eritrócitos infectados *(*Pf-EIs) (Schoefield & Grau, 2005). Como resultado desse processo, observa-se a obstrução microvascular (Dondorp et al., 2004; Dondorp et al., 2008), distúrbios metabólicos, como a acidose (Planche & Krishna, 2006) e liberação de mediadores inflamatórios (Schoefield, 2007; van der Heyde et al., 2006), que podem combinar-se causando manifestações clínicas graves e até morte do hospedeiro (Rowe et al., 2009).

Entre as formas graves da doença a PAM é a manifestação na qual a patogênese é melhor compreendida. O acúmulo de Pf-EI no espaço interviloso da placenta (Rogerson et al., 2007), em muitos casos associado com infiltrados de monócitos e macrófagos (Ordi et al., 2001), e alta expressão de citocinas pró-inflamatórias (Ordi et al., 1998; Rogerson et al., 2003) está associado a patogênese na PAM levando a abortos, retardo de crescimento intra-uterino, partos prematuros e baixo peso da criança ao nascimento (Brabin et al., 2004). Sabe-se que esse sequestro parasitário é mediado pela forma $VAR2_{CSA}$ de PfEMP1 que se liga a CSA no tecido placentário, e que os parasitas da placenta quase sempre transcrevem o gene que codifica esta proteína (Duffy et al., 2006; Tuikue Ndam et al., 2005; Salanti et al., 2003). Como o parasita na placenta, quase sempre, apresenta o mesmo fenótipo ($VAR2_{CSA),}$ uma imunidade específica contra as formas placentárias do parasita é adquirida após sucessivas gestações, podendo prevenir o sequestro e, dessa maneira, evitar a cascata de eventos inflamatórios que conduzem ao agravamento da doença (Schoefield & Grau, 2005, Beeson & Duffy, 2005).

Já a MC, responsável pelas complicações mais sérias e pelo maior número de mortes das infecções por *P. falciparum* (Mackintosh et al., 2004), tem sua patogênese pouco compreendida e muitos trabalhos discutem os fenômenos patológicos por detrás desta doença. Neste sentido, existem duas principais teorias para explicar a patogênese da MC humana. A primeira, teoria da obstrução mecânica, proposta há mais de cem anos e confirmada por numerosos estudos posteriores, sugere que a MC é uma conseqüência direta do seqüestro de eritrócitos, que acarreta na obstrução do fluxo cerebral e hipóxia cerebral (Berendt et al., 1994). A segunda, a teoria da inflamação, sugere que a MC é resultado de uma resposta imune exacerbada, na qual citocinas do tipo Th1, especialmente TNF-α (fator de necrose tumoral alfa) e IFN-γ (interferon gama),

apresentam um papel central no processo (Clark & Rockett, 1994). O mérito relativo destas duas teorias tem sido extensivamente debatido, e existe um consenso em considerar uma hipótese conciliatória (van der Heyde et al., 2006). De fato, parece que a combinação da obstrução mecânica, imunopatologia e outros mecanismos como a disfunção da homeostase e complicações metabólicas seja fundamental no desencadeamento da síndrome neurológica (Combes et al., 2006).

O aumento da permeabilidade dos capilares alveolares, resultando em perda de fluído intravascular para os pulmões parece ser a chave do mecanismo fisiopatológico por detrás das manifestações graves pulmonares (lesão pulmonar aguda- ALI, e ARDS) (Mohan et al., 2008). Neste sentido, acredita-se que o sequestro de EI nos capilares pulmonares e a consequente liberação de proteínas parasitárias na região, atrairiam neutrófilos, macrófagos e monócitos para o tecido, ativando-os e a consequente produção de citocinas pró-inflamatórias provocaria dano no endotélio vascular levando ao aumento da permeabilidade dos capilares alveolares (Mohan et al., 2008).

As outras manifestações de gravidade têm sua patogênese pouco compreendida e estudos mais aprofundados em pacientes ou modelos são necessários para elucidar os mecanismos fisiopatológicos envolvidos na doença.

Apesar de ser mais descrito em *P. falciparum*, o fenômeno de citoaderência é comum em outras espécies de *Plasmodium*. Por exemplo, a formação de rosetas já foi descrita em todas as espécies que infectam o homem (Handunnetti et al., 1989; Angus et al., 1996; Lowe et al., 1998; Udomsangpetch et al., 1995; Chotivanich et al., 1998; Russell et al., 2011) e em espécies que infectam outros mamíferos (Mackinnon et al., 2002; Udomsangpetch et al., 1991). A autoaglutinação, foi descrita não só em *P. falciparum* (Roberts et al., 1992), mas também em *P.*

knowlesi (Knisely et al., 1941). Dentre os parasitas que infectam o homem o sequestro foi verificado somente em *P. falciparum*, embora já tenha sido descritas para algumas espécies de roedores (Franke-Fayard et al., 2010; Cox et al., 1987; Coquelin et al, 1999; Mota et al., 2000; Neres et al., 2008; Franke-Fayarad et al., 2006).

2. Malária vivax e gravidade

Embora a mortalidade por *P. vivax* seja considerada baixa quando comparada à infecção por *P. falciparum*, sua morbidade é elevada. Neste sentido, ao contrário dos 70-80 milhões de casos anuais previamente descritos (Mendis et al., 2001) para a infecção por *P. vivax*, dados recentes estimam que esse número alcance de 130 a 435 milhões de casos a cada ano (Hay et al., 2004). Além disso, estudos recentes sugerem que o impacto clínico causado por essa infecção tem sido subestimado e que seu impacto econômico nunca foi adequadamente considerado (WHO, 2011).

Nos últimos 10 anos, as complicações graves observadas em infecções por *P. falciparum*, que incluem insuficiência renal, icterícia, ARDS, MC, convulsões, anemia, hiperparasitemia, trombocitopenia, edema pulmonar, ruptura do baço e morte, também foram relatadas para *P. vivax* em vários países (Figura 22) (Kochar et al., 2005; Kochar et al., 2007; Kochar et al., 2009; Tjitra et al., 2008; Tanwar et al., 2011; Lomar et al., 2005; Alexandre et al., 2006; Andrade et al., 2010; Lacerda et al., 2011; Anstey et al., 2007). Apesar do diagnóstico na maioria dos casos ter sido feito através da coloração de Giemsa, alguns destes trabalhos utilizou a técnica de PCR (reações de polimerase em cadeia) para exclusão de infecção mista.

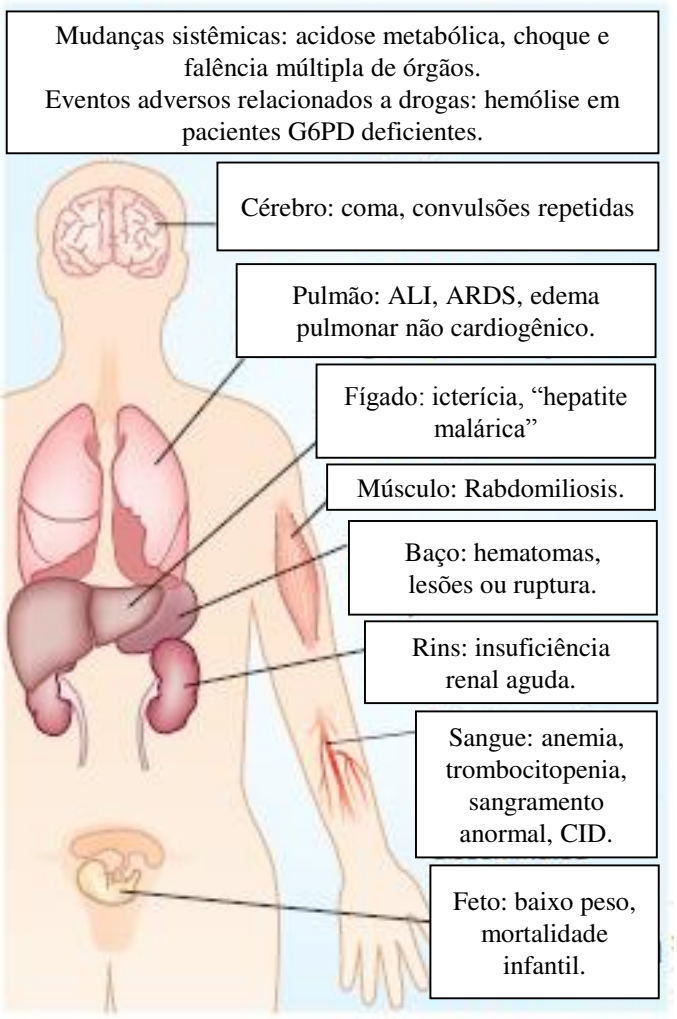

Figura 22: Clínica associada a infecções por *P. vivax*. CID: Coagulação Intravascular Disseminada. Adaptado de Bassat & Alonso (2011).

No Brasil, por exemplo, 234 mortes por malária vivax foram oficialmente reportadas entre os anos de 1998 e 2008 (Oliveira-Ferreira et al., 2010). Ainda, um estudo recente na cidade de Manaus, Amazonas, mostrou um aumento do número de internações hospitalares de pacientes infectados por *P. vivax* (Santos-Cinimera et al., 2007). Neste sentido, estudo clínico com 17 pacientes hospitalizados em Manaus, com diagnóstico microscópico e molecular (PCR) para *P. vivax*, revelou gravidade clínica, em sua maioria, relacionadas a icterícia e anemia grave

(Alexandre et al., 2010). Em outro estudo na cidade de Buritis, Rondônia, além de anemia grave e icterícia, a maior causa de gravidade nas infecções exclusivas (confirmada por PCR) por *P. vivax* foi insuficiência respiratória (Andrade et al., 2010). Neste estudo, dos 19 pacientes apresentando malária grave (segundo critério da OMS para *P. falciparum*), 6 sucumbiram à infecção, 4 por ARDS e 2 por insuficiência renal (Andrade et al., 2010). Também, foi observada uma associação entre a gravidade e o aumento nas citocinas inflamatórias TNF-α, IFN-γ e no balanço IFN-γ/IL-10, bem como nos níveis de proteína C reativa, creatinina sérica e bilirrubina (Andrade et al., 2010).

Estudo na Papua, Indonésia, entre os anos de 2004 e 2007, demonstrou que nesta região pacientes com malária vivax apresentam mais chances de desenvolver malária grave que pacientes com malária falciparum, e mais a taxa de mortalidade foi similar entre pacientes com monoinfecção por uma das duas espécies (Tjitra et al., 2008). Similarmente aos relatos no Brasil, os principais quadros de gravidade na Papua foram a anemia grave (83%) e insuficiência respiratória (11%), embora também tenha sido elevada a frequência de coma entre esses pacientes (6%) (Tjitra et al., 2008).

Na Índia, diversos estudos vem relatando manifestações graves em infecções por *P. vivax* (Kochar et al., 2005; Kochar et al., 2007; Kochar et al., 2009; Tanwar et al., 2011; Yadav et al., 2011; Mohapatra et al., 2002; Sarkar et al., 2010; Kute et al., 2011; Singh et al., 2011). Neste sentido, um primeiro estudo publicado em 2002 relatou algumas manifestações consideradas na época atípicas em infecções por *P. vivax*, como icterícia (7,2%), anemia grave (7,2%) e manisfestações cerebrais (0,9%) (Mohapatra et al., 2002), no entanto estas manifestações pareciam ser esporádicas e não foi descartada a possibilidade de infecções mistas por PCR. Estudos na região do Rajastão, India, reportaram diversas manifestações grave em mono-infecções por *P.*

vivax, confirmada por PCR (Kochar et al., 2005; Kochar et al., 2007; Kochar et al., 2009; Tanwar et al., 2011). Entre os quadros de gravidade encontrados estão anemia grave, icterícia, MC, ARDS, insuficiência renal, sangramentos anormais e inclusive disfunção múltipla de órgãos. Nesses estudos, o pior prognóstico (quatro mortes entre sete pacientes com esse quadro) está relacionado a ARDS (Kochar et al., 2005; Kochar et al., 2009).

Semelhante ao que tem sido observado no Brasil, Papua e Índia, um número crescente de casos com complicações na malária vivax têm sido relatadas em todo o mundo (Baird et al., 2007; Bassat & Alonso, 2011; Anstey et al., 2009; Costa F.T., 2011), confirmando que há de fato um aumento do número de casos graves exclusivamente associado com infecção por *P. vivax*. No entanto, se essa gravidade está diretamente associada a mudanças na biologia do parasita, aumento de resistência às drogas antimaláricas ou a presença de comorbidades ainda é uma questão de debate (Bassat & Alonso, 2011; Costa et al., 2011).

Devido à ausência de *knobs*, de genes *var* e de proteínas homólogas à PfEMP-1, por muito tempo inferiu-se que *P. vivax* não era capaz de citoaderir ao endotélio. Atualmente, com a crescente documentação de casos graves de infecções por *P. vivax* (Price et al., 2007; Kochar et al., 2005; Kochar et al., 2007; Kochar et al., 2009; Barcus et al., 2007; Tjitra et al., 2008; Genton et al., 2008; Tanwar et al., 2011) inclusive na Amazônia Brasileira (Alexandre et al., 2010, Andrade et al., 2010; Lacerda et al., 2011) e também a existência no genoma de *P. vivax* de família de genes subteloméricos variantes (*vir*) (del Portillo et al., 2001), essa inferência vem sendo questionada. Ainda, mesmo sem apresentar *knobs*, Pv-EIs são capazes de formar rosetas (Udomsangpetch et al., 1995, Chotivanich et al., 1998; Russell et al., 2011); bem como, alguns plasmódios de roedores, como *P. berghei* e *P. chabaudi,* que também não possuem *knobs*, são capazes de citoaderir ao endotélio (Franke-Fayard et al., 2010; Cox et al., 1987; Coquelin et al.,

1999; Mota et al., 2000; Neres et al., 2008; Franke-Fayard et al., 2006). Estes achados sugerem que a citoadesão ao endotélio é uma característica antiga dentro deste gênero, não restrita ao *P. falciparum,* aos *knobs* ou à PfEMP-1.

Ainda, apesar de estudos documentando a presença de todos os estágios de *P. vivax* no sangue periférico de pacientes infectados, o sequestro de *P. vivax* chegou a ser proposto há mais de 50 anos, ao se observar que apenas uma pequena proporção de estágios maduros do parasita (esquizontes) é encontrada na circulação periférica (Leeson et al., 1957).

Neste sentido, estudo recente de nosso grupo demonstrou que Pv-EI obtidos de pacientes com malária não grave da Amazônia brasileira foram capazes de citoaderir em condições estáticas e de fluxo em criocortes de placenta, células endoteliais de pulmão (HLEC) e de cérebro (SBEC). Apesar do número de Pv-EI aderidos por mm^2, sob condições estáticas, ter sido 10-15 vezes menor do que de Pf-EI, a força de interação da adesão sob condições fisiológicas (ensaios de fluxo) foi similar nos dois parasitas (Carvalho et al., 2010).

Neste mesmo trabalho, para identificar os potenciais receptores endoteliais envolvidos na citoadesão de Pv-EI, células CHO transfectadas com diferentes receptores endoteliais foram utilizadas em ensaios de adesão. Neste sentido, foi encontrada maior adesão de Pv-EI a células transfectadas com ICAM-1 em comparação a células transfectadas com CD36 ou sem transfecção. Além disso, a adesão de parasitas a células endoteliais foi inibida pela adição de CSA solúvel, embora o tratamento das mesmas células com condroitinase-ABC não tenha afetado a adesão (Carvalho et al., 2010). Estes resultados sugerem que a CSA não está diretamente envolvida na adesão de Pv-EI, e como a CSA possui carga altamente negativa devido à presença de grupos sulfato, é possível que a inibição da citoadesão por CSA tenha sido

sustentada por interações eletrostáticas e não através da competição pelo receptor (Costa et al., 2011).

Como em *P. falciparum* a citoadesão é mediada por proteínas codificadas através de genes variáveis (*var*), foi questionado se as proteínas VIR expressas a partir de uma superfamília gênica variante (*vir*) poderiam mediar a citoadesão de Pv-EI. Neste sentido anticorpos policlonais contra proteínas VIR de duas diferentes subfamílias (VIR_{E4} e VIR_{A5}) foram capazes de inibir a adesão *ex vivo* de Pv-EI a células endoteliais (Carvalho et al., 2010). Além disso, devido a ausência de um método de cultivo prolongado de *P. vivax*, Bernabeu et al. (2011) utilizaram *P. falciparum* 3D7 transfectados com 3 antígenos VIR de diferentes subfamílias (subfamília A- VIR_{17}; subfamília C- VIR_{14} e subfamília D- VIR_{10}) para acessar o papel dessas proteínas no fenômeno de citoadesão. Dos três antígenos testados, dois (VIR_{14} e VIR_{10}) foram exportados para a membrana eritrocitária, e somente um deles (VIR_{14}) foi capaz de mediar adesão a diferentes receptores endoteliais expressos em CHO em condições estáticas. Entretanto em condições fisiológicas, esses parasitas só foram capazes de aderir a CHO_{ICAM} (Bernabeu et al., 2011).

Ainda, trabalho com autópsias de 17 pacientes, que sucumbiram à infecção por malária vivax no Brasil, observou que ARDS é uma das principais patologias associadas a complicações nesse parasita e mais a presença de Pv-EIs nos capilares pulmonares desses pacientes, mesmo na ausência de parasitemia periférica, sugere fortemente a existência de citoadesão *in vivo* (Lacerda M. V. G., comunicação pessoal).

Coletivamente esses dados indicam que Pv-EI são capazes de citoaderir à diferentes receptores endoteliais e que pelo menos em parte essa citoadesão é mediada por proteínas VIR; e mais juntos esses trabalhos dão suporte a ideia de que essa citoadesão, ao menos parcialmente, exerce algum papel na patogênese observada na malária vivax.

Objetivos

1. Objetivo Geral

Avaliar o fenômeno de citoadesão em isolados de *P. vivax* da Amazônia brasileira, quanto a capacidade adesiva em células endoteliais e a formação de rosetas antes e após o amadurecimento de Pv-EI *in vitro*.

2. Objetivos Específicos

2.1 Avaliar a capacidade adesiva de Pv-EI coletados diretamente de pacientes infectados ou amadurecidos *in vitro* em células endoteliais e seus receptores.

2.2 Comparar as taxas de citoadesão de Pv-EI coletados diretamente de pacientes infectados e amadurecidos *in vitro*.

2.3 Verificar se os receptores envolvidos na citoadesão de Pv-EI amadurecidos *in vitro* são similares ou não aos receptores envolvidos na citoadesão de Pv-EI coletados diretamente de pacientes infectados.

2.4 Verificar e quantificar a formação de rosetas por Pv-EI coletados diretamente de pacientes infectados ou amadurecidos *in vitro*.

Métodos

1. Procedimentos com o paciente

A unidade de saúde de seleção de pacientes foi a FMT-HVD, situada em Manaus- AM, unidade de referência da SUSAM para o atendimento de pacientes com doenças infecciosas.

Os indivíduos que obedeceram aos critérios de inclusão foram informados e convidados a participar do projeto. Os pacientes que aceitaram participar do estudo fizeram a leitura e concordância do TCLE (Anexo). Posteriormente foi preenchido um Questionário (Anexo) e coletados cerca de 10 ml de sangue por punção venosa (um tubo com EDTA). Após o preenchimento do questionário e coleta de sangue, o paciente recebeu atendimento médico e tratamento antimalárico apropriado dando seguimento a rotina ambulatorial da FMT-HVD.

1.1 Critérios de inclusão

- Pacientes com mais de 18 anos.
- Pacientes com diagnóstico microscópico positivo para malária vivax com parasitemia maior ou igual a uma cruz (1- parasito/campo).

1.2 Critérios de não inclusão.

- Pacientes com diagnóstico microscópico ou molecular (por PCR) para malária por *P. falciparum*, ou co-infecção vivax e falciparum (malária mista).
- Mulheres com história de gestação atual.
- Paciente na vigência de tratamento antimalárico.

1.3 Critério de exclusão *a posteriori*

- Pacientes com diagnóstico molecular para *P. falciparum* ou infecção mista

2. Isolamento e amadurecimento de Pv-EI

Os eritrócitos foram ressuspensos em PBS (50% hematócrito) e a suspensão foi passada por duas colunas de celulose CF11 (seringa de 10 ml com 1 cm^3 de lã de vidro e 5 cm^3 de celulose CF11) sucessivamente para a remoção de leucócitos (Sriprawat et al., 2009). O obtido da coluna foi então lavado uma vez em meio RPMI, e o pellet ressuspenso em meio RPMI (hematócrito 20%). Em seguida, 5 mL dessa suspensão foi transferida para tubo plástico cônico de 15 mL. Com auxílio de uma pipeta *Pasteur* longa, 5 mL de uma solução de *Percoll*® 45% (GE Healthcare, USA) em RPMI foram depositados abaixo da suspensão de eritrócitos. O material foi centrifugado a 1.500g por 20 minutos, em baixa aceleração e sem freio para formação de gradiente (Carvalho et al., 2010). Após a centrifugação os eritrócitos sadios estavam depositados no fundo do tubo, enquanto as formas maduras eram encontradas na interface. Os eritrócitos infectados foram retirados da interface e três lavagens em meio RPMI foram feitas para a completa remoção da solução de *Percoll*®. Após a última lavagem os eritrócitos infectados foram ressuspensos em meio RPMI, o número de eritrócitos totais contado com o auxílio da Câmara de Neubauer e a parasitemia pós *Percoll*® determinada por meio de esfregaços corados com Panótico Rápido (Laborclin). Parte dos Pv-EI foi utilizado logo após o enriquecimento por *Percoll*® e parte foi mantida em cultivo (37°C, atmosfera de 5% de O_2) para amadurecimento, em meio McCoy 5A (Sigma, USA) contendo D-glicose e 20% de plasma humano AB+

decomplementado, até que a maioria dos parasitas tenha atingido o estágio de esquizonte (16-24h) (Russell et al., 2011)

3. Cultivo de Células

Nos ensaios de citoadesão foram utilizadas células endoteliais do pulmão humano (HLEC) e células de ovário de hamster chinês (CHO). HLEC foi cultivada em meio DMEM/HAM F12 (Nutricell, Brasil) suplementado com 30 μg/mL de fator de crescimento de células endoteliais (ECGS, Sigma, USA) e 10% de SFB, e CHO cultivadas em meio RPMI 1640 suplementado com 10% de SFB. As células foram mantidas em incubadora com pressão constante de 5% de CO_2 e 37°C.

4. Ensaio de Citoadesão

As células foram cultivadas em lâminas de oito poços com 0,69 cm^2 cada (*Culture Slides*, Becton & Dickinson), até atingirem confluência total. Após 3 lavagens com meio de adesão (Meio RPMI pH 6,8), foram adicionados 5 x 10^4 Pv-EI em cada poço, amadurecidos ou não, seguido de incubação durante 1h. Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens em meio de adesão em condições estáticas. Em seguida, as lâminas foram fixadas e coradas com o *kit Panótico Rápido*. O número de eritrócitos infectados, ou não, aderidos a monocamada endotelial foi contado com auxílio de microscópio, e a adesão foi expressa como a média ± desvio padrão do número de eritrócitos por mm^2 observados em 3 poços.

Como as células HLEC expressam diferentes receptores (ICAM-1, CSA, CD36, E-Selectina, entre outros), a fim de averiguar quais deles estão envolvidos na citoadesão de *P. vivax*, foram adicionados em alguns poços, juntamente com Pv-EI, 10 µg/ml de anticorpos monoclonais (mAb) contra CD36 (clone FA6-152, Santa Cruz) ou contra ICAM-1 (clone 84H10, Chemicon) ou ainda 100 µg/ml de CSA solúvel (*CSA salt from bovine trachea*- Sigma, USA). Em alguns ensaios de citoadesão em HLEC, foi adicionado plasma do próprio paciente (autólogo) durante a incubação das células com Pv-EI.

Nos ensaios com as células CHO foram utilizados dois clones celulares diferentes: CHO_{745} que não expressa CD36, CSA e ICAM-1 (Andrews et al., 2005), e CHO_{K1} que não expressa CD36 e ICAM-1 (Golnitz et al., 2008), mas expressa fortemente CSA. Além disso, foram utilizadas células CHO_{745} transfectadas com CD36 ou ICAM-1.

5. Ensaio de Rosetas

Para o ensaio de rosetas, Pv-EI amadurecidos ou não foram incubados em placa de 96 poços a 37°C por 1h em meio RPMI com 20% de soro AB (Sigma, USA) ou plasma do próprio paciente (volume total de 50 µL, hematócrito de 4%, parasitemia de 2%). Após a incubação, foi adicionado 5 µL de Acridine Orange (200 µM) e a porcentagem de rosetas [100x (n° de parasitas em rosetas/ n° de parasitas totais)] foi quantificada com auxílio de microscópócito de fluorescência Nikon. Foram considerados parasitas em rosetas quando mais de três eritrócitos sadios estavam aderidos ao eritrócito parasitado.

6. Tipagem sanguínea

Foi feita a tipagem sanguínea de todos os isolados utilizados nos ensaios de roseta com auxílio de um kit (Ebram). Resumidamente os eritrócitos foram ressuspensos em solução salina (hematócrito 20%) e uma gota dessa solução foi colocada junto a uma gota de solução contendo os soros com os anticorpos (anti-A, anti-B, anti-AB) sobre lâmina. Após homogeneizar com o auxílio de uma ponteira, foi verificada a aglutinação do sangue na presença de cada um dos anticorpos. A tipagem foi feita de acordo com a Tabela 3.

Tabela 3: Tipagem sanguínea ABO.

Tipo sanguíneo	Anti-A	Anti-B	Anti-AB
A	Aglutinado	Não aglutinado	Não aglutinado
B	Não aglutinado	Aglutinado	Não aglutinado
AB	Aglutinado	Aglutinado	Aglutinado
O	Não aglutinado	Não aglutinado	Não aglutinado

7. Citometria de fluxo

Para verificar a porcentagem de Pv-EI com IgG aderidos antes e pós-amadurecimento realizamos a marcação dupla de Pv-EI com brometo de etídeo (Sigma, USA), para marcar o parasita, e anti-IgG conjugado com Alexa 488 (Invitrogen, USA) para marcação do anticorpo. Para construção do *gate*, utilizamos Pv-EI sem marcação e para eliminação de *background* nos diferentes filtros Pv-EI também foi marcado com cada fluoroféro separadamente. Após 5

lavagens, Pv-EI marcado ou não foi fixado em Solução 2% de Paraformaldeído (Sigma, USA) em PBS e a leitura no citômetro se deu em no máximo 24h.

8. Análise Estatística

A análise estatítica deste trabalho foi efetuada com o auxílio do software Prism™ versão 5.0 (Graphpad, USA). Para comparar a frequência dos estágios de desenvolvimento de *P. vivax* na circulação periférica utilizamos o teste *Kuskall-Wallis*. O teste utilizado para comparação de porcentagem de esquizontes antes e pós o amadurecimento foi o teste t pareado. Para comparar as taxas de adesão em diferentes condições utilizamos o teste t pareado ou *Teste U Mann-Whitney*. A ánalise da correlação foi feita através do teste de *Spearman*.

Resultados e discussão

1. Amadurecimento de Pv-EI

Recentemente, nosso grupo demonstrou que formas circulantes de Pv-EI, diferente do que se acreditava, apresentam capacidade adesiva *ex vivo* a diferentes células endoteliais e em criocortes de placenta, no entanto essa adesão foi 10-15 vezes menor que em *P. falciparum* (Carvalho et al. 2010). Em *P. falciparum* somente os trofozoítos jovens estão circulantes na corrente periférica dos pacientes, o restante dos estágios estão sequestrados na microvasculatura de diferentes órgãos, e assim os ensaios de adesão com isolados desse parasita são efetuados após algum tempo de cultivo para o seu amadurecimento (Cojean et al., 2008). Diante disto, nos questionamos se ao utilizar as formas que estão na circulação periférica do paciente, não estávamos excluindo dos ensaios os estágios que realmente tem capacidade adesiva.

Embora diversos estudos afirmem que todos os estágios de desenvolvimento de *P. vivax* encontram-se na circulação periférica, nossas observações, que corroboram com as de Leeson (Leeson, 1957), demonstram uma baixa porcentagem de esquizontes na circulação periférica de pacientes com malária vivax em relação a outros estágios de desenvolvimento (Figura 23A). A porcentagem de Pv-EI na circulação periférica de pacientes em esquizonte foi em média de 9,5% (variando entre 0 e 50%), a de trofozoítas jovens foi de 68,5% e de trofozoítas maduros de 22,0% (Figura 23B). Esta baixa porcentagem de esquizontes de *P. vivax* nos leva a pensar que estes estágios estão se desenvolvendo em algum outro local que não a circulação periférica e, portanto podemos especular que estas formas devam estar sequestradas, aderidas em algum órgão deste paciente.

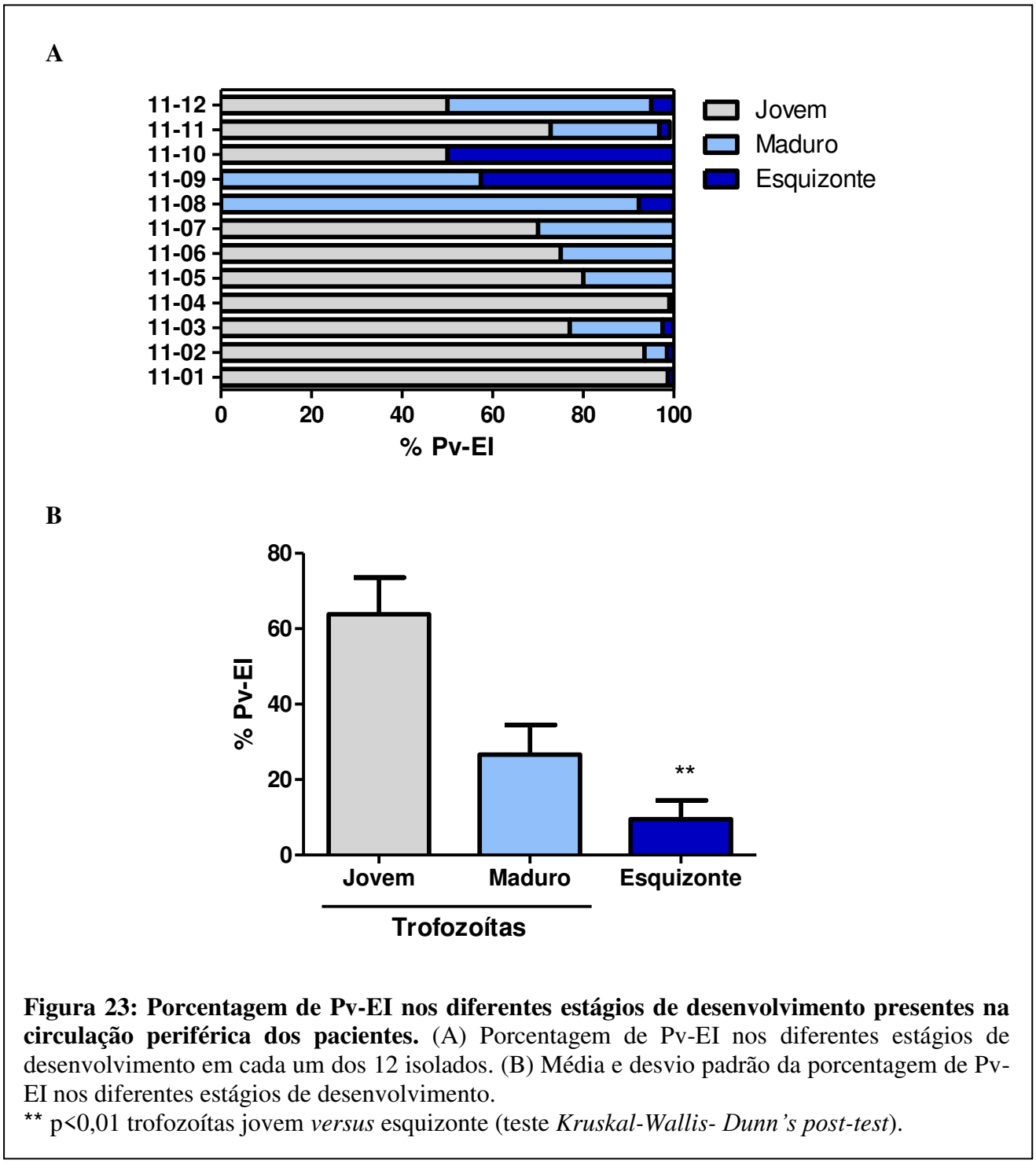

Figura 23: Porcentagem de Pv-EI nos diferentes estágios de desenvolvimento presentes na circulação periférica dos pacientes. (A) Porcentagem de Pv-EI nos diferentes estágios de desenvolvimento em cada um dos 12 isolados. (B) Média e desvio padrão da porcentagem de Pv-EI nos diferentes estágios de desenvolvimento.
** p<0,01 trofozoítas jovem *versus* esquizonte (teste *Kruskal-Wallis- Dunn's post-test*).

Para verificar a capacidade adesiva dos esquizontes de *P. vivax*, que são encontrados em baixas porcentagens na circulação periférica de pacientes, desenvolvemos uma técnica para

amadurecimento de Pv-EI. Como podemos observar na Figura 24, após 16-24h de cultivo *in vitro*, a porcentagem de esquizontes nos isolados aumentou (9,6% antes de amadurecer para 57,2% pós-amadurecimento). Entretanto, em alguns isolados, mesmo após 24h de amadurecimento, não foi possível a obtenção de esquizontes; em outros, no entanto, conseguimos obter 100% dos parasitas no estágio de esquizonte (Figura 24).

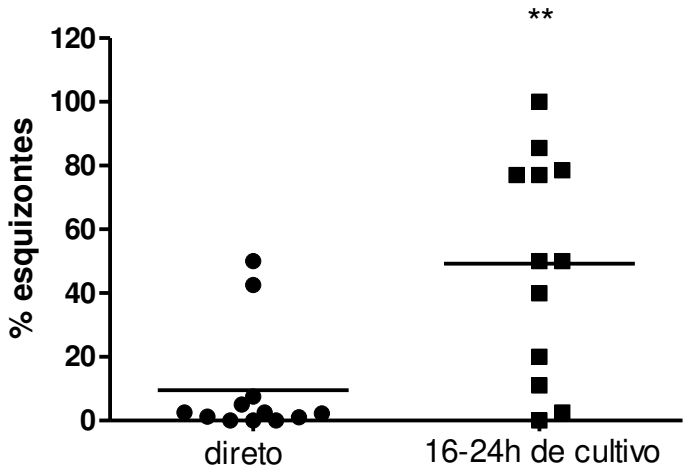

Figura 24: Porcentagem de Pv-EI esquizontes antes e após cultivo de 16-24 horas para amadurecimento. Cada ponto corresponde a um isolado e o traço central é a média dos 12 isolados testados.
** p<0,01 esquizontes (%) direto *versus* esquizontes (%) após 16-24h de cultivo (teste t pareado).

2. Citoadesão de Pv-EI amadurecidos

Para verificar se esquizontes de *P. vivax* apresentam maior capacidade adesiva que trofozoítos, formas que estão circulantes na corrente periférica dos pacientes, realizamos ensaios de citoadesão em HLEC com Pv-EI em dois diferentes momentos: direto da circulação periférica

e pós-amadurecimento (18-24 h de cultivo). Como podemos observar na Figura 25, a citoadesão de Pv-EI amadurecidos *in vitro* em HLEC foi cerca de 4 vezes maior que a citoadesão de Pv-EI retirados da circulação de pacientes (em média 27,8 EI/mm^2 e 7,0 EI/mm^2, respectivamente).

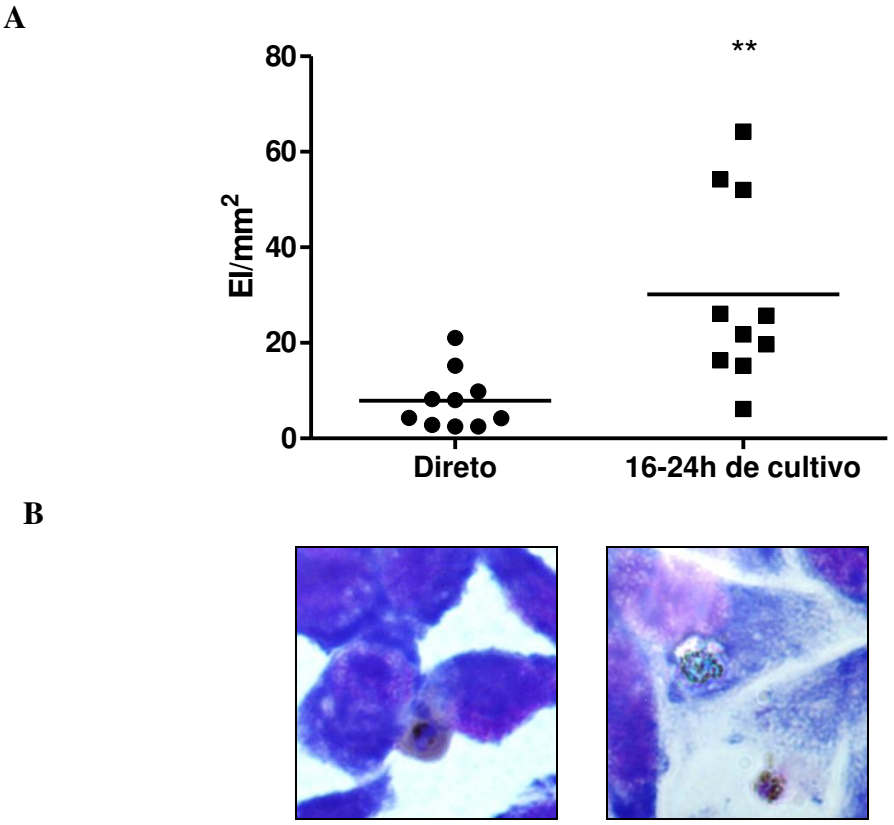

Figura 25: Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de células HLEC durante 1 hora a 37 °C. Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e Pv-EI aderidos foram contados. (A) Citoadesão expressa em EI/mm2 de Pv-EI em HLEC antes e pós amadurecimento. Cada ponto representa um isolado e o traço central a média dos 10 isolados testados. (B) Fotomicrografia da citoadesão de Pv-EI em HLEC antes e pós-amadurecimento.
** p<0,01 Citoadesão de Pv-EI direto *versus* Citoadesão de Pv-EI após 16-24h de cultivo (teste t pareado).

Uma vez que o amadurecimento propiciou o aumento da porcentagem de esquizontes e também o aumento na capacidade adesiva de Pv-EI, entendemos que os esquizontes de *P. vivax* obtidos no amadurecimento foram os responsáveis pela maior capacidade adesiva de Pv-EI. Neste sentido, estudos *in vivo* utilizando o *Plasmodium* murino *P. berghei* ANKA demonstraram que os esquizontes ficam sequestrados na microvasculatura tecidual do animal e mais, que esse processo é finamente regulado, uma vez que o sequestro só ocorre em parasitas que já iniciaram a divisão nuclear (Franke-Fayard et al., 2010). Neste trabalho, os animais foram infectados com esquizontes de *P. berghei* ANKA sincronizados *in vitro*, e logo após a infecção já era possível observar trofozoítos jovens na circulação periférica dos animais, no entanto após 18-22h horas da infecção não era possível encontrar parasitas circulantes, exatamente no momento que *in vitro* o parasita estava no estágio de esquizonte, levando a conclusão de que os parasitas estavam sequestrados e não mais circulantes quando neste estágio (Franke-Fayard et al., 2010).

Nossos dados sugerem que esquizontes são as formas responsáveis pela citoadesão, no entanto, em alguns isolados não encontramos esta relação. Neste sentido, em dois isolados (11-05 e 11-10), que mesmo após 24 horas de amadurecimento não houve aumento na porcentagem de esquizontes, a citoadesão também foi potencializada (Figura 26A, Tabela 4). E ainda em outros dois isolados (11-01 e 11-03), mesmo o amadurecimento aumentando a porcentagem de esquizontes, a citoadesão antes e pós amadurecimento foi similar (Figura 26B, Tabela 4).

Tabela 4: Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento. Quantidade de Pv-EI em esquizontes em porcentagem e citoadesão expressa em EI/mm2 antes e pós amadurecimento. Em cinza, pacientes nos quais não há relação entre o aumento da porcentagem de esquizontes e o aumento na citoadesão.

	Direto		16-24h de cultivo	
Isolados	**Esquizontes (%)**	**Citoadesão (EI/mm^2)**	**Esquizontes (%)**	**Citoadesão (EI/mm^2)**
11-01	1,3	21.0 ± 5.6	85,6	21.7 ± 11.9
11-02	1,5	15.2 ± 3.7	77,0	54.2 ± 5.5*
11-03	2,5	8.0 ± 2.1	78,5	15.2 ± 7.4
11-04	1,0	2.5 ± 1.0	39,9	25.7 ± 8.9*
11-05	0,0	4.2 ± 3.4	0,0	52.0 ± 11.3*
11-06	0,0	4.3 ± 1.1	50,0	64.2 ± 20.8*
11-09	42,6	9.9 ± 1.1	50,0	16.3 ± 0.7*
11-10	50,0	8.2 ± 2.0	11,0	19.7 ± 6.8*
11-11	2,3	2.5 ± 1.8	20,0	6.2 ± 2.2*
11-12	5,0	2.8 ± 1.3	77,0	26.1 ± 7.0*

* $p<0,05$ Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann Whitney*).

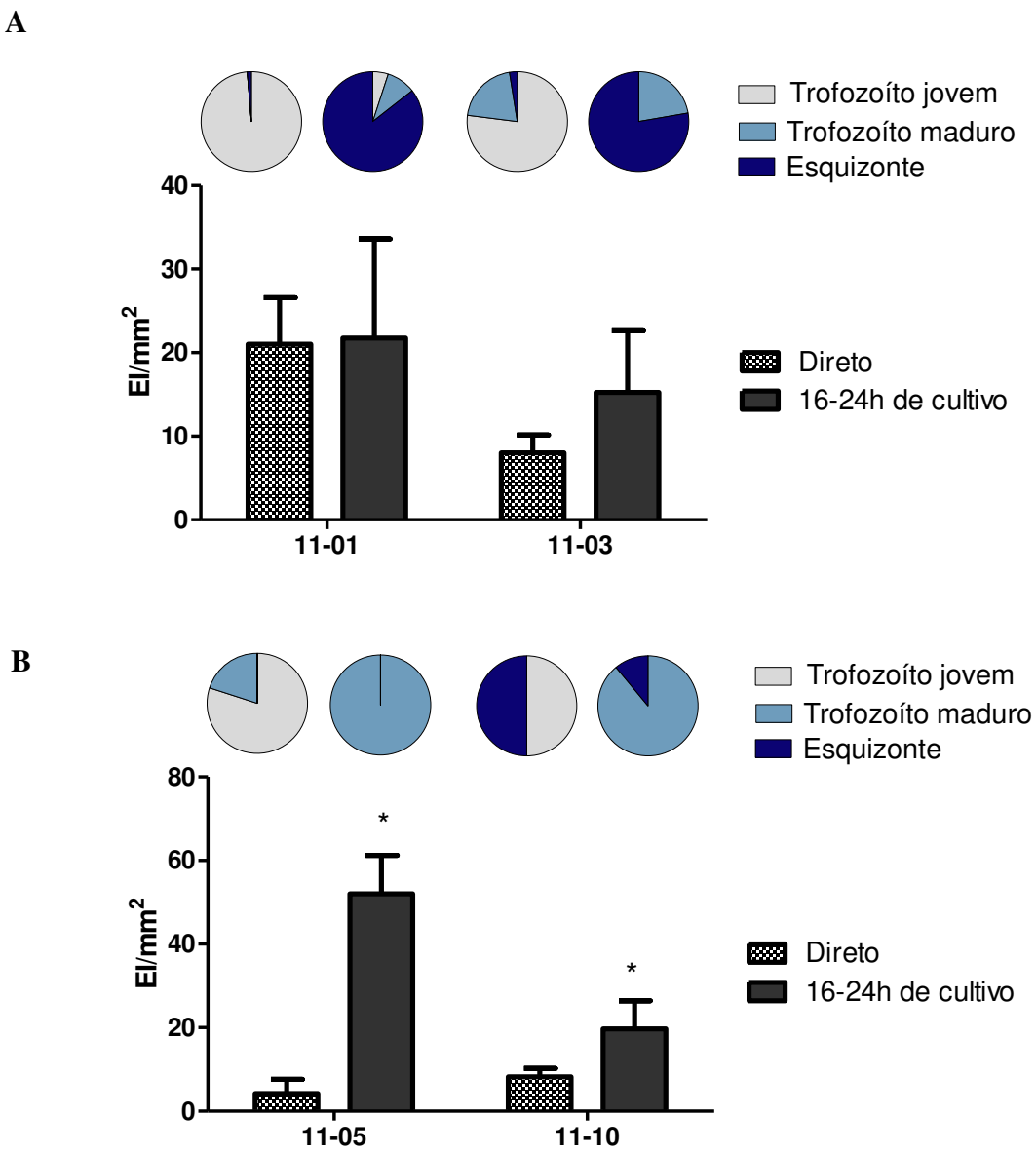

Figura 26: Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento, relacionando com os estágios de desenvolvimento dos isolados nos dois momentos. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de células HLEC durante 1 hora a 37 °C. Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão. (A) Citoadesão dos isolados 11-01 e 11-03 em HLEC. Nestes isolados, mesmo com o aumento da porcentagem de esquizontes pós-amadurecimento, a citoadesão foi não foi potencializada. (B) Citoadesão dos isolados 11-05 e 11-10 em HLEC. Nestes isolados a citoadesão foi potencializada pós-amadurecimento mesmo sem ter havido aumento na porcentagem de esquizontes.
* $p<0,05$ Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).

Assim nos questionamos, se algum outro fator propiciado pelo amadurecimento de Pv-EI *in vitro* seria o responsável pelo aumento na citoadesão. Neste sentido, sabemos que a presença de anticorpos contra os antígenos expressos na superfície de Pf-EI são capazes de bloquear e também reverter a citoadesão de Pf-EI a receptores endoteliais *in vitro* (Sharling et al., 2004). Como o amadurecimento poderia propiciar não só uma mudança no padrão de antígenos expressos na superfície de Pv-EI, mas também na presença de anticorpos capazes de bloquear os ligantes parasitários responsáveis pela adesão, investigamos o quanto a presença de anticorpos aderidos a Pv-EI poderia estar influenciando a capacidade adesiva desse parasita nos dois momentos avaliados: direto da circulação periférica e pós-amadurecimento (18-24 h de cultivo). Como podemos observar na Figura 27, a porcentagem de IgG aderidos a Pv-EI foi significativamente menor pós-amadurecimento (74,4% e 20,8%, antes e pós amadurecimento respectivamente). E, portanto, a menor quantidade de anticorpos aderidos a Pv-EI, além do aumento na porcentagem de esquizontes, pode ser responsável pela maior capacidade adesiva de Pv-EI amadurecido *in vitro*.

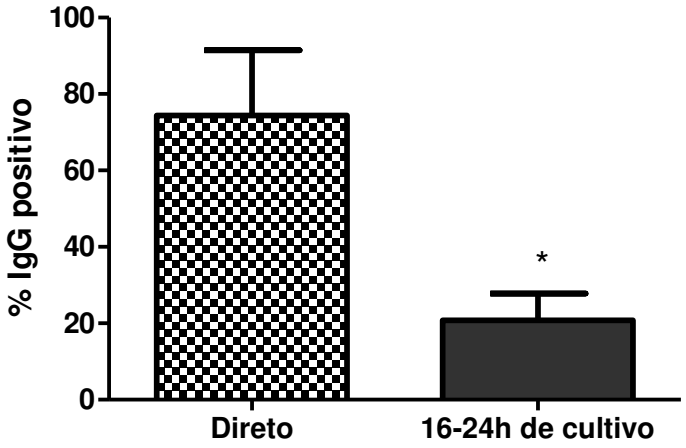

Figura 27: Porcentagem de IgG aderidos a Pv-EI antes e após cultivo de 16-24 horas para amadurecimento. Pv-EI obtidos pós Percoll ou pós-amadurecimento foram duplamente marcados com brometo de etídeo e anti-IgG conjugado com Alexa e por citometria de fluxo foi determinada a porcentagem de Pv-EI positivos para Alexa (com IgG aderidos). Resultados expressos são a média e desvio padrão de 3 diferentes isolados.
* p<0,05 Direto *versus* 16-24h de cultivo (teste U de *Mann-Whitney*).

No entanto, se existem anticorpos contra as proteínas expressas em Pv-EI antes de amadurecer circulantes no soro de pacientes, existem anticorpos circulantes contra as proteínas expressas nos estágios obtidos pós-amadurecimento *in vitro*. E assim, se IgG aderidas aos antígenos Pv-EI retiradas diretamente da circulação periférica estão realmente bloqueando a citoadesão destes a HLEC, ao incubarmos os parasitas pós-amadurecimento com soro provenientes do mesmo paciente que o isolado estaríamos aumentando a porcentagem de IgG aderidos a Pv-EI e consequentemente diminuindo a capacidade adesiva do mesmo. Então, realizamos novos ensaios de citoadesão em HLEC com Pv-EI nos dois momentos avaliados, direto da circulação periférica e pós-amadurecimento (16-24 h de cultivo), no entanto na presença de soro do próprio paciente (autólogo). Como podemos observar na Figura 28, a citoadesão de Pv-EI amadurecidos não foi reduzida na presença de soro autólogo, mas sim potencializada. Isto

nos leva a acreditar que não é a presença de IgG aderidas a Pv-EI que está bloqueando a citoadesão dos parasitas coletados diretamente da circulação.

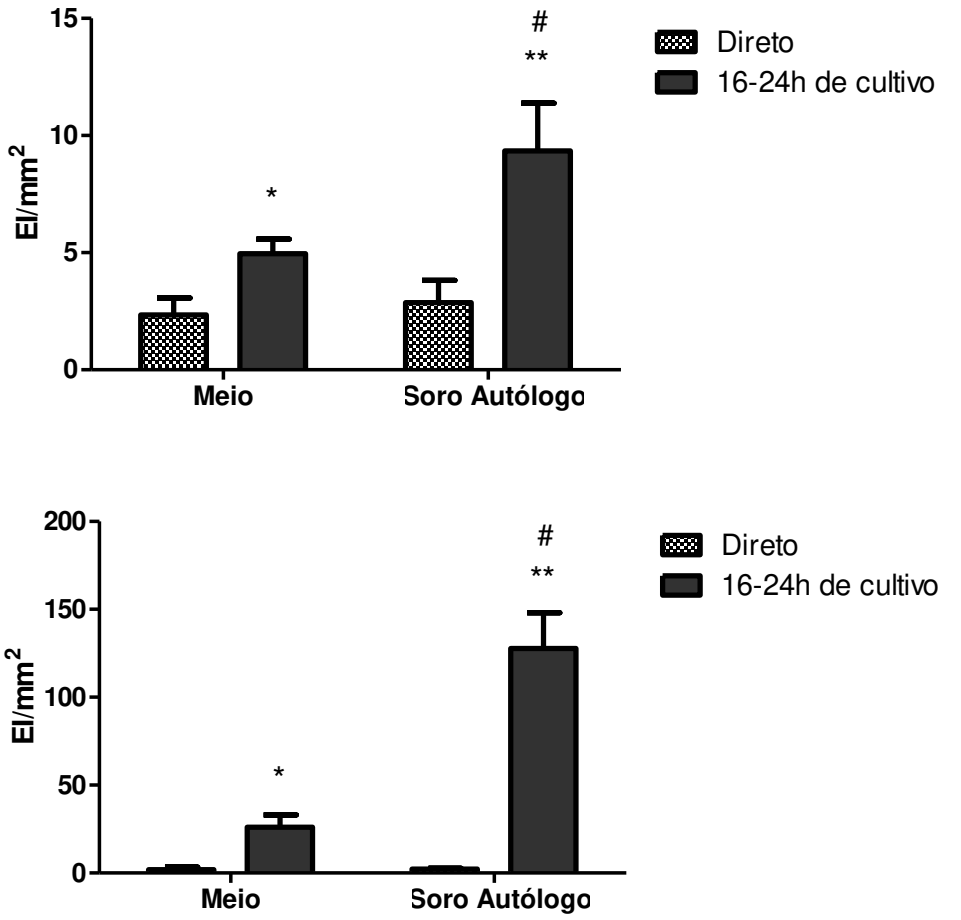

Figura 29: Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento, na presença ou não de soro autólogo. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de células HLEC durante 1 hora a 37 °C, na ausência, ou na presença de soro autólgo (1/50). Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão. (A) Citoadesão do isolado 11-11 em HLEC. (B) Citoadesão do isolado 11-12 em HLEC.
* p<0,05 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
** p<0,01 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
# p<0,05 Citoadesão após 16-24h de cultivo meio *versus* soro autólogo (teste t Mann-Whitney).

Portanto, a citoadesão de Pv-EI após o amadurecimento não foi potencializada pela ausência de anticorpos que bloqueassem esse fenômeno, mas sim pela presença de formas mais maduras nos isolados pós cultivo. Neste sentido, de acordo com o coeficiente de relação de *Spearman* observou-se que existe correlação significativa entre a porcentagem de esquizontes e a taxa de citoadesão em HLEC nos diferentes isolados em ambos os momentos avaliados (R_S = 0,47; p = 0,01; n = 20) (Figura 29).

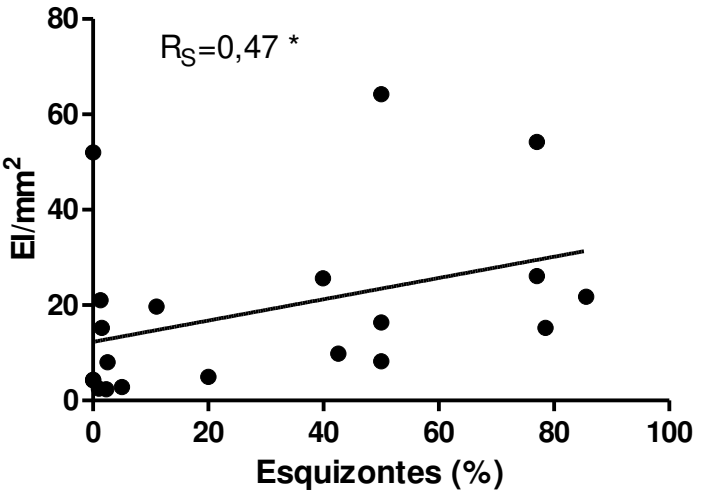

Figura 29: Correlação de Spearman entre porcentagem de esquizontes e citoadesão de Pv-EI em HLEC expressa em EI/mm^2.
* p<0,05 (Correlação de Spearman)

Assim, nossos dados indicam que o aumento na frequência de esquizontes de *P. vivax* está relacionado com o aumento na capacidade adesiva de Pv-EI, sugerindo que a presença de antígenos com expressão elevada ou exclusiva neste estágio sejam os responsáveis pela maior capacidade adesiva do mesmo. Neste sentido, estudo do transcriptoma de formas sanguíneas de *P. vivax*, que avaliou a expressão de RNA_m em diferentes estágios do parasita, mostrou que 52 genes *vir*, 13 *pvtrag* (família gênica associada à evasão do sistema imune) e outros 156 genes

hipotéticos tem sua expressão aumentada justamente durante a esquizogonia, diferente de *P. falciparum* no qual os genes de famílias antigênicas tem sua expressão silenciada em estágios mais maduros (Bozdech et al., 2008).

Além disso, o fato da citoadesão de Pv-EI amadurecidos ter sido potencializada pela presença de soro autólogo (Figura 28), nos leva ao fato da existência de algum mediador da citoadesão no soro desses pacientes. Neste sentido, ensaios *in vitro* com *P. falciparum* mostraram que a presença de TNF-α, LT-α, plaquetas e micropartículas de diferentes origens são capazes de potencializar a citoadesão (Schoefield & Grau, 2005). Qual o papel desses na citoadesão de *P. vivax* é incerto, mas nossos dados abrem perspectiva para estudos posteriores investigando o papel de cada um deles nesse fenômeno.

3. Receptores envolvidos na citoadesão de Pv-EI amadurecidos

Uma vez verificada a maior capacidade adesiva de Pv-EI amadurecido *in vitro*, provavelmente pela presença de estágios mais avançados do desenvolvimento de *P. vivax*, nos questionamos se a citoadesão de Pv-EI amadurecido *in vitro* poderia se dar em receptores diferentes daqueles observados no parasita coletado diretamente da circulação periférica do paciente.

Para isso, além de utilizarmos inibidores específicos ou competidor da citoadesão a alguns dos receptores presentes na HLEC (mAb contra CD36 e ICAM-1, CSA solúvel), utilizamos linhagens celulares expressando majoritariamente um dos receptores avaliados (CHO_{K1}, expressando CSA e CHO_{745} transfectadas com CD36 ou ICAM-1). A citoadesão na presença dos

inibidores ou competidor e nas células CHO foram realizados com Pv-EI antes e pós amadurecimento e a taxa de citoadesão foi quantificada e comparada nos dois momentos.

Como podemos observar na Figura 30, a CSA solúvel inibiu a citoadesão de Pv-EI após amadurecimento em HLEC nos três isolados testados, mas só foi capaz de inibir a citoadesão de Pv-EI circulantes na corrente periférica no isolado 11-10 (Figura 30C). Como podemos observar na Figura 30C, o paciente 11-10, diferente da maioria dos pacientes aqui analisados, apresentava elevada porcentagem de esquizontes circulantes na corrente sanguínea (50%). E, portanto, o fato da CSA solúvel ter competido com receptores nas células endoteliais e inibido a citoadesão deste isolado antes do amadurecimento deve-se, provavelmente, a essa elevada porcentagem de esquizontes. Em outro dos isolados testados (11-05), o cultivo de 16 a 24 horas não foi eficaz na obtenção de maior porcentagem de esquizontes, entretanto da mesma maneira que nos outros isolados a CSA solúvel inibiu significativamente a adesão de Pv-EI pós amadurecimento em HLEC (Figura 31B). Contudo, apesar do cultivo ter sido ineficaz na obtenção de esquizontes no isolado 11-05, os parasitas amadureceram durante a incubação e passaram do estágio de trofozoíto jovem para trofozoíto maduro. Portanto, a CSA solúvel foi capaz de inibir não só a citoadesão de esquizontes de Pv-EI (Figura 30), mas também trofozoítas maduros (Figura 30B e 30C).

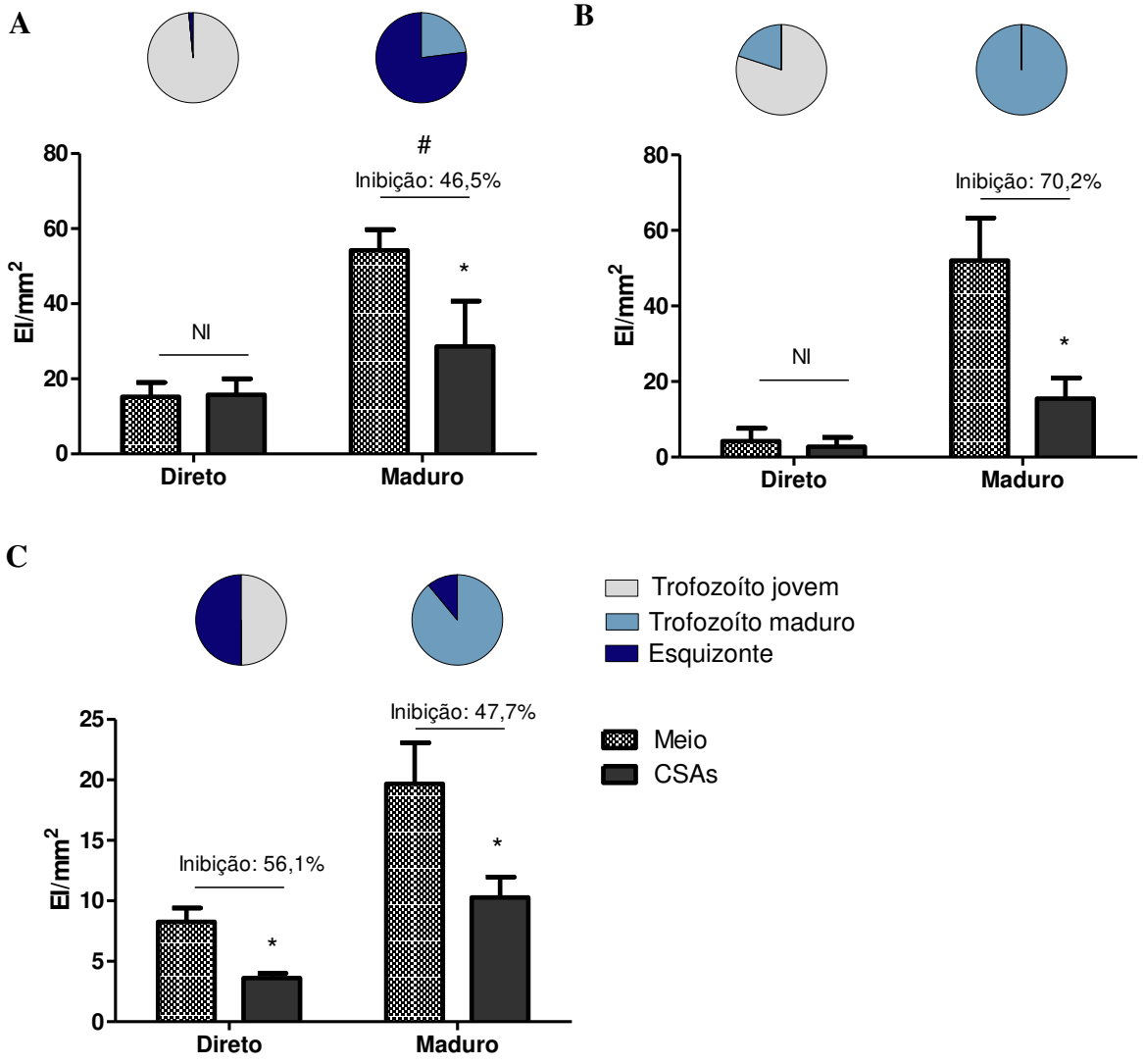

Figura 30: Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento, na presença ou não de CSA solúvel (CSAs). 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de células HLEC durante 1 hora a 37°C, na ausência, ou na presença de CSAs (100µg/mL). Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão. (A) Citoadesão do isolado 11-02 em HLEC. (B) Citoadesão do isolado 11-05. (C) Citoadesão do isolado 11-10..
NI: Não inibiu.
* p<0,05 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
# p<0,05 Inibição antes de amadurecer *versus* inibição pós-amadurecimento (teste U de *Mann-Whitney*).

A citoadesão ao receptor CSA também foi avaliada através de ensaios de adesão de PvEI a CHO_{K1}, célula que expressa CSA e não expressa CD36 e ICAM-1. No isolado 11-08, a citoadesão a CHO_{K1} foi similar antes e pós o cultivo por 16-24 horas *in vitro* (Figura 31). O isolado 11-08 já apresentava 100% das formas maduras antes do amadurecimento (92,3% trofozoítas maduros e 7,6% esquizontes). E, portanto o fato da citoadesão não ter sido diferente nos dois momentos avaliados deve-se a presença de formas maduras antes e pós-amadurecimento. Isso corrobora com os dados encontrados nos ensaios com a CSA solúvel, nos quais o receptor CSA parece estar envolvido na citoadesão tanto de trofozoítos maduros quanto de esquizontes. Entretanto, no isolado 11-07 a citoadesão de Pv-EI a CHO_{K1} foi menor pós amadurecimento *in vitro* que a citoadesão de Pv-EI retirados diretamente da circulação, mesmo tendo atingido 100% de formas maduras (trofozoítas e esquizontes) depois do amadurecimento, em comparação a somente 30% (trofozoítas maduros) antes do cultivo (Figura 31). Isso contrapõe os dados obtidos anteriormente, que mostraram que a adesão ao receptor CSA se dá por formas maduras de *P.vivax.* Uma explicação para esses dados seria a existência de outros receptores existentes nas células CHO_{K1} que possam estar envolvidos na citoadesão de formas jovens de Pv-EI. Outra possibilidade é a da CSA solúvel estar atuando, não só competindo com o receptor CSA de HLEC, mas também inibindo a adesão a outros receptores através de ligações eletrostáticas (Costa et al., 2011). Neste sentido, trabalho anterior do nosso grupo mostrou que a CSAs foi capaz de inbir a citoadesão de PvEI, enquanto o tratamento das células com condroitinase ABC, enzima que cliva as condrotinas A,B e C, não inibiu (Carvalho et al., 2010), sugerindo que a CSA solúvel esteja inibindo a adesão a outros receptores que não a CSA.

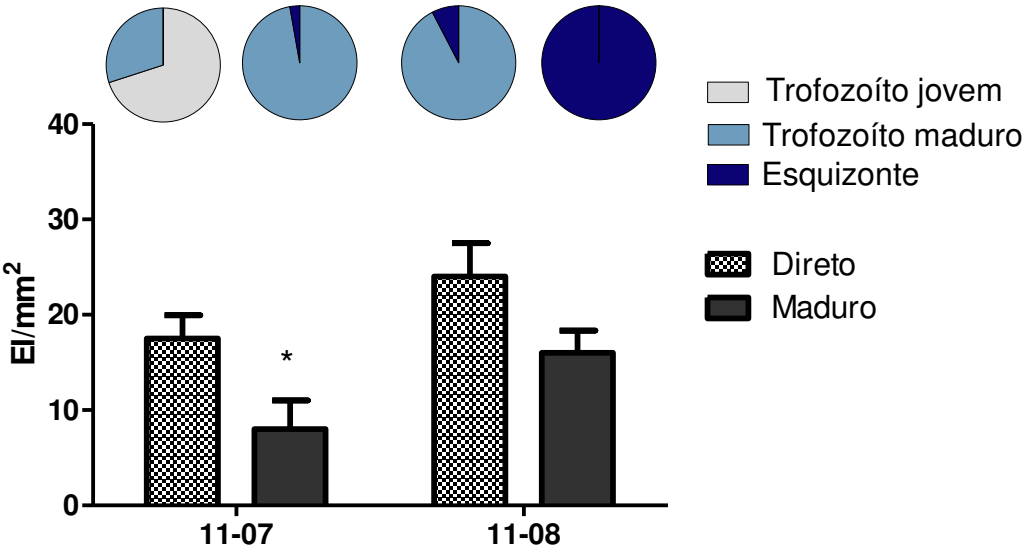

Figura 31: Citoadesão de Pv-EI em CHO_{K1} antes e após cultivo de 16-24 horas para amadurecimento. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de CHO_{K1} durante 1 hora a 37 °C. Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e os Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão.
* $p<0,05$ Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).

A capacidade adesiva de PvEI, antes e pós-amadurecimento, ao receptor CD36 das células HLEC foi investigada utilizando mAb contra CD36 para bloquear a citoadesão a esse receptor. Como podemos observar na Figura 32, o mAb CD36 foi capaz de inibir a citoadesão de PvEI somente pós o amadurecimento *in vitro* nos dois isolados testados, sugerindo que as formas maduras obtidas no cultivo são capazes de aderir a esse receptor, enquanto as formas mais jovens não. A inibição da citoadesão por mAb CD36 foi de 70,5% no isolado 11-02, que apresentava 77% dos PvEI em esquizonte e de 47,1% no isolado 11-05, que apresentava 100% dos PvEI em trofozoíta maduro.

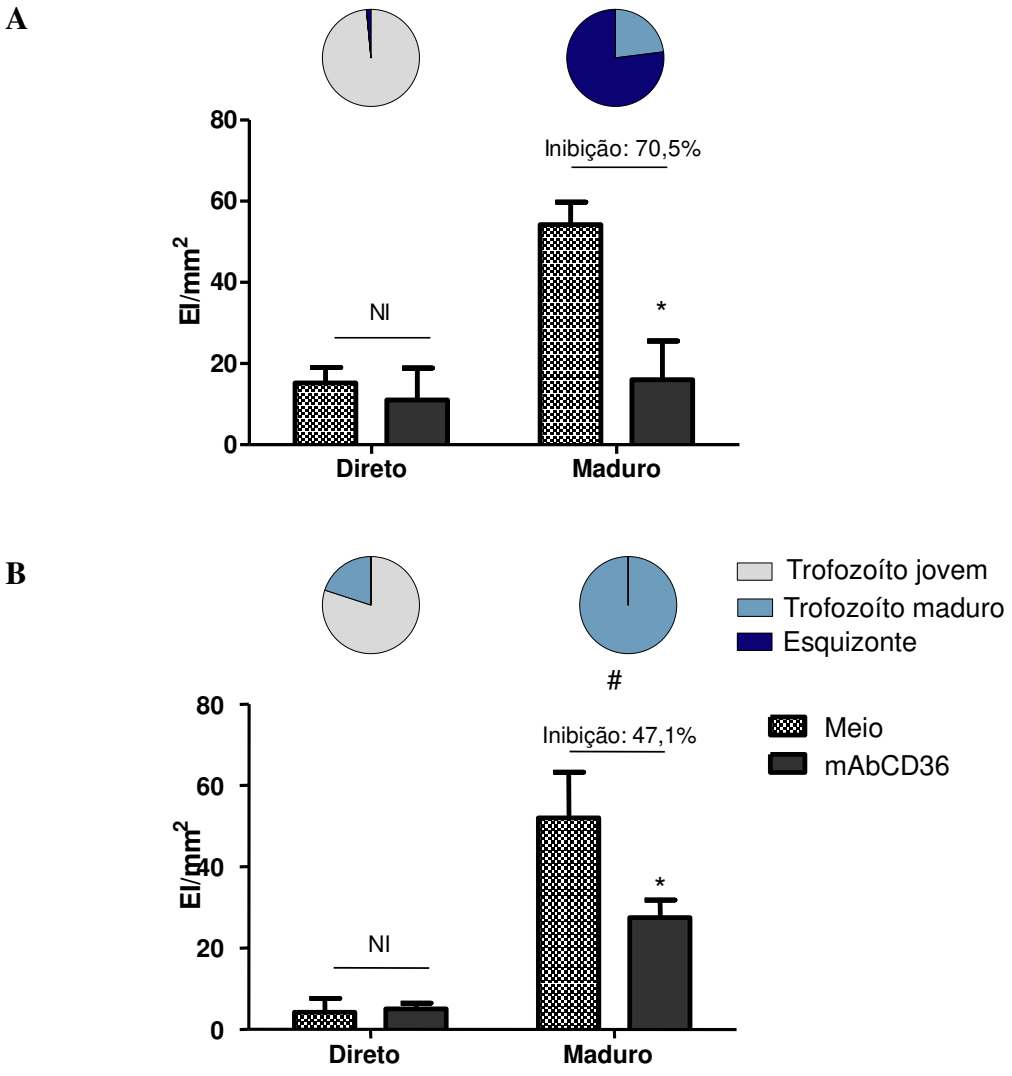

Figura 32: Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento, na presença ou não de mAb CD36. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de células HLEC durante 1 hora a 37 °C, na ausência, ou na presença de mAb CD36 (10µg/mL). Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão. (A) Citoadesão do isolado 11-02 em HLEC. (B) Citoadesão do isolado 11-05.
NI: Não inibiu.
* p<0,05 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
# p<0,05 Inibição antes de amadurecer *versus* inibição pós-amadurecimento (teste U de *Mann-Whitney*).

Nos ensaios utilizando células CHO transfectadas com CD36 (CHO_{CD36}), em um dos isolados testados (11-08) a capacidade adesiva de Pv-EI após 16 a 24 horas de cultivo *in vitro* foi 4 vezes maior que do que antes do cultivo (Figura 33). Importante ressaltar que neste isolado, o amadurecimento resultou em 100% de esquizontes contra 7,6% antes do amadurecimento, sugerindo que a maior capacidade adesiva se deva ao desenvolvimento de esquizontes pós-cultivo (Figura 33). No outro isolado (11-07) a capacidade adesiva de Pv-EI a CHO_{CD36} antes e pós-amadurecimento foi similar (Figura 33) e o amadurecimento resultou em 100% de formas maduras, mas uma baixa porcentagem de esquizontes (2,5%).

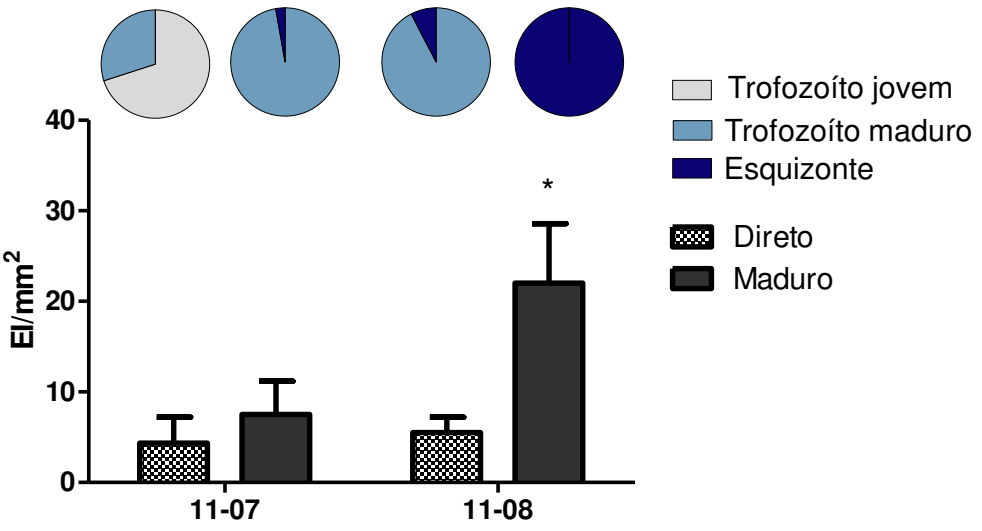

Figura 33: Citoadesão de Pv-EI em CHO_{CD36} antes e após cultivo de 16-24 horas para amadurecimento. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de CHO_{CD36} durante 1 hora a 37 °C. Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e os Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão.
* $p<0,05$ Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).

Estes dados somados sugerem fortemente que as formas maduras de *P. vivax* apresentam capacidade adesiva a CD36, e mais que os esquizontes parecem ser o principal envolvido na adesão a esse receptor. Esses dados corroboram com os achados em *P. berghei* ANKA que mostram o envolvimento desse receptor no sequestro de esquizontes nos pulmões e tecido adiposo dos animais, uma vez que animais deficientes na expressão de CD36 apresentaram redução na quantidade de parasitas aderidos nesses tecidos em relação a animais que expressavam o receptor (Franke-Fayard et al., 2005). Ainda, o fato das complicações em pacientes com malária vivax serem muitas vezes por manifestações pulmonares, do receptor CD36 estar relacionado à citoadesão de esquizontes de parasitas murinos nesse órgão e da capacidade adesiva de esquizontes de Pv-EI nesse receptor sugerem que o CD36 pode estar envolvido na imunopatogênese da malária grave por *P. vivax*.

Ao avaliar a capacidade adesiva de Pv-EI ao receptor ICAM-1 das células HLEC, verificamos que o mAb ICAM-1 foi capaz de inibir a adesão dos isolados que apresentavam maior porcentagem de Pv-EI em esquizonte, 11-02 pós-amadurecimento (77% de esquizontes, inibição de 44,3%) e 11-10 antes de amadurecer (50% de esquizontes, inibição de 42,1%), mas não dos isolados com baixa porcentagem deste estágio, 11-02 antes do amadurecimento (1,5% de esquizontes) e 11-10 pós-amadurecimento (11% de esquizontes) (Figura 34). Estes dados sugerem que assim como CD36, o receptor ICAM-1 estaria envolvido pricipalmente na citoadesão de esquizontes de Pv-EI.

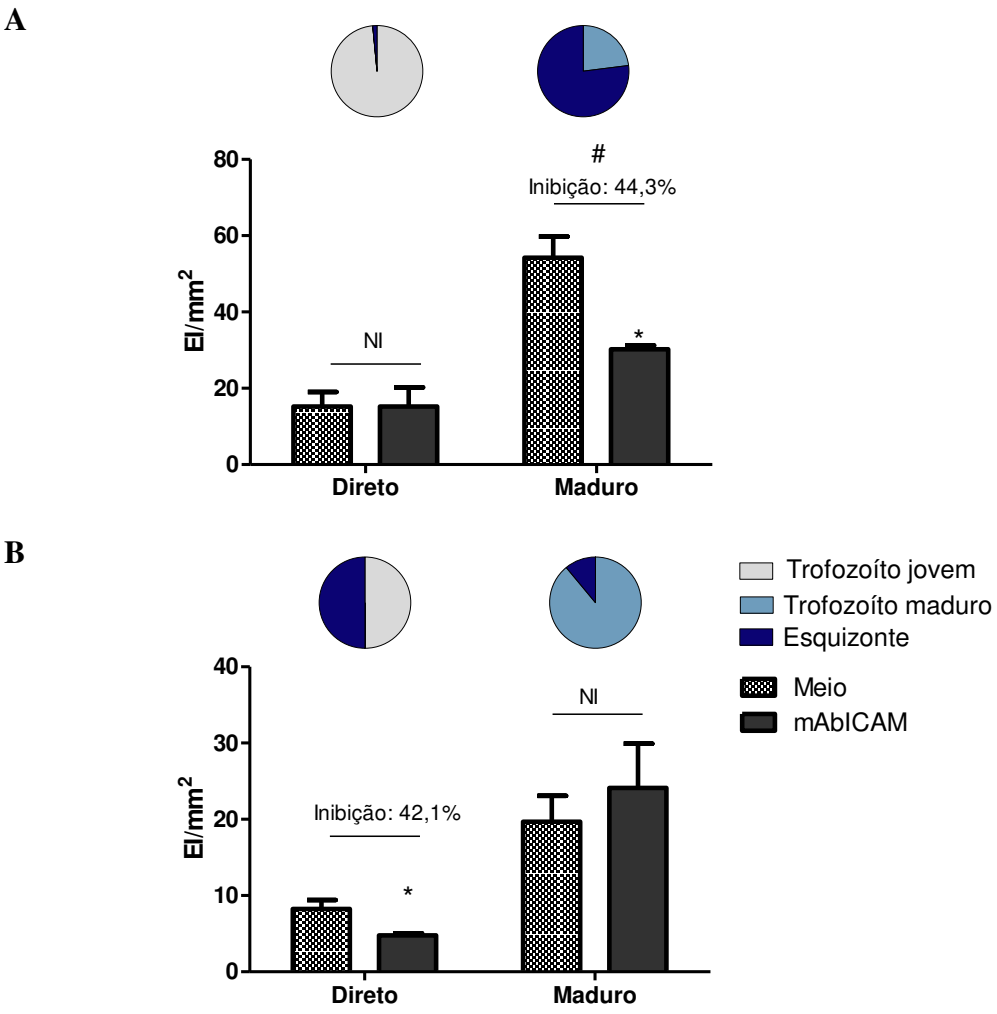

Figura 34. Citoadesão de Pv-EI em HLEC antes e após cultivo de 16-24 horas para amadurecimento, na presença ou não de mAb ICAM. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de células HLEC durante 1 hora a 37 °C, na ausência, ou na presença de mAb ICAM (10µg/mL). Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão. (A) Citoadesão do isolado 11-02 em HLEC. (B) Citoadesão do isolado 11-10 em HLEC.
NI: Não inibiu.
* p<0,05 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
# p<0,05 Inibição antes de amadurecer *versus* inibição pós-amadurecimento (teste U de *Mann-Whitney*).

Quando realizados ensaios de citoadesão de Pv-EI em células CHO_{ICAM}, a citoadesão não foi potencializada pós-amadurecimento nos dois isolados testados (Figura 35). Esses dados sugerem que todos os estágios de Pv-EI possuem capacidade de aderir a ICAM-1. Neste sentido, trabalho anterior do nosso grupo mostrou que Pv-EI coletados diretamente da corrente periférica de pacientes, ou seja com baixa proporção de esquizontes em relação a outros estágios do parasita, apresentava cerca de 2 vezes mais capacidade de aderir a CHO_{ICAM} que a CHO_{CD36} (Carvalho et al., 2010).

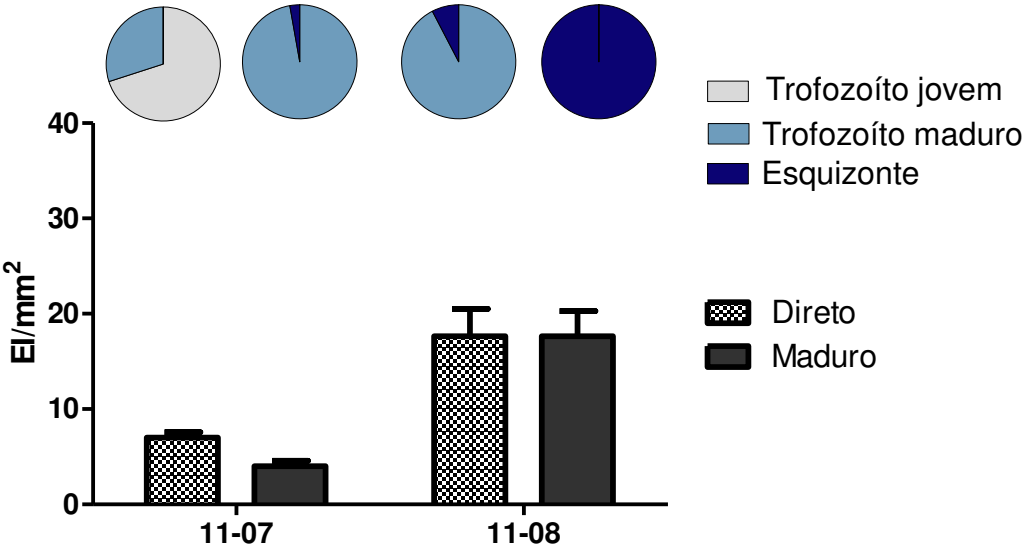

Figura 35: Citoadesão de Pv-EI em CHO_{ICAM} antes e após cultivo de 16-24 horas para amadurecimento. 5 x 10^4/poço Pv-EI amadurecidos ou não foram deixados aderir a monocamada de CHO_{ICAM} durante 1 hora a 37 °C. Após este período, os eritrócitos não aderidos foram removidos por meio de extensivas lavagens e os Pv-EI aderidos foram contados. Resultados são expressos como a média de 4 poços ± desvio padrão.

Esta informação somada aos dados aqui obtidos indicam que o ICAM-1 é receptor na citoadesão de Pv-EI inclusive em estágios mais jovens do desenvolvimento; já o receptor CD36

também está envolvido na citoadesão de Pv-EI, entretanto essa capacidade adesiva parece ser restrita as formas maduras de *P. vivax*, em especial, os esquizontes. Já o envolvimento do receptor CSA necessita de mais investigações, uma vez que a célula CHO_{K1} apresenta outros receptores que possam estar envolvidos na citoadesão de Pv-EI e a CSA solúvel parece não ser um competidor específico da ligação a esse receptor (Carvalho et al., 2010; Costa et al., 2011).

4. Formação de Rosetas

Trabalhos anteriores já descreveram a capacidade de Pv-EI em formar rosetas (Udomsangpetch et al., 1995; Chotivanich et al., 1998; Russell et al., 2011), mas nenhum deles avaliou os estágios ou outros fatores envolvidos nesse fenômeno. Neste estudo, a formação de rosetas por Pv-EI foi avaliada em 20 diferentes isolados antes e pós-amadurecimento *in vitro*, na presença de soro AB comercial (*Serum AB*, Sigma, USA) ou na presença de soro do próprio paciente (autólogo). Como podemos observar na Figura 36, a formação de rosetas por Pv-EI também foi potencializada pelo amadurecimento *in vitro* do parasita, tanto na presença de soro comercial (0,3% para 6,0%, antes e pós-amadurecimento, respectivamente) quanto na presença de soro autólogo (5,3% para 43,6%, antes e pós-amadurecimento, respectivamente). Além disso, a porcentagem de parasitas em rosetas foi significativamente maior na presença de soro autólogo, tanto antes de amadurecer (0,3% para 5,3%, soro AB e soro autólogo, respectivamente), quanto depois do amadurecimento (6,0% para 43,6%, soro AB e soro autólogo, respectivamente) (Figura 36).

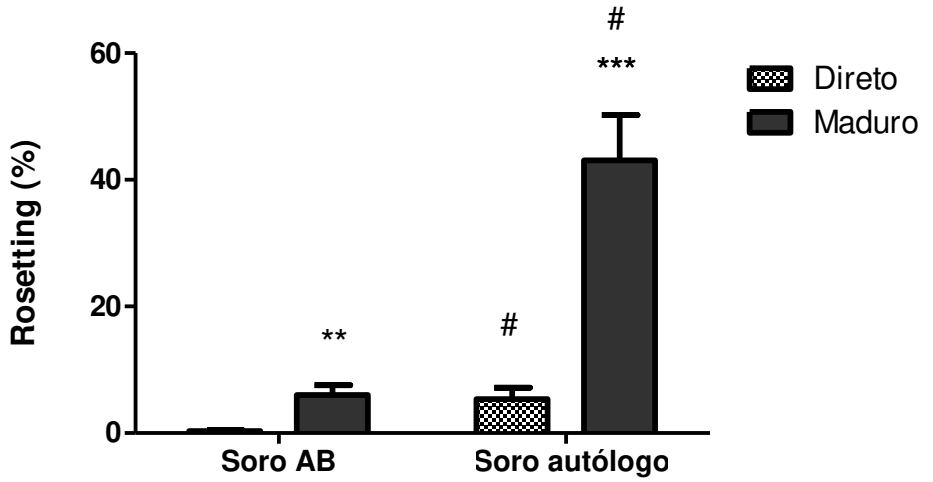

Figura 36: Formação de rosetas por Pv-EI antes e após cultivo de 16-24 horas para amadurecimento, na presença de soro AB ou soro autólogo. Média e desvio padrão da porcentagem de rosetas formada em 20 isolados diferentes.
** p<0,01 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
*** p<0,001 Citoadesão direto *versus* Citoadesão após 16-24h de cultivo (teste U de *Mann-Whitney*).
# p<0,05 Citoadesão meio *versus* soro autólogo (teste U de *Mann-Whitney*).

Sabe-se que a formação de rosetas em *P. falciparum* também é mediada por PfEMP-1. Neste sentido, diferente da citoadesão na placenta que sabidamente é mediada por uma única variante de PfEMP-1 codificada por *var*$_2$*CSA*, uma gama de genes *var* já foram identificados na formação de rosetas em *P. falciparum* (Mercereau-Puijalon et al., 2008). E mais, alguns dos receptores celulares envolvidos na formação de rosetas, como CD36, CSA e PECAM, também estão envolvidos na citoadesão ao endotélio (Mercereau-Puijalon et al., 2008). Sendo assim, é possível que os ligantes parasitários e os receptores celulares envolvidos na formação de rosetas e na citoadesão ao endotélio sejam compartilhados. Portanto, como a citoadesão de Pv-EI a receptores endoteliais *in vitro* foi aumentada com o amadurecimento do parasita, era esperado que a formação de rosetas também fosse potencializada. Assim, o aumento da formação de

rosetas por Pv-EI após o amadurecimento, provavelmente, deve-se também ao aumento na proporção de formas maduras, inclusive esquizontes, após o cultivo.

A maior capacidade em formar rosetas de Pv-EI na presença de soro do próprio paciente nos leva ao fato da existência de algum mediador do fenômeno nesse soro. Sabe-se que no soro existem alguns componentes essenciais para a formação de rosetas em *P. falciparum.* Recentemente, um trabalho identificou dois desses componentes: o fator D do complemento e albumina (Luginbuhl et al., 2007). Alguns trabalhos também consideram essencial a presença de IgG e/ou IgM obtidas em soros não-imunes a malária (Clough et al., 1998; Fernandez et al., 1998). Entretanto, não sabemos que fator está presente no soro dos pacientes e não no soro AB comercial que tenha propiciado a maior quantidade de rosetas. Talvez o aumento na formação de rosetas na presença de soro autólogo não se deva a presença de uma única molécula, mas sim de uma combinação de diferentes componentes, e estudos mais direcionados são necessários para identificar o que está envolvido na formação de rosetas por Pv-EI.

Por fim, avaliamos também o papel do sistema sanguíneo ABO na formação de rosetas, uma vez que em *P. falciparum* alguns trabalhos indicam que pacientes com o tipo sanguíneo A e B apresentem mais rosetas que pacientes com sangue tipo O (Mercereau-Puijalon et al., 2008). Como podemos observar na Figura 37, não houve diferença significativa na formação de rosetas em nenhum dos quatro tipos sanguíneos ABO nas diferentes condições testadas: antes e pós-amadurecimento e na presença de soro autólogo ou soro AB.

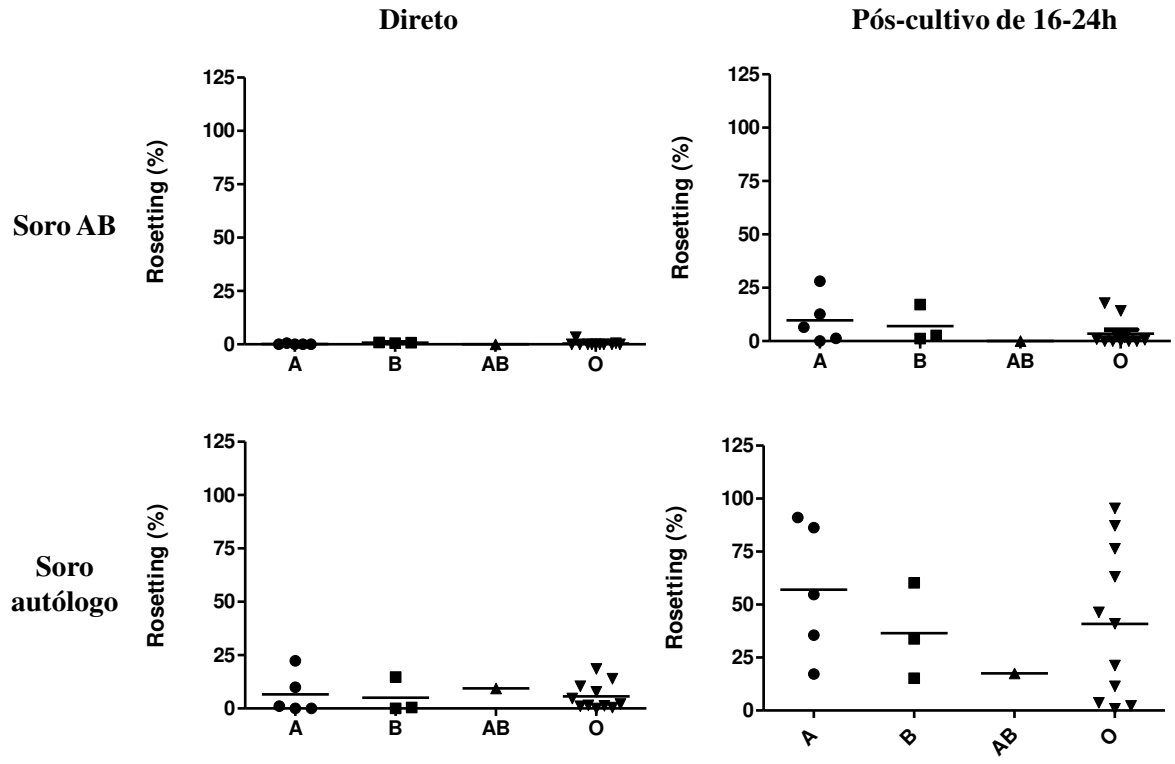

Figura 37. Formação de rosetas em Pv-EI e sistema sanguíneo ABO. Envolvimento do sistema sanguíneo ABO na formação de rosetas por Pv-EI nos 20 isolados testados nas diferentes condições: antes e pós-amadurecimento e na presença de soro AB ou soro autólogo.

Embora, neste trabalho, não tenhamos elucidado os fatores envolvidos na formação de rosetas em *P. vivax*, os ensaios *in vitro* conduzidos na presença de soro autólogo são mais próximos ao que ocorre na circulação dos pacientes *in vivo* do que na presença de um soro comercial (sem os mediadores produzidos na infecção malárica), e assim a elevada proporção de rosetas formadas pelo parasita amadurecido na presença desse soro (43,6%) nos leva a acreditar que esse fenômeno possa ter algum papel na patogênese desse parasita.

Conclusões

Com os dados apresentados neste capítulo, podemos concluir que:

1. Em infecções por *P. vivax*, a proporção de parasitas circulantes no estágio de esquizontes é mais baixa que em outros estágios (trofozoítas jovens e trofozoítas maduros).
2. O cultivo *in vitro* de Pv-EI por período de 16 a 24 horas permite o desenvolvimento do parasita e culmina com o aumento na proporção de formas mais maduras do parasita, principalmente esquizontes.
3. A citoadesão de Pv-EI em HLEC foi maior nos parasitas que foram cultivados *in vitro* por 16 a 24 horas que nos parasitas retirados diretamente da circulação periférica dos pacientes.
4. A porcentagem de IgG aderidos ao eritrócito infectado é maior em Pv-EI retirados diretamente da circulação periférica de pacientes que em Pv-EI cultivados *in vitro* por 16 a 24 horas.
5. A taxa de citoadesão de Pv-EI em HLEC está correlacionada positivamente com a porcentagem de Pv-EI no estágio de esquizonte.
6. A citoadesão de Pv-EI em HLEC foi potencializada na presença de soro autólogo durante o ensaio, tanto nos parasitas retirados diretamente da circulação periférica de pacientes, quanto nos parasitas cultivados *in vitro* por 16 a 24 horas.
7. A CSA solúvel foi capaz de inibir a citoadesão de Pv-EI em HLEC antes (1 entre 3 isolados) e após o cultivo *in vitro* (3 de 3 isolados). A inibição por CSA solúvel só se deu quando 50% ou mais dos parasitas estavam em estágios maduros (trofozoítas maduros e/ou esquizontes).
8. A citoadesão de Pv-EI em CHO_{K1} foi menor após o amadurecimento em 1 de 2 isolados testados, mas não foi possível relacionar a citoadesão a CHO_{K1} com o amadurecimento *in vitro* e/ou estágio de desenvolvimento parasitário.

9. O mAb CD36 inibiu a citoadesão de Pv-EI em HLEC nos dois isolados testados após o cultivo *in vitro*, mas não antes do cultivo, sugerindo que o amadurecimento *in vitro* propiciou a adesão a esse receptor.
10. A citoadesão em CHO_{CD36} foi maior após o cultivo *in vitro* de 16 a 24 horas no isolado 11-08 e isso se deve, provavelmente, ao aumento na proporção de esquizontes.
11. O mAb ICAM inibiu a citoadesão de Pv-EI em HLEC antes (1 entre 2 isolados) e após o cultivo *in vitro* (1 entre 2 isolados testados). Entretanto em ambos os isolados nos quais houve inibição pelo mAb ICAM a porcentagem de esquizontes era elevada (maior que 50%), sugerindo que esse estágio seja responsável pela citoadesão a esse receptor.
12. A citoadesão em CHO_{ICAM} foi similar antes e após o cultivo *in vitro* de 16 a 24 horas nos dois isolados testados e, portanto não foi possível relacionar a citoadesão CHO_{ICAM} com o amadurecimento *in vitro* e/ou estágio de desenvolvimento parasitário.
13. A formação de rosetas por Pv-EI foi maior nos parasitas cultivados *in vitro* por 16 a 24 horas, provavelmente devido a obtenção de formas mais maduras do desenvolvimento de *P. vivax*.
14. A formação de rosetas por Pv-EI foi potencializada na presença de soro autólogo, , tanto nos parasitas retirados diretamente da circulação periférica de pacientes, quanto nos parasitas cultivados *in vitro* por 16 a 24 horas.
15. A formação de rosetas por Pv-EI não foi diferente nos diferentes grupos sanguíneos do Sistema ABO.

Referências Bibliográficas

Alexandre, M.A., et al., Severe Plasmodium vivax malaria, Brazilian Amazon. Emerg Infect Dis, 2010. 16(10): p. 1611-4.

Amino, R., et al., Imaging malaria sporozoites in the dermis of the mammalian host. Nat Protoc, 2007. 2(7): p. 1705-12.

Amino, R., et al., Quantitative imaging of Plasmodium sporozoites in the mammalian host. C R Biol, 2006. 329(11): p. 858-62.

Andrade, B.B., et al., Severe Plasmodium vivax malaria exhibits marked inflammatory imbalance. Malar J, 2010. 9: p. 13.

Andrews, K.T., et al., Adherence of Plasmodium falciparum infected erythrocytes to CHO-745 cells and inhibition of binding by protein A in the presence of human serum. Int J Parasitol, 2005. 35(10): p. 1127-34.

Andrighetti-Frohner, C.R., et al., Cytotoxicity and potential antiviral evaluation of violacein produced by Chromobacterium violaceum. Mem Inst Oswaldo Cruz, 2003. 98(6): p. 843-8.

Andrighetti-Frohner, C.R., et al., In vitro testing for genotoxicity of violacein assessed by Comet and Micronucleus assays. Mutat Res, 2006. 603(1): p. 97-103.

Angus, B.J., et al., Short report: Rosette formation in Plasmodium ovale infection. Am J Trop Med Hyg, 1996. 55(5): p. 560-1.

Anstey, N.M., et al., Lung injury in vivax malaria: pathophysiological evidence for pulmonary vascular sequestration and posttreatment alveolar-capillary inflammation. J Infect Dis, 2007. 195(4): p. 589-96.

Anstey, N.M., et al., The pathophysiology of vivax malaria. Trends Parasitol, 2009. 25(5): p. 220-7.

Ashley, A.W. and White N.J. Antimalarial Agents, in Antimicrobial Pharmacodynamics in Theory and Clinical Practice, Niightingale C.H.; Ambrose P.G.; Dursano G.L.; Murakawa T., Editor 2007, Healthcare Informa.

Fernandez, V., Sorbitol-synchronization of Plasmodium falciparum-infected erythrocytes., in Methods in malaria research, I.P. Ljungström, H.; Schlichtherle, M.; Scherf, A.; Wahlgren M. , Editor 2004, MR4/ATCC, Manassas, VA.

Baird, J.K., et al., Diagnosis of resistance to chloroquine by Plasmodium vivax: timing of recurrence and whole blood chloroquine levels. Am J Trop Med Hyg, 1997. 56(6): p. 621-6.

Baird, J.K., et al., In vivo resistance to chloroquine by Plasmodium vivax and Plasmodium falciparum at Nabire, Irian Jaya, Indonesia. Am J Trop Med Hyg, 1997. 56(6): p. 627-31.

Baird, J.K., et al., Treatment of chloroquine-resistant Plasmodium vivax with chloroquine and primaquine or halofantrine. J Infect Dis, 1995. 171(6): p. 1678-82.

Baird, J.K., Neglect of Plasmodium vivax malaria. Trends Parasitol, 2007. 23(11): p. 533-9.

Baird, J.K., Severe and fatal vivax malaria challenges 'benign tertian malaria' dogma. Ann Trop Paediatr, 2009. 29(4): p. 251-2.

Ballantine, J.A., et al., The Chemistry of Bacteria .8. The Synthesis of Violacein and Related Compounds. Journal of the Chemical Society, 1960(May): p. 2292-2299.

Barcus, M.J., et al., Demographic risk factors for severe and fatal vivax and falciparum malaria among hospital admissions in northeastern Indonesian Papua. Am J Trop Med Hyg, 2007. 77(5): p. 984-91.

Barreto, E.S., et al., Diversity in antifungal activity of strains of Chromobacterium violaceum from the Brazilian Amazon. J Ind Microbiol Biotechnol, 2008. 35(7): p. 783-90.

Baruch, D.I., Adhesive receptors on malaria-parasitized red cells. Baillieres Best Pract Res Clin Haematol, 1999. 12(4): p. 747-61.

Basco, L.K., et al., Plasmodium falciparum and Plasmodium vivax: lactate dehydrogenase activity and its application for in vitro drug susceptibility assay. Exp Parasitol, 1995. 80(2): p. 260-71.

Bassat, Q. and P.L. Alonso, Defying malaria: Fathoming severe Plasmodium vivax disease. Nat Med, 2011. 17(1): p. 48-9.

Beeson, J.G. and G.V. Brown, Pathogenesis of Plasmodium falciparum malaria: the roles of parasite adhesion and antigenic variation. Cell Mol Life Sci, 2002. 59(2): p. 258-71.

Beeson, J.G. and P.E. Duffy, The immunology and pathogenesis of malaria during pregnancy. Curr Top Microbiol Immunol, 2005. 297: p. 187-227.

Berendt, A.R., D.J. Ferguson, and C.I. Newbold, Sequestration in Plasmodium falciparum malaria: sticky cells and sticky problems. Parasitol Today, 1990. 6(8): p. 247-54.

Berendt, A.R., et al., Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. Nature, 1989. 341(6237): p. 57-9.

Berendt, A.R., G.D. Tumer, and C.I. Newbold, Cerebral malaria: the sequestration hypothesis. Parasitol Today, 1994. 10(10): p. 412-4.

Bernabeu, M., et al., Functional analysis of Plasmodium vivax VIR proteins reveals different subcellular localizations and cytoadherence to the ICAM-1 endothelial receptor. Cell Microbiol, 2011.

Bozdech, Z., et al., The transcriptome of Plasmodium vivax reveals divergence and diversity of transcriptional regulation in malaria parasites. Proc Natl Acad Sci U S A, 2008. 105(42): p. 16290-5.

Brabin, B.J., et al., The sick placenta-the role of malaria. Placenta, 2004. 25(5): p. 359-78.

Brazilian National Genome Project, C., The complete genome sequence of Chromobacterium violaceum reveals remarkable and exploitable bacterial adaptability. Proc Natl Acad Sci USA, 2003. 100(20): p. 11660-5.

Bromberg, N. and N. Duran, Violacein biotransformation by basidiomycetes and bacteria. Lett Appl Microbiol, 2001. 33(4): p. 316-9.

Bromberg, N., Biotransformação da Violaceína, in Insituto de Química2000, Unicamp.

Bromberg, N., et al., Growth inhibition and pro-apoptotic activity of violacein in Ehrlich ascites tumor. Chem Biol Interact, 2010. 186(1): p. 43-52.

Bromberg, N., et al., Violacein cytotoxicity on human blood lymphocytes and effect on phosphatases. J Enzyme Inhib Med Chem, 2005. 20(5): p. 449-54.

Campbell, C.C., et al., Chloroquine-resistant Plasmodium falciparum from East Africa: cultivation and drug sensitivity of the Tanzanian I/CDC strain from an American tourist. Lancet, 1979. 2(8153): p. 1151-4.

Carvalho, B.O., et al., On the cytoadhesion of Plasmodium vivax-infected erythrocytes. J Infect Dis, 2010. 202(4): p. 638-47.

Castillo, C.M., L.E. Osorio, and G.I. Palma, Assessment of therapeutic response of Plasmodium vivax and Plasmodium falciparum to chloroquine in a Malaria transmission free area in Colombia. Mem Inst Oswaldo Cruz, 2002. 97(4): p. 559-62.

Chang, K.H. and M.M. Stevenson, Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. Int J Parasitol, 2004. 34(13-14): p. 1501-16.

Chotivanich, K., et al., Antimalarial drug susceptibility of Plasmodium vivax in the Republic of Korea. Am J Trop Med Hyg, 2009. 80(6): p. 902-4.

Chotivanich, K., et al., Ex-vivo short-term culture and developmental assessment of Plasmodium vivax. Trans R Soc Trop Med Hyg, 2001. 95(6): p. 677-80.

Chotivanich, K.T., et al., Characteristics of Plasmodium vivax-infected erythrocyte rosettes. Am J Trop Med Hyg, 1998. 59(1): p. 73-6.

Clark, I.A. and K.A. Rockett, The cytokine theory of human cerebral malaria. Parasitol Today, 1994. 10(10): p. 410-2.

Clough, B., et al., Plasmodium falciparum: the importance of IgM in the rosetting of parasite-infected erythrocytes. Exp Parasitol, 1998. 89(1): p. 129-32.

Coatney, G.R., Pitfalls in a discovery: the chronicle of chloroquine. Am J Trop Med Hyg, 1963. 12: p. 121-8.

Cojean, S., et al., Cytoadherence characteristics to endothelial receptors ICAM-1 and CD36 of Plasmodium falciparum populations from severe and uncomplicated malaria cases. Parasite, 2008. 15(2): p. 163-9.

Combes, V., et al., Cerebral malaria: role of microparticles and platelets in alterations of the blood-brain barrier. Int J Parasitol, 2006. 36(5): p. 541-6.

Congpuong, K., et al., Sensitivity of Plasmodium vivax to chloroquine in Sa Kaeo Province, Thailand. Acta Trop, 2002. 83(2): p. 117-21.

Coquelin, F., et al., Final stage of maturation of the erythrocytic schizonts of rodent Plasmodium in the lungs. C R Acad Sci III, 1999. 322(1): p. 55-62.

Costa, F.T., et al., On cytoadhesion of Plasmodium vivax: raison d'etre? Mem Inst Oswaldo Cruz, 2011. 106 Suppl 1: p. 79-84.

Cox, J., S. Semoff, and M. Hommel, Plasmodium chabaudi: a rodent malaria model for in-vivo and in-vitro cytoadherence of malaria parasites in the absence of knobs. Parasite Immunol, 1987. 9(5): p. 543-61.

Craig, A. and A. Scherf, Molecules on the surface of the Plasmodium falciparum infected erythrocyte and their role in malaria pathogenesis and immune evasion. Mol Biochem Parasitol, 2001. 115(2): p. 129-43.

Cui, F., et al., Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: preparation, in vitro characterization and in vivo evaluation. J Control Release, 2006. 114(2): p. 242-50.

de Carvalho, D.D., et al., Cytotoxic activity of violacein in human colon cancer cells. Toxicol In Vitro, 2006. 20(8): p. 1514-21.

de Santana Filho, F.S., et al., Chloroquine-resistant Plasmodium vivax, Brazilian Amazon. Emerg Infect Dis, 2007. 13(7): p. 1125-6.

De Souza, A.O.A., D.C.G.; Sato, D.N.; Durán, N., Atividade da violaceína in vitro sobre o Mycobacterium tuberculosis H37RA. Revista do Insituto Adolfo Lutz, 1999. 58: p. 59-62.

de Souza, J.B. and E.M. Riley, Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. Microbes Infect, 2002. 4(3): p. 291-300.

Deitsch, K.W. and L. Hviid, Variant surface antigens, virulence genes and the pathogenesis of malaria. Trends Parasitol, 2004. 20(12): p. 562-6.

del Portillo, H.A., et al., A superfamily of variant genes encoded in the subtelomeric region of Plasmodium vivax. Nature, 2001. 410(6830): p. 839-42.

Denis, M.B., et al., Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. Trop Med Int Health, 2006. 11(9): p. 1360-6.

Dondorp, A.M., E. Pongponratn, and N.J. White, Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology. Acta Trop, 2004. 89(3): p. 309-17.

Dondorp, A.M., et al., Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med, 2009. 361(5): p. 455-67.

Dondorp, A.M., et al., Artemisinin resistance: current status and scenarios for containment. Nat Rev Microbiol, 2010. 8(4): p. 272-80.

Dondorp, A.M., et al., Direct in vivo assessment of microcirculatory dysfunction in severe falciparum malaria. J Infect Dis, 2008. 197(1): p. 79-84.

Druilhe, P., et al., A colorimetric in vitro drug sensitivity assay for Plasmodium falciparum based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. Am J Trop Med Hyg, 2001. 64(5-6): p. 233-41.

Druilhe, P., et al., Improved assessment of plasmodium vivax response to antimalarial drugs by a colorimetric double-site plasmodium lactate dehydrogenase antigen capture enzyme linked immunosorbent assay. Antimicrob Agents Chemother, 2007. 51(6): p. 2112-6.

Duffy, M.F., et al., Transcribed var genes associated with placental malaria in Malawian women. Infect Immun, 2006. 74(8): p. 4875-83.

Duran, N. and C.F. Menck, Chromobacterium violaceum: a review of pharmacological and industiral perspectives. Crit Rev Microbiol, 2001. 27(3): p. 201-22.

Duran, N., et al., Bacterial chemistry-III: preliminary studies on trypanosomal activities of Chromobacterium violaceum products. An Acad Bras Cienc, 1989. 61(1): p. 31-6.

Duran, N., et al., Evaluation of the antiulcerogenic activity of violacein and its modulation by the inclusion complexation with beta-cyclodextrin. Can J Physiol Pharmacol, 2003. 81(4): p. 387-96.

Duran, N., et al., Violacein: properties and biological activities. Biotechnol Appl Biochem, 2007. 48(Pt 3): p. 127-33.

Duran, N., S. Erazo, and V. Campos, Bacterial Chemistry-Ii - Anti-Microbial Photoproduct from Pigment of Chromobacterium-Violaceum. An Acad Bras Cienc, 1983. 55(3): p. 231-234.

Durán, N.H.M., Production process of preparation and purification of antitumoral (3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene)-1,3-dihydro-2H-indol-2-one). , 1997: Brazil.

Engwerda, C., et al., Experimental models of cerebral malaria. Curr Top Microbiol Immunol, 2005. 297: p. 103-43.

Fernandez, V., et al., Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in Plasmodium falciparum malaria. Infect Immun, 1998. 66(6): p. 2969-75.

Fernandez, V., Sorbitol-synchronization of Plasmodium falciparum-infected erythrocytes., in Methods in malaria research, I.P. Ljungström, H.; Schlichtherle, M.; Scherf, A.; Wahlgren M. , Editor 2004, MR4/ATCC, Manassas, VA.

Ferreira, C.V., et al., Molecular mechanism of violacein-mediated human leukemia cell death. Blood, 2004. 104(5): p. 1459-64.

Fogh, S., S. Jepsen, and P. Effersoe, Chloroquine-resistant Plasmodium falciparum malaria in Kenya. Trans R Soc Trop Med Hyg, 1979. 73(2): p. 228-9.

Foote, S.J. and A.F. Cowman, The mode of action and the mechanism of resistance to antimalarial drugs. Acta Trop, 1994. 56(2-3): p. 157-71.

Franke-Fayard, B., A.P. Waters, and C.J. Janse, Real-time in vivo imaging of transgenic bioluminescent blood stages of rodent malaria parasites in mice. Nat Protoc, 2006. 1(1): p. 476-85.

Franke-Fayard, B., et al., Murine malaria parasite sequestration: CD36 is the major receptor, but cerebral pathology is unlinked to sequestration. Proc Natl Acad Sci U S A, 2005. 102(32): p. 11468-73.

Franke-Fayard, B., et al., Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria? PLoS Pathog, 2010. 6(9): p. e1001032.

Gamain, B., et al., Pregnancy-associated malaria: parasite binding, natural immunity and vaccine development. Int J Parasitol, 2007. 37(3-4): p. 273-83.

Genton, B., et al., Plasmodium vivax and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. PLoS Med, 2008. 5(6): p. e127.

Golnitz, U., L. Albrecht, and G. Wunderlich, Var transcription profiling of Plasmodium falciparum 3D7: assignment of cytoadherent phenotypes to dominant transcripts. Malar J, 2008. 7: p. 14.

Good, M.F., et al., Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. Annu Rev Immunol, 2005. 23: p.69-99.

Handunnetti, S.M., et al., Involvement of CD36 on erythrocytes as a rosetting receptor for Plasmodium falciparum-infected erythrocytes. Blood, 1992. 80(8): p. 2097-104.

Handunnetti, S.M., et al., Uninfected erythrocytes form "rosettes" around Plasmodium falciparum infected erythrocytes. Am J Trop Med Hyg, 1989. 40(2): p. 115-8.

Harinasuta, T., P. Suntharasamai, and C. Viravan, Chloroquine-resistant falciparum malaria in Thailand. Lancet, 1965. 2(7414): p. 657-60.

Haun, M., et al., Bacterial chemistry. VI. Biological activities and cytotoxicity of 1,3-dihydro-2H-indol-2-one derivatives. Biol Res, 1992. 25(1): p. 21-5.

Hay, S.I., et al., The global distribution and population at risk of malaria: past, present, and future. Lancet Infect Dis, 2004. 4(6): p. 327-36.

Ho, M. and N.J. White, Molecular mechanisms of cytoadherence in malaria. Am J Physiol, 1999. 276(6 Pt 1): p. C1231-42.

Justo, G.Z.F., C. V.; Peppelenbosch, M. P.; Durán, N. , Cellular and molecular targets in the evaluation of the antitumor potential of biocompounds. Annual Review Biomedical Science, 2005. 7 (Special Issue 1).

Kaewpongsri, S., et al., The presence of leukocytes in ex vivo assays significantly increases the 50-percent inhibitory concentrations of artesunate and chloroquine against Plasmodium vivax and Plasmodium falciparum. Antimicrob Agents Chemother, 2011. 55(3): p. 1300-4.

Kam, N.W., Z. Liu, and H. Dai, Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. Angew Chem Int Ed Engl, 2006. 45(4): p. 577-81.

Kappe, S.H., et al., That was then but this is now: malaria research in the time of an eradication agenda. Science, 2010. 328(5980): p. 862-6.

Knisely, M.H.S.-T., W.K.; Eliot, T.S., Observations on circulating blood in the small vessels of internal organs in living Macacus rhesus infected with malarial parasites. Anat Rec 1941. 79 p. 90.

Kochar, D.K., et al., Plasmodium vivax malaria. Emerg Infect Dis, 2005. 11(1): p. 132-4.

Kochar, D.K., et al., Post-malaria neurological syndrome--a case of bilateral facial palsy after Plasmodium vivax malaria. J Vector Borne Dis, 2007. 44(3): p. 227-9.

Kochar, D.K., et al., Severe Plasmodium vivax malaria: a report on serial cases from Bikaner in northwestern India. Am J Trop Med Hyg, 2009. 80(2): p. 194-8.

Kodach, L.L., et al., Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells. Carcinogenesis, 2006. 27(3): p. 508-16.

Kolaczinski, K., et al., Sulfadoxine-pyrimethamine plus artesunate compared with chloroquine for the treatment of vivax malaria in areas co-endemic for Plasmodium falciparum and P. vivax: a randomised non-inferiority trial in eastern Afghanistan. Trans R Soc Trop Med Hyg, 2007. 101(11): p. 1081-7.

Kraemer, S.M. and J.D. Smith, A family affair: var genes, PfEMP1 binding, and malaria disease. Curr Opin Microbiol, 2006. 9(4): p. 374-80.

Krudsood, S., et al., Clinical efficacy of chloroquine versus artemether-lumefantrine for Plasmodium vivax treatment in Thailand. Korean J Parasitol, 2007. 45(2): p. 111-4.

Kumar, N. and H. Zheng, Stage-specific gametocytocidal effect in vitro of the antimalaria drug qinghaosu on Plasmodium falciparum. Parasitol Res, 1990. 76(3): p. 214-8.

Kute, V.B., et al., Unusual presentation of Plasmodium vivax: a neglected human malaria parasite. Parasitol Res, 2011.

Lacerda, M.V., et al., Understanding the clinical spectrum of complicated Plasmodium vivax malaria: a systematic review on the contributions of the Brazilian literature. Malar J, 2012. 11(1): p. 12.

Landau, I.G., P., Animal models: Rodents., in Malaria: Parasite biology, pathogenesis, and protection. , I.W. Sherman, Editor 1998, ASM Press, Washington D.C.

Leeson, H.S., Microscopic Diagnosis of Human Malaria, Vol 2, a Morphological-Study of the Erythrocytic Parasites - Field,Jw, Schute,Pg. Royal Society of Health Journal, 1957. 77(10): p. 694-694.

Leon, L.L., et al., Antileishmanial activity of the violacein extracted from Chromobacterium violaceum. J Antimicrob Chemother, 2001. 48(3): p. 449-50.

Leslie, T., et al., Sulfadoxine-pyrimethamine, chlorproguanil-dapsone, or chloroquine for the treatment of Plasmodium vivax malaria in Afghanistan and Pakistan: a randomized controlled trial. JAMA, 2007. 297(20): p. 2201-9.

Lichstein, H.C. and V.F. Van De Sand, The Antibiotic Activity of Violacein, Prodigiosin, and Phthiocol. J Bacteriol, 1946. 52(1): p. 145-6.

Lomar, A.V., et al., Acute respiratory distress syndrome due to vivax malaria: case report and literature review. Braz J Infect Dis, 2005. 9(5): p. 425-30.

Lopes, S.C., et al., Violacein extracted from Chromobacterium violaceum inhibits Plasmodium growth in vitro and in vivo. Antimicrob Agents Chemother, 2009. 53(5): p. 2149-52.

Lowe, B.S., M. Mosobo, and P.C. Bull, All four species of human malaria parasites form rosettes. Trans R Soc Trop Med Hyg, 1998. 92(5): p. 526.

Luginbuhl, A., et al., Complement factor D, albumin, and immunoglobulin G anti-band 3 protein antibodies mimic serum in promoting rosetting of malaria-infected red blood cells. Infect Immun, 2007. 75(4): p. 1771-7.

Mackinnon, M.J., P.R. Walker, and J.A. Rowe, Plasmodium chabaudi: rosetting in a rodent malaria model. Exp Parasitol, 2002. 101(2-3): p. 121-8.

Mackintosh, C.L., J.G. Beeson, and K. Marsh, Clinical features and pathogenesis of severe malaria. Trends Parasitol, 2004. 20(12): p. 597-603.

MacPherson, G.G., et al., Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol, 1985. 119(3): p. 385-401.

Malleret, B., et al., A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development. Scientific Reports, 2011. 1.

Martin, C.R. and P. Kohli, The emerging field of nanotube biotechnology. Nat Rev Drug Discov, 2003. 2(1): p. 29-37.

Martins, D., et al., Antitumoral activity of L-ascorbic acid-poly- D,L-(lactide-co-glycolide) nanoparticles containing violacein. Int J Nanomedicine, 2010. 5: p. 77-85.

McLean, S.A., C.D. Pearson, and R.S. Phillips, Plasmodium chabaudi: antigenic variation during recrudescent parasitaemias in mice. Exp Parasitol, 1982. 54(3): p. 296-302.

Melo, P.S., et al., Nanocytotoxicity: violacein and violacein-loaded poly (D, L-lactide-co-glycolide) nanoparticles acting on human leukemic cells. J Biomed Nanotechnol, 2009. 5(2): p. 192-201.

Melo, P.S., et al., Violacein and its beta-cyclodextrin complexes induce apoptosis and differentiation in HL60 cells. Toxicology, 2003. 186(3): p. 217-25.

Melo, P.S., et al., Violacein cytotoxicity and induction of apoptosis in V79 cells. In Vitro Cell Dev Biol Anim, 2000. 36(8): p. 539-43.

Mendis, K., et al., The neglected burden of Plasmodium vivax malaria. Am J Trop Med Hyg, 2001. 64(1-2 Suppl): p. 97-106.

Mercereau-Puijalon, O., M. Guillotte, and I. Vigan-Womas, Rosetting in Plasmodium falciparum: a cytoadherence phenotype with multiple actors. Transfus Clin Biol, 2008. 15(1-2): p. 62-71.

Miller, L.H., et al., The pathogenic basis of malaria. Nature, 2002. 415(6872): p. 673-9.

Mohan, A., S.K. Sharma, and S. Bollineni, Acute lung injury and acute respiratory distress syndrome in malaria. J Vector Borne Dis, 2008. 45(3): p. 179-93.

Mohapatra, M.K., et al., Atypical manifestations of Plasmodium vivax malaria. Indian J Malariol, 2002. 39(1-2): p. 18-25.

Moore, D.V. and J.E. Lanier, Observations on two Plasmodium falciparum infections with an abnormal response to chloroquine. Am J Trop Med Hyg, 1961. 10: p. 5-9.

Mota, M.M., et al., Plasmodium chabaudi-infected erythrocytes adhere to CD36 and bind to microvascular endothelial cells in an organ-specific way. Infect Immun, 2000. 68(7): p. 4135-44.

Nandy, A., et al., Monitoring the chloroquine sensitivity of Plasmodium vivax from Calcutta and Orissa, India. Ann Trop Med Parasitol, 2003. 97(3): p. 215-20.

Nateghpour, M., S.A. Ward, and R.E. Howells, Development of halofantrine resistance and determination of cross-resistance patterns in Plasmodium falciparum. Antimicrob Agents Chemother, 1993. 37(11): p. 2337-43.

Neres, R., et al., Pregnancy outcome and placenta pathology in Plasmodium berghei ANKA infected mice reproduce the pathogenesis of severe malaria in pregnant women. PLoS One, 2008. 3(2): p. e1608.

Newbold, C., et al., Cytoadherence, pathogenesis and the infected red cell surface in Plasmodium falciparum. Int J Parasitol, 1999. 29(6): p. 927-37.

Noedl, H., et al., Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med, 2008. 359(24): p. 2619-20.

Nosten, F., et al., Effects of artesunate-mefloquine combination on incidence of Plasmodium falciparum malaria and mefloquine resistance in western Thailand: a prospective study. Lancet, 2000. 356(9226): p. 297-302.

O'Brien, C., et al., Recent clinical and molecular insights into emerging artemisinin resistance in Plasmodium falciparum. Curr Opin Infect Dis, 2011. 24(6): p. 570-7.

Ockenhouse, C.F., C. Magowan, and J.D. Chulay, Activation of monocytes and platelets by monoclonal antibodies or malaria-infected erythrocytes binding to the CD36 surface receptor in vitro. J Clin Invest, 1989. 84(2): p. 468-75.

Oliveira-Ferreira, J., et al., Malaria in Brazil: an overview. Malar J, 2010. 9: p. 115.

Olliaro, P.L. and W.R. Taylor, Antimalarial compounds: from bench to bedside. J Exp Biol, 2003. 206(Pt 21): p. 3753-9.

Olliaro, P., Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacology & Therapeutics, 2001. 89: p. 207-19.

Ordi, J., et al., Massive chronic intervillositis of the placenta associated with malaria infection. Am J Surg Pathol, 1998. 22(8): p. 1006-1

Ordi, J., et al., Placental malaria is associated with cell-mediated inflammatory responses with selective absence of natural killer cells. J Infect Dis, 2001. 183(7): p. 1100-7.

Pain, A., et al., Platelet-mediated clumping of Plasmodium falciparum-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. Proc Natl Acad Sci U S A, 2001. 98(4): p. 1805-10.

Pantarotto, D., et al., Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chem Commun (Camb), 2004(1): p. 16-7.

Planche, T. and S. Krishna, Severe malaria: metabolic complications. Curr Mol Med, 2006. 6(2): p. 141-53.

Price, R.N., et al., Plasmodium vivax treatments: what are we looking for? Curr Opin Infect Dis, 2011. 24(6): p. 578-85.

Price, R.N., et al., Vivax malaria: neglected and not benign. Am J Trop Med Hyg, 2007. 77(6 Suppl): p. 79-87.

Ratcliff, A., et al., Therapeutic response of multidrug-resistant Plasmodium falciparum and P. vivax to chloroquine and sulfadoxine-pyrimethamine in southern Papua, Indonesia. Trans R Soc Trop Med Hyg, 2007. 101(4): p. 351-9.

Rettori, D. and N. Duran, Production, extraction and purification of violacein: an antibiotic pigment produced by Chromobacterium violaceum. World Journal of Microbiology & Biotechnology, 1998. 14(5): p. 685-688.

Rieckmann, K.H., D.R. Davis, and D.C. Hutton, Plasmodium vivax resistance to chloroquine? Lancet, 1989. 2(8673): p. 1183-4.

Robert, C., et al., Chondroitin-4-sulphate (proteoglycan), a receptor for Plasmodium falciparum-infected erythrocyte adherence on brain microvascular endothelial cells. Res Immunol,1995. 146(6): p. 383-93.

Roberts, D.J., et al., Autoagglutination of malaria-infected red blood cells and malaria severity. Lancet, 2000. 355(9213): p. 1427-8.

Roberts, D.J., et al., Rapid switching to multiple antigenic and adhesive phenotypes in malaria. Nature, 1992. 357(6380): p. 689-92.

Rogerson, S.J., et al., Chondroitin sulfate A is a cell surface receptor for Plasmodium falciparum-infected erythrocytes. J Exp Med, 1995. 182(1): p. 15-20.

Rogerson, S.J., et al., Malaria in pregnancy: pathogenesis and immunity. Lancet Infect Dis, 2007. 7(2): p. 105-17.

Rogerson, S.J., et al., Placental monocyte infiltrates in response to Plasmodium falciparum malaria infection and their association with adverse pregnancy outcomes. Am J Trop Med Hyg, 2003. 68(1): p. 115-9.

Rowe, A., et al., Plasmodium falciparum rosetting is associated with malaria severity in Kenya. Infect Immun, 1995. 63(6): p. 2323-6.

Rowe, J.A., et al., Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. Expert Rev Mol Med, 2009. 11: p. e16.

Ruebush, T.K., 2nd, et al., Chloroquine-resistant Plasmodium vivax malaria in Peru. Am J Trop Med Hyg, 2003. 69(5): p. 548-52.

Russell, B., et al., A reliable ex vivo invasion assay of human reticulocytes by Plasmodium vivax. Blood, 2011. 118(13): p. e74-81.

Russell, B.M., et al., Simple in vitro assay for determining the sensitivity of Plasmodium vivax isolates from fresh human blood to antimalarials in areas where P. vivax is endemic. Antimicrob Agents Chemother, 2003. 47(1): p. 170-3.

Salanti, A., et al., Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Mol Microbiol, 2003. 49(1): p. 179-91.

Santos-Ciminera, P.D., et al., Molecular epidemiology of Plasmodium vivax in the State of Amazonas, Brazil. Acta Trop, 2007. 102(1): p. 38-46.

Saraiva, V.S., et al., Cytotoxic effects of violacein in human uveal melanoma cell lines. Melanoma Res, 2004. 14(5): p. 421-4.

Sarkar, S. and P. Bhattacharya, Cerebral malaria caused by Plasmodium vivax in adult subjects. Indian J Crit Care Med, 2008. 12(4): p. 204-5.

Sarkar, S., K. Saha, and C.S. Das, Three cases of ARDS: An emerging complication of Plasmodium vivax malaria. Lung India, 2010. 27(3): p. 154-7.

Schmidt, B.A., [3H] Hypoxanthine incorporation assay for the study of growth inhibition by drugs, I.P. Ljungström, H.; Schlichtherle, M.; Scherf, A.; Wahlgren M. , Editor 2004, MR4/ATCC, Manassas, VA.

Schofield, L. and G.E. Grau, Immunological processes in malaria pathogenesis. Nat Rev Immunol, 2005. 5(9): p. 722-35.

Schofield, L., Intravascular infiltrates and organ-specific inflammation in malaria pathogenesis. Immunol Cell Biol, 2007. 85(2): p. 130-7.

Sharling, L., et al., Antibodies from malaria-exposed pregnant women recognize trypsin resistant epitopes on the surface of Plasmodium falciparum-infected erythrocytes selected for adhesion to chondroitin sulphate A. Malar J, 2004. 3: p. 31.

Sharma, Y.D., Knobs, knob proteins and cytoadherence in falciparum malaria. Int J Biochem, 1991. 23(9): p. 775-89.

Sherman, I.W., S. Eda, and E. Winograd, Cytoadherence and sequestration in Plasmodium falciparum: defining the ties that bind. Microbes Infect, 2003. 5(10): p. 897-909.

Shirata, A., et al., Isolation of bacteria producing bluish-purple pigment and use for dyeing. Jarq-Japan Agricultural Research Quarterly, 2000. 34(2): p. 131-140.

Singh, B., et al., A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet, 2004. 363(9414): p. 1017-24.

Singh, H., et al., Plasmodium vivax malaria: is it actually benign? J Infect Public Health, 2011. 4(2): p. 91-5.

Soto, J., et al., Plasmodium vivax clinically resistant to chloroquine in Colombia. Am J Trop Med Hyg, 2001. 65(2): p. 90-3.

Sriprawat, K., et al., Effective and cheap removal of leukocytes and platelets from Plasmodium vivax infected blood. Malar J, 2009. 8: p. 115.

Srivastava, I.K., H. Rottenberg, and A.B.Vaidya, Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J Biol Chem, 1997. 272: p. 3961-6.

Stepniewska, K. and N.J. White, Some considerations in the design and interpretation of antimalarial drug trials in uncomplicated falciparum malaria. Malar J, 2006. 5: p. 127.

Stepniewska, K., et al., In vivo parasitological measures of artemisinin susceptibility. J Infect Dis, 2010. 201(4): p. 570-9.

Sturm, A., et al., Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science, 2006. 313(5791): p. 1287-90.

Sumawinata, I.W., et al., Very high risk of therapeutic failure with chloroquine for uncomplicated Plasmodium falciparum and P. vivax malaria in Indonesian Papua. Am J Trop Med Hyg, 2003. 68(4): p. 416-20.

Sutanto, I., et al., Resistance to chloroquine by Plasmodium vivax at Alor in the Lesser Sundas Archipelago in eastern Indonesia. Am J Trop Med Hyg, 2009. 81(2): p. 338-42.

Talisuna, A.O., P. Bloland, and U. D'Alessandro, History, dynamics, and public health importance of malaria parasite resistance. Clin Microbiol Rev, 2004. 17(1): p. 235-54.

Tanwar, G.S., et al., Clinical profiles of 13 children with Plasmodium vivax cerebral malaria. Ann Trop Paediatr, 2011. 31(4): p. 351-6.

Tasanor, O., et al., Clinical-parasitological response and in-vitro sensitivity of Plasmodium vivax to chloroquine and quinine on the western border of Thailand. Trans R Soc Trop Med Hyg, 2006. 100(5): p. 410-8.

Tjitra, E., et al., Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med, 2008. 5(6): p. e128.

Trager, W. and J.B. Jensen, Human malaria parasites in continuous culture. 1976. J Parasitol, 2005. 91(3): p. 484-6.

Trigg, P.I. and A.V. Kondrachine, Commentary: malaria control in the 1990s. Bull World Health Organ, 1998. 76(1): p. 11-6.

Tuikue Ndam, N.G., et al., High level of var2csa transcription by Plasmodium falciparum isolated from the placenta. J Infect Dis, 2005. 192(2): p. 331-5.

Udomsangpetch, R., et al., Cultivation of Plasmodium vivax. Trends Parasitol, 2008. 24(2): p. 85-8.

Udomsangpetch, R., et al., Rosette formation by Plasmodium coatneyi-infected red blood cells. Am J Trop Med Hyg, 1991. 44(4): p. 399-401.

Udomsanpetch, R., et al., Rosette formation by Plasmodium vivax. Trans R Soc Trop Med Hyg, 1995. 89(6): p. 635-7.

Valecha, N., et al., Therapeutic efficacy of chloroquine in Plasmodium vivax from areas with different epidemiological patterns in India and their Pvdhfr gene mutation pattern. Trans R Soc Trop Med Hyg, 2006. 100(9): p. 831-7.

Valibayov, A., et al., Clinical efficacy of chloroquine followed by primaquine for Plasmodium vivax treatment in Azerbaijan. Acta Trop, 2003. 88(1): p. 99-102.

van der Heyde, H.C., et al., A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. Trends Parasitol, 2006. 22(11): p. 503-8.

Vijaykadga, S., et al., Assessment of therapeutic efficacy of chloroquine for vivax malaria in Thailand. Southeast Asian J Trop Med Public Health, 2004. 35(3): p. 566-9.

Vijaykadga, S., et al., In vivo sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. Trop Med Int Health, 2006. 11(2): p. 211-9.

Wells, T.N., P.L. Alonso, and W.E. Gutteridge, New medicines to improve control and contribute to the eradication of malaria. Nat Rev Drug Discov, 2009. 8(11): p. 879-91.

Wernsdorfer, W.H. and D. Payne, The dynamics of drug resistance in Plasmodium falciparum. Pharmacol Ther, 1991. 50(1): p. 95-121.

White, N.J. and P. Olliaro, Artemisinin and derivatives in the treatment of uncomplicated malaria. Med Trop (Mars), 1998. 58(3 Suppl): p. 54-6.

White, N.J., Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia, 1999. 41(1-3): p. 301-8.

White, N.J., Qinghaosu (artemisinin): the price of success. Science, 2008. 320(5874): p. 330-4.

World Health Organization, Global Partnership to Roll Back Malaria., Antimalarial drug combination therapy : report of a WHO technical consultation, 4-5 April 2001, Geneva: World Health Organization. 36 p.

World Health Organization, Communicable Diseases Cluster. Severe falciparum malaria.Trans R Soc Trop Med Hyg, 2000. 94 Suppl 1: p. S1-90.

World Health Organization, World Malaria Report 2008.

World Health Organization, UNICEF and Global Partnership to Roll Back Malaria., World malaria report : 2005, 294 p.

World Health Organization, Global report on antimalarial drug efficacy and drug resistance: 2000-2010, Geneva: World Health Organization. 115 p.

World Health Organization, World malaria report 2011, Geneva: World Health Organization. xii, 246 p.

World Health Organization, Update on artemisinin resistance September 2011.

Wyler, D.J., Splenic functions in malaria. Lymphology, 1983. 16(2): p. 121-7.

Yadav, D., et al., Changing Profile of Severe Malaria in North Indian Children. Indian J Pediatr, 2011.

Anexos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

1. DADOS DE IDENTIFICAÇÃO

Nome (Paciente): .....
Documento de Identidade: .....
Endereço: ..... CEP: .....
Cidade: ..... Estado..... Fone:.....

2. DADOS SOBRE A PESQUISA CIENTÍFICA

2.1 **Titulo:** Estudo da biologia de *Plasmodium vivax*: imunopatogênese, citoadesão e invasão.

2.2 **Investigador Principal:** Dr Marcus Vinicius Guimarães Lacerda

2.3 **Colaboradores:** MSc Stefanie Costa Pinto Lopes, Dra. Letusa Albrecht, Dr. Fabio Trindade Maranhão Costa.

3. EXPLICAÇÕES DO PESQUISADOR AO PACIENTE

Você está sendo convidado a participar da pesquisa **"Estudo da biologia de *Plasmodium vivax*: imunopatogênese, citoadesão e invasão"** na Fundação de Medicina Tropical do Amazonas, Brasil. Você foi selecionado por ter visitado a FMT-HVD, hospital de referência para o tratamento da malária em Manaus, e apresentar essa doença. A qualquer momento você pode desistir de participar e retirar seu consentimento. Sua recusa não trará nenhum prejuízo em sua relação com o pesquisador ou com a Instituição. O acompanhamento da sua doença e tratamento continuará sendo realizado independentemente de você estar na pesquisa ou não.

A cidade de Manaus apresenta, anualmente, um grande número de pessoas acometidas com malária vivax e algumas dessas pessoas apresentam complicações decorrentes da doença. Pouco se sabe a respeito da biologia do parasita causador da malária, sua patogênese e a resposta imune envolvida durante a infecção. Esse estudo tem como objetivo entender melhor a biologia do *Plasmodium vivax*, um dos parasitas causadores da malária sendo o mais freqüente em nossa região, e assim ajudar a compreender os mecanismos por trás da doença malária.

Você não precisa fazer qualquer coisa especial para participar desse projeto e receberá o mesmo tratamento e acompanhamento estabelecido pelo Ministério da Saúde. Como parte desse estudo nós coletaremos uma amostra de sangue (cerca de 15 ml) através da punção da veia do antebraço (venopunção) feita por técnico de saúde da equipe.

Nós gostaríamos de sua permissão para fazer testes com os parasitas de malária nas amostras de sangue coletadas e para guardar o restante de sangue para ser usado em estudos sobre malária no futuro. Isto pode exigir o armazenamento mais prolongado de seu sangue em laboratórios da FMT-HVD. Para isso seu sangue será guardado com um código e não deverá identificar seu nome, resguardando sua privacidade.

Você pode sentir alguma dor ou desconforto por causa da coleta da amostra de sangue. Mas qualquer dor deve durar poucos instantes. A amostra de sangue coletada é muito pequena e não representa nenhum risco à sua saúde. O benefício em estar participando deste estudo será o aumento de informações sobre malária vivax e você não receberá nenhum incentivo financeiro paras esse estudo.

Se você concordar em participar, todas as informações coletadas serão confidenciais, usadas somente no estudo. Nós não compartilharemos suas informações e não tornaremos público qualquer detalhe sobre você.

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4. CONTATO

Se você tiver qualquer pergunta ou preocupação sobre o estudo, por favor, vamos esclarecer isso agora. Mesmo assim se você desejar esclarecer suas dúvidas sobre a pesquisa, sinta-se a vontade para contatar o pesquisador principal do projeto Dr. Marcus Vinícius Guimarães de Lacerda na Fundação de Medicina Tropical, situada no endereço Avenida Pedro Teixeira, n°25, Bairro Dom Pedro e telefone (92) 3656 0620. O endereço do comitê de ética em pesquisa da FMT-HVD (que é grupo de pessoas que avaliam este projeto e acompanham a pesquisa) é o mesmo acima e o telefone para contato é o (92) 2127 3432. O presidente deste comitê é o Dr Luiz Carlos de Lima Ferreira.

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5. CONSENTIMENTO PÓS-INFORMADO

Declaro que recebi a explicação de que serei um dos participantes dessa pesquisa e entendo todas as suas etapas e objetivos. Se eu não souber ler ou escrever, uma pessoa de minha confiança lerá este documento para mim e depois escreverá nesta página o meu nome e a data do preenchimento.

E por estar devidamente informado e esclarecido sobre o conteúdo deste termo, livremente, sem qualquer pressão por parte dos pesquisadores, expresso meu consentimento para minha inclusão nesta pesquisa.

_____	___/___/___
Assinatura do participante	Data

_____	_____	___/___/___
Nome do entrevistador	Assinatura do entrevistador	Data

QUESTIONÁRIO

Dados do paciente:
1. Nome: ⊥⊥
2. Sobrenome: ⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥
⊥⊥
3. Data de nascimento (dd/mm/aa): ⊥⊥⊥/⊥⊥⊥/⊥⊥⊥ Idade (em anos): ⊥⊥⊥
4. Sexo (1= Homem, 2=Mulher): ⊥⊥
5. Se for mulher, está grávida? (1=sim, 2=não, 3=não sabe, 99= NA) ⊥⊥⊥
6. Residência:
Bairro: ⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥
Cidade: ⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥⊥ Estado: ⊥⊥⊥
7. Tempo de residência nesse local (em anos): ⊥⊥⊥

Dados malária:
8. Malária (1=vivax, 2=falciparum, 3=malariae, 99= NA): ⊥⊥⊥
9. Parasitemia (1= <+/2, 2= +/2, 3= +, 4= ++, 5= +++): ⊥⊥
10. Foi diagnosticado com malária anteriormente (1=sim, 2=não): ⊥⊥
Se a resposta for sim:
11. Episódios anteriores de malária: ⊥⊥⊥ 11. No último ano (99=NA): ⊥⊥⊥
12. Data da última infecção malárica (mm/aa): ⊥⊥⊥/⊥⊥⊥
13. Tomou medicação antimalárica no último mês (1=sim, 2=não): ⊥⊥
14. Sintomas (1=sim, 2=não)
Febre ⊥⊥ Calafrio ⊥⊥ Cefaléia ⊥⊥ Dor abdominal ⊥⊥ Diarréia ⊥⊥
Vômitos ⊥⊥

Artigos publicados relacionados ao trabalho

On cytoadhesion of *Plasmodium vivax*: *raison d'être*?

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It is generally accepted that Plasmodium vivax, *the most widely distributed human malaria parasite, causes mild disease and that this species does not sequester in the deep capillaries of internal organs. Recent evidence, however, has demonstrated that there is severe disease, sometimes resulting in death, exclusively associated with* P. vivax *and that* P. vivax*-infected reticulocytes are able to cytoadhere in vitro to different endothelial cells and placental cryosections. Here, we review the scarce and preliminary data on cytoadherence in* P. vivax, *reinforcing the importance of this phenomenon in this species and highlighting the avenues that it opens for our understanding of the pathology of this neglected human malaria parasite.*

Key words: *Plasmodium vivax* - malaria - cytoadherence - severe disease

Plasmodium vivax: the last malaria parasite? - The renewed momentum for global malaria eradication has generated the need for further study of *P. vivax* (Alonso et al. 2011). In fact, the burden of *P. vivax* seems to be increasing from 70-80 million to 130-435 million clinical cases per year and threatens 2.6 billion people (Mendis et al. 2001, Hay et al. 2004, Guerra et al. 2010). Frequent relapses of infection with this species and the emergence of chloroquine resistance are two major factors likely responsible for this increasing burden of *P. vivax* malaria (Kochar et al. 2005, Baird 2007, Price et al. 2007). Notably, in South and Southeast Asia, *P. vivax* infections represent more than 50% of malaria cases, whereas in Central and South America infections by *P. vivax* can reach up to 70-90%. Thus, *P. vivax* is most prevalent malaria outside Sub-Saharan Africa.

Notably, the life cycle and biology of *P. vivax* differ from those of *Plasmodium falciparum* (Table). The following differences are worth highlighting: (i) *P. vivax* sporozoites can enter hepatocytes and remain dormant as hypnozoites, which are responsible for clinical relapses (Krotoski 1985), (ii) *P. vivax* preferentially, if not exclusively, invades reticulocytes (Kitchen 1938), and this is arguably the reason why parasitemias are low in natural *vivax* infection, (iii) gametocytes appear in the blood before clinical symptoms are manifest, which complicates and compromises clinical treatment and control of the disease (Boyd & Kitchen 1937), and (iv) mosquito vectors from areas where *P. vivax* is more prevalent are outdoor-biting and anthropophilic, which makes control measures, such as impregnated bed nets, of limited value. Experts therefore agree that due to this unique biology, the present tools used to control *P. falciparum* will not suffice for *P. vivax*, which reinforces the importance of understanding the biology, epidemiology and pathology of *P. vivax* (Mueller et al. 2009).

Pathology of P. vivax - Pathogenesis in *falciparum* malaria is multi-factorial and involves numerous host and parasite factors. However, central to this pathogenesis are the phenomena of antigenic variation, rosetting and cytoadherence (Miller et al. 2002). Antigenic variation refers to the capacity of parasites to clonally express variant proteins at the surface of infected red blood cells (IRBCs) and this function to evade the host immune response and allow for the establishment of chronic infection (Brown & Brown 1965). Rosetting is the formation of rosettes resulting from the adhesion of erythrocytes infected with mature forms of the parasite to uninfected erythrocytes and this process has been shown to increase the microvascular obstruction of blood flow (David et al. 1988). Cytoadherence refers to the capacity of IRBC to adhere to host cells. This process has been described for the endothelium and for the placenta and it causes the disappearance of mature trophozoites and schizonts from the peripheral blood (Bignami & Bastianelli 1889, Miller et al. 1994). Remarkably, the original search for genes responsible for these phenomena identified a single subtelomeric multigene variant family named the *var* gene family (Baruch et al. 1995, Smith et al. 1995, Su et al. 1995). *P. falciparum var* genes encode proteins collectively known as PfEMP1 that contain ligand domains for different endothelial receptors, such as CD36, intercellular adhesion molecule-1 (ICAM-1) and chondroitin sulphate

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TABLE

Main biological differences between *Plasmodium vivax* and *Plasmodium falciparum*

P. vivax	*P. falciparum*
Seventy-300 million yearly clinical cases (Mendis et al. 2001, Hay et al. 2004).	Three hundred-500 million yearly clinical cases (Guerra et al. 2007).
Usually non-lethal and non-severe; yet this situation has radically changed recently (Kochar et al. 2009).	Lethal and severe, responsible for close to one million deaths each year mostly in children under five years old.
Sporozoites can remain within hepatocytes in dormant stages known as hypnozoites (Krotoski 1985).	Sporozoites within hepatocytes undergo immediate schizogony with no dormant stages.
Hypnozoites cause clinical relapses.	There are no clinical relapses.
Invades preferentially, if not exclusively, reticulocytes (Kitchen 1938).	Invades all red blood cells.
Requires mainly (Chitnis & Sharma 2008), but not exclusively (Mercereau-Puijalon & Menard 2010), the Duffy Blood Group as the receptor for entrance into reticulocytes.	Has multiple entrance pathways and receptors (Cowman & Crabb 2006).
Surface membrane of infected reticulocytes suffers invaginations called caveola vesicles.	Surface membrane of infected red blood cells suffers protrusions called knobs.
Continuous in vitro culture not available.	Continuous in vitro culture available since 1976 (Trager & Jensen 1976).
Gametocytes appear in peripheral blood before clinical symptoms (Boyd & Kitchen 1937).	Gametocytes appear in peripheral blood after clinical symptoms.
Mosquito vectors are mostly outdoor bitting.	Mosquito vectors are mostly indoor bitting.
Chloroquine resistance first reported in 1989.	Chloroquine resistance first reported in late 1950s.
Complete genome sequence first reported in 2008 (Carlton et al. 2008).	Complete genome sequence first reported in 2002 (Gardner et al. 2002).

A (CSA). Furthermore, *var* gene expression is associated with various clinical syndromes of severe *falciparum* disease (Pasternak & Dzikowski 2009). In addition to *var* genes, the full genome sequence of *P. falciparum* has revealed additional subtelomeric multigene families that are likely involved in the pathology of malaria (Gardner et al. 2002). These data therefore indicate that expression of *P. falciparum* multigene families, which are clustered in subtelomeric regions, is associated with the pathology of *falciparum* malaria and the phenomena of antigenic variation, rosetting and cytoadherence.

Studies from *P. vivax* patients in Sri Lanka have shown that *P. vivax* undergoes antigenic variation and that the mature forms have the capacity for rosetting (Udagama et al. 1987, Udomsanpetch et al. 1995). Thus, using immune sera from primary-infected or multiply infected *P. vivax* patients, immunofluorescence assays from different isolates have revealed that only patients with multiple infections were able to recognise several different isolates (Udagama et al. 1987). Moreover, rosetting experiments using blood and wild isolates revealed that close to 70% of the isolates showed rosetting (Udomsanpetch et al. 1995). However, approaches similar to those used for studies of cytoadhesion in *P. falciparum* (Hasler et al. 1993) failed to observe the cytoadhesion of *P. vivax*-infected reticulocytes to human umbilical vein endothelial cells, C32 melanoma cells, platelets or to purified CD36. Notably, sequence analysis of a *P. vivax* isolate revealed the presence of a subtelomeric multigene variant superfamily termed *vir* (*P. vivax* variant genes), which is likely involved in antigenic variation in this species (del Portillo et al. 2001). Further analyses have indicated that *vir* genes could have additional functions and it was hypothesised that they mediate adhesion to facilitate the establishment of chronic infection (del Portillo et al. 2004). The complete genome sequence of the Sal I strain revealed the presence of additional subtelomeric gene families (Carlton et al. 2008), which further indicated that *P. vivax*, like *P. falciparum*, has clustered multigene families at its chromosome ends. However, the relationship between these gene families and pathology has been mostly overlooked, as *vivax* malaria had been predominantly accepted to be clinically benign and not to cytoadhere.

In the last 10 years, the severe complications observed in *P. falciparum* infection, which include renal failure, jaundice, acute respiratory distress syndrome, cerebral malaria, seizures, anemia, hyperparasitemia, thrombocytopenia, pulmonary edema, splenic rupture and death, have also been reported worldwide for *P. vivax* (Kochar et al. 2005, 2009, Baird 2007, Price et al. 2007, Alexandre et al. 2010). However, diagnosis was made in most cases using Giemsa staining, which cast reasonable doubts as to the potential for undetectable infection with *P. falciparum*. The use of nested polymerase chain reactions (PCRs) to exclude the possibility of mixed infection, however, has confirmed sole infection with *P. vivax* in some of these reports. In addition, the clinical exclusion of other syndromes, such as those caused by respiratory

pathogens, human immunodeficiency virus, histoplasma, coccidians and paracoccidians, as well as the adoption of World Health Organization criteria used for severe *falciparum* disease, are now being adopted for the reports of new cases of severe *vivax* disease. In Brazil, for instance, a recent study from the city of Manaus, Amazonas, showed an increased number of hospital admissions for *P. vivax*-infected patients (Santos-Ciminera et al. 2007) with increased clinical severity, which were mostly related to jaundice and severe anaemia after *P. falciparum* or other possible causes of disease were excluded (Alexandre et al. 2010). Similar to what has been observed in Brazil, an increasing number of cases with complications have been reported worldwide (Anstey et al. 2009). These data illustrate and confirm that there is indeed an augmentation of the number of severe cases exclusively associated with *P. vivax* infection across different endemic regions of the world. Nevertheless, whether this severity is directly associated with changes in parasite biology, increasing resistance to antimalarial drugs or with the presence of comorbidities is still a matter of debate.

Although it has long been axiomatic that *P. vivax* is less virulent than *P. falciparum* due to its lack of cytoadherence, the existence of subtelomeric variant families in the genome of *P. vivax* and the recent confirmed reports of severe *vivax* disease have led us to question the validity of this view. Moreover, in spite of studies that have documented the presence of all stages of *P. vivax* in the peripheral blood of infected patients, previous studies revealed that the presence of mature forms were disproportionably lower than young stages, indicating a segmentation of these mature forms somewhere else than in peripheral blood (Field et al. 1963).

Cytoadherence of P. vivax-infected reticulocytes - In an attempt to elucidate whether cytoadhesion is a *P. falciparum*-restricted phenomenon, a recent collaborative study by our groups (Carvalho et al. 2010) demonstrated that *P. vivax*-IRBC (Pv-IRBCs) obtained from Brazilian patients with non-severe disease were able to cytoadhere under static or flow conditions to brain, lung (Fig. 1), placental and endothelial receptors, which have previously been implicated in the binding of *P. falciparum* (Beeson & Brown 2002). Under static conditions, the number per mm^2 of adherent Pv-IRBC was 10-15-fold lower than that of *P. falciparum*-IRBC (Pf-IRBC). However, the percentage of parasites able to bind endothelial receptors under flow conditions did not differ significantly. These results differ from those previously reported in Sri Lanka (Udomsanpetch et al. 1995). One explanation for this discrepancy is that our cytoadhesion assays used percoll-enriched mature forms of the parasite at pH 6.8, whereas this other studies has used forms obtained directly from the blood at pH 7.4.

To identify potential endothelial receptors involved in *P. vivax* adherence, CHO cells expressing different endothelial receptors were used for adhesion assays. We found greater adhesion to ICAM-1-transfected cells in comparison to CD36-transfected or untransfected cells. However, we cannot rule out the role of additional host receptors in Pv-IRBC. Moreover, adhesion of parasites to endothelial cells was significantly inhibited by the addition of soluble CSA, although treatment of the same cells with chondroitinase-ABC (CaseABC) did not affect adhesion (Carvalho et al. 2010). These results indicated that the CSA backbone was not directly involved in parasite adhesion. As CSA is highly negatively charged due to the presence of sulphate groups, the inhibition of cytoadhesion may be sustained by a charge interaction. Indeed, it has been shown that the binding of several *P. falciparum* VAR2CSA domains to CSA at various salt concentrations is not different from their binding to non-placental domains, which demonstrates that binding to CSA is highly dependent on ionic strength (Resende et al. 2009).

We hypothesised that Vir proteins, due to their variant nature, sub-cellular localisation and genome organisation, could mediate *P. vivax* cytoadherance. By using two different antibodies raised against two different Vir proteins, the specific inhibition of the IRBC-endothelial cell interaction was demonstrated (Carvalho et al. 2010). To perform functional binding assays in the absence of a continuous in vitro culture system for *P. vivax*, 3D7 *P. falciparum* transgenic lines expressing different Vir proteins, motifs, protein domains and secondary structures have been generated (M Bernabeu & C Fernandez-Becerra, unpublished observations). Notably, one of these transgenic lines that expressed Vir proteins on the surface of the IRBC mediated cytoadherence to ICAM-1, VCAM and E-selectin. Moreover, two polyclonal, mono-specific anti-Vir antibodies significantly inhibited this adhesion. Together, these data further support our findings and indicate that *P. vivax*-infected reticulocytes can cytoadhere to a variety of endothelial receptors and that this adherence is at least partially mediated specifically by Vir proteins.

Cytoadherence and severe vivax disease - The recent findings that infection with *P. vivax* can lead to severe complications in endemic areas worldwide (Kochar et al. 2005, Lomar et al. 2005, Tjitra et al. 2008) have led the scientific community to search for the mechanisms of pathogenesis related to this infection, which have been neglected thus far. One of the major arguments against the severity of *P. vivax* infection was based on the lack of cytoadherence and the formation of knobs by this parasite. However, robust evidence points to the existence of severe *falciparum* disease without the presence of the aforementioned mechanisms, which therefore suggests that these are not the *sine qua non* for severity (Maegraith 1948). As reviewed above, cytoadhesion was demonstrated for *P. vivax* using an in vitro approach (Carvalho et al. 2010) and unpublished data on adhesion to the placenta (FTM Costa & MVG Lacerda, unpublished observations) (Fig. 2), lungs and spleen (MVG Lacerda & H del Portillo, unpublished observations) have recently been obtained. When IRBCs are found inside of blood vessels in human tissues, there is no way to confirm that cytoadhesion has taken place, and this possibility should also be taken into account for previous studies of *P. falciparum*-infected tissues.

There is not much information available on autopsies related to *vivax* infection, which could lend support to cytoadherence during severe *vivax* disease. As reviewed elsewhere (Anstey et al. 2009), however, early XX century autopsies for which *P. vivax* diagnosis was based on

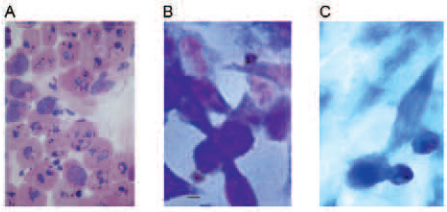

Fig. 1: adhesion of *Plasmodium vivax*-infected erythrocytes maturing forms to endothelial cells. A: Giemsa staining of *P. vivax* mature forms obtained after Percoll enrichment. Representative photomicrographs of *P. vivax*-infected red blood cells adhesion to human lung; B: Saimiri brain; C: endothelial cells.

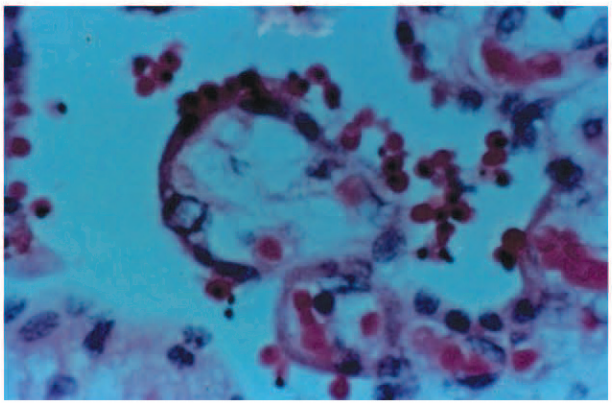

Fig. 2: presence of *Plasmodium vivax*-infected red blood cells attached to the synciotrophoblast of a placenta from a patient diagnosed with *P. vivax* in the 36th week of pregnancy (optical microscopy, 1,000X, Thomas' stain).

thick blood smears reported the presence of intracapillary masses of swollen, infected erythrocytes and pigment (Billings & Post 1915). In addition, these autopsies reported the frequent presence of mature malaria parasites within a red blood cell taking up the entire lumen and being in immediate contact with endothelial cells in some vessels in the brain, as well as an unusually large number of infected red cells and young plasmodia and a large amount of pigment in intestinal submucosal vessels (Bruetsch 1932). In these cases, however, comorbidities and mixed infection with *P. falciparum* were not properly discarded. More recently, one autopsy from India, which had confirmed *P. vivax* monoinfection by PCR, found congestion of alveolar capillaries by monocytic infiltrates and diffuse damage to alveolar membranes consistent with acute respiratory distress syndrome. Parasites were observed in lung tissue as well as in other organs without lesions (Valecha et al. 2009). Despite the fact that this is evidence from a single case, this resembles the findings of cytoadhesion in *P. falciparum* pulmonary disease (Corbett et al. 1989). From measurements of gas transfer and lung volume in non-severe *vivax* patients, the progressive alveolar-capillary dysfunction following treatment was found to be consistent with the greater inflammatory response induced by this parasite (Anstey et al. 2007). As a major complication of severe *vivax* disease, the pathogenesis of respiratory distress requires further investigation into the cytoadhesion that takes place, as suggested by studies of in vitro adhesion and endothelial activation of lung microvasculature. In fact, the host inflammatory response, the endothelial Weibel-Palade body release, and the plasma concentrations of angiopoietin-2, ICAM-1, E-selectin, interleukin (IL)-6 and IL-10 are greater in patients with *vivax* compared to *P. falciparum* malaria (Yeo et al. 2010). As the addition of lipopolysaccharide was found to increase the adhesion of IRBCs to human lung endothelial cells in vitro (Carvalho et al. 2010), it is possible that secondary bacterial infections may release endotoxins that could contribute to the enhanced endothelial activation and cytoadhesion. Thus, coinfections and pathogenesis during *vivax* infection require further investigation.

The role of platelets in cytoadhesion is well-documented for severe *falciparum* malaria. Here, platelets participate in strengthening the adhesion of IRBCs to CD36 (known as glycoprotein IV in platelets), which most likely explains their accumulation in brain microvessels (Grau et al. 2003, Wassmer et al. 2004). Platelets can also participate in tumor necrosis factor (TNF)-induced microvascular pathology (Lou et al. 1997, Wassmer et al. 2006) and platelet-derived microparticles seem to play an important role in endothelial activation (Combes et al. 2006). Despite strong evidence that *vivax* disease triggers greater thrombocytopenia than does *falciparum* disease (Kochar et al. 2010), there are no data available on the ability of these particles to enhance *vivax* cytoadhesion.

As reviewed elsewhere (Clark & Alleva 2009), sequestration in the bone marrow has been shown to cause the dyserythropoiesis observed in *P. falciparum* malaria (Wickramasinghe et al. 1987). Dyserythropoiesis was demonstrated in the bone marrow during *P. vivax* infection, where cytoadhesion was not thought to play a role (Wickramasinghe et al. 1989). As a result, the idea of cytoadhesion as the cause of severe anaemia was abandoned and the presence of increased TNF levels was shown to induce dyserythropoiesis in both species (Clark & Chaudhri 1988). The presence of *P. vivax* parasites in the bone marrow prompts the need for clarification on its implications for disease pathogenesis (Lacerda et al. 2008).

Perspectives and concluding remarks - Demonstration of the cytoadherence of *P. vivax* opens new investigational lines of research. First, proteomic analysis of adhesion proteins and Vir proteins on the surface of infected reticulocytes should further the identification of new parasite ligands. In addition, microscopic analyses from autopsies where coinfections have been ruled out will reinforce the presence of parasite cytoadherence. The role of cytoadherence to the spleen, lungs and placenta in the establishment of chronic infection will further reveal the molecular basis of pathology in *P. vivax* malaria and the demonstration of cytoadhesion in other tissues, such as the kidneys and bone marrow will confirm existing observations of severe disease and the association between cytoadhesion and severe disease syn-

dromes. Furthermore, technological breakthroughs in noninvasive imaging will demonstrate whether *P. vivax*-infected reticulocytes adhere to different organs in vivo and whether this adhesion is associated with severe *vivax* malaria. Ultimately, understanding the factors that determine *P. vivax*-associated morbidity and severe disease will contribute to the control, elimination and eradication of malaria worldwide (Bassat & Alonso 2011).

REFERENCES

Alexandre MA, Ferreira CO, Siqueira AM, Magalhaes BL, Mourao MP, Lacerda MV, Alecrim MG 2010. Severe *Plasmodium vivax* malaria, Brazilian Amazon. *Emerg Infect Dis 16*: 1611-1614.

Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, Doumbo OK, Greenwood B, Hall BF, Levine MM, Mendis K, Newman RD, Plowe CV, Rodriguez MH, Sinden R, Slutsker L, Tanner M 2011. A research agenda to underpin malaria eradication. *PLoS Med 8*: e1000406.

Anstey NM, Handojo T, Pain MC, Kenangalem E, Tjitra E, Price RN, Maguire GP 2007. Lung injury in vivax malaria: pathophysiological evidence for pulmonary vascular sequestration and posttreatment alveolar-capillary inflammation. *J Infect Dis 195*: 589-596.

Anstey NM, Russell B, Yeo TW, Price RN 2009. The pathophysiology of *vivax* malaria. *Trends Parasitol 25*: 220-227.

Baird JK 2007. Neglect of *Plasmodium vivax* malaria. *Trends Parasitol 23*: 533-539.

Baruch DI, Pasloske BL, Singh HB, Bi X, Ma XC, Feldman M, Taraschi TF, Howard RJ 1995. Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell 82*: 77-87.

Bassat Q, Alonso PL 2011. Defying malaria: fathoming severe *Plasmodium vivax* disease. *Nat Med 17*: 48-49.

Beeson JG, Brown GV 2002. Pathogenesis of *Plasmodium falciparum* malaria: the roles of parasite adhesion and antigenic variation. *Cell Mol Life Sci 59*: 258-271.

Bignami A, Bastianelli G 1889. Observations of estivo-autumnal malaria. *Riforma Medica 6*: 1334-1335.

Billings F, Post W 1915. Fatal malaria due to the tertian parasite. *Trans Chicago Path Soc 9*: 209-215.

Boyd MF, Kitchen SF 1937. On the infectiousness of patients infected with *Plasmodium vivax* and *Plasmodium falciparum*. *Am J Trop Med Hyg 17*: 253-262.

Brown KN, Brown IN 1965. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature 208*: 1286-1288.

Bruetsch W 1932. The histopathology of therapeutic (tertian) malaria. *Am J Psychiatry 12*: 19-65.

Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P, Cheng Q, Coulson RM, Crabb BS, Del Portillo HA, Essien K, Feldblyum TV, Fernandez-Becerra C, Gilson PR, Gueye AH, Guo X, Kang'a S, Kooij TW, Korsinczky M, Meyer EV, Nene V, Paulsen I, White O, Ralph SA, Ren Q, Sargeant TJ, Salzberg SL, Stoeckert CJ, Sullivan SA, Yamamoto MM, Hoffman SL, Wortman JR, Gardner MJ, Galinski MR, Barnwell JW, Fraser-Liggett CM 2008. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature 455*: 757-763.

Carvalho BO, Lopes SC, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, Mamoni R, Leite JA, Rodrigues MM, Soares IS, Oliveira TR, Wunderlich G, Lacerda MV, del Portillo HA, Araujo MO, Russell B, Suwanarusk R, Snounou G, Renia L, Costa FT 2010. On the cytoadhesion of *Plasmodium vivax*-infected erythrocytes. *J Infect Dis 202*: 638-647.

Chitnis CE, Sharma A 2008. Targeting the *Plasmodium vivax* Duffy-binding protein. *Trends Parasitol 24*: 29-34.

Clark IA, Alleva LM 2009. Is human malarial coma caused, or merely deepened, by sequestration? *Trends Parasitol 25*: 314-318.

Clark IA, Chaudhri G 1988. Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. *Br J Haematol 70*: 99-103.

Combes V, Coltel N, Faille D, Wassmer SC, Grau GE 2006. Cerebral malaria: role of microparticles and platelets in alterations of the blood-brain barrier. *Int J Parasitol 36*: 541-546.

Corbett CE, Duarte MI, Lancellotti CL, Silva MA, Andrade Junior HF 1989. Cytoadherence in human *falciparum* malaria as a cause of respiratory distress. *J Trop Med Hyg 92*: 112-120.

Cowman AF, Crabb BS 2006. Invasion of red blood cells by malaria parasites. *Cell 124*: 755-766.

David PH, Handunnetti SM, Leech JH, Gamage P, Mendis KN 1988. Rosetting: a new cytoadherence property of malaria-infected erythrocytes. *Am J Trop Med Hyg 38*: 289-297.

del Portillo HA, Fernandez-Becerra C, Bowman S, Oliver K, Preuss M, Sanchez CP, Schneider NK, Villalobos JM, Rajandream MA, Harris D, Pereira da Silva LH, Barrell B, Lanzer M 2001. A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. *Nature 410*: 839-842.

del Portillo HA, Lanzer M, Rodriguez-Malaga S, Zavala F, Fernandez-Becerra C 2004. Variant genes and the spleen in *Plasmodium vivax* malaria. *Int J Parasitol 34*: 1547-1554.

Field JW, Sandosham AA, Fong YL 1963. *The microscopial diagnosis of human malaria*, The Government Press, Kuala Lampur, p. 20-117.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature 419*: 498-511.

Grau GE, Mackenzie CD, Carr RA, Redard M, Pizzolato G, Allasia C, Cataldo C, Taylor TE, Molyneux ME 2003. Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J Infect Dis 187*: 461-466.

Guerra CA, Hay SI, Lucioparedes LS, Gikandi PW, Tatem AJ, Noor AM, Snow RW 2007. Assembling a global database of malaria parasite prevalence for the Malaria Atlas Project. *Malaria J 6*: 17.

Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, Kabaria CW, Tatem AJ, Manh BH, Elyazar IR, Baird JK, Snow RW, Hay SI 2010. The international limits and population at risk of *Plasmodium vivax* transmission in 2009. *PLoS Negl Trop Dis 4*: e774.

Hasler T, Albrecht GR, Van Schravendijk MR, Aguiar JC, Morehead KE, Pasloske BL, Ma C, Barnwell JW, Greenwood B, Howard RJ 1993. An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. *Am J Trop Med Hyg 48*: 332-347.

Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW 2004. The global distribution and population at risk of malaria: past, present, and future. *Lancet Infect Dis 4*: 327-336.

Kitchen SK 1938. The infection of reticulocytes by *Plasmodium vivax*. *Am J Trop Med Hyg 18*: 347.

Kochar DK, Das A, Kochar A, Middha S, Acharya J, Tanwar GS, Gupta A, Pakalapati D, Garg S, Saxena V, Subudhi AK, Boopathi PA, Sirohi P, Kochar SK 2010. Thrombocytopenia in *Plasmodium falciparum*, *Plasmodium vivax* and mixed infection malaria: a study from Bikaner (Northwestern India). *Platelets 21*: 623-627.

Kochar DK, Das A, Kochar SK, Saxena V, Sirohi P, Garg S, Kochar A, Khatri MP, Gupta V 2009. Severe *Plasmodium vivax* malaria: a report on serial cases from Bikaner in northwestern India. *Am J Trop Med Hyg 80*: 194-198.

Kochar DK, Saxena V, Singh N, Kochar SK, Kumar SV, Das A 2005. *Plasmodium vivax* malaria. *Emerg Infect Dis 11*: 132-134.

Krotoski WA 1985. Discovery of the hypnozoite and a new theory of malarial relapse. *Trans R Soc Trop Med Hyg 79*: 1-11.

Lacerda MV, Hipolito JR, Passos LN 2008. Chronic *Plasmodium vivax* infection in a patient with splenomegaly and severe thrombocytopenia. *Rev Soc Bras Med Trop 41*: 522-523.

Lomar AV, Vidal JE, Lomar FP, Barbas CV, Matos GJ, Boulos M 2005. Acute respiratory distress syndrome due to *vivax* malaria: case report and literature review. *Braz J Infect Dis 9*: 425-430.

Lou J, Donati YR, Juillard P, Giroud C, Vesin C, Mili N, Grau GE 1997. Platelets play an important role in TNF-induced microvascular endothelial cell pathology. *Am J Pathol 151*: 1397-1405.

Maegraith BG 1948. *Pathological processes in malaria and blackwater fever*, Charles C Thomas, Springfield, 430 pp.

Mendis K, Sina BJ, Marchesini P, Carter R 2001. The neglected burden of *Plasmodium vivax* malaria. *Am J Trop Med Hyg 64*: 97-106.

Mercereau-Puijalon O, Menard D 2010. *Plasmodium vivax* and the Duffy antigen: a paradigm revisited. *Transfus Clin Biol 17*: 176-183.

Miller LH, Baruch DI, Marsh K, Doumbo OK 2002. The pathogenic basis of malaria. *Nature 415*: 673-679.

Miller LH, Good MF, Milon G 1994. Malaria pathogenesis. *Science 264*: 1878-1883.

Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, del Portillo HA 2009. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect Dis 9*: 555-566.

Pasternak ND, Dzikowski R 2009. PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite *Plasmodium falciparum*. *Int J Biochem Cell Biol 41*: 1463-1466.

Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM 2007. *Vivax* malaria: neglected and not benign. *Am J Trop Med Hyg 77*: 79-87.

Resende M, Ditlev SB, Nielsen MA, Bodevin S, Bruun S, Pinto VV, Clausen H, Turner L, Theander TG, Salanti A, Dahlback M 2009. Chondroitin sulphate A (CSA)-binding of single recombinant duffy-binding-like domains is not restricted to *Plasmodium falciparum* erythrocyte membrane protein 1 expressed by CSA-binding parasites. *Int J Parasitol 39*: 1195-1204.

Santos-Ciminera PD, Alecrim MG, Roberts DR, Quinnan GV Jr 2007. Molecular epidemiology of *Plasmodium vivax* in the state of Amazonas, Brazil. *Acta Trop 102*: 38-46.

Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Peterson DS, Pinches R, Newbold CI, Miller LH 1995. Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell 82*: 101-110.

Su XZ, Heatwole VM, Wertheimer SP, Guinet F, Herrfeldt JA, Peterson DS, Ravetch JA, Wellems TE 1995. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell 82*: 89-100.

Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, Lampah DA, Price RN 2008. Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med 5*: e128.

Trager W, Jensen JB 1976. Human malaria parasites in continuous culture. *Science 193*: 673-675.

Udagama PV, David PH, Peiris JS, Ariyaratne YG, Perera KL, Mendis KN 1987. Demonstration of antigenic polymorphism in *Plasmodium vivax* malaria with a panel of 30 monoclonal antibodies. *Infect Immun 55*: 2604-2611.

Udomsanpetch R, Thanikkul K, Pukrittayakamee S, White NJ 1995. Rosette formation by *Plasmodium vivax*. *Trans R Soc Trop Med Hyg 89*: 635-637.

Valecha N, Pinto RG, Turner GD, Kumar A, Rodrigues S, Dubhashi NG, Rodrigues E, Banaulikar SS, Singh R, Dash AP, Baird JK 2009. Histopathology of fatal respiratory distress caused by *Plasmodium vivax* malaria. *Am J Trop Med Hyg 81*: 758-762.

Wassmer SC, Lepolard C, Traore B, Pouvelle B, Gysin J, Grau GE 2004. Platelets reorient *Plasmodium falciparum*-infected erythrocyte cytoadhesion to activated endothelial cells. *J Infect Dis 189*: 180-189.

Wassmer SC, Souza JB, Frere C, Candal FJ, Juhan-Vague I, Grau GE 2006. TGF-beta1 released from activated platelets can induce TNF-stimulated human brain endothelium apoptosis: a new mechanism for microvascular lesion during cerebral malaria. *J Immunol 176*: 1180-1184.

Wickramasinghe SN, Looareesuwan S, Nagachinta B, White NJ 1989. Dyserythropoiesis and ineffective erythropoiesis in *Plasmodium vivax* malaria. *Br J Haematol 72*: 91-99.

Wickramasinghe SN, Phillips RE, Looareesuwan S, Warrell DA, Hughes M 1987. The bone marrow in human cerebral malaria: parasite sequestration within sinusoids. *Br J Haematol 66*: 295-306.

Yeo TW, Lampah DA, Tjitra E, Piera K, Gitawati R, Kenangalem E, Price RN, Anstey NM 2010. Greater endothelial activation, Weibel-Palade body release and host inflammatory response to *Plasmodium vivax*, compared with *Plasmodium falciparum*: a prospective study in Papua, Indonesia. *J Infect Dis 202*: 109-112.

MALARIA JOURNAL

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In vitro and *in vivo* assessment of the anti-malarial activity of *Caesalpinia pluviosa*

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Abstract

Background: To overcome the problem of increasing drug resistance, traditional medicines are an important source for potential new anti-malarials. *Caesalpinia pluviosa*, commonly named "sibipiruna", originates from Brazil and possess multiple therapeutic properties, including anti-malarial activity.

Methods: Crude extract (CE) was obtained from stem bark by purification using different solvents, resulting in seven fractions. An MTT assay was performed to evaluate cytotoxicity in MCF-7 cells. The CE and its fractions were tested *in vitro* against chloroquine-sensitive (3D7) and -resistant (S20) strains of *Plasmodium falciparum* and *in vivo* in *Plasmodium chabaudi*-infected mice. *In vitro* interaction with artesunate and the active *C. pluviosa* fractions was assessed, and mass spectrometry analyses were conducted.

Results: At non-toxic concentrations, the 100% ethanolic (F4) and 50% methanolic (F5) fractions possessed significant anti-malarial activity against both 3D7 and S20 strains. Drug interaction assays with artesunate showed a synergistic interaction with the F4. Four days of treatment with this fraction significantly inhibited parasitaemia in mice in a dose-dependent manner. Mass spectrometry analyses revealed the presence of an ion corresponding to *m/z* 303.0450, suggesting the presence of quercetin. However, a second set of analyses, with a quercetin standard, showed distinct ions of *m/z* 137 and 153.

Conclusions: The findings show that the F4 fraction of *C. pluviosa* exhibits anti-malarial activity *in vitro* at non-toxic concentrations, which was potentiated in the presence of artesunate. Moreover, this anti-malarial activity was also sustained *in vivo* after treatment of infected mice. Finally, mass spectrometry analyses suggest that a new compound, most likely an isomer of quercetin, is responsible for the anti-malarial activity of the F4.

Background

One of the principal reasons for malaria's high morbidity and mortality is the widespread presence of drug-resistant strains of the parasite, resulting in the dramatically decreased efficacy of the available anti-malarial drugs, such as chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) [1].

The compounds most widely used to treat malaria, quinine and artemisinin, are derived from traditional medicine and plant extracts [2]. Quinine was the first drug successfully used to treat malaria. However, this alkaloid has a high level of toxicity and a short pharmacological half-life, which limit its use [2,3]. Currently, artemisinin-based combination treatment (ACT) is the therapy of choice for uncomplicated *Plasmodium falciparum* malaria in areas of widespread parasite CQ-resistance [4]. However, failure to clear parasites after ACT treatment has recently been reported on the Cambodia-Thailand border, and genes related to artemisinin resistance have been discovered [5-9]. Furthermore, no new class of anti-malarial has been introduced since 1996 [10], and the most successful malaria vaccine was only partially efficient and short lived [11]. Therefore, the discovery of new potential anti-malarial compounds is urgently needed.

Caesalpinia pluviosa, commonly named "sibipiruna", is a leguminous of the Fabaceae family that is originated from Brazil. This genus is a rich source of furanoditerpenoids and has demonstrated multiple therapeutic properties, including antiviral [12-14], antimicrobial

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[15,16], anti-inflammatory [17,18], and antioxidant [19,20] activities. Preliminary studies have shown that *C. pluviosa* crude extract (CE) had *in vitro* anti-malarial activity against a CQ-resistant strain [21]. However, that work focused only on the CE, and no parasite inhibition was observed in an *in vivo* test. In the present study, the *in vitro* anti-malarial activities of *C. pluviosa* extracts and the fractions effective against CQ- resistant and -sensitive *P. falciparum* strains, alone or in combination with artesunate, have been evaluated and identified.

The cytotoxic properties of these plant-derived materials were determined and the *in vivo* effects in *Plasmodium chabaudi*-infected mice were also studied. Finally, mass spectrometry analyses were carried out to characterize new potential molecules with anti-malarial activity.

Methods

Plant extraction and fractionation

Stem bark from *C. pluviosa* was collected and deposited at the campus of Universidade Estadual de Maringá, Brazil, in September 2006 as voucher #HUEM 12492. All plant material was ground and subjected to a turbo-extraction process with 50% ethanol-water for 15 min at a T<40°C. After evaporation of the organic solvent using a rotavapor under reduced pressure at 40°C, the CE (50 g) was lyophilized. CE was dissolved in water (500 mL) and extracted with ethyl acetate (10 × 500 mL). After removing the organic solvents by rotavapor under reduced pressure, aqueous (F1) and ethyl acetate (F2) fractions were formed. The F2 fraction was chromatographed by CC (chromatographic column) on Sephadex LH-20, resulting in five sub-fractions: 50% ethanolic-water (F3; 2.17 g), 100% ethanolic (F4; 0.21 g), 50% methanolic-water (F5; 0.06 g), 100% methanolic (F6; 0.17 g) and 70% acetone-water (F7; 0.08 g). All fractions were concentrated under reduced pressure at 40°C for solvent evaporation. These samples were lyophilized and used in biological tests, as described below. Concentrations of *C. pluviosa* CE/fractions were calculated on a dry material basis. Figure 1 summarizes the *C. pluviosa* extraction and its fractionation process.

Cultivation of *Plasmodium falciparum* infected-erythrocytes *P. falciparum*

Plasmodium falciparum chloroquine-sensitive (3D7) [22] and chloroquine-resistant (S20) [23] strains were cultured in a candle jar as described elsewhere [24]. Briefly, *P. falciparum* infected-erythrocytes (Pf-iE) were cultivated in fresh-type O^+ human erythrocytes (UNICAMP, Blood Bank, Brazil) suspended at 4% final haematocrit in complete medium (RPMPI-1640 containing 10% homologous human plasma and 25 mM HEPES buffer, pH 7.4).

Cytotoxicity assays

The cytotoxicities of the plant CE/fractions were assessed by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to evaluate their effects on the proliferation of a human breast cancer cell line, MCF-7 [25,26]. Cells were cultivated in DMEM/HAM-F12 medium supplemented with 10% heat-inactivated foetal bovine serum, penicillin (1 unit/mL) and streptomycin (1 unit/mL) in a humidified atmosphere of 5% CO_2 at 37°C. MCF-7 cells were seeded in 96-well plates at a density of 2×10^4 cells per well and incubated with different concentrations of plant samples. After 48 h, 5 mg/mL of MTT solution was added for 4 h and formazan crystals were resuspended in 50 μL of isopropanol. The absorbances (A_{590}) of cells containing medium (control) or in the presence of different concentrations of the CE/fractions (tested) were determined using an ELISA reader (Asys-Expertplus, UK). Data were calculated as the percentage of inhibition using the following formula: % inhibition = $[1 - (A_t/A_c)] \times 100$. A_t and A_c refer to the absorbance of the tested *C. pluviosa* CE/fractions and the control, respectively.

The toxicity of the *C. pluviosa* CE/fractions was also determined in non-infected erythrocytes (niE) in the presence of different plant sample concentrations or untreated cells (control). The niE were cultured in complete medium at 37°C in candle jars, and the red blood cell density (RBCD) was determined after 48 h incubation with the aid of a Neubauer chamber. The percentage of RBCD, relative to 0 h, was calculated by the following formula: % RBCD = [1 - (n° niE treated with CE and fractions per hour after incubation/n° niE per or at 0 h)] × 100. Non-toxic samples were those in which no significant inhibition on MCF-7 growth or RBCD, relative to the control, was observed.

In vitro anti-malarial activity of *C. pluviosa* extract and its fractions

[^{3}H] hypoxanthine incorporation assays were used to determine *C. pluviosa* anti-malarial activity against Pf-iE growth as previously described [27]. Background values were determined by incubation with niE. Parasite growth in the presence of *C. pluviosa* extract and fractions (sample) was compared to control cultures (medium only). Inhibition of parasite growth was calculated according to the following formula: % inhibition = [1 - (n° niE treated with CE and fractions per hour after incubation/n° niE per or at 0 h)] × 100. The anti-malarial activities of the CE/fractions were classified according to their IC_{50} (μg/mL) values, defined as that concentration of compound which inhibits growth by 50% relative to untreated controls [21]. Fractions of $IC_{50} < 5$ were considered active, fractions of $10 > IC_{50} > 5$ represented moderate activity and fractions with IC_{50} values > 10 were classified as inactive.

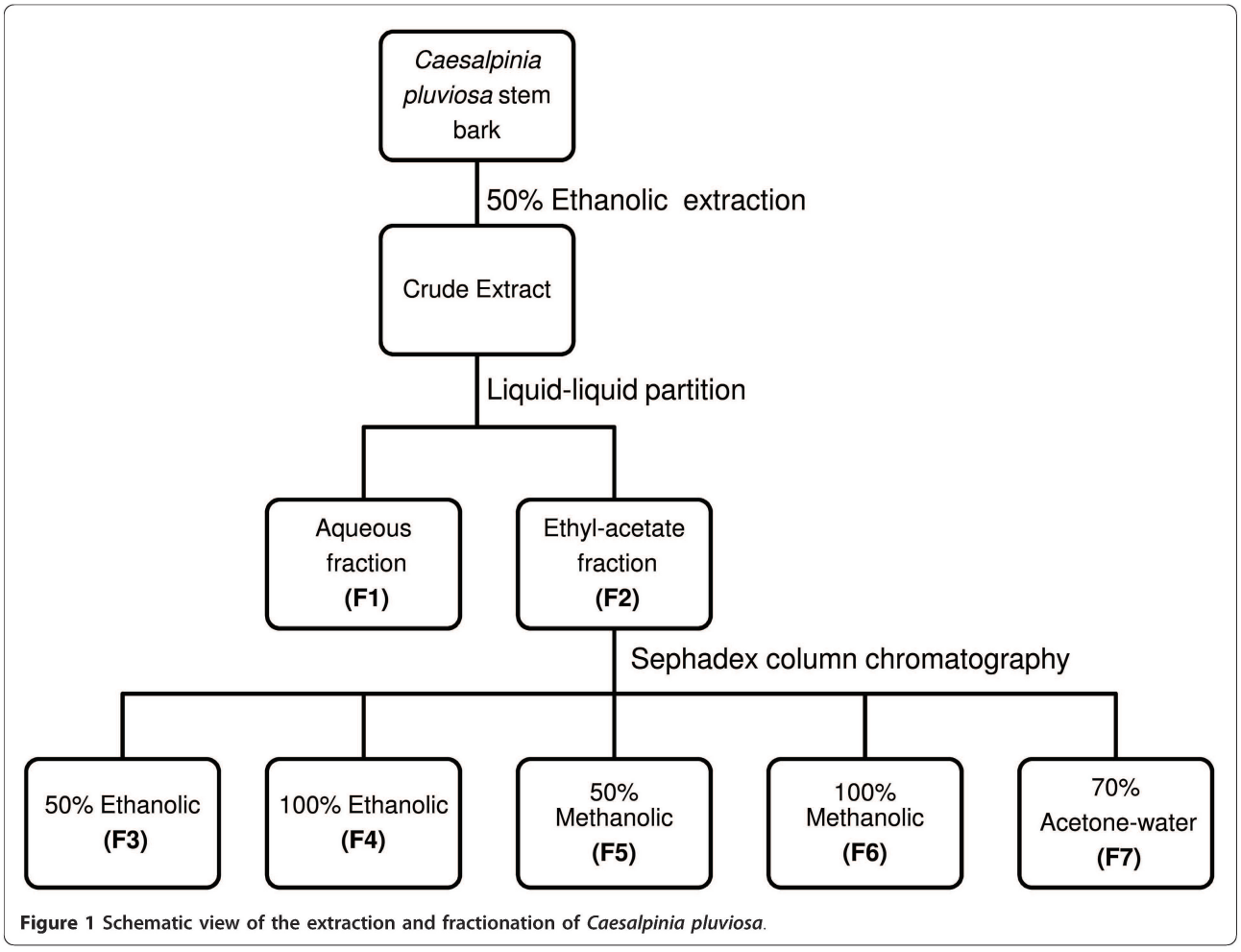

Figure 1 Schematic view of the extraction and fractionation of *Caesalpinia pluviosa*.

In vivo anti-malarial activity of plant extract/fractions

In vivo anti-malarial activity of plant fractions was verified in C57BL/6 mice (7-10 weeks-old, weighing 20 ± 3 g) purchased from the Centro de Bioterismo-UNICAMP, Brazil, and maintained in specific pathogen-free animal facility. All experiments and procedures were approved by the UNICAMP Ethical Committee for Animal Research (protocol # 1806-1). Groups of 10 mice were infected intraperitoneally (i.p) with 10^6 iE of *P. chabaudi chabaudi* AS (PchAS). PchAs is a non-lethal strain kindly provided by Hernando Del Portillo (Department of Parasitology, ICB, USP, São Paulo-SP, Brazil and currently at CRESIB, Barcelona, Spain). One hour post-infection (p.i), groups of 10 animals were injected i.p with different concentrations (75, 50, 25 mg/kg/day) of the F4 and F5 fractions (50, 25 mg/kg/day) diluted in 200 μL PBS with 1.25% DMSO. Mice were treated for 4 days (day 0-3 p.i) according to previous studies [28,29]. Parasitaemia was monitored daily by microscopic examination of Giemsa-stained thin blood smears prepared from mouse tail blood beginning on the third day p.i. Mice in the control group received 200 μL of 1.25% DMSO diluted in PBS.

In vitro compounds interaction on the Pf-iE

The artesunic acid (artesunate) used for combined treatment with F4 fractions was derived from artemisinin isolated from 1 kg of dried-plant material collected from CPQBA's experimental field (hybrid Ch × Viet 55) and extracted with ethanol as previously described [30]. Subsequent semi-synthesis procedures provided pure artesunic acid (98%), which was further dissolved in sodium bicarbonate solution (0.5%) prior to use as sodium artesunate on Pf-iE. Artesunate was identified by comparing the experimental product to a commercial sample (Aldrich® 98%, Sigma-Aldrich, USA). The 3D7 and S20 strains of *P. falciparum* (4% parasitaemia and 2% haematocrit) were incubated for 48 h in the presence of artesunate and the F4 fraction, solubilized in 1.25% DMSO. *C. pluviosa* fractions were dispensed into the 96-well micro-titre plates at different concentration (ng/mL) combinations of the F4 fraction and artesunate as

follows: 4000-1.8, 2000-0.9, 1000-0.45, 500-0.225. The combination was performed by adding 50 μL of *C. pluviosa* fraction (4000 ng/mL) to 50 μL artesunate (1.8 ng/mL) and so on. Parasitaemia was analysed in thin blood smears, and the inhibition was compared to that of the control (100 μL of 1.25% DMSO) that represented 100% of Pf-iE growth. Corresponding IC_{50} values were determined for each drug alone and in combination [31]. The synergism degree was evaluated as described previously [32]. The sum of fractional inhibition concentration (SFIC) was calculated using the formula: $K = A_c/A_e + B_c/B_e$, where K is the value corresponding to SFIC, A_c and B_c are the equally effective concentrations (IC_{50}) when used in combination, and A_e and B_e are the equally effective concentrations used alone. The *in vitro* drug interaction was classified as follows: SFIC < 1 denotes synergism, 1 < SFIC < 2 denotes additive interaction, and SFIC≥2 denotes antagonism [33].

Mass spectrometry

Samples of the F4 fraction were dissolved in HPLC grade MeOH, and 10 μL of this solution was diluted in 1 mL of solvent ($MeOH/H_2O$ [1:1] with 0.1% formic acid [v/v]). A Q-TOF mass spectrometer (Micromass, Manchester, UK) with an electrospray source was used to perform Electrospray Ionization/Mass Spectrometry (ESI-MS) and ESI-MS/MS analyses. The mass spectrometer was operated in the positive ion mode. The ESI source unit was operated at a desolvation temperature of 100°C, with a capillary voltage of 3.5 kV and cone voltage of 40 eV. Samples were directly infused at a rate of 10 μL min^{-1} into the ion source using a syringe pump (Pump 11, Harvard Apparatus, Holliston, USA). The spectra were acquired in the interval of 100 to 2000 *m/z* and accumulated for 1 min. ESI-MS/MS were obtained for ions of interest using collision energies ranging from 10 to 50 eV. The collision gas pressure (argon) was optimized to produce extensive fragmentation of the ions under investigation. To calculate the theoretical masses of the compounds, MassLynx 4.1 software was used. The error among the theoretical and experimental masses was calculated according to the following formula: $E = (m_{ex} - m_t/m_t) \times 10^6$; where m_{ex} is an experimental mass and m_t is a theoretical mass.

Statistical analysis

Toxicity data were analysed using ANOVA or Mann-Whitney tests. Statistical significance between treated and non-treated *P. falciparum*-infected erythrocytes was determined using the Mann-Whitney *U* test. The inhibition of parasitaemia, resulting from sole or combined treatment with artesunate and F4, was compared using the Kruskal-Wallis test. For analysis of *in vivo* treatment with the F4 fraction, an ANOVA test was used. Calculations were performed using BioEstat™ version 3.0 (CNPq, Brazil), and values were considered significant when $p < 0.05$.

Results

Cytotoxicity assessment of *C. pluviosa*

The cytotoxic potential of the CE and fractions (F1-F7) was determined on MCF-7 cells by means of MTT assays. As shown in Table 1, plant CE from all fractions was highly cytotoxic at concentrations of 1600 and 400 μg/mL, significantly inhibiting cell growth from 20% (F4) to 66% (F6) ($p < 0.05$). In contrast, at 100 μg/mL the F4 and the F7 fractions did not significantly reduce cell growth compared to the control. The F1 and F5 fractions slightly, but significantly, inhibited MCF-7 cell development. No inhibition was observed by 25 μg/mL of any fraction. To determine if this low toxicity could be extant to niE, the RBCD percentage of plant CE and fractions relative to the control was determined at concentrations varying from 0.19 to 25 μg/mL.

In vitro determination of *C. pluviosa* anti-malarial activity

To evaluate the anti-malarial effects of plant CE/fractions against chloroquine-sensitive (3D7) and -resistant (S20) *P. falciparum* strains, parasite growth inhibition was measured by determining [^{3}H] hypoxanthine incorporation at different concentrations (0.19 - 25 μg/mL). The anti-malarial activity IC_{50} values were compared for *C. pluviosa* CE and fractions. As shown in Table 2 and consistent with a previous study [21], the F1 and the F7 fractions were inactive (> 10 μg/mL) against both strains of parasites. However, the IC_{50} values of the CE and the other fractions were < 5 μg/mL, indicating inhibition activity. Of these, the F3 fraction and the CE presented higher IC_{50} values than the F4 and F5 fractions.

The inhibition curve of the CE and the fractions that presented anti-malarial activity (IC_{50} < 5 μg/mL) are shown in Figure 2(A-D). The CE and the F2, F4 and F5

Table 1 Growth inhibition (%)[a] of MCF-7 cells *in vitro* treatment of *Caesalpinia pluviosa* assessed by MTT assay.

	Concentrations (μg/mL)			
Samples	1600	400	100	25
CE	33.13 ± 10.88*	26.42 ± 19.26	21.95 ± 14.06	NT[a]
F1	45.43 ± 5.60*	44.21 ± 8.48*	6.98 ± 6.11	NT
F2	50.81 ± 13.25*	27.98 ± 7.15*	18.83 ± 17.15	NT
F3	41.67 ± 10.26*	44.31 ± 13.98*	45.73 ± 6.88*	NT
F4	55.90 ± 9.37*	20.19 ± 3.38*	NT	NT
F5	46.98 ± 11.73*	37.85 ± 7.86*	8.28 ± 2.56*	NT
F6	66.25 ± 5.45*	53.82 ± 4.69*	17.39 ± 14.27	NT
F7	62.66 ± 9.91*	44.93 ± 18.61*	NT	NT

[a]Values are expressed as the mean of triplicates ± SD.

[b]NT: Non toxic.

*$p < 0.05$

Table 2 *C. pluviosa* IC_{50} values (μg/mL) for crude extract and its fractions

Plant Samples	IC_{50} 3D7	IC_{50} S20
CE	4.84 ± 0.17	3.41 ± 2.45
F1	10.98 ± 6.01	13.29 ± 2.70
F2	2.13 ± 0.94	2.07 ± 1.38
F3	4.55 ± 2.05	5.49 ± 1.26
F4	0.72 ± 0.29	1.25 ± 0.38
F5	0.59 ± 0.33	1.72 ± 0.27
F6	1.30 ± 0.43	3.61 ± 2.46
F7	17.19	ND

The data shown are expressed as the mean of quadruplicates ± SD.
ND: Not determined.

fractions were capable of inhibiting the growth of both parasite strains in a dose-dependent manner. The CE and all three fractions inhibited parasite development at concentrations of 25 and 12.5 μg/mL. At 6.25 μg/mL, inhibition was observed in the fractionated, but not crude extract. Notably, only the F4 fraction was able to sustain the inhibition on parasite growth throughout several concentrations, as shown in Figure 2C.

Evaluation of the *in vivo* anti-malarial activity of *C. pluviosa*

After demonstrating the capacity of F4 and F5 fractions to control parasite burden *in vitro*, we evaluated whether this activity could be sustained in a mouse model of infection. *Plasmodium chabaudi*-infected mice were treated with different concentrations of both fractions for four days (day 0-3 p.i), starting at 1 h p.i. As shown in Figure 3, the parasitaemia of infected-mice was significantly reduced in a dose-dependent manner when the F4 fraction was administered at doses of 50 and 25 mg/kg/day during days 5-8 p.i. The highest dose (50 mg/kg/day) inhibited parasitaemia on days 6 and 7 p.i by 79.4% and 74.1%, respectively (Table 3). At the lower dose of 25 mg/kg/day, a significant reduction of parasite growth was also observed, although the percentage of inhibition achieved on day 6 p.i was 63.6% (Table 3). Doses of 75 mg/kg/day of the F4 and 50 mg/kg/day of the F5 were fatal to 40% of animals (n = 10) on days 2 and 4 p.i, respectively. In another set of experiments, 50 mg/kg/day of the F4 fraction (during days 3-7 p.i) inhibited parasite growth by 51% to 81% (Table 3).

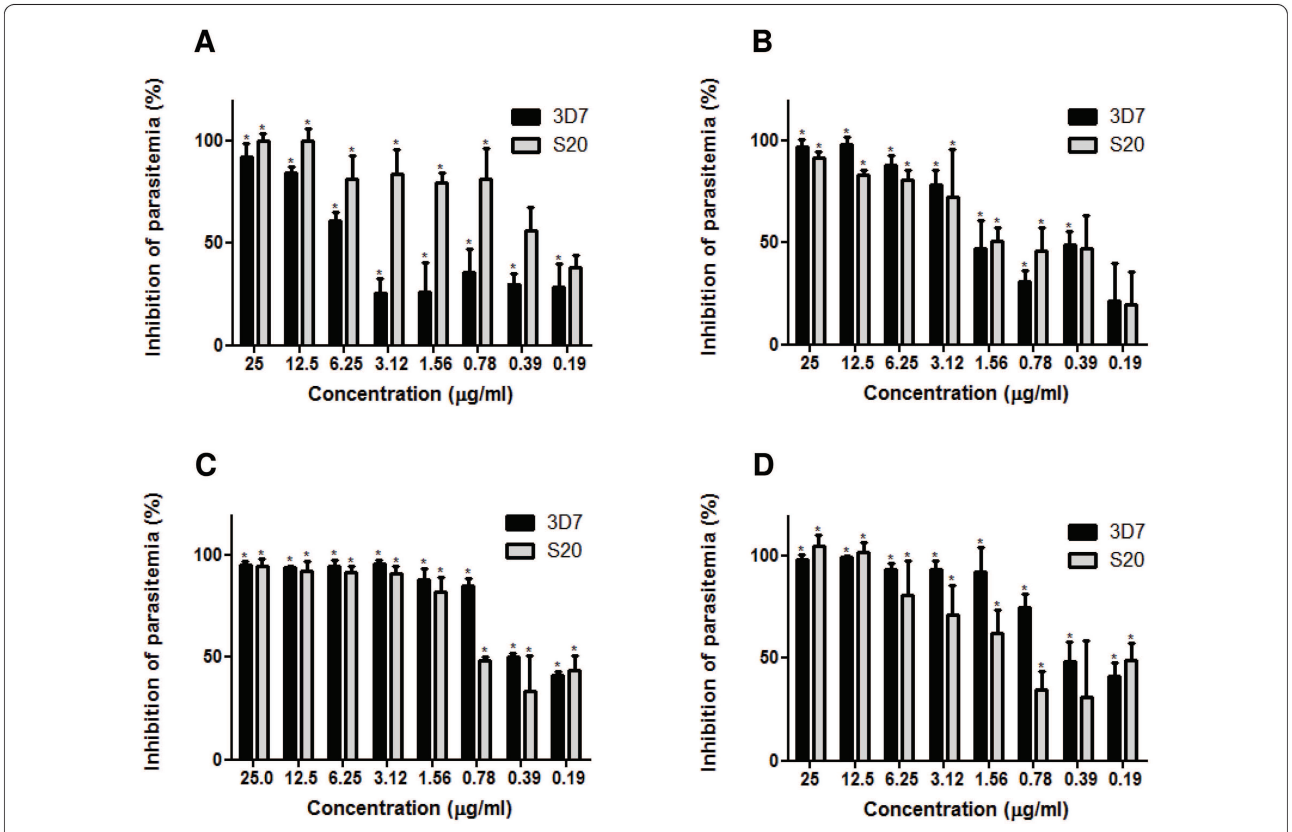

Figure 2 Assessment of *C. pluviosa* anti-malarial activity against *P. falciparum*. Inhibition (%) of parasite growth of chloroquine-sensitive (3D7) and -resistant (S20) strains of *P. falciparum* cultivated 48 h at 37°C with *C. pluviosa* (**A**) crude extract and (**B**) ethyl acetate, (**C**) 100% ethanolic, (**D**) 50% methanolic fractions. The results are expressed as the mean of quadruplicates ± SD. (*$p < 0.05$ vs. untreated parasites).

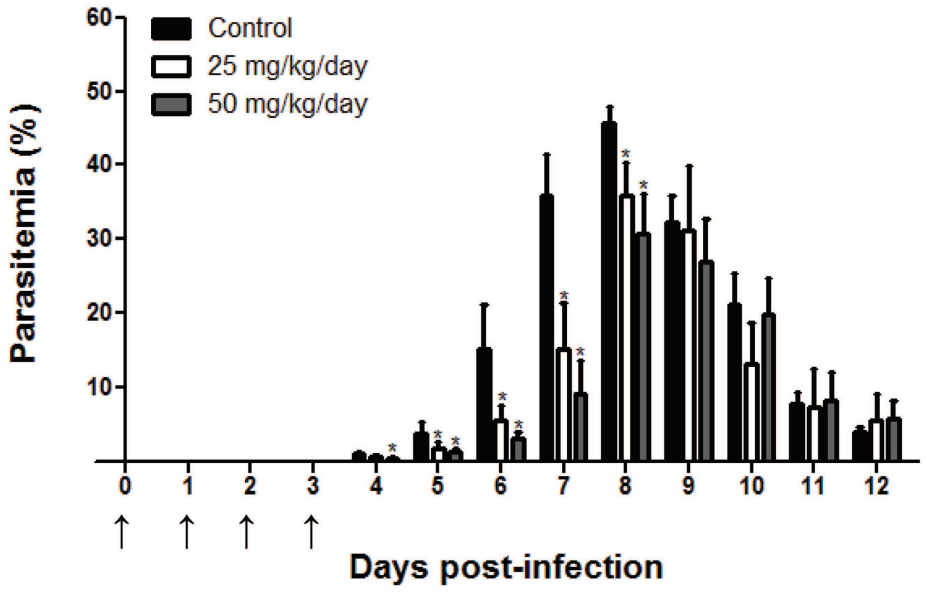

Figure 3 Effect of the *C. pluviosa* F4 fraction on murine-derived *Plasmodium*. Groups of ten C57BL/6 mice infected i.p with 10^6 iE *Plasmodium chabaudi chabaudi* were left untreated or treated with different doses of the F4 fraction administered i.p for 4 consecutive days (days 0 to 3 p.i), starting on day 0 at 1 h p.i. The parasitaemia levels were determined daily until day 12 p.i. Results are expressed as the mean of a group mice ± SD. (*$p < 0.05$ vs. untreated mice group). The administration of F4 fraction is indicated by arrows.

Evaluation of the interaction between F4 fraction and artesunate

As shown in Figure 4, artesunate combined with the F4 fraction significantly reduced parasite development compared to the reduction observed when either compound was tested alone. The IC_{50} values (ng/mL) of the F4 fraction and artesunate alone were 3237 and 1.324, respectively, whereas the combination of the two led to values of 1402 and 0.630 respectively. Calculation of the SFIC value (0.908) indicated a synergic effect of the F4 fraction with artesunate.

Molecular composition analysis of fractions from *C. pluviosa*

After assessing anti-malarial activity *in vitro* and *in vivo*, mass spectrometry was performed to characterize and identify the possible molecules involved in the anti-malarial activity. To accomplish this, the F4 (active) and F7 (inactive) fractions were ionized, and their spectra were analysed. As shown in Figure 5, their spectra displayed similarities, including *m/z* 102.1314, 150.1387, 195.0256, 288.3157 and 415.2506. However, the most active fraction (F4) presented a distinct signal of *m/z* 303.0450.

As these compounds were protonated $[M+H]^+$, substances with a molecular mass (MM) of 302 in *Caesalpinia* spp. were searched, corresponding to the 303.0450. Table 4 shows all compounds found in the literature with 302 MM, which include ellagic acid [34], protosappanin C [35], sappanone B [36], 3'-deoxy-4-*O*-methylepisappanol [36], quercetin [37], and voucapen-5α-ol [38]. The high resolution and accuracy of mass

Table 3 Parasitemia inhibition [a] of *Plasmodium chabaudi*-infected mice left untreated or treated with different doses of the 100% ethanolic fraction for 4 days (0-3 post-infection) from two independent experiments

	Doses (mg/kg/day)	Days post-infection					
		D3	D4	D5	D6	D7	D8
Experiment							
# 1	50	ND	54.22 ± 14.20*	66.72 ± 13.16*	79.44 ± 5.77*	72.17 ± 12.28*	32.74 ± 11.59*
	25	ND	29.10 ± 11.51	51.46 ± 23.74*	63.64 ± 13.71*	57.45 ± 17.15*	21.50 ± 9.82*
# 2	50	81.16 ± 7.23*	90.45 ± 5.53*	91.06 ± 5.40*	86.04 ± 5.38*	51.04 ± 16.45*	NI

[a] Values are expressed as the mean of parasitemia inhibition (%).

NI: No inhibition.

ND: Not determined.

* $p < 0.05$ vs. control

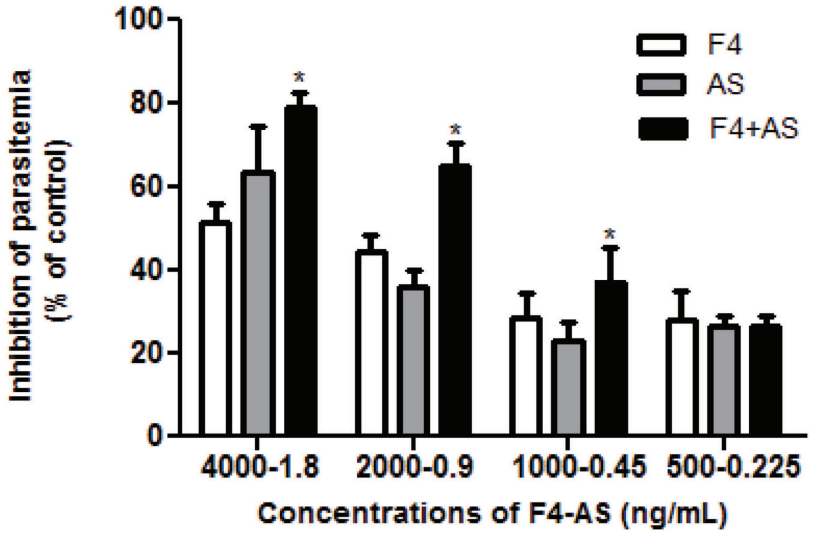

Figure 4 Evaluation of the interaction between the F4 fraction and artesunate. Inhibition (%) of parasite development (3D7) cultivated 48 h at 37°C with different concentration (ng/mL) combinations of the F4, artesunate (AS) or as a combined solution (F4+AS). The results are expressed as the mean of triplicates ± SD. (*$p < 0.05$ vs. non-combined treatment).

spectrometry allows up to 20 portions per million (ppm) masses error. After calculating the theoretical masses of all retrieved compounds and the error among theoretical and experimental masses, quercetin was the only compound with an acceptable error (E = 18.15 ppm).

To confirm the hypothesis that quercetin was the corresponding molecule for the *m/z* 303.0450 signal found in the F4 fraction, a new set of ESI-MS analyses using a quercetin standard (Sigma-Aldrich, USA) and the F4 fraction was performed (Figure 6). Although both

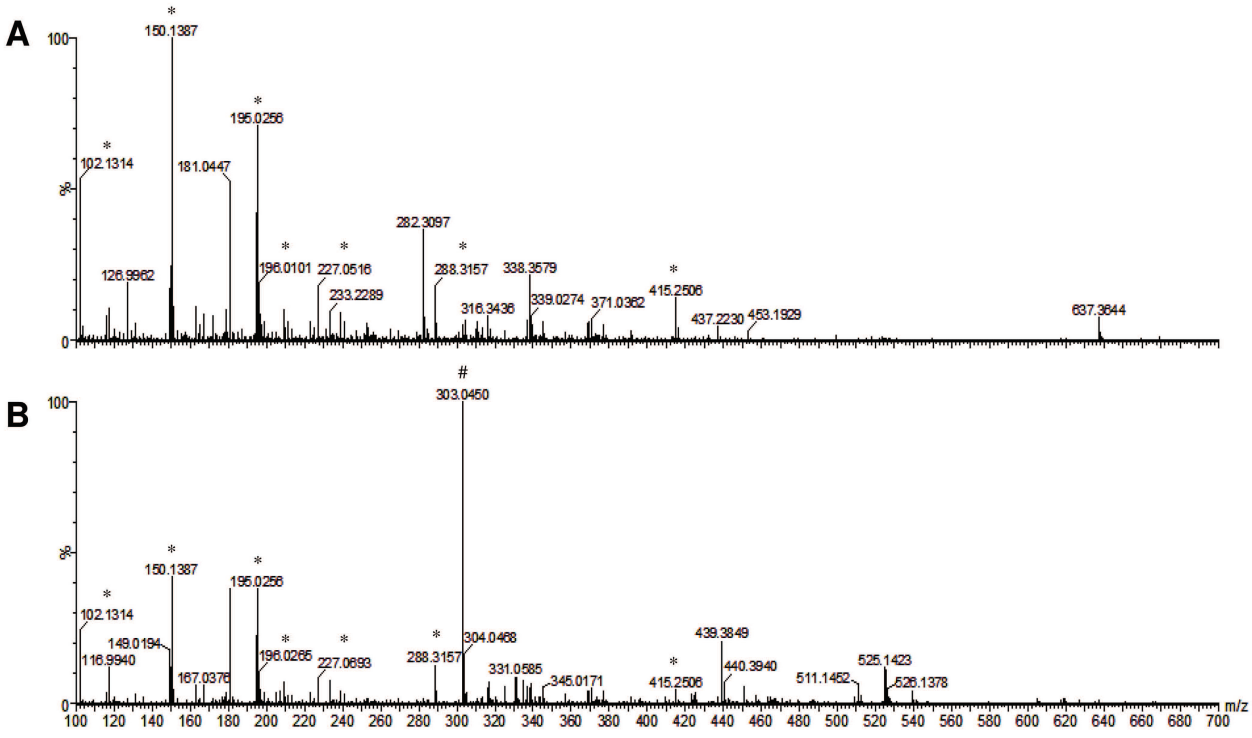

Figure 5 Electrospray Ionization/Mass Spectrometry (ESI-MS) analyses of the (A) 70% acetone-water and (B) 100% ethanolic fractions. Ions are marked either as those detected in both spectra (*) or only in the F4 fraction (#).

Table 4 Molecular composition identified in *Caesalpinia* spp

Composition	Molecular formula	$[M+1]^+$	E (ppm)	References
1	$C_{14}H_6O_8$	303.0141	101.97	[34]
2	$C_{16}H_{14}O_6$	303.0869	138.24	[35]
3	$C_{16}H_{14}O_6$	303.0869	138.24	[36]
4	$C_{17}H_{18}O_5$	303.1233	258.31	[36]
5	$C_{15}H_{10}O_7$	303.0505	18.15	[37]
6	$C_{20}H_{30}O_2$	303.2324	618.01	[38]

1. Ellagic acid, 2. Protosappanin C, 3. Sapponone B, 4. 3-deoxy-4-O-methylepisappanol, 5. Quercetin, 6. Voucapen-5α-ol. The compounds 2 and 3 are isomeric forms. Compounds with molar masses at 302 and its molecular formula were obtained from literature. $[M+1]^+$, molar masses plus a proton. E: Error between theoretical and experimental masses.

compounds presented some similarities in their spectra, distinct signs of *m/z* 137 and 153 were observed only in the quercetin standard.

Discussion

Medicinal plants present a promising source of novel therapeutic agents for the treatment of many tropical diseases, including those caused by protozoa. These data confirmed the anti-malarial activity of the CE [21,39] and indicate that the F4 fraction possibly contains the main compound related to this activity.

When assessing anti-malarial activity *in vitro*, as classified previously [21], the CE/fractions of *C. pluviosa* have been presented inhibitory activity against chloroquine-sensitive (3D7) and -resistant (S20) strains of *P. falciparum*. These findings differ from previous studies [21,39], which reported that the CE is inactive against the chloroquine-sensitive strain (IC_{50} = 15 μg/mL). Moreover, the anti-malarial activity of the CE found here was twofold more efficient against the chloroquine-resistant strain (IC_{50} = 3.41 μg/mL) when compared with that found in the same previous studies (IC_{50} = 8.3 μg/mL). The discrepancy in IC_{50} values might be due to differences in the phytochemical and pharmacokinetic properties of the extract, which can vary depending on the origin, genotype and harvest period of the plants [40,41].

Because the F4 and F5 fractions presented the strongest inhibitory activity *in vitro*, these two fractions were chosen for evaluation in *P. chabaudi*-infected mice. After

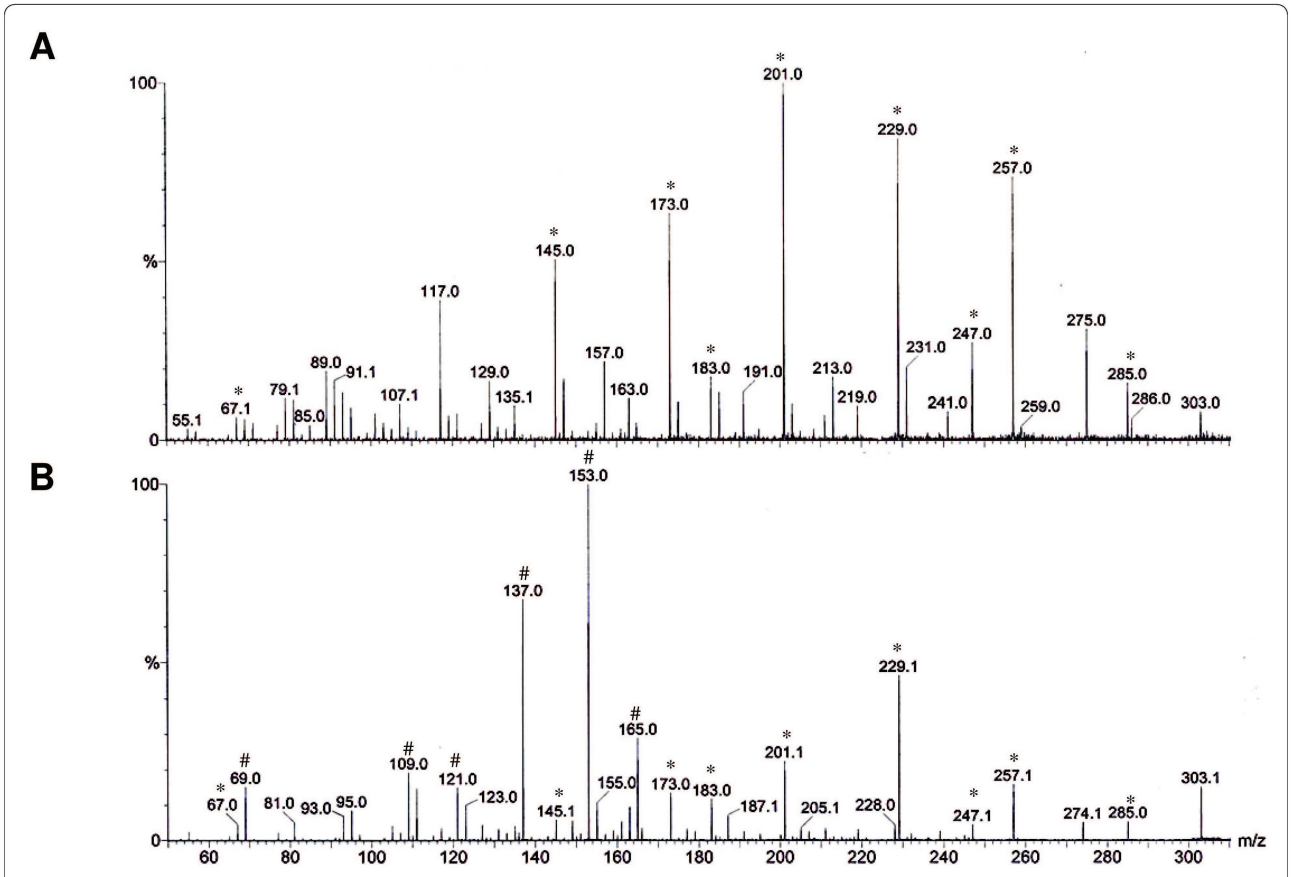

Figure 6 ESI-MS/MS of the ion of *m/z* 303 from the (A) 100% ethanolic fraction or from a (B) solution of quercetin standard. Major fragment ions are marked as either detectable in both spectra (*) or only the spectra of the quercetin standard (#).

four days of treatment, our results showed that the anti-malarial activity of the F4 fraction was also very efficient *in vivo*. These data show that even after the fractionation and purification processes of the extract, no significant anti-malarial activity modification was observed. This is noteworthy because previous studies have reported that certain extracts and fractions have strong activity *in vitro*, but no inhibitory activity *in vivo* [42,43].

As ACT has been employed in areas with higher ratios of anti-malarial treatment failure, and interaction with artesunate has been a major step in drug discovery [44], the anti-malarial activity of the F4 fraction in the presence of artesunate was evaluated. Although few studies have shown *in vitro* synergistic interactions of natural compounds with artesunate [45], these analyses showed that artesunate was able to potentiate the reduction of Pf-iE development when combined with the F4 fraction, indicating that these distinct compounds had a synergistic effect. The fact that the interaction between F4 fraction and artesunate did not exhibit antagonistic interactions should prompt further exploration of novel therapeutic concentrations and combinations of other compounds from plants extracts for the treatment of malaria. As studies of drug combinations may reduce the risk of developing drug resistance and may lead to more effective therapeutic regimens for the treatment of malaria [44], a detailed evaluation of this synergic effect *in vivo* will certainly bring to light pertinent issues such as pharmacokinetics and pharmacodynamics of these compounds, solely or combined.

Mass spectrometry analyses of the F4 fraction detected an ion of *m/z* 303.0450, similar to the fragmentation profile of quercetin (MM = 302), a natural flavonoid very common in edible fruits and vegetables [46]. Indeed, previous studies on the composition of *Caesalpinia* spp. have led to the isolation of several compounds, such as diterpenes [47-49], flavonoids [50], biflavonoids [51] and tannins [52]. Analyses of 480 plant-derived compounds have revealed that diterpenoids and flavonoids isolated from Caesalpiniaceae family are associated with anti-malarial activity against *P. falciparum* [53]. However, a second set of analyses using a quercetin standard showed distinct ions of *m/z* 137 and 153. Furthermore, the IC_{50} value of the F4 fraction (0.72 μg/mL) obtained in these work was nine-fold lower than that of quercetin (6.5 μg/mL), as previously described [54], thus reinforcing the notion of a new compound related to the anti-malarial activity of F4 fraction, both *in vitro* and *in vivo* assays.

Conclusion

The present study indicates that the F4 fraction of *C. pluviosa* has no cytotoxic effect and exhibits anti-malarial activity, both *in vitro* and *in vivo*. When combined with artesunate, this fraction potentiated the activity by significantly inhibiting parasitaemia. The presented findings suggest that a new compound, most likely an isomer of quercetin, is related to the anti-malarial activity of the F4 fraction.

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Authors' contributions

ACAVK carried out laboratory work, analysed the data and helped to draft the manuscript. SCPL contributed to the *in vitro* and *in vivo* anti-malarial activity assays. FGB and JCPM collected and fractionated the plant specimens. ECC and MNE participated in the mass spectrometry analysis and critically revised the manuscript. WCSN participated in the data analyses and helped to draft the manuscript, LMY helped in the design of experiments, and MAF participated in the experiments of drug combination. FTMC contributed to the study design and coordination, helped to interpret the data and drafted the final version of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Le Bras J, Durand R: **The mechanisms of resistance to antimalarial drugs in *Plasmodium falciparum*.** *Fundam Clin Pharmacol* 2003, 17:147-153.
2. Camargo LMA, Oliveira S, Basano S, Garcia CRS: **Antimalarials and the fight against malaria in Brazil.** *Ther Clin Risk Manag* 2009, 5:311-317.
3. Muregi FW: **Antimalarial drugs and their useful therapeutic lives: Rational drug design lessons from pleiotropic action of quinolines and artemisinins.** *Curr Drug Discov Technol* 2010, 7:280-316.
4. Mackinnon MJ, Marsh K: **The selection landscape of malaria parasites.** *Science* 2010, 328:866-871.
5. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM: **Evidence of artemisinin-resistant malaria in western Cambodia.** *N Engl J Med* 2008, 359:2619-2620.

6. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ: **Artemisinin resistance in *Plasmodium falciparum* malaria.** *N Engl J Med* 2009, **361**:455-467.
7. Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, Rutvisuttinunt W, Bethell D, Surasri S, Fukuda MM, Socheat D, Chan Thap L: **Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia.** *Clin Infect Dis* 2010, **51**:82-89.
8. Saralamba S, Pan-Ngum W, Maude RJ, Lee SJ, Taming J, Lindegårdh N, Chotivanich K, Nosten F, Day NP, Socheat D, White NJ, Dondorp AM, White LJ: **Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*.** *Proc Natl Acad Sci USA* 2011, **108**:397-402.
9. Rodrigues LA, Henriques G, Borges ST, Hunt P, Sanchez CP, Martinelli A, Cravo P: **Experimental evolution of resistance to artemisinin combination therapy results in amplification of the *mdr1* gene in a rodent malaria parasite.** *PLoS One* 2010, **5**:e11593.
10. Ekland EH, Fidock DA: ***In vitro* evaluations of antimalarial drugs and their relevance to clinical outcomes.** *Int J Parasitol* 2008, **38**:743-747.
11. Snounou G, Grüner AC, Müller-Graf CD, Mazier D, Rénia L: **The *Plasmodium* sporozoite survives RTS, S vaccination.** *Trends Parasitol* 2005, **21**:456-461.
12. Jiang RW, Ma SC, But PP, Mak TC: **New antiviral cassane furanoditerpenes from *Caesalpinia minax*.** *J Nat Prod* 2001, **64**:1266-1272.
13. Chiang LC, Chiang W, Liu MC, Lin CC: ***In vitro* antiviral activities of *Caesalpinia pulcherrima* and its related flavonoids.** *J Antimicrob Chemother* 2003, **52**:194-198.
14. Liu AL, Shu SH, Qin HL, Lee SM, Wang YT, Du GH: ***In vitro* anti-influenza viral activities of constituents from *Caesalpinia sappan*.** *Planta Med* 2009, **75**:337-339.
15. Pereira MSV, Dias CS, Costa VCO, Conde NCO, Buzalaf MAR: ***In vitro* antimicrobial activity of *Caesalpinia ferrea* Martius fruits against oral pathogens.** *J Ethnopharm* 2009, **124**:289-294.
16. Arif T, Mandal TK, Kumar N, Bhosale JD, Hole A, Sharma GL, Padhi MM, Lavekar GS, Dabur R: ***In vitro* and *in vivo* antimicrobial activities of seeds of *Caesalpinia bonduc* (Lin.) Roxb.** *J Ethnopharm* 2009, **123**:177-180.
17. Rao YK, Fang S-H, Tzeng Y-M: **Anti-inflammatory activities of flavonoids isolated from *Caesalpinia pulcherrima*.** *J Ethnopharm* 2005, **100**:249-253.
18. Yodsaoue O, Karalai C, Ponglimanont C, Tewtrakul S, Chantrapromma S: **Potential anti-inflammatory diterpenoids from the roots of *Caesalpinia mimosoides* Lamk.** *Phytochem* 2010, **71**:1756-1764.
19. Mandal S, Hazra B, Sarkar R, Biswas S, Mandal N: **Assessment of the antioxidant and reactive oxygen species scavenging activity of methanolic extract of *Caesalpinia crista* leaf.** *eCAM* 2009.
20. Shukla S, Mehta A, John J, Singh S, Mehta P, Vyas SP: **Antioxidant acitivity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds.** *Food Chem Toxicol* 2009, **47**:1848-1851.
21. Deharo E, Bourdy G, Munõz V, Ruiz G, Sauvain M: **A search for natural bioactive compounds in Bolivia through a multidisciplinary approach. Part V. Evaluation of the antimalarial activity of plants used by the Tacana Indians.** *J Ethnopharm* 2001, **77**:91-98.
22. Walliker D, Quakyi IA, Wellems TE, McCutchan TF, Szarfman A, London WT, Corcoran LM, Burkot TR, Carter R: **Genetic analysis of the human malaria parasite *Plasmodium falciparum*.** *Science* 1987, **236**:1661-1666.
23. Di Santi SM, Boulos M, Vasconcelos MA, Oliveira S, Couto A, Rosário VE: **Characterization of *Plasmodium falciparum* strains of the State of Rondonia, Brazil, using microtests of sensitivity to antimalarials, enzyme typing and monoclonal antibodies.** *Rev Inst Med Trop São Paulo* 1987, **29**:142-147.
24. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193**:673-675.
25. Mosmann T: **Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.** *J Immunol Methods* 1983, **65**:55-63.
26. Soule HD, Vazguez J, Long A, Albert S, Brennan M: **A human cell line from a pleural effusion derived from a breast carcinoma.** *J Natl Cancer Inst* 1973, **51**:1409-1416.
27. Schlichtherle S, Gandjour A, Neugebauer A, Rüssmann W, Lauterbach KW: **The cost-effectiveness of screening strategies for amblyopia: a preliminary report.** *Strabismus* 2000, **8**:291-295.
28. Peters W, Robinson BL: **The chemotherapy of rodent malaria. XLVII. Studies on pyronaridine and other Mannich base antimalarials.** *Ann Trop Med Parasitol* 1992, **86**:455-465.
29. Soh PN, Witkowski B, Olagnier D, Nicolau ML, Garcia-Alvarez MC, Berry A, Benoit-Vical F: ***In vitro* and *in vivo* properties of ellagic acid in malaria treatment.** *Antimicrob Agents Chemother* 2009, **53**:1100-1106.
30. Lopes SC, Blanco YC, Justo GZ, Nogueira PA, Rodrigues FL, Goelnitz U, Wunderlich G, Facchini G, Brocchi M, Duran N, Costa FT: **Violacein extracted from *Chromobacterium violaceum* inhibits *Plasmodium* growth *in vitro* and *in vivo*.** *Antimicrob Agents Chemother* 2009, **53**:2149-2152.
31. Sixsmith DG, Watkins WM, Chulay JD, Spencer HC: ***In vitro* antimalarial activity of tetrahydrofoliate dehydrogenase inhibitors.** *Am J Trop Med Hyg* 1984, **33**:772-776.
32. Berenbaum MC: **A method for testing for synergy with any number of agents.** *J Infect Dis* 1978, **137**:122-130.
33. Gupta S, Thapar MM, Wernsdorfer WH, Björkman A: ***In vitro* interactions of artemisinin with atovaquone, quinine, and mefloquine against *Plasmodium falciparum*.** *Antimicrob Agents Chemother* 2002, **46**:1510-1515.
34. Ueda H, Tachibana Y, Moriyasu M, Kawanishi K, Alves SM: **Aldose reductase inhibitors from the fruits of *Caesalpinia ferrea* Mart.** *Phytomedicine* 2001, **8**:377-381.
35. Nagai M, Nagumo S: **Protosappanin C from *Sappan lignum* and absolute configuration of protosappanins.** *Chem Pharm Bull* 1987, **35**:3002-3005.
36. Fu L-C, Huang X-an, Lai Z-yuan, Hu Y-jie, Liu H-jiao, Cai X-ling: **A new 3-benzylchroman derivative from *Sappan lignum (Caesalpinia sappan)*.** *Molecules* 2008, **13**:1923-1930.
37. Prasad GR: **Chemical examination of the flowers of *Caesalpinia pulcherrima*.** *Indian J Pharm Sci* 1978, **40**:103-104.
38. McPherson DD, Che C-T, Cordell GA, Soejarto DD, Pezzuto JM, Fong HHS: **Diterpenoids from *Caesalpinia pulcherrima*.** *Phytochemistry* 1985, **25**:167-170.
39. Baelmans R, Deharo E, Bourdy G, Muñoz V, Quenevo C, Sauvain M, Ginsburg H: **A search for natural bioactive compounds in Bolivia through a multidisciplinary approach. Part IV. Is a new haem polymerisation inhibition test pertinent for the detection of antimalarial natural products?** *J Ethnopharmacol* 2000, **73**:271-275.
40. Parlatan A, Sariçoban C, Özcan MM: **Chemical composition and antimicrobial activity of the extracts of Kefe cumin (*Laser trilobum L.*) fruits from different regions.** *Int J Food Sci Nutr* 2008, **26**:1-12.
41. Tan XJ, Li Q, Chen XH, Wang ZW, Shi ZY, Bi KS, Jia Y: **Simultaneous determination of 13 bioactive compounds in Herba *Artemisia scopariae* (Yin Chen) from different harvest seasons by HPLC-DAD.** *J Pharm Biomed Anal* 2008, **47**:847-853.
42. Garavito G, Rincón J, Arteaga L, Hata Y, Bourdy G, Gimenez A, Pinzón R, Deharo E: **Antimalarial activity of some Colombian medicinal plants.** *J Ethnopharmacol* 2006, **107**:460-462.
43. Dell'Agli M, Galli GV, Corbett Y, Taramelli D, Lucantoni L, Habluetzel A, Maschi O, Caruso D, Giavarini F, Romeo S, Bhattacharya D, Bosisio E: **Antiplasmodial activity of *Punica granatum* L. fruit rind.** *J Ethnopharmacol* 2009, **125**:279-285.
44. Enserink M: **Malaria's drug miracle in danger.** *Science* 2010, **328**:844-846.
45. Mishra K, Chakraborty D, Pal A, Dey N: ***Plasmodium falciparum: in vitro* interaction of quassin and neo-quassin with artesunate, a hemisuccinate derivative of artemisinin.** *Exp Parasitol* 2010, **124**:421-427.
46. Chiang LC, Chiang W, Liu MC, Lin CC: ***In vitro* antiviral activities of *Caesalpinia pulcherrima* and its related flavonoids.** *J Antimicrob Chemother* 2003, **52**:194-198.
47. Wu ZH, Huang J, Li WD, Wu LJ, Gao HY: **Three new cassane diterpenes from the seeds of *Caesalpinia minax* Hance.** *J Asian Nat Prod Res* 2010, **12**:781-787.
48. Yodsaoue O, Karalai C, Ponglimanont C, Tewtrakul S, Chantrapromma S: **Potential anti-inflammatory diterpenoids from the roots of *Caesalpinia mimosoides* Lamk.** *Phytochemistry* 2010, **71**:1756-1764.
49. Das B, Srinivas Y, Sudhakar C, Mahender I, Laxminarayana K, Reddy PR, Raju TV, Jakka NM, Rao JV: **New diterpenoids from *Caesalpinia* species and their cytotoxic activity.** *Bioorg Med Chem Lett* 2010, **20**:2847-2850.
50. Das B, Thirupathi P, Ravikanth B, Aravind Kumar R, Sarma AV, Basha SJ: **Isolation, synthesis, and bioactivity of homoisoflavonoids from *Caesalpinia pulcherrima*.** *Chem Pharm Bull* 2009, **57**:1139-1141.

51. Bahia MV, Dos Santos JB, Juceni PD, David JM: **Biflavonoids and other phenolics from *Caesalpinia pyramidalis* (Fabaceae).** *J Braz Chem Soc* 2005, 16:1402-1405.
52. Pawar CR, Surana SJ: **Optimizing conditions for gallic acid extraction from *Caesalpinia decapetala* wood.** *Pak J Pharm Sci* 2010, **23**:423-425.
53. Bero J, Frédérich M, Quentin-Leclercq J: **Antimalarial compounds isolated from plants used in traditional medicine.** *J Pharm Pharmacol* 2009, 61:1401-1433.
54. del Rayo Camacho M, Phillipson JD, Croft SL, Marley D, Kirby GC, Warhurst DC: **Assessment of the antiprotozoal activity of *Galphimia glauca* and the isolation of new nor-secofriedelanes and nor-friedelanes.** *J Nat Prod* 2002, 65:1457-1461.

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MAJOR ARTICLE

On the Cytoadhesion of *Plasmodium vivax*–Infected Erythrocytes

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Background. *Plasmodium falciparum* and *Plasmodium vivax* are responsible for most of the global burden of malaria. Although the accentuated pathogenicity of *P. falciparum* occurs because of sequestration of the mature erythrocytic forms in the microvasculature, this phenomenon has not yet been noted in *P. vivax*. The increasing number of severe manifestations of *P. vivax* infections, similar to those observed for severe falciparum malaria, suggests that key pathogenic mechanisms (eg, cytoadherence) might be shared by the 2 parasites.

Methods. Mature *P. vivax*–infected erythrocytes (Pv-iEs) were isolated from blood samples collected from 34 infected patients. Pv-iEs enriched on Percoll gradients were used in cytoadhesion assays with human lung endothelial cells, *Saimiri* brain endothelial cells, and placental cryosections.

Results. Pv-iEs were able to cytoadhere under static and flow conditions to cells expressing endothelial receptors known to mediate the cytoadhesion of *P. falciparum*. Although Pv-iE cytoadhesion levels were 10-fold lower than those observed for *P. falciparum*–infected erythrocytes, the strength of the interaction was similar. Cytoadhesion of Pv-iEs was in part mediated by VIR proteins, encoded by *P. vivax* variant genes (*vir*), given that specific antisera inhibited the Pv-iE–endothelial cell interaction.

Conclusions. These observations prompt a modification of the current paradigms of the pathogenesis of malaria and clear the way to investigate the pathophysiology of *P. vivax* infections.

It has long been recognized that the directly attributable morbidity and mortality differ for the 2 most prevalent malarial parasite species, *Plasmodium falciparum* and *Plasmodium vivax*. The higher multiplicative potential of *P. falciparum*–infected erythrocytes (Pf-iEs) no doubt contributes to their increased virulence. However, it is the withdrawal of mature Pf-iEs (parasites older than 24 h) from the peripheral circulation to that of the internal organs—a phenomenon known as *sequestration* [1]—that is considered to be the key pathogenic event. *P. falciparum* is characterized by almost total sequestration, such that few if any mature Pf-iE forms

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are observed in peripheral blood samples during infection. Given that mature *P. vivax*–infected erythrocytes (Pv-iEs) are frequently observed in peripheral blood samples, it was concluded that sequestration did not occur with this parasite. As a consequence, the paradigm was formulated that sequestration of Pf-iEs in specific organs is the principal initial cause of pathology and that, when sequestration occurs in the brain or placenta, the likelihood of cerebral malaria and pregnancy-associated malaria increases. Indeed, most forms of severe malaria and nearly all mortality have been almost exclusively recorded for falciparum cases.

P. vivax, the most prevalent malarial species outside sub-Saharan Africa, imposes a substantial global public health burden [2], with recent estimates of 130–435 million infected persons per year among the 2.6 billion people at risk. Of equal importance was the observation that many types of severe malaria—long considered to be specific to *P. falciparum*—also commonly occur in *P. vivax*–infected persons. For instance, infection with *P. vivax* during pregnancy was found to be associated with a substantial reduction in birth weight [3]. Furthermore, in some areas of endemicity progression of vivax malaria to clinically severe forms, including cerebral malaria and acute respiratory distress syndrome, was found to occur as frequently as for falciparum infections, with similar levels of fatality [4–7]. This raises the possibility that pathological processes linked to cytoadhesion might also operate in *P. vivax*.

In *P. falciparum* infection, cytoadhesion of Pf-iEs to endothelial cells is mediated by interactions between members of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) family, polymorphic proteins encoded by the *var* multigene family [8, 9], and defined host receptors on endothelial cells. Of the 10 or so receptors identified to date [10, 11], 3 have been extensively investigated: CD36, intercellular adhesion molecule 1 (ICAM-1), and chondroitin sulfate A (CSA). The last has been specifically associated with the binding of Pf-iEs to the placenta [12].

We wished to establish whether *P. vivax* parasites are able to cytoadhere under static or flow conditions to endothelial cells and placental cryosections and whether the receptors for Pf-iEs were also implicated. Furthermore, we assessed the involvement of VIR proteins as a potential Pv-iE ligand.

METHODS

Ethical approval. Informed consent was sought and granted from all patients attending the Tropical Medicine Foundation of Amazonas (FMT-AM), Amazonas, in northern Brazil. The procedures were approved by the Ethics Committee Board of the FMT-AM (process 2758/2008-FMT-AM; approval no. 1943).

Parasite isolation and enrichment. Once microscopic diagnosis of uncomplicated vivax or falciparum malaria was made and before the treatment was initiated, 5–10 mL of blood were collected into citrate-coated Vacutainer tubes (BD). Parasitemia levels rarely exceeded 5000 parasites/μL of blood. The blood was immediately processed to obtain enriched Pv-iEs. On average, a total of $\sim 1 \times 10^6$ Pv-iEs could be obtained, allowing only a limited number of cytoadhesion assays to be conducted. Patients who had received antimalarial treatment 3–4 weeks before the test were excluded. Immediately after collection, the red blood cells containing trophozoites and schizonts were separated from the younger forms on a 45% Percoll (Amersham) gradient, as described elsewhere [13] with minor modifications. Briefly, after plasma separation by centrifugation, blood pellets were washed 3 times and then resuspended in RPMI 1640 medium (Sigma) to a final hematocrit of 10%. Five milliliters of this suspension was overlaid on a 5-mL 45% Percoll solution

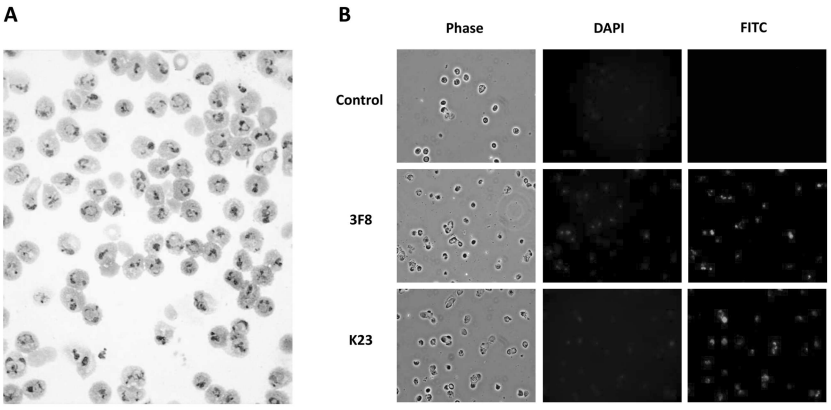

Figure 1. Percoll gradient enrichment of *Plasmodium vivax* maturing forms. *A,* Giemsa staining of the mature trophozoites and young schizonts obtained after Percoll gradient enrichment, yielding cell suspensions with 85%–97% *P. vivax*–infected erythrocytes. *B,* Parasite species and maturity confirmed by immunofluorescence assay using anti–PvMSP-1_{19} conformational monoclonal antibodies (3F8 and K23) diluted at 1:50. Normal mouse serum samples were used as negative controls. DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate.

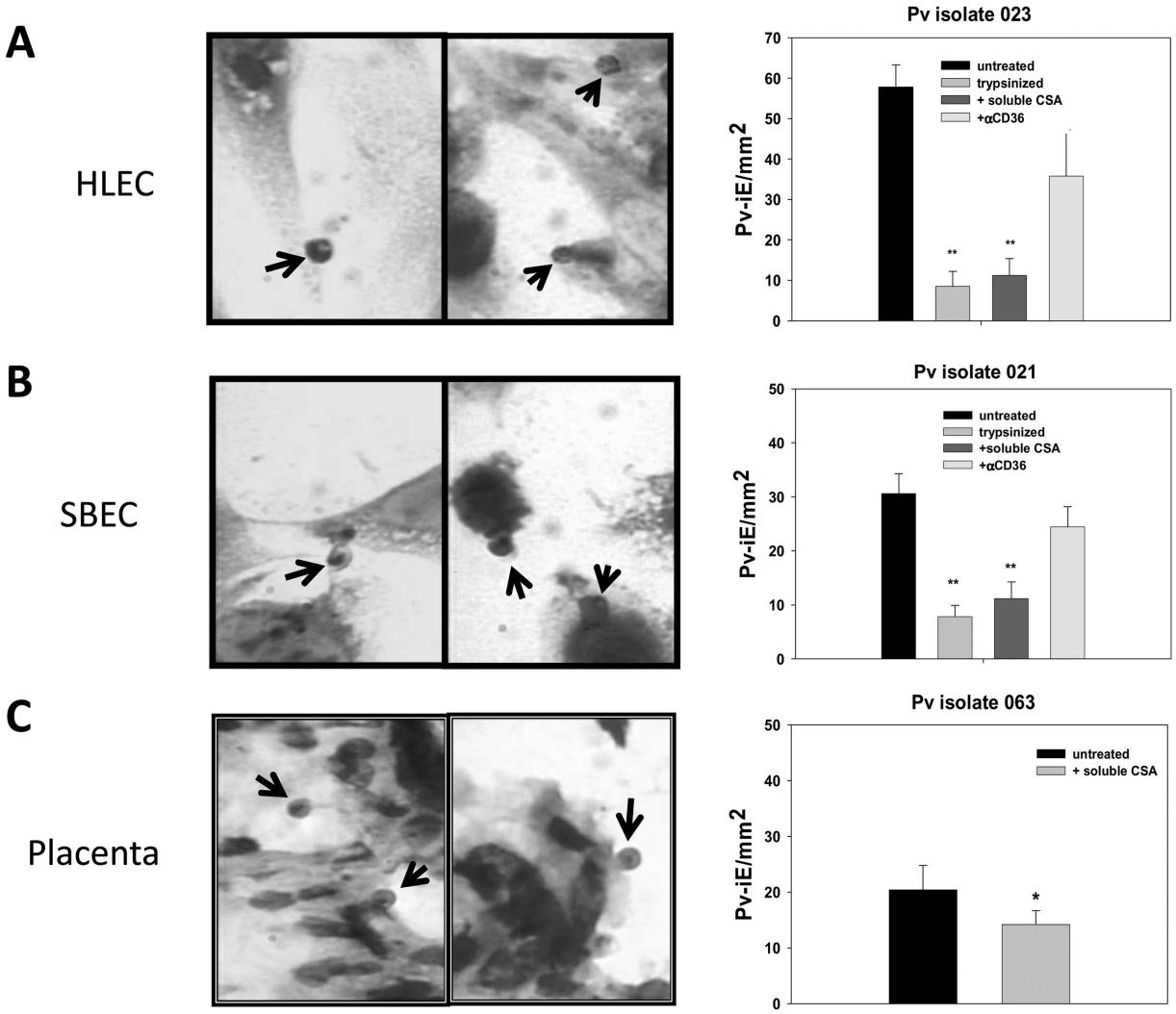

Figure 2. Adhesion of *Plasmodium vivax*–infected erythrocytes (Pv-iEs) to lung and brain endothelial cells or placental cryosections. Shown are representative photomicrographs of (*left*) and cytoadhesion assays for (*right*) Pv-iEs binding to human lung endothelial cells (HLECs) (*A*), *Saimiri* brain endothelial cells (SBECs) (*B*), and placental cryosections (*C*) stained with Giemsa stain and visualized using a Nikon microscope at ×100 magnification. Arrows indicate Pv-iEs bound to host cells. Representative results for Pv-iEs obtained from different patients and used for cytoadhesion assays conducted on untreated cells, on Pv-iEs pretreated with trypsin, or in the presence of soluble chondroitin sulfate A (CSA) or anti-CD36 antibody are shown. In all cases, parasites (5×10^4) were left for 1 h at 37°C in 8-well culture slides (0.69 cm^2/well) and then extensively washed, and adhered Pv-iEs were counted. Data are the mean number of bound Pv-iEs per square millimeter, normalized to an input of 1×10^3 Pv-iEs/mm^2; error bars indicate standard deviations. *$.05 > P > .01$ and **$0.01 > P > .001$ (Kruskal-Wallis test).

(2.25 mL of Percoll, 0.5 mL of RPMI 1640 [×10], and 2.25 mL of distilled water) in a 15-mL tube. After centrifugation, floating mature iEs were collected and resuspended in RPMI 1640. Ex vivo, Pf-iEs were enriched in Percoll gradient at 60%, as described elsewhere [14]. Giemsa-stained thick smears (before enrichment) and thin smears (after enrichment) were examined to determine the *Plasmodium* species and the percentage of mature stages, respectively. A sensitive nested polymerase chain reaction (PCR) assay was applied to the samples to confirm the diagnosis [15].

P. vivax *slide preparation and immunofluorescence assays.* Immunofluorescence assays (IFAs) were performed in 8-well slides containing mature stages of *P. vivax*. Immediately after enrichment on Percoll, Pv-iEs were washed and resuspended in 10% fetal calf serum (FCS) (Nutricell) and then deposited on IFA slides (50 μL/well), fixed in acetone for 10 min, air-dried, and stored at −20°C until use. Ten micrograms per milliliter of each monoclonal antibody (3F8 or K23) against *P. vivax* merozoite surface protein 1 (PvMSP1) [16] was diluted in phosphate-buffered saline (PBS) and applied to slides for 30

Table 1. Cytoadhesion Assays for *Plasmodium vivax*–Infected Erythrocytes (Pv-iEs) and *Plasmodium falciparum*–Infected Erythrocytes (Pf-iEs) Collected from 24 Patients

	HLECs					SBECs					
		% of inhibition[b]					% of inhibition[b]			Placental cryosections	
Parasite, patient	Adhesion[a]	Trypsinized	sCSA	Anti-CD36	CaseABC	Adhesion[a]	Trypsinized	sCSA	Anti-CD36	Adhesion[a]	sCSA, % of inhibition[b]
Pv-iEs											
008						58.2 ± 9.8	19 ± 10				
015						32.4 ± 11.04	52 ± 11*				
016	52.4 ± 8.5	70 ± 7*									
019	36.1 ± 10.6	59 ± 10 **	25 ± 21								
021						30.6 ± 3.7	74 ± 7**	63 ± 10**	20 ± 12		
022	28.3 ± 4.5	29 ± 21**	52 ± 8**	2 ± 27							
023	57.8 ± 5.5	85 ± 6**	81 ± 7**	38 ± 20							
024	33.9 ± 3.7	76 ± 7**	65 ± 8**	20 ± 11							
030	16.6 ± 3.4	46 ± 10***				12.8 ± 2.8	42 ± 18*				
031	26.1 ± 4.5	47 ± 15***									
034	56.7 ± 7.4		18 ± 10*			49.5 ± 7.9		28 ± 9*			
035	37.5 ± 9.4		41 ± 13**			23.9 ± 8.0		31 ± 30			
036										22.3 ± 5.4	23 ± 19
063										20.4 ± 4.4	30 ± 12*
065	33.5 ± 8.8		51 ± 13***								
066										9.9 ± 3.2	15 ± 40
076	19.7 ± 4.0		34 ± 18***		7 ± 25						
083	38.6 ± 7.3		49 ± 15**		4 ± 21						
086	30.1 ± 6.5		40 ± 16**		13 ± 22						
087	41.3 ± 5.2		16 ± 23		3 ± 15						
Pf-iEs											
002	130.1 ± 23.7										
003	636.7 ± 119.2										
005										773.5 ± 179.5	87 ± 7*
008	454.4 ± 45.4										

NOTE. Data from *P. falciparum* isolates confirmed that the host cells and placental cryosections used were able to sustain parasite cytoadhesion. Asterisks indicate statistical significance with respect to adhesion values, as follows: *.05 > *P* > .01; **.01 > *P* > .001; and *P* < .001. HLECs, human lung endothelial cells; SBECs, *Saimiri* brain endothelial cells; SD, standard deviation.

[a] Mean no. (±SD) of bound infected erythrocytes per square millimeter, normalized to an input of 1×10^3 infected erythrocytes/mm^2.

[b] Percentage of inhibition (±SD) of adhesion was calculated after enumeration of adherent infected erythrocytes either following trypsinization of infected erythrocytes or prior to the assay, or when the assay was conducted in the presence of soluble CSA (sCSA), anti-CD36 monoclonal antibody, or in endothelial cells pretreated with chondroitinase ABC (CaseABC).

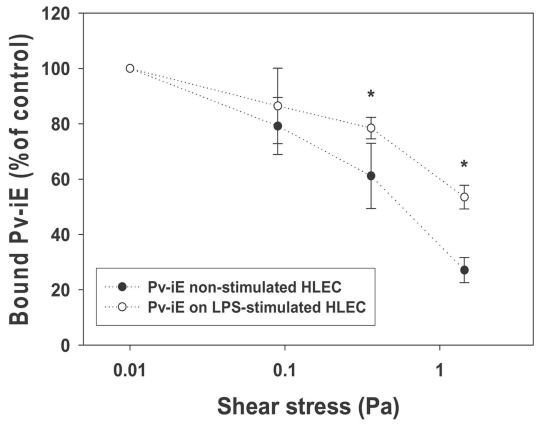

Figure 3. Resistance of *Plasmodium vivax*–infected erythrocyte (Pv-iE) cytoadhesion to flow conditions. Enriched Pv-iEs (5×10^5) were allowed to adhere to human lung endothelial cells (HLECs) during a 1-h incubation at 37°C in single-well microslides (8.6 cm^2) mounted in a flow chamber system, and cytoadhesion medium (RPMI 1640 [pH 6.8]) was flowed through at a wall shear stress of 0.09, 0.36, and 1.44 Pa for 10, 5, and 2.5 min, respectively; medium was not flowed through the control chamber. The Pv-iEs that still bound at the end of the flow period were counted in 20 randomly selected fields, and the percentage of binding compared with that in the control chamber were calculated. Data are mean binding percentages for 2 patient isolates (104 and 106) on HLECs previously treated with lipopolysaccharide (LPS) (1.0 μg/mL) or left untreated; error bars indicate standard deviations. *$P < .05$ for the comparison of adhesion to nonstimulated vs LPS-stimulated HLECs (Mann-Whitney *U* test).

min at 37°C. After washing with PBS, slides were incubated with 10 μg/mL fluorescein isothiocyanate–conjugated sheep anti–mouse immunoglobulin G (Sigma) and 100 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes) for 30 min at 37°C and then washed several times. Positive monoclonal antibody recognition was detected with the aid of an immunofluorescence microscope (Nikon). For IFAs conducted using VIR antisera, Pv-iEs were left in suspension (liquid phase). Briefly, parasites were fixed in 2% paraformaldehyde and washed and diluted in FCS, and then mouse VIR-A4 and VIR-E5 antisera were added at a final dilution of 1:20 and incubated for 60 min. After 2 washes with FCS, cells were incubated with 100 μg/mL Alexa Fluor 488–conjugated goat anti–mouse IgG (Molecular Probes) and 500 μg/mL DAPI for 30 min at 37°C and then washed twice with FCS. In both assays, positive recognition by monoclonal antibody, VIR-A4, VIR-E5, or MSP-1_{19} antisera was detected with the aid of an immunofluorescence microscope.

Polyclonal VIR antisera were generated by injecting mice with VIR-A4 or VIR-E5 glutathione S-transferase (GST) fusion proteins belonging to the A or E *vir* subfamilies, respectively [17, 18] and raised after 2 immunizations (21-day interval) with each recombinant protein (5 μg/animal/dose) emulsified in complete or incomplete Freund adjuvant. No Pv-iE–positive labeling was visualized by means of GST antisera.

Selection of monophenotypic cultured* P. falciparum *parasites and cells. The *P. falciparum* lines FCR3 [19] and S20 [20] were cultured in candle jars. Briefly, Pf-iEs were cultivated in fresh type O^+ human erythrocytes (Blood Center, Universidade Estadual de Campinas) suspended at a final hematocrit of 4% in complete medium (RPMI 1640 [pH 7.2]) (Sigma) and supplemented with 10% homologous human plasma.

The following cell lines were used in this study: *Saimiri* brain endothelial cells (SBECs) and human lung endothelial cells (HLECs) [21, 22], adapted from cultured primary explants, and CHO-ICAM, CHO-CD36 and CHO-K1 cells [23]. Selection of FCR3 parasites to CSA ($FCR3^{CSA}$) was performed by panning (5 rounds) of mature-stage iEs on endothelial cells [21, 24] in the presence of soluble CSA (100 μg/mL; Sigma).

Mature S20 trophozoites were selected on CHO-ICAM cells (5 rounds) and then by a further 2 rounds on plastic plates coated with recombinant ICAM-1 [25]. The selected $S20^{ICAM}$ parasite line bound strongly to CHO-ICAM cells but poorly to nontransfected CHO-K1 cells (data not shown).

Static cytoadhesion assays. We assessed the ability of Pv-iEs or Pf-iEs obtained from infected patients to adhere to placenta cryosections, HLECs, and SBECs by performing static cytoadhesion assays as described elsewhere [19, 21, 26] with minor modifications. Briefly, HLECs or SBECs were grown to confluence on 8-well culture slides (0.69 cm^2/well; BD), and 5×10^4 Percoll-enriched iEs were then added to each well in a total volume of 200 μL of cytoadhesion medium (RPMI 1640 [pH 6.8]), either alone or in the presence of 100 μL of soluble CSA (100 μg/mL) or anti-CD36 (5 μg/mL). Culture slides were incubated for 1 h at 37°C and then extensively washed in cytoadhesion medium. To confirm that the ligands on the surface of Pv-iEs were proteins, Pv-iEs were treated with trypsin (1 mg/mL) for 45 min at 37°C before incubation over endothelial cells. For confirmation of CSA as a receptor, HLECs were previously incubated with chondroitinase ABC (CaseABC) (0.5 U/mL; Sigma) for 2 h at 37°C. Involvement of VIR antigens in the cytoadhesion of Pv-iEs to HLECs was evaluated in an assay where inhibition of parasite binding was tested with antisera (diluted at 1:5 or 1:10) to VIR-A4 or VIR-E5 GST-fused proteins. Serum samples from mice immunized solely with GST in complete or incomplete Freund adjuvant were used as specificity controls. For these assays, inhibition was determined as a percentage of the negative control and was expressed as the mean value for 3 wells ± standard deviation.

Assays of adhesion to placental trophoblasts were performed as described elsewhere [19, 26, 27] with minor modifications. Placental biopsy samples from 3 human immunodeficiency virus–negative Brazilian women with malaria were collected immediately after delivery, snap-frozen in liquid nitrogen/*n*-hex-

Table 2. Cytoadhesion Assays for *Plasmodium vivax*–Infected Erythrocytes (Pv-iEs) Obtained from 7 Patients or Cultured *Plasmodium falciparum*–Infected Erythrocytes (Pf-iEs) to Human Lung Endothelial Cells (HLECs) under Flow Conditions

Parasite, patient (LPS treatment)	Adhesion[a] at 0.00 Pa	Shear stress					
		0.09 Pa		0.36 Pa		1.44 Pa	
		Adhesion[a]	Binding,[b] %	Adhesion[a]	Binding,[b] %	Adhesion[a]	Binding,[b] %
Pv-iEs							
090 (no)	67.4 ± 14.4	34.2 ± 8.6	50.8	30.4 ± 9.6	45.2	ND	…
090 (yes)	55.6 ± 9.6	48.0 ± 17.8	86.3	42.7 ± 15.4	76.9	ND	…
091 (yes)	53.0 ± 6.1	39.0 ± 7.7	73.6	38.5 ± 10.1	72.6	ND	…
092 (no)	17.6 ± 9.6	13.4 ± 8.2	76.1	10.8 ± 2.9	61.1	ND	…
093 (no)	47.9 ± 12.0	29.9 ± 8.2	62.4	ND	…	ND	…
094 (no)	60.4 ± 13.8	51.3 ± 9.1	84.9	ND	…	ND	…
104 (no)	30.4 ± 9.1	26.3 ± 5.3	86.5	16.1 ± 5.3	52.9	9.2 ± 7.7	30.3
104 (yes)	32.5 ± 13.8	25.0 ± 5.3	76.9	24.6 ± 5.3	75.7	16.4 ± 6.7	50.5
106 (no)	28.6 ± 8.6	20.5 ± 14.9	71.7	19.8 ± 6.1	69.2	6.8 ± 5.8	23.8
106 (yes)	29.1 ± 8.2	27.9 ± 10.6	96.2	23.6 ± 7.2	81.1	16.4 ± 6.7	56.4
Pf-iEs							
$S20^{ICAM}$ (no)	39.8 ± 15.8	19.2 ± 13.0	47.9	12.0 ± 5.8	30.0	9.6 ± 8.2	24.3
$FCR3^{CSA}$ (no)	370.9 ± 50.9	333.1 ± 25.0	89.8	294.5 ± 36.9	79.4	190.8 ± 34.7	51.4

NOTE. *P. vivax* or cultured *P. falciparum*, selected on intercellular adhesion molecule 1 ($S20^{ICAM}$) or chondroitin sulfate A ($FCR3^{CSA}$), were allowed to adhere to HLECs either previously incubated with 1.0 μg/mL lipopolysaccharide (LPS) or not incubated with LPS, and then cytoadhesion medium was flowed through at a wall shear stress of 0.09, 0.36, or 1.44 Pa for 10, 5, and 2.5 min, respectively. ND, not determined; SD, standard deviation.

[a] No. of remaining adherent parasites after application of a flow condition. Data are the mean no. (±SD) of infected erythrocytes per square millimeter, normalized to an input of 1×10^3 infected erythrocytes/mm^2.

[b] Percentage of parasites that remained cytoadherent after being subjected to wall shear stress.

ane (Merck), and then stored frozen in Tissue-Teck (Thermo) before use. Serial placenta cryosections (5–7 μm) were cut with a cryostome and mounted on individual glass slides. Cryosections were washed and air-dried, and an area of ~1 cm^2 was delimited with a Dako Pen device. Assays of the adhesion of Pv-iEs to placental cryosections were performed as for endothelial cells. Three to 4 placenta cryosections were used for each adhesion assay. After a 1-h incubation at 37°C and following Giemsa staining, the number of infected erythrocytes that adhered to the endothelial cell monolayer or to placenta cryosections was counted under the microscope.

Involvement of CD36 or ICAM-1 host receptors was verified by allowing Pv-iEs to adhere under static conditions to CHO-CD36 cells, CHO-ICAM cells, or CHO-745 cells, a cell line that does not express either of these receptors [23]. In these experiments, we performed adhesion assays without using human serum in the medium because it has been shown that human immunoglobulins present in normal serum can mediate binding of Pf-iEs to CHO-745 cells [28].

Flow-based cytoadhesion assays. To assess the resistance of Pv-iEs to shear stress, we performed flow-based cytoadhesion assays according to a modified version of a method that has been described elsewhere [19, 26, 27]. Briefly, HLECs were cultured to confluence in single-well culture microslides (8.6 cm^2, corresponding to 12.5 times the area of each well in a 8-well culture slide), to which 1.5 mL of 5×10^5 Pv-iEs enriched on 45% Percoll gradient was added. After a 1-h incubation at 37 °C, microslides were mounted in a flow chamber system (Immunetics), and cytoadhesion medium (RPMI 1640 [pH 6.8]) was flowed through at a wall shear stress of 0.09, 0.36, or 1.44 Pa for 10, 5, and 2.5 min, respectively. After this, the

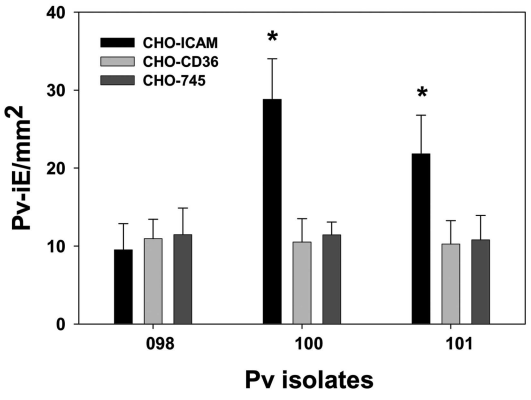

Figure 4. Adhesion of *Plasmodium vivax*–infected erythrocytes (Pv-iEs) to specific receptors. Pv-iEs (5×10^4 cells/well) were allowed to adhere to CHO–intercellular adhesion molecule (ICAM), CHO-CD36, or control CHO-745 cells. Data are the mean number of bound Pv-iEs per square millimeter, normalized to an input of 1×10^3 Pv-iEs/mm^2; error bars indicate standard deviations. *$P < .001$ for the comparison of adhesion to CHO-ICAM cells vs CHO-CD36 or CHO-745 cells (Kruskal-Wallis test).

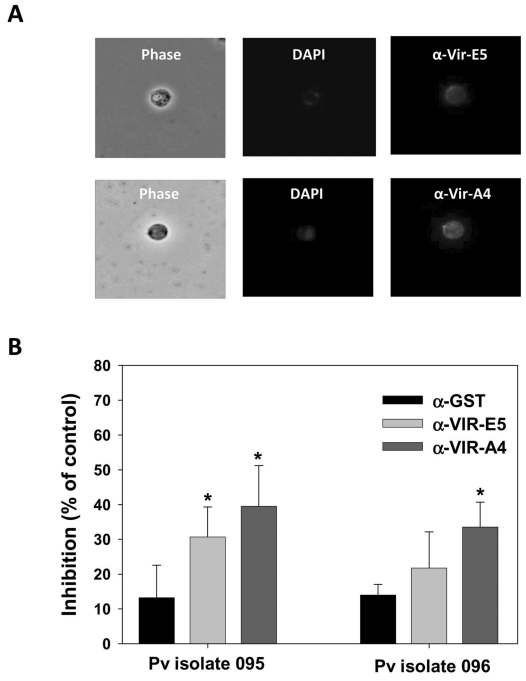

Figure 5. Recognition and blocking of *Plasmodium vivax*–infected erythrocyte (Pv-iE) cytoadhesion to human lung endothelial cells (HLECs) by specific VIR antisera. *A*, Immunofluorescence of a Pv-iE labeled with antisera against VIR-A4 (1:20) or VIR-E5 (1:20). Shown are results for phase contrast, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, and mouse anti–VIR-E5 (*top*) or anti–VIR-A4 (*bottom*) with Alexa Fluor 488–conjugated goat anti–mouse immunoglobulin G. *B*, Results of incubation of Pv-iEs (5×10^4 cells/well) for 1 h at 37°C alone or in the presence of VIR-A4 or VIR-E5 antisera diluted at 1:5 (isolate 095) or 1:10 (isolate 096). In both assays, serum samples from mice immunized solely with glutathione S-transferase (GST) in Freund adjuvant were used as a negative control. For these assays, inhibition was determined as the percentage of negative control counts, expressed as mean values for triplicate wells; error bars indicate standard deviations. *$P < .05$ for the comparison of inhibition with GST antisera (Kruskal-Wallis test).

remaining bound Pv-iEs were counted in 20 randomly selected fields through a digital camera attached to a microscope (Moticam 2500; Motic). Results were expressed as the mean number of infected erythrocytes per square millimeter ± standard deviation. In some experiments, microslides were stained with Giemsa after a determined shear stress condition, to visualize bound Pv-iEs. Alternatively, HLECs were incubated with lipopolysaccharide (LPS) (1.0 μg/mL; Sigma) for 4 h at 37°C and then washed before parasite cytoadhesion. Cultured *P. falciparum* panned isolates ($FCR3^{CSA}$ and $S20^{ICAM}$) were used as controls. To ascertain whether *P. vivax* adhesion to HLECs occurred under flow conditions, 1×10^6 enriched Pv-iEs diluted in cytoadhesion medium were flowed at a wall shear stress of 0.09 or 0.36 Pa for 1 h and then filmed. For both the static and flow-based assay, parasite adhesion was normalized to 1×10^3 infected erythrocytes/mm^2, taking into account the area and the number of infected erythrocytes used in each assay (5×10^4 infected erythrocytes in 0.69 cm^2 or 5×10^5 infected erythrocytes in 8.6 cm^2).

Flow cytometry. Levels of ICAM-1 expression on HLECs were assessed by flow cytometry. Briefly, 1×10^6 cells either treated with LPS (1.0 μg/mL) or left untreated were harvested, incubated with anti–human CD54 (ICAM-1) (phycoerythrin; BD Bioscience) for 30 min at 4°C, washed, and fixed in 2% formaldehyde. Analysis was performed using a FACScanto flow cytometer (BD), and the mean fluorescence intensity and the percentage of positive cells were analyzed with the aid of FCS Express software (version 3.00.0320; De Novo Software). For each sample, a minimum of 100,000 events were acquired.

Statistical analysis. The statistical significance of adhesion to different cells types at various conditions was determined using the Mann-Whitney *U* test or the Kruskal-Wallis test. Calculations were performed using BioEstat software (version 3.0; CNPq, Brazil) and Prism software (version 3.02; GraphPad Software). Differences were considered significant at $P < .05$.

RESULTS

Our investigation of Pf-iE cytoadherence was substantially facilitated by the availability of in vitro–cultured *P. falciparum*, an avenue not open for *P. vivax*. We conducted our experiments with Pv-iEs using samples obtained directly from patients presenting with uncomplicated *P. vivax* malaria in Manaus, Brazil. The limitations imposed by reliance on clinical samples with parasites of diverse genotypes and of varying suitability for cytoadherence assays were compensated for by the collection of a relatively large number of samples ($n = 34$). As controls for the cytoadhesion assays, *P. falciparum*–infected blood was also obtained from 4 patients attending the same hospital. In all cases, a sensitive PCR assay was used to exclude the presence of mixed-species infections. As an inital step, cytoadherence was assessed using HLECs, SBECs, and human placental cryosections. These cell lines and placental cryosections have been characterized and validated for assays of *P. falciparum* cytoadhesion [19, 21, 22, 26, 27]. In *P. falciparum* infection, mature forms of the parasite cytoadhere more strongly than do freshly invaded forms. We hypothesized that this could be also the case for *P. vivax* infection. Given the different densities of mature-stage and early-stage parasites, we subjected *P. vivax* parasites collected from the patients directly and without prior short-term culturing to a Percoll gradient, enabling the recovery of almost-pure trophozoite- and schizont-stage Pv-iEs (Figure 1). Of note, after enrichment the percentage of $MSP\text{-}1_{19}$–expressing forms observed in IFA varied from 85% to 97%.

Pv-iEs were first tested for their ability to cytoadhere to HLECs, SBECs, and placental cryosections under static conditions. For some patients, aliquots of the same Pv-iE suspension were tested after digestion with trypsin or in the presence

of soluble CSA, anti-CD36 monoclonal antibody in the cytoadhesion medium, or HLECs pretreated with CaseABC (Figure 2 and Table 1). All the Pv-iEs tested displayed some level of cytoadherence to HLECs and/or SBECs or to placental cryosections. Pv-iE binding to areas not containing endothelial cells was not observed. Pretreatment of Pv-iEs with trypsin generally decreased adhesion to HLECs or SBECs by 19%–85% (mean, 54%), depending on the parasite isolate. Significant inhibition of cytoadherence was also observed in the presence of soluble CSA (range, 16%–81%; mean, 42%). By contrast, when assays were conducted in the presence of anti-CD36, the extent of inhibition of cytoadherence was not statistically significant (Table 1), although only a few isolates could be tested. Moreover, unlike the higher inhibition observed with soluble CSA, HLECs pretreated with CaseABC did not significantly abolish adhesion of the same Pv-iEs (Table 1). Cytoadhesion assays were also conducted in parallel with similarly enriched mature Pf-iEs derived from 4 patients. The levels of cytoadherence observed were ~10-fold higher than those recorded for Pv-iEs (Table 1).

Having demonstrated that Pv-iEs could cytoadhere to endothelial cells under static conditions, it was important to evaluate whether the observed cytoadherence was biologically relevant and whether it could be maintained under the flow conditions that parasites are subjected to in the bloodstream. Shear stress in postcapillary venules is close to 0.08 Pa [29]. Thus, we enriched Pv-iEs from 7 individuals and conducted flow-based cytoadhesion assays on HLECs at shear stress conditions varying from 0.09 to 1.44 Pa. The parasites that remained attached after being subjected to increasing flow rates for defined periods were enumerated by microscopic examination after Giemsa staining (Figure 3 and Table 2). Given that HLECs express CSA and ICAM-1 on their surface, we performed parallel assays using *P. falciparum* FCR3 and the S20 iEs preselected on CSA ($FCR3^{CSA}$) and ICAM-1 ($S20^{ICAM}$). As for Pf-iEs ($FCR3^{CSA}$), even at a relatively high shear stress (1.44 Pa) a substantial proportion (30%) of the cytoadherent Pv-iEs could not be detached (Table 2). Furthermore, stimulation of the HLECs with LPS significantly strengthened the cytoadherence, because 56% of the cytoadherent Pv-iEs could not be detached at 1.44 Pa (Figure 3). Of note, LPS treatment augmented ICAM-1 expression levels by 3-fold (data not shown). The strength of Pv-iE adhesion was similar to that observed for *P. falciparum* $FCR3^{CSA}$ to unstimulated HLECs (Table 2). The behavior of the cytoadherent Pv-iEs to HLECs under flow conditions was recorded in real time and can be seen in Videos 1 and 2, which are available in the online version of the *Journal* and which show that cytoadherent Pv-iEs display the rolling characteristics of cytoadherent *P. falciparum* parasites [30]. Video 1 shows the rolling and binding of a Pv-iE to an HLEC; parasites were diluted in medium and flowed at a wall shear stress of 0.09 Pa. Video 2 shows 2 Pv-iEs bound to HLECs under a flow condition of 0.36 Pa.

We then tested whether CD36 or ICAM-1, both present on the HLEC surface [21, 22], were implicated in the observed Pv-iE cytoadherence. For this, CHO lines transfected with CD36 or ICAM-1 were used. None of the Pv-iEs from the 3 samples tested showed binding to CD36 (Figure 4), because the levels of cytoadherent Pv-iEs were not significantly different from background (binding of Pv-iEs to untransfected CHO-745 cells). By contrast, a 2.1–2.7-fold increase in cytoadhesion to ICAM-1 over background was observed for Pv-iEs from 2 of the 3 samples tested (Figure 4). Low cytoadhesion of Pv-iEs isolated from patient 098 may indicate variations in the binding phenotypes of different isolates.

Finally, we investigated potential parasite ligands involved in Pv-iE cytoadhesion. A multigene family orthologous to the *P. falciparum var* genes does not occur in the genome of *P. vivax* [31]. However, a superfamily of surface-expressed variant antigens genes (*vir*) is present in *P. vivax*; it has ~350 members, which can be subdivided into 10 subfamilies and unclustered members [17, 31, 32]. Hence, we tested 2 specific polyclonal antisera, VIR-A4 and VIR-E5, from the A and E subfamilies, respectively [18]. Pv-iEs were specifically recognized by both antisera in IFAs (Figure 5*A*) but not by the control GST antisera. Significant inhibition of Pv-iE cytoadherence on HLECs was observed when either VIR antisera was included in the assay medium but not in the presence of the GST antisera (Figure 5*B*).

DISCUSSION

Our observations provide the first evidence, to our knowledge, that mature Pv-iEs are capable of cytoadhering to endothelial cells and placental cryosections. Two receptors used by *P. falciparum* for binding to endothelial cells, ICAM-1 and CSA, were also implicated in the cytoadhesion of *P. vivax* parasites, at least to some extent. However, CaseABC treatment suggested that cytoadhesion to CSA, a highly negative charged molecule, might be due to charge interaction. Indeed, it has been shown that binding to CSA is highly dependent on ionic strength [33]. The fraction of Pv-iEs that cytoadhered was up to 10-fold lower than that for Pf-iEs. Importantly, cytoadhesion of Pv-iEs, once established, is as strong as that of CSA-selected Pf-iEs, as demonstrated by the flow assays (Table 2). Because the parasites we assayed were directly obtained from patients, our data suggest that only a minor fraction of *P. vivax* would have the capacity to cytoadhere in vivo. Partial retention of *P. vivax* from the peripheral circulation would explain why the mature forms of this parasite are generally found in peripheral blood. This is consistent with the observation of partial and differential accumulation of Pv-iEs in organs of *P. vivax*–infected squirrel monkeys [34], as well as with observations of partial depletion

of mature *P. vivax* in the peripheral blood of humans [35, 36]. Our observation of Pv-iE cytoadhesion to placental tissue from sections of the placenta obtained after delivery (data not shown) suggests that the phenomenon can occur in vivo. However, the histopathological studies of postmortem tissues that would be required to indicate whether this phenomenon extends to other organs in infected individuals are limited.

For all isolates for which trypsin treatment was tested, the only partially abrogated Pv-iE cytoadhesion suggests that trypsin-resistant ligands commonly occur on the Pv-iE surface. It would be important to establish whether other receptors are implicated in Pv-iE cytoadhesion and explore the precise roles played by ICAM-1 and CSA. Finally, detailed investigations of the role played by VIR proteins as cytoadhesive ligands, for which we present indirect evidence, are complicated because (1) multiple members of the VIR protein family are expressed on the infected red blood cell surface [17], unlike the clonally expressed *var* genes of *P. falciparum* [37], and (2) the high number of *vir* genes present in the genome (346 *vir* genes for the Sal line of *P. vivax* [31], as opposed to 59 *var* genes for the 3D7 line of *P. falciparum*) [38]. Nevertheless, our data call for further investigation of the role played by VIR proteins in Pv-iE cytoadhesion.

Although infections with *P. vivax* are less life-threatening than those with *P. falciparum*, morbidity in *P. vivax* infection is associated with anemia and a pronounced cytokine-mediated inflammatory response [4]. Differential accumulation of a proportion of parasites to some organs, such as the lungs or placenta, might be targeted by the inflammatory response to this organ, leading to a more severe clinical presentation [4, 39, 40].

In conclusion, our observations add a new aspect to the pathophysiology of a major (yet mostly neglected) human pathogen, which could lead to novel therapeutic approaches to alleviate the increasingly recognized health burden globally imposed by this distinctly not-so-benign parasite.

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References

1. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature **2002**; 415:673–679.
2. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg **2001**; 64:97–106.
3. Nosten F, McGready R, Simpson JA, et al. Effects of *Plasmodium vivax* malaria in pregnancy. Lancet **1999**; 354:546–549.
4. Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivax malaria. Trends Parasitol **2009**; 25:220–227.
5. Genton B, D'Acremont V, Rare L, et al. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. PLoS Med **2008**; 5:e127.
6. Kochar DK, Saxena V, Singh N, Kochar SK, Kumar SV, Das A. *Plasmodium vivax* malaria. Emerg Infect Dis **2005**; 11:132–134.
7. Tjitra E, Anstey NM, Sugiarto P, et al. Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med **2008**; 5:e128.
8. Kyes S, Horrocks P, Newbold C. Antigenic variation at the infected red cell surface in malaria. Annu Rev Microbiol **2001**; 55:673–707.
9. Scherf A, Lopez-Rubio JJ, Riviere L. Antigenic variation in *Plasmodium falciparum.* Annu Rev Microbiol **2008**; 62:445–470.
10. Cserti CM, Dzik WH. The ABO blood group system and *Plasmodium falciparum* malaria. Blood **2007**; 110:2250–2258.
11. Fairhurst RM, Wellems TE. Modulation of malaria virulence by determinants of *Plasmodium falciparum* erythrocyte membrane protein-1 display. Curr Opin Hematol **2006**; 13:124–130.
12. Duffy PE. *Plasmodium* in the placenta: parasites, parity, protection, prevention and possibly preeclampsia. Parasitology **2007**; 134:1877–1881.
13. Ihalamulla RL, Mendis KN. *Plasmodium vivax*: isolation of mature asexual stages and gametocytes from infected human blood by colloidal silica (Percoll) gradient centrifugation. Trans R Soc Trop Med Hyg **1987**; 81:25–28.
14. Fernandez V. Purification and synchronization of erythrocytic stages. In: Ljungström I, Perlmann H, Schlichtherle M, Scherf A, Wahlgren M. Methods in malaria research. 4th ed. Manassas, Virginia: MR4/ATCC, **2004**:26–7.
15. Snounou G, Singh B. Nested PCR analysis of *Plasmodium* parasites. Methods Mol Med **2002**; 72:189–203.
16. Bargieri DY, Rosa DS, Braga CJ, et al. New malaria vaccine candidates based on the *Plasmodium vivax* merozoite surface protein-1 and the TLR-5 agonist *Salmonella* Typhimurium FliC flagellin. Vaccine **2008**; 26:6132–6142.
17. Fernandez-Becerra C, Pein O, de Oliveira TR, et al. Variant proteins of *Plasmodium vivax* are not clonally expressed in natural infections. Mol Microbiol **2005**; 58:648–658.
18. Oliveira TR, Fernandez-Becerra C, Jimenez MC, Del Portillo HA, Soares IS. Evaluation of the acquired immune responses to *Plasmodium vivax* VIR variant antigens in individuals living in malaria-endemic areas of Brazil. Malar J **2006**; 5:83.
19. Costa FT, Fusai T, Parzy D, et al. Immunization with recombinant duffy binding–like–γ3 induces pan-reactive and adhesion-blocking antibodies against placental chondroitin sulfate A–binding *Plasmodium falciparum* parasites. J Infect Dis **2003**; 188:153–164.
20. di Santi SM, Boulos M, Vasconcelos MA, Oliveira S, Couto A, Rosario VE. Characterization of *Plasmodium falciparum* strains of the state of Rondonia, Brazil, using microtests of sensitivity to antimalarials, enzyme typing and monoclonal antibodies [in Portuguese]. Rev Inst Med Trop Sao Paulo **1987**; 29:142–147.
21. Gay F, Robert C, Pouvelle B, Peyrol S, Scherf A, Gysin J. Isolation and characterization of brain microvascular endothelial cells from *Saimiri* monkeys: an in vitro model for sequestration of *Plasmodium falciparum*-infected erythrocytes. J Immunol Methods **1995**; 184:15–28.
22. Muanza K, Gay F, Behr C, Scherf A. Primary culture of human lung microvessel endothelial cells: a useful in vitro model for studying *Plasmodium falciparum*-infected erythrocyte cytoadherence. Res Immunol **1996**; 147:149–163.
23. Hasler T, Albrecht GR, Van Schravendijk MR, et al. An improved microassay for *Plasmodium falciparum* cytoadherence using stable trans-

formants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. Am J Trop Med Hyg **1993**; 48:332–347.
24. Pouvelle B, Fusai T, Lepolard C, Gysin J. Biological and biochemical characteristics of cytoadhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin-4-sulfate. Infect Immun **1998**; 66:4950–4956.
25. Noviyanti R, Brown GV, Wickham ME, Duffy MF, Cowman AF, Reeder JC. Multiple var gene transcripts are expressed in *Plasmodium falciparum* infected erythrocytes selected for adhesion. Mol Biochem Parasitol **2001**; 114:227–237.
26. Pouvelle B, Traore B, Nogueira PA, Pradines B, LePolard C, Gysin J. Modeling of *Plasmodium falciparum*–infected erythrocyte cytoadhesion in microvascular conditions: chondroitin-4-sulfate binding, a competitive phenotype. J Infect Dis **2003**; 187:292–302.
27. Avril M, Traore B, Costa FT, Lepolard C, Gysin J. Placenta cryosections for study of the adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A in flow conditions. Microbes Infect **2004**; 6:249–255.
28. Andrews KT, Adams Y, Viebig NK, Lanzer M, Schwartz-Albiez R. Adherence of *Plasmodium falciparum* infected erythrocytes to CHO-745 cells and inhibition of binding by protein A in the presence of human serum. Int J Parasitol **2005**; 35:1127–1134.
29. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. Blood **1996**; 88:1525–1541.
30. Cooke BM, Berendt AR, Craig AG, MacGregor J, Newbold CI, Nash GB. Rolling and stationary cytoadhesion of red blood cells parasitized by *Plasmodium falciparum*: separate roles for ICAM-1, CD36 and thrombospondin. Br J Haematol **1994**; 87:162–170.
31. Carlton JM, Adams JH, Silva JC, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature **2008**; 455:757–763.
32. del Portillo HA, Fernandez-Becerra C, Bowman S, et al. A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. Nature **2001**; 410:839–842.
33. Resende M, Ditlev SB, Nielsen MA, et al. Chondroitin sulphate A (CSA)-binding of single recombinant Duffy-binding-like domains is not restricted to *Plasmodium falciparum* erythrocyte membrane protein 1 expressed by CSA-binding parasites. Int J Parasitol **2009**; 39:1195–1204.
34. Fremount HN, Rossan RN. The sites of sequestration of the Uganda-Palo Alto strain of *Plasmodium falciparum*-infected red blood cells in the squirrel monkey, *Samimiri sciureus*. J Parasitol **1974**; 60:534–536.
35. Rudolf GD, Ramsay JC. Enumeration of parasites in therapeutic malaria. J Trop Med Hyg **1927**; 30:1–8.
36. Field JW, Shute PG. The microscopic diagnosis of human malaria. II. A morphological study of the erythrocytic parasites. Kuala Lumpur: Government Press, **1956**.
37. Chen Q, Fernandez V, Sundstrom A, et al. Developmental selection of var gene expression in *Plasmodium falciparum*. Nature **1998**; 394:392–395.
38. Gardner MJ, Hall N, Fung E, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **2002**; 419:498–511.
39. Anstey NM, Handojo T, Pain MC, et al. Lung injury in vivax malaria: pathophysiological evidence for pulmonary vascular sequestration and posttreatment alveolar-capillary inflammation. J Infect Dis **2007**; 195:589–596.
40. Tan LK, Yacoub S, Scott S, Bhagani S, Jacobs M. Acute lung injury and other serious complications of *Plasmodium vivax* malaria. Lancet Infect Dis **2008**; 8:449–454.

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Violacein Extracted from *Chromobacterium violaceum* Inhibits *Plasmodium* Growth In Vitro and In Vivo▽

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Violacein is a violet pigment extracted from the gram-negative bacterium *Chromobacterium violaceum*. It presents bactericidal, tumoricidal, trypanocidal, and antileishmanial activities. We show that micromolar concentrations efficiently killed chloroquine-sensitive and -resistant *Plasmodium falciparum* strains in vitro; inhibited parasitemia in vivo, even after parasite establishment; and protected *Plasmodium chabaudi chabaudi*-infected mice from a lethal challenge.

Violacein is a violet pigment isolated from *Chromobacterium violaceum*, a gram-negative betaproteobacterium found in the Amazon River in Brazil. It has been reported to kill bacteria (4) and induces apoptosis in various types of cancer cells (1, 5, 7, 8, 10, 11). Moderate activity against *Trypanosoma cruzi* and *Leishmania amazonensis* promastigotes has also been observed (3, 9). Due to the widespread presence of drug resistance in the malaria parasite, resulting in dramatically decreased efficacy of available antimalarial drugs (15), and the fact that immunoprotection achieved by the most successful malaria vaccine is only partial and short-lived (14), we evaluated the in vitro and in vivo effects of violacein on human and murine blood stage forms of *Plasmodium* parasites.

Isolation and purification of violacein, 3-[1,2-dihydro-5-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-3*H*-pyrrol-3-ilydeno]-1,3-dihydro-2*H*-indol-2-one (Fig. 1), from *C. violaceum* (CCT3496) were performed as previously described (12). Toxicity was measured as the concentration-dependent lysis of normal erythrocytes (NE) by counting red blood cells per milliliter with the aid of a Neubauer chamber. After 48 h of exposure to various concentrations of violacein, the percent red blood cell density (RBCD) relative to that of the control (without violacein) was monitored and calculated according to the formula (RBCD per milliliter in the presence of violacein/RBCD per milliliter without violacein) × 100. As shown in Fig. 2A, a slight reduction in the RBCD percentage at violacein concentrations of >8.0 μM was observed. Significant (Mann-Whitney U test, $P < 0.05$) toxicity to NE occurred at a concentration of 14.0 μM.

Next, we performed dose-response assays to obtain the 50% inhibitory concentrations (IC_{50}s) of violacein against erythrocytes infected with chloroquine-sensitive or -resistant strains of *P. falciparum* (3D7 [16] or S20 [2], respectively) at 1% parasitemia and a 2% final hematocrit. We used [^{3}H]hypoxanthine (Amersham Biosciences, Amersham, United Kingdom) incorporation to assess parasite growth according to a protocol described elsewhere (13). Violacein was tested in triplicate at least three times with different batches and cells, and parasite growth was compared to that in nontreated infected erythrocytes (IE), which represented 100% parasite growth. Percent parasite growth inhibition was calculated according to the formula [1 − (cpm of treated IE − cpm of NE/cpm of nontreated IE − cpm of NE)] × 100. After a 48-h incubation, violacein inhibited parasite development even at the lowest tested concentration of 0.06 μM and completely abrogated parasite viability at concentrations of >1.0 μM (Fig. 2B).

The IC_{50} of violacein against *P. falciparum* strain 3D7 was calculated as 0.85 ± 0.11 μM. We then tested whether the effect of violacein was directed against young (rings, 0 to 24 h) or mature (trophozoites and schizonts, 24 to 48 h) blood forms by using synchronized parasites (± 6 h) obtained by repeated 5% sorbitol treatment as previously described (6). After a 24-h incubation, inhibition of the different parasite stages by violacein was measured. As shown in Fig. 2C, there was no statistically significant difference (Mann-Whitney U test, $P > 0.05$) between the inhibitory values of violacein in either parasite stage. We then asked if susceptibility or resistance to chloroquine also predicts parasite sensitivity to violacein. As shown in Fig. 2D, the violacein IC_{50}s for strains 3D7 and S20 did not differ significantly (0.85 ± 0.11 and 0.63 ± 0.13 μM, respectively; Mann-Whitney U test, $P > 0.05$).

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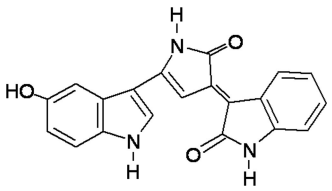

FIG. 1. Chemical structure of violacein.

We then investigated whether violacein antimalarial activity could be sustained in a mouse model, where other characteristics such as bioavailability and pharmacokinetics have to be taken in account. For the in vivo assays, C57BL/6 mice (7 to 10 mice per group, aged 7 to 10 weeks, and with a body weight of 20 ± 3 g) were infected with a nonlethal (AS) or a lethal (AJ) strain of *Plasmodium chabaudi chabaudi* by intraperitoneal (i.p.) injection with 10^6 IE. Parasitemia levels were determined daily by counting the IE among at least 1,000 erythrocytes in Giemsa-stained blood smears. As shown in Fig. 3A, daily administration of violacein i.p. for 11 consecutive days (0 to 10 days postinfection [p.i.]) reduced the parasitemia of *P. chabaudi chabaudi* AS-infected mice. Thirty-nine percent inhibition was observed on day 7 p.i. (parasitemia peak), in comparison to nontreated mice (control; Table 1), even at a low dose of 0.75 mg/kg/day. Moreover, the two highest doses of violacein (3.75 and 7.5 mg/kg/day) almost completely abolished parasitemia on day 7 p.i., corresponding, respectively, to 82 and 87% inhibition of parasite development (Table 1). In addition, violacein doses of 0.75 to 7.5 mg/kg/day were able to inhibit the peak parasitemia in a dose-dependent manner (Table 1).

Since violacein did not completely abrogate parasitemia early in infection and drug pressure was removed by day 10 p.i., we monitored the parasitemia levels of *P. chabaudi chabaudi* AS-infected mice treated with the highest dose of violacein daily until day 22 p.i. Notably, on day 16 p.i., which represents the sixth day after the end of violacein treatment, parasite development was still significantly (Mann-Whitney U test, $P <$

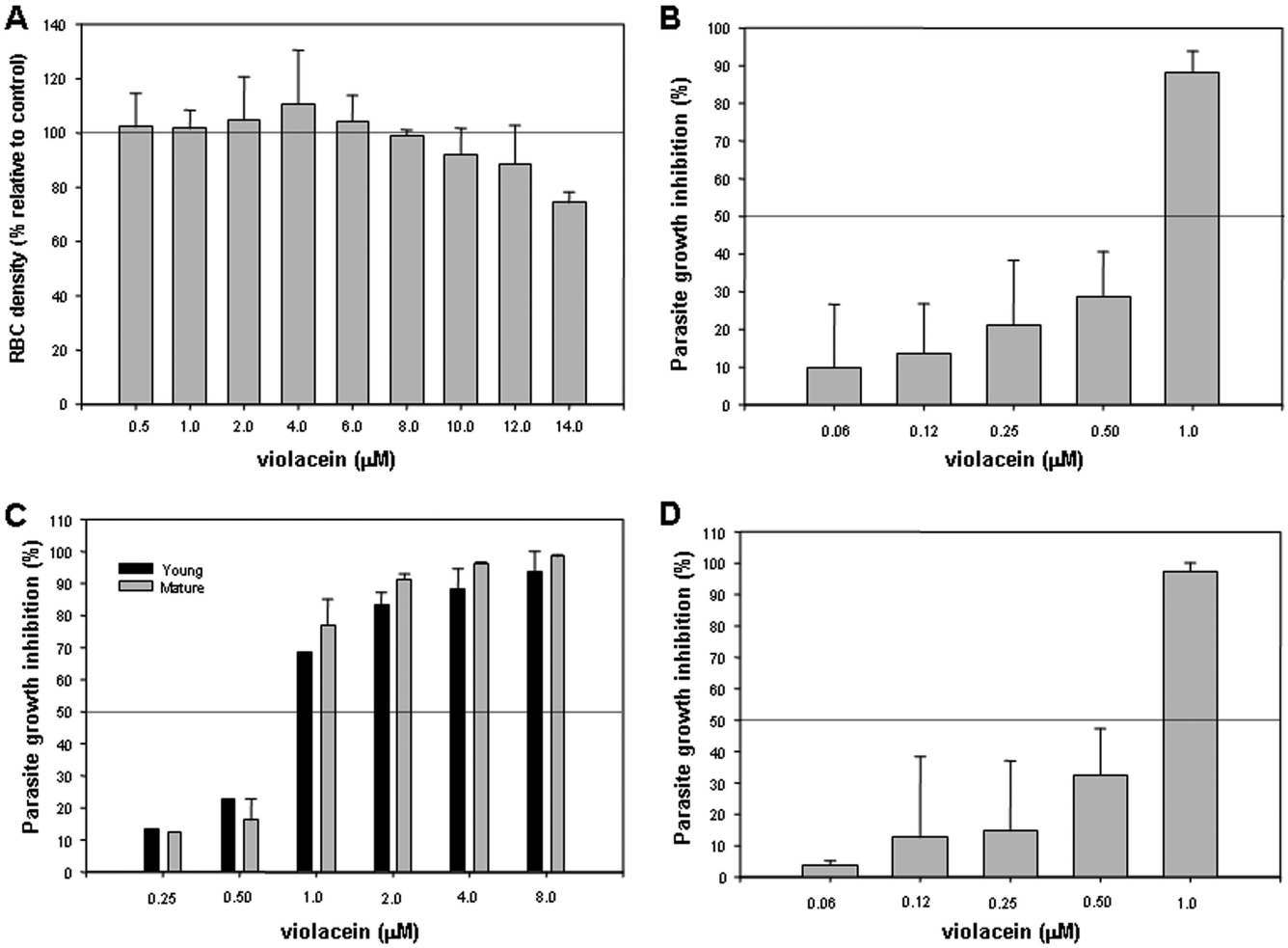

FIG. 2. Evaluation of violacein antimalarial activity against *P. falciparum*. (A) Noninfected erythrocytes (RBC) were cultivated for 48 h at 37°C in the presence of different concentrations of violacein. The RBCD was determined as a percentage of that the control (without drug). Inhibition of the growth of *P. falciparum* 3D7 IE cultivated for 48 h at 37°C with different concentrations of violacein (B) and of young or mature stages of this parasite (C) is also shown. (D) Effect of violacein against a chloroquine-resistant strain (S20) of *P. falciparum* in IE. Results are expressed as the mean of triplicate measurements ± the standard deviation.

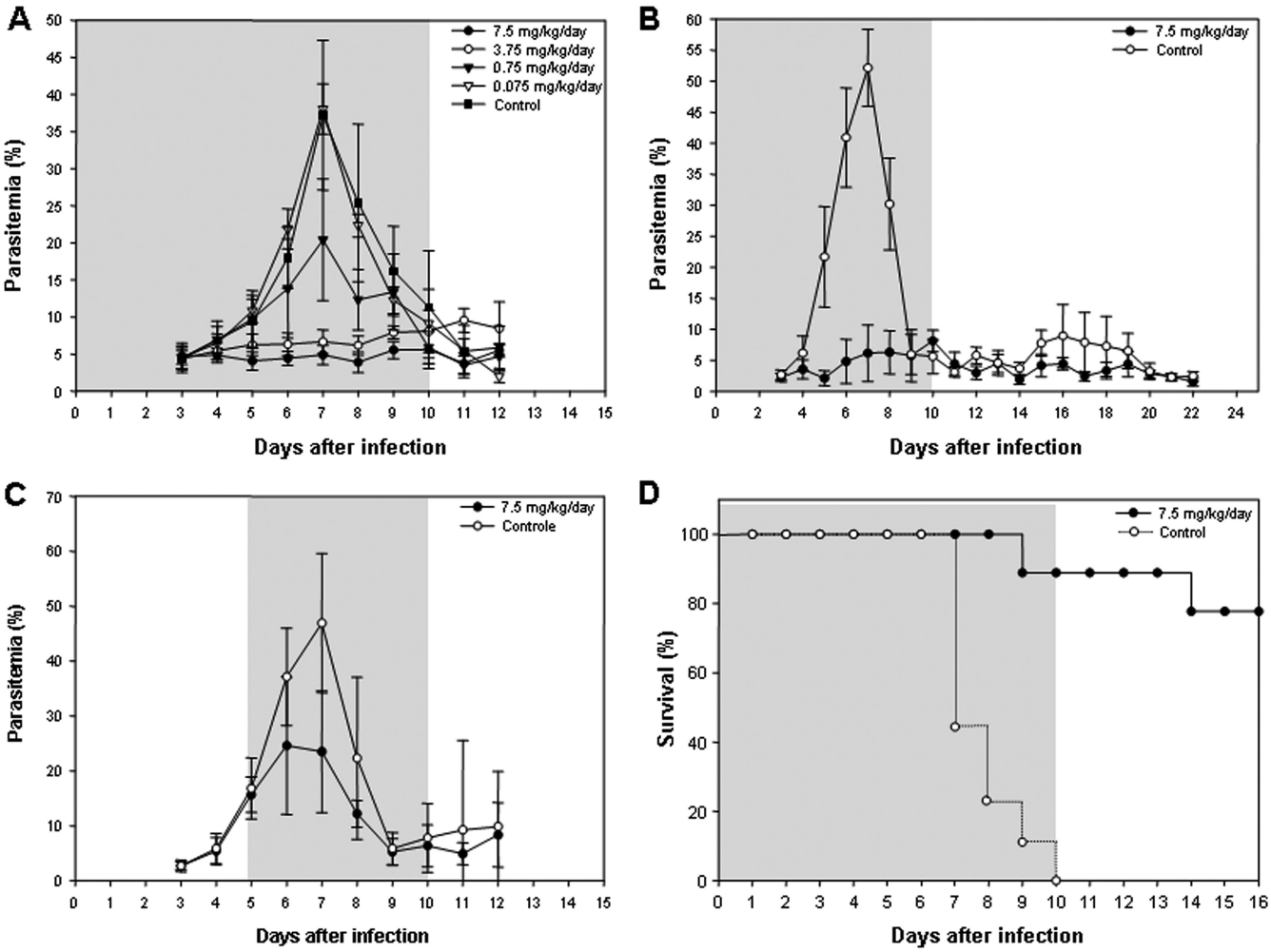

FIG. 3. Effect of violacein on mouse-derived *Plasmodium* parasites. Groups of 7 to 10 C57BL/6 mice were infected i.p. with 10^6 *P. chabaudi chabaudi* AS IE and then left untreated or treated with different doses of violacein administered i.p. for 11 consecutive days (days 0 to 10 p.i.), starting on day 0 at 1 h p.i. Parasitemia levels were determined daily until day 12 p.i. (A) or up to day 22 p.i. (B) after treatment with the highest dose (7.5 mg/kg/day). (C) Analysis of violacein antimalarial activity after parasite establishment and treatment for 6 consecutive days (days 5 to 10 p.i.) with the highest dose of violacein in *P. chabaudi chabaudi* AS-infected mice. Results are expressed as the mean of a group of mice ± the standard deviation. (D) Analysis of the survival of groups of 10 C57BL/6 mice infected with *P. chabaudi chabaudi* AJ (lethal strain) by i.p. injection of 10^6 IE and then treated i.p. with violacein at 7.5 mg/kg/day for 11 consecutive days (days 0 to 10 p.i.). The drug administration period is indicated by shading in each graph.

0.05) inhibited (up to 59%; Fig. 3B). To verify whether violacein had an effect on parasite growth after the establishment of infection, *P. chabaudi chabaudi* AS-infected mice received violacein from day 5 (16% parasitemia) to day 10 p.i. This can reflect the time point when malaria therapy is given to patients. As shown in Fig. 3C, violacein administration during patent parasitemia was able to reduce parasite growth significantly (Mann-Whitney U test, $P < 0.01$), by up to 50.1%, in compar-

TABLE 1. Inhibition of parasitemia in *P. chabaudi chabaudi* AS-infected mice under violacein treatment

Violacein dose (mg/kg/day)	Mean % inhibition[a] ± SD (*P* value)			
	Day 5	Day 6	Day 7	Day 8
7.5	56.48 ± 12.84 (<0.005)	75.40 ± 5.17 (<0.005)	86.90 ± 3.53 (<0.005)	84.67 ± 5.43 (<0.05)
3.75	33.23 ± 14.72 (NS)[b]	64.89 ± 8.00 (<0.005)	82.12 ± 4.33 (<0.005)	75.73 ± 13.93 (<0.05)
0.75	3.58 ± 33.80 (NS)	20.42 ± 36.30 (NS)	39.25 ± 14.15 (<0.05)	48.85 ± 15.08 (NS)
0.075	NI[c] (ND)[d]	NI (ND)	NI (ND)	12.04 ± 6.11 (NS)

[a] The data shown are percentages of the parasitemia of nontreated mice, which was defined as 100%. Values for groups of 7 to 10 mice are shown.
[b] NS, not statistically significant.
[c] NI, no inhibition.
[d] ND, not determined.

ison to nontreated animals on the seventh day of infection. Next, to determine the protective effect of violacein, mice were infected with the lethal AJ strain of *P. chabaudi chabaudi* and their survival rate was evaluated. As shown in Fig. 3D, 100% of the nontreated mice died by day 10 p.i., with 50% of the deaths occurring early on day 7 p.i. In contrast, animals treated with violacein at 7.5 mg/kg/day did not succumb to infection until days 9 (10%) and 14 (10%) p.i., reaching 80% survival on day 16; clearly demonstrating the significant (log rank test, $P < 0.0001$) protective effect of violacein.

This study demonstrates for the first time the antimalarial activity of violacein by showing inhibition of the growth of human- and mouse-derived *Plasmodium* parasites. Also, violacein was effective against young and mature forms of the human parasite and its activity extended to chloroquine-sensitive and -resistant strains of *P. falciparum*. Our data call for new formulations based on violacein nanoparticles to improve solubility, bioavailability, and activity and to decrease drug toxicity.

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REFERENCES

1. **de Carvalho, D. D., F. T. M. Costa, N. Duran, and M. Haun.** 2006. Cytotoxic activity of violacein in human colon cancer cells. Toxicol. In Vitro **20:**1514–1521.
2. **di Santi, S. M., M. Boulos, M. A. Vasconcelos, S. Oliveira, A. Couto, and V. E. Rosário.** 1987. Characterization of Plasmodium falciparum strains of the State of Rondonia, Brazil, using microtests of sensitivity to antimalarials, enzyme typing and monoclonal antibodies. Rev. Inst. Med. Trop. Sao Paulo **29:**142–147.
3. **Durán, N., V. Campos, R. Riveros, A. Joyas, M. F. Pereira, and M. Haun.** 1989. Bacterial chemistry. III. Preliminary studies on trypanosomal activities of Chromobacterium violaceum products. An. Acad. Bras. Cienc. **61:**31–36.
4. **Durán, N., and C. F. Menck.** 2001. *Chromobacterium violaceum*: a review of pharmacological and industrial perspectives. Crit. Rev. Microbiol. **27:**201–222.
5. **Durán, N., G. Z. Justo, C. V. Ferreira, P. S. Melo, L. Cordi, and D. Martins.** 2007. Violacein: properties and biological activities. Biotechnol. Appl. Biochem. **48:**127–133.
6. **Fernandez, V.** 2004. Sorbitol-synchronization of *Plasmodium falciparum*-infected erythrocytes, p. 24. *In* I. Ljungström, H. Perlmann, M. Schlichtherle, A. Scherf, and M. Wahlgren (ed.), Methods in malaria research, 4th ed. MR4/ATCC, Manassas, VA.
7. **Ferreira, C. V., C. L. Bos, H. H. Versteeg, G. Z. Justo, N. Duran, and M. P. Peppelenbosch.** 2004. Molecular mechanism of violacein-mediated human leukemia cell death. Blood **104:**1459–1464.
8. **Kodach, L. L., C. L. Bos, N. Duran, M. P. Peppelenbosch, C. V. Ferreira, and J. C. Hardwick.** 2006. Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells. Carcinogenesis **27:**508–516.
9. **Leon, L. L., C. C. Miranda, A. O. De Souza, and N. Duran.** 2001. Antileishmanial activity of the violacein extracted from Chromobacterium violaceum. J. Antimicrob. Chemother. **48:**449–450.
10. **Melo, P. S., G. Z. Justo, M. B. de Azevedo, N. Duran, and M. Haun.** 2003. Violacein and its β-cyclodextrin complexes induce apoptosis and differentiation in HL60 cells. Toxicology **186:**217–225.
11. **Melo, P. S., S. S. Maria, B. C. Vidal, M. Haun, and N. Duran.** 2000. Violacein cytotoxicity and induction of apoptosis in V79 cells. In Vitro Cell Dev. Biol. Anim. **36:**539–543.
12. **Rettori, D., and N. Durán.** 1998. Production, extraction and purification of violacein: an antibiotic produced by *Chromobacterium violaceum*. World J. Microbiol. Biotechnol. **14:**685–688.
13. **Schmidt, B. A. 2004.** ^{3}H-hypoxanthine incorporation assay for the study of growth inhibition by drugs, p. 87–89. *In* I. Ljungström, H. Perlmann, M. Schlichtherle, A. Scherf, and M. Wahlgren (ed.), Methods in malaria research, 4th ed. MR4/ATCC, Manassas, VA.
14. **Snounou, G., A. C. Grüner, C. D. Müller-Graf, D. Mazier, and L. Rénia.** 2005. The *Plasmodium* sporozoite survives RTS,S vaccination. Trends Parasitol. **21:**456–461.
15. **Talisuna, A.O., P. E Okello, A. Erhart, M. Coosemans, and U. D′Alessandro.** 2007. Intensity of malaria transmission and the spread of *Plasmodium falciparum* resistant malaria: a review of epidemiologic field evidence. Am. J. Trop. Med. Hyg. **77:**170–180.
16. **Walliker, D., I. A. Quakyi, T. E. Wellems, T. F. McCutchan, A. Szarfman, W. T. London, L. M. Corcoran, T. R. Burkot, and R. Carter.** 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science **236:**1661–1666.

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Regulatory T Cell Induction during *Plasmodium chabaudi* Infection Modifies the Clinical Course of Experimental Autoimmune Encephalomyelitis

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Abstract

Background: Experimental autoimmune encephalomyelitis (EAE) is used as an animal model for human multiple sclerosis (MS), which is an inflammatory demyelinating autoimmune disease of the central nervous system characterized by activation of Th1 and/or Th17 cells. Human autoimmune diseases can be either exacerbated or suppressed by infectious agents. Recent studies have shown that regulatory T cells play a crucial role in the escape mechanism of *Plasmodium* spp. both in humans and in experimental models. These cells suppress the Th1 response against the parasite and prevent its elimination. Regulatory T cells have been largely associated with protection or amelioration in several autoimmune diseases, mainly by their capacity to suppress proinflammatory response.

Methodology/Principal Findings: In this study, we verified that $CD4^+CD25^+$ regulatory T cells (T regs) generated during malaria infection (6 days after EAE induction) interfere with the evolution of EAE. We observed a positive correlation between the reduction of EAE clinical symptoms and an increase of parasitemia levels. Suppression of the disease was also accompanied by a decrease in the expression of IL-17 and IFN-γ and increases in the expression of IL-10 and TGF-β1 relative to EAE control mice. The adoptive transfer of $CD4^+CD25^+$ cells from *P. chabaudi*-infected mice reduced the clinical evolution of EAE, confirming the role of these T regs.

Conclusions/Significance: These data corroborate previous findings showing that infections interfere with the prevalence and evolution of autoimmune diseases by inducing regulatory T cells, which regulate EAE in an apparently non-specific manner.

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Introduction

The determinants underlying the heterogeneity of multiple sclerosis (MS) remain unclear. However, current evidence indicates the involvement of a complex genetic trait that probably requires an environmental factor, such as an infection, to be triggered [1]. Classical studies have demonstrated that experimental autoimmune encephalomyelitis (EAE), an experimental model of MS, can be either exacerbated or suppressed by infectious agents [2–6]. EAE is an inflammatory demyelinating autoimmune disease of the CNS that is characterized by the activation of Th1 and/or Th17 cells [7,8]. Amplification of the response of these lymphocytes leads to tissue injury, which can result in demyelination. Regulatory mechanisms might be activated to down regulate exacerbated inflammatory responses. Indeed, regulatory cells that are positive for the expression of the transcription factor Foxp3 have a crucial function in activating immune suppression and in the maintenance of immune homeostasis [9–11]. A deficiency in either the number or function of Foxp3-positive T cells has been described in both the MS and the EAE model [12–15]. The $Foxp3^+$ IL-10-producing cells are associated with the recovery phase of EAE, and *in vitro*-generated or purified natural regulatory T cells prevent the induction of EAE by producing IL-10 [16].

On the other hand, some studies have shown that the activation of T regs, either in experimental models of malaria or in humans infected by the malaria parasite, suppresses the Th1 response and prevents elimination of the parasite. The enhancement of $CD4^+CD25^+$ regulatory T cells probably plays a crucial role in this escape mechanism [17–22]. These natural regulatory cells seem to be associated with a burst of TGF-β production and decreases in antigen-specific responses and proinflammatory cytokine production [23].

In the present study, we used experimental malaria infection and the murine MS model to investigate how the regulatory

mechanisms induced during *Plasmodium* infection interfere with the clinical course and immune responses in the EAE model.

Materials and Methods

Animals

C57BL/6 mice (6–8 weeks old) were purchased from the University of Campinas (Campinas, SP, Brazil) and maintained in a specific pathogen-free animal facility. All experiments and procedures were approved by the UNICAMP Committee for Ethics in Animal Research (Protocol No. 857-1).

Induction of EAE

Briefly, mice were injected subcutaneously (s.c.) with 100 μg/animal of $pMOG_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK) or $pCIR_{180-198}$ (NPYCNVLTNLKN DYDKIRK) (Genemed Synthesis, CA, USA) emulsified in complete Freund's adjuvant containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). Each immunized animal receives a total of 100 μl of the emulsion in the both upper flanks and 200 ng/mouse of Pertussis toxin intraperitoneal (i.p.) (List Biochemicals, Campbell, CA, USA) on days 0 and 2 after immunization. Clinical expression of the disease was graded on a clinical index scale of 0 to 5 as previously described [24].

Malaria infection

Mice were infected i.p. with 10^6 infected red blood cells (iRBCs) of the non-lethal line of *P. chabaudi chabaudi* AS or were injected with saline only (negative control group), 6 days after EAE induction (6 d.a.i) or 25 days prior the EAE induction (post-infection with *P. chabaudi*). The group injected with *P. chabaudi* 6 d.a.i. presented a peak of parasitemia in the same day of EAE maximum clinical score.

The blood stage forms of both parasites were stored in liquid nitrogen after *in vivo* passages in C57BL/6 mice according to a protocol described elsewhere [25]. The percentage of parasitemia was determined daily by counting the number of iRBCs among at least 1,000 erythrocytes in Giemsa-stained blood smears. The corporal temperatures and relative body weights of the mice were evaluated daily, starting on day 1 post-infection, by rectal introduction of a precision digital thermometer (model TE-300, Instrucamp, Brazil) and with a precision balance (Metter Toledo), respectively.

Flow Cytometry

All analyses were performed using a flow cytometer (FACScanto or FACSCalibur) (BD Bioscience, San Jose, CA, USA). For Foxp3 labeling, permeabilization buffer (PBS 10% rat serum and 1% Triton) was used. The antibodies were as follows: anti-CD4 FITC, anti-CD4 PE, anti-CD25 PE (BD Bioscience, San Jose, CA, USA) and anti-Foxp3 APC (eBioscience, San Diego, CA, USA). The data were analyzed using FACSDiva (BD Bioscience, San Jose, CA, USA) or MDI 2.9 software.

Cell sorting

All sorting were performed using a cell sorter flow cytometer FACSAria (BD Bioscience, San Jose, CA, USA). Cells were kept on ice before and after sorting analysis. Cell purity was confirmed immediately after sorting and cell viability was assured before the transfer. Each animal was injected (i.v.) with 1×10^6 viable cells.

Quantitative real-time PCR (qRT-PCR)

mRNA was extracted using Trizol and reverse transcribed to generate cDNA. Taqman analysis was performed using a Taqman ABI Prism 7500 Sequence Detector (PE Applied Biosystems, Darmstadt, Germany). The primers GAPDH, IL-17A, IL-10, TGF-β_1, IL-27 and Foxp3 were obtained from Applied Biosciences (Mm00439619_m1 (IL-17A), Mm99999915_m1 (GAPDH), Mm00475156_m1 (Foxp3), Mm03024053 (TGFβ1), Mm00461164 (IL-27), Mm00439616_m1 (IL-10)). The specific mRNAs were normalized to the expression of a housekeeping gene (GAPDH). The data were obtained using independent duplicate measurements. The threshold cycle value of the individual measurements did not exceed 0.5 amplification cycles.

Statistical Analysis

The statistical significance of the results was determined using a non-parametric analysis of variance (Kruskal-Wallis test), the Mann-Whitney test (U-test) or a non-parametric correlation (Spearman's rank). A p value less than 0.05 was considered significant.

Results

Modulation of EAE and P. chabaudi infection

The inductions of EAE and *P. chabaudi* infection were designed to coincide with the peak of the clinical symptoms of EAE. EAE was more severe approximately 14 days after immunization with $pMOG_{35-55}$, and the peak of parasitemia occurred 7 days after infection (Fig. 1A). The presence of an autoimmune response during EAE induction did not change the parasitemia levels (Fig. 1B) or the corporal temperature of the animals during the course of malaria infection (Fig. 1C). However, the clinical course of EAE was significantly diminished until 28 days post-EAE induction (Fig. 1D). The reduction in clinical signs correlated with a loss of body weight. As shown in Figure 1E, animals with EAE and infected with *P. chabaudi* 6 d.a.i presented a modest loss of body weight relative to the group with EAE alone. Of note, parasitemia and clinical symptoms of EAE were significantly negatively correlated during the clinical course of the disease in the group of mice infected with *P. chabaudi* 6 d.a.i (Fig. 1F).

Next, we measured cytokine expression by qRT-PCR to verify whether the reduction in the clinical signs of EAE observed in infected with *P. chabaudi* 6 d.a.i was related to differences in the cytokine expression profile. As shown in Figure 2, a significant reduction in IL-17A expression was noted in the regional lymph nodes in the EAE-malaria group in comparison to the EAE group. Moreover, corroborating the reduction in the pro-inflammatory profile, we discovered higher expression of IL-10, TGF-β_1 and IL-27 in both lymph nodes in the EAE-malaria mice relative to the EAE-alone mice (Fig. 2).

Involvement of regulatory T cells

Corroborating with previous studies investigating experimental malaria infection models [17], the percentages of $CD4^+CD25^+$ regulatory T cells increased in the spleen as the parasitemia evolved, with a peak observed on day 4 after infection (6.4%) (Fig. 3A). By contrast, naïve mice presented $CD4^+CD25^+$ percentages (2.0%) were three fold lower than those detected in malaria-infected mice. A significant difference in the percentage of T regs was noted on day 4 post-infection in the spleen of EAE mice when compared to EAE-malaria-infected animals (Fig. 3B). Indeed, whereas only 6.0% of the cells harvested from EAE mice were $CD4^+CD25^+Foxp3^+$ T cells, in the EAE animals harboring a malaria infection, up to 14.1% of the CD4+ cells expressed $CD4^+CD25^+Foxp3^+$. Analyses of peripheral blood cells revealed a minor increase in the percentage of T reg cells in the EAE-malaria

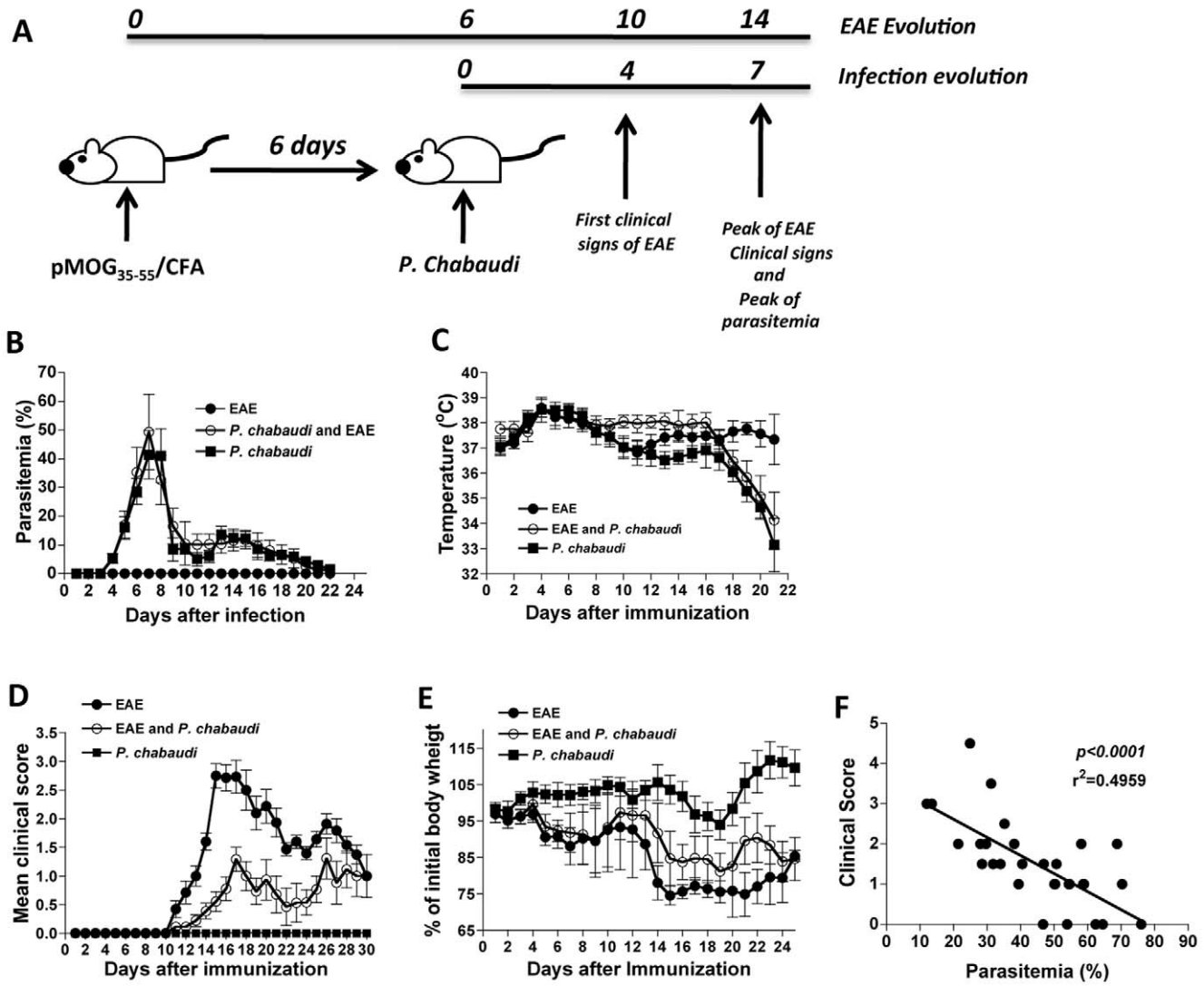

Figure 1. Malaria infection *P. chabaudi*-infected mice 6 d.a.i ameliorates clinical signs of autoimmunity. (**A**) Experimental design of EAE induction and *P. chabaudi* infection. (**B**) Parasitemia levels, (**C**) corporal temperature, (**D**) EAE clinical score and (**E**) weight loss in groups of mice infected with *P. chabaudi* alone (*P. chabaudi* - black squares), *P. chabaudi* infected 6 days after EAE induction (*P. chabaudi* + EAE - white circles), and in mice harboring EAE alone (EAE - black circles). The results are expressed as the mean of each group of mice (n = 5–8) ± SD. (**F**) Correlation between parasitemia level and EAE clinical score in the pool of mice harboring a malaria infection 6 days after EAE induction.
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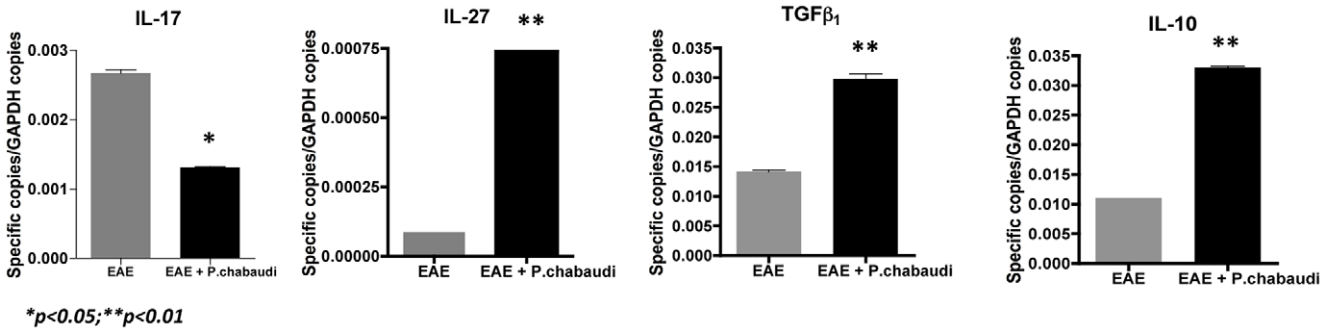

Figure 2. Induction of EAE in *Plasmodium chabaudi*-infected mice modulates their cytokine expression profile. Expression levels of IL-17A, IL-10, IL-27 and TGF-β_1 measured by qRT-PCR in *P. chabaudi* infected 6 days after EAE induction (black bars) and the EAE alone (gray bars) group. The values represent the mean number of specific cytokine gene copies relative to GAPDH in three-five mice ± SD.
doi:10.1371/journal.pone.0017849.g002

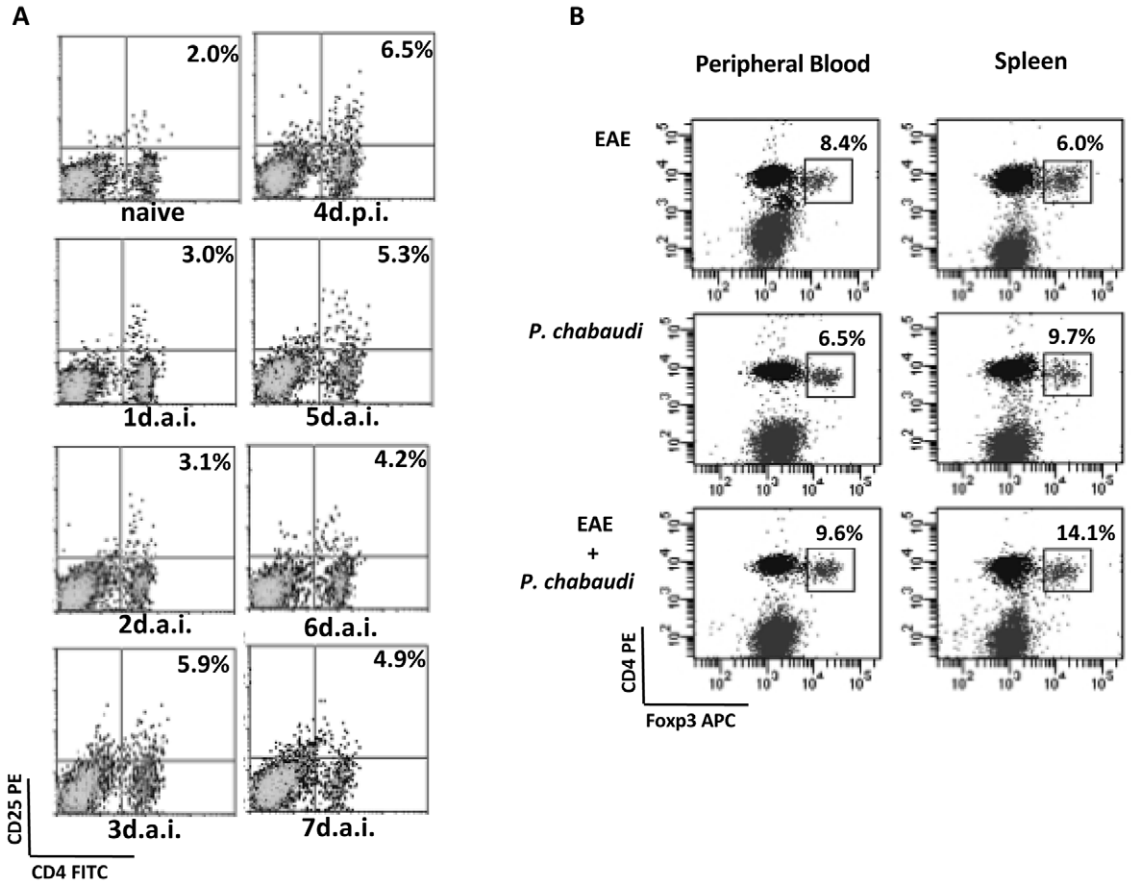

Figure 3. Mice harboring a malaria infection display an increase in the percentage of T regs during EAE evolution. (**A**) Daily quantification of $CD4^+CD25^+$ cells in *P. chabaudi*-infected mice on days 0–7 post-infection. (**B**) Quantification of $CD4^+CD25^+Foxp3^+$ cells 4 days post-infection and/or 10 days after EAE induction. The percentages are relative to the total number of $CD4^+$ cells and are representative of three independent assays.
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(9.6%) relative to the EAE-only mice (8.4%). Nevertheless, these values did not reach statistical significance (Fig. 3C).

$CD4^+CD25^+$ regulatory T cell transfer

To verify the participation of $CD4^+CD25^+$ cells in the regulation of autoimmune responses during EAE, we transferred purified $CD4^+CD25^+$ T cells from *P. chabaudi*-infected mice four days after infection into MOG_{35-55}-immunized mice. No parasites were detected in the $CD4^+CD25^+$ cell preparation. Sorting analyses revealed that $CD4^+CD25^-$ (Fig. 4C) and $CD4^+CD25^+$ (Fig. 4D) T cells were highly pure (95%±2%) and expressed high levels of Foxp3, as revealed by testing an aliquot of 10^6 sorted cells (Fig. 4E). Thus, each set ($CD4^+CD25^-$ or $CD4^+CD25^+$) of T cells were transferred intravenously into a different group of MOG35-55 immunized mice 10 days after EAE induction. The transfer of $CD4^+CD25^+$ cells harvested and sorted from EAE-malaria mice significantly diminished the evolution of clinical signs of EAE ($p<0.01$) as compared to animals that were immunized only, or even to the group that received $CD4^+CD25^-$ cells (Fig. 4F). Of note, the anti-inflammatory effect of the T regs was temporary, because 10 days after the transfer, no significant difference in EAE clinical scores were found between the two groups.

Longevity of the regulatory T cell effect

To determine whether the malaria-induced regulatory T cells were able to sustain their anti-inflammatory effect and thereby prevent mice from developing clinical signs of EAE, we induced EAE 25 days after infection with *P. chabaudi* (Fig. 5A). At this time point, almost no parasites were detected in the peripheral blood (Fig. 1B). No significant differences in the evolution of the disease were observed between the group with previous *P. chabaudi* infection and the group that was immunized with MOG peptide only (EAE) (Fig. 5B). Indeed, this observation is consistent with the low percentage of $CD4^+CD25^+$ T cells (2.2%) harvested from the mice 35 days p.i. (Fig. 5C), which did not differ significantly from the percentage observed in naïve animals. These results indicate that the mechanism that suppresses the autoimmune response in *P. chabaudi* infection is transitory and does not generate a specific memory.

Discussion

In the present study, we used *P. chabaudi* infection and EAE induction to demonstrate that the immune response to *Plasmodium* spp. modulates the autoimmune response.

One of the main challenges of multiple sclerosis is an understanding its etiology. It is currently well accepted that the disease results from a genetic predisposition combined with an environmental factor such as an infection [26]. However, many recent studies have demonstrated an immunoregulatory role of infectious agents or their products during the evolution of EAE or MS [4–6]. In all likelihood, both observations are compatible

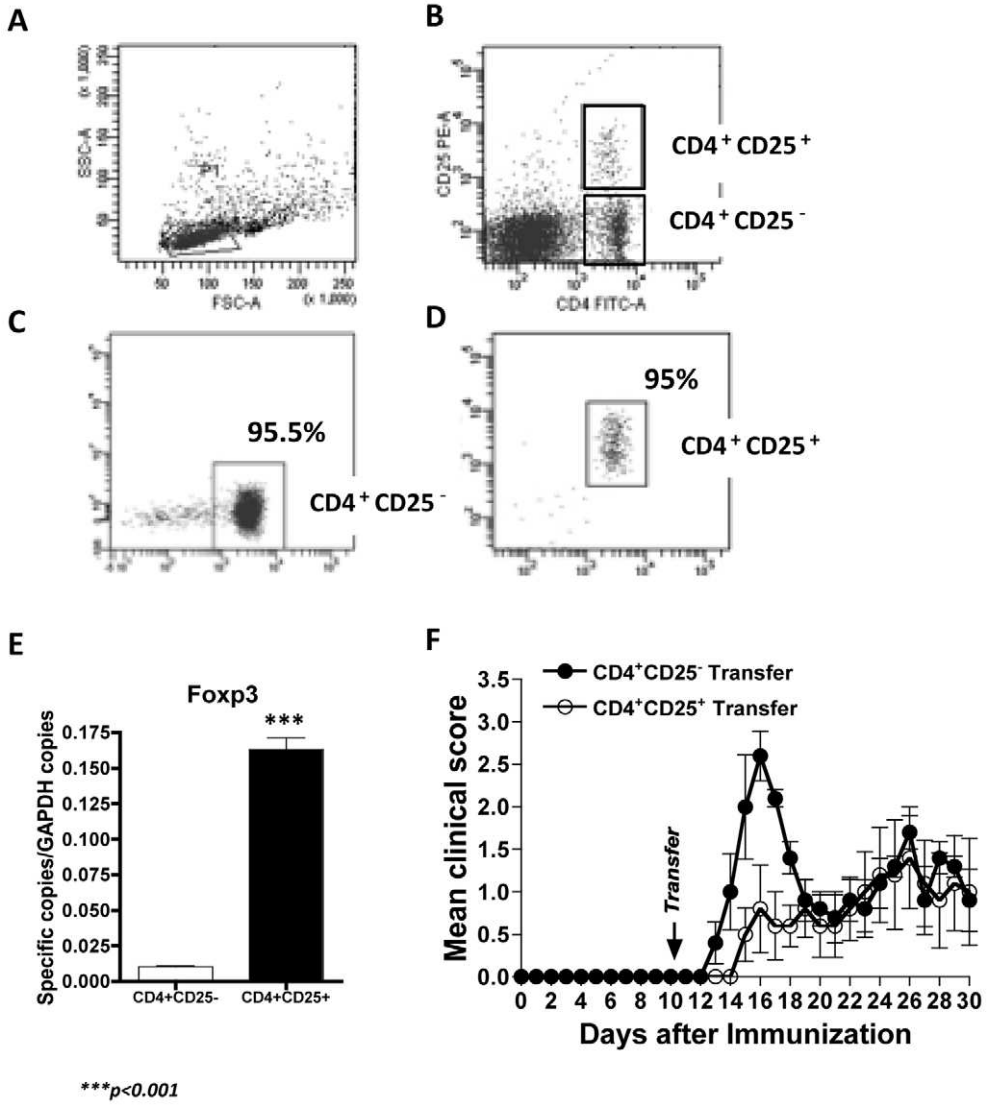

Figure 4. Transfer of $CD4^+CD25^+$ regulatory T cells diminishes the clinical signs of EAE. (**A**) Lymphocytes are gated from whole spleen cells from a pool of 3–4 mice infected with *P. chabaudi*. (**B**) Selection of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells before sorting. (**C**) Purity of the sorted $CD4^+CD25^-$ and (**D**) $CD4^+CD25^+$ cells. (**E**) qRT-PCR assessment of expression levels of Foxp3 in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells after sorting. The values represent the mean number of specific cytokine gene copies relative to GAPDH ± SD. **(F)** Evolution of the clinical scores of EAE following the transfer of $CD4^+CD25^+$ (white circles) or $CD4^+CD25^-$ cells (black circles). The results represent the mean of three-five mice ± SD.
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depending on the genetic predisposition of each individual, the specific action of each infectious agent and the specific timing of the infection.

Here, we conducted a study to understand the effects of malaria infection on the evolution of MS in a murine experimental model. When EAE induction coincided with the peak of infection in *P. chabaudi*-infected mice, we observed a reduction in the clinical signs of EAE, including weight loss. Of note, we discovered a negative correlation between the clinical score for EAE and the percentage of iRBCs. The protective effect of malaria infection on EAE evolution was correlated with the high expression levels of IL-10, IL-27 and TGF-β_1 and reduced levels of IL-17. Moreover, an elevated percentage of T regs on day 4 p.i. with *P. chabaudi* seemed to play a pivotal role in EAE amelioration. Interestingly, the presence of a proinflammatory response evoked during EAE evolution did not alter the evolution of parasitemia. The specific proinflammatory response to MOG_{35-55} might not influence reactivity to malarial antigens. The suppression of immune responses by *Plasmodium* parasites might be explained, at least in part, by the capacity of human or murine iRBCs to convert latent TGF-β into its bioactive form [27]. The activation of TGF-β by the parasite might induce the conversion of naïve T cells into T reg cells, or it might directly suppress the autoimmune response [28–29].

In recent years, a growing body of evidence has demonstrated the importance of T regs in the immunological response induced during malaria infection in humans [19–22], and in several experimental models [17,30]. The enhancement of T regs apparently contributes to the immune evasion mechanism of *Plasmodium spp.* and allows parasite development, although this phenomenon may not hold true for all murine-derived *Plasmodium* species [31]. In vitro co-cultures of PBMCs and *P. falciparum*-infected red blood cells induce $CD4^+CD25^+Foxp3^+$ T cells [32], and a positive correlation between the absolute number of

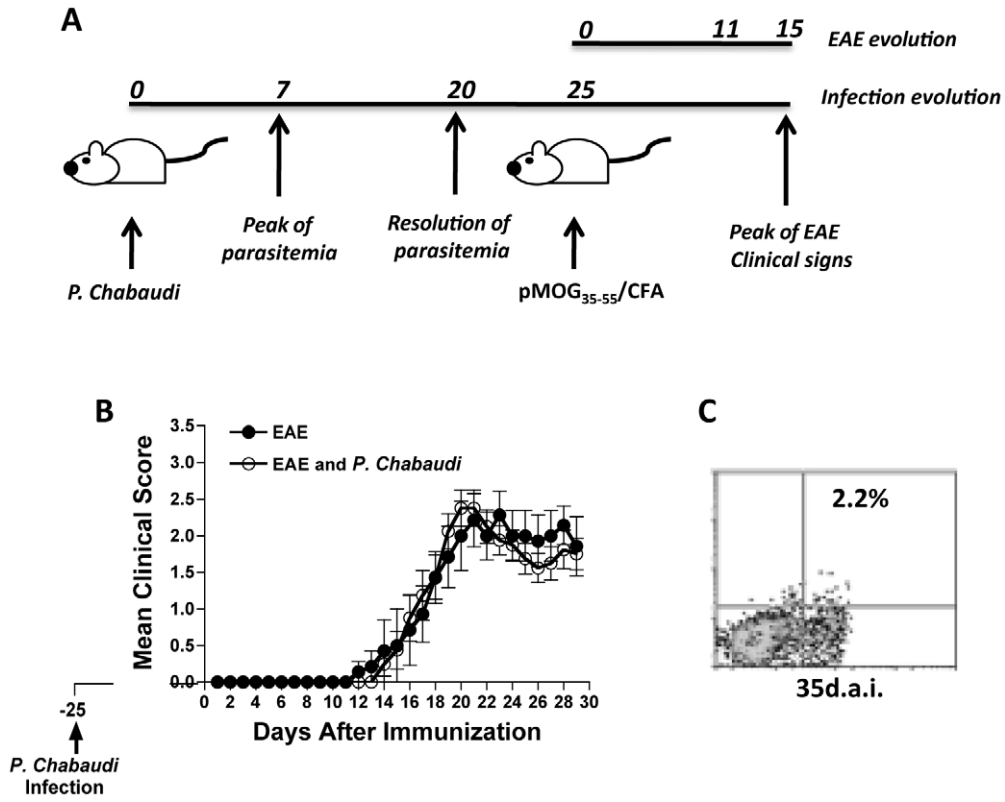

Figure 5. Induction of EAE after recovery from malaria infection does not reduce clinical signs. (**A**) Experimental design of post *P. chabaudi* infection EAE induction in *P. chabaudi*-infected mice (n = 5–7). (**B**) EAE induction 25 days post-*P. chabaudi* infection (white circles) or EAE induction alone (black circles). The results represent the mean of three-five mice ± SD. (**C**) $CD4^+CD25^+$ quantification 25 days post-*P. chabaudi* infection.
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circulating T regs and the parasite burden during acute *P. vivax* infection has been recently observed [22].

Our data demonstrate a progressive increase in the percentage of $CD4^+CD25^+$ T cells during *P. chabaudi* infection, with a peak observed on day 4 after infection. These $CD4^+CD25^+$ T cells express high amounts of Foxp3 in both *P. chabaudi*-infected mice and in *P. chabaudi*-infected mice 6 d.a.i when compared with EAE-alone mice. This finding may explain the observed suppressive effect of malaria infection on the course of EAE. A large number of reports have highlighted the role of regulatory T cells both in the prevention of EAE induction [33–34] and in the resolution of the disease [16,35], mainly via IL-10 and/or TGF-β induction in these cells [36].

Because $CD4^+CD25^+Foxp3^+$ T regs are capable of inhibiting proinflammatory responses by releasing IL-10 and TGF-β_1, in our model and in other autoimmune models [10], it is conceivable that these $CD4^+CD25^+Foxp3^+$ T regs might play a central role in regulating the autoimmune response during *Plasmodium* infection. To test this hypothesis, we transferred highly pure sorted $CD4^+CD25^+$ T cells from *P. chabaudi*-infected mice four days after infection into MOG_{35-55} immunized mice, avoiding the introduction of parasites into the system. The adoptive transfer of $CD4^+CD25^+$ T cells, which express higher amounts of Foxp3, but not of $CD4^+CD25^-$ cells, significantly diminished the clinical signs of EAE for approximately 10 days, similar to the signs observed with *P. chabaudi*-infected mice 6 d.a.i. These results unequivocally demonstrated the central role of malaria-induced T regs in the control of EAE development. However, when EAE was induced in a group of mice with controlled parasitemia, no amelioration of the clinical signs of EAE was observed, and the percentage of $CD4^+CD25^+$ cells was comparable to that in naïve animals. Our findings indicate that the process of T reg cell enhancement is not long lasting and probably does not induce immunological memory. Indeed, these T regs are rapidly inducible and short-lived, as previously described [37]. Nonetheless, malaria-induced T regs are able to suppress responses to non-malarial antigens [38].

Despite these indications, we tried to establish some cross-specificity between the MOG protein and *P. chabaudi* antigens. Using the Sanger Plasmodium databank, we were able to demonstrate significant homology between the MOG protein (NP_034944) and the CIR protein (PC500044.00.0). Five different regions presented some similarity when the two proteins were aligned (Fig. S1A). One of these five regions exactly matched the MOG_{35-55} peptide (Fig. S1B). However, no cross-reactivity was observed when this peptide ($CIR_{180-198}$) was used either to induce EAE in naïve animals or to treat MOG_{35-55} immunized animals (data not shown). However, we cannot exclude the possibility of polyclonal expansion of lymphocytes promoted by the malaria infection, which may enhance MOG_{35-55} specific T regs and thereby cause the phenomena described herein, albeit in smaller numbers. Nevertheless, our data provide strong evidence that once activated by the TCR or during polyclonal expansion, T regs may exert their suppression in a completely non-specific manner. Indeed, Thornton and Shevach have proposed this non-specific suppressor mechanism using an *in vitro* model system [39]. Moreover, the regulatory functions of these T regs are probably due to their expression and release of inhibitory cytokines such as TGF-β and IL-10 in a standard suppressive manner.

In the present study we describe for the first time the ability of *Plasmodium* infection to interfere with the EAE autoimmune response. Our findings indicate that the T reg cells generated during *P. chabaudi* infection act in an apparently non-specific manner to control the proinflammatory response, mainly via increased expression of IL-10 and TGF-β_1.

Supporting Information

Figure S1 (**A**) Amino acid sequence alignment of the MOG (black) and CIR proteins (http://www.ebi.ac.uk: Accession number: needle-20100715-1546033194.output) (red). Green symbols indicate identical amino acids; yellow symbols represent conservative changes and blue symbols represent semi-conservative changes. (**B**) Sequence comparison of the MOG_{35-55} and $CIR_{180-198}$ peptides.
(TIF)

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Author Contributions

Conceived and designed the experiments: FTMC ASF. Performed the experiments: ASF RLT YCB SCPL ALFL FP. Analyzed the data: FTMC ASF. Contributed reagents/materials/analysis tools: LMBS. Wrote the paper: FTMC ASF.

References

1. Hauser SL, Oksenberg JR (2006) The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. Neuron 52: 61–76.
2. Brocke S, Gaur A, Piercy C, Gautam A, Gijbels K, et al. (1993) Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. Nature 365: 642–644.
3. Peacock JW, Elsawa SF, Petty CC, Hickey WF, Bost KL (2003) Exacerbation of experimental autoimmune encephalomyelitis in rodents infected with murine gammaherpesvirus-68. Eur J Immunol 33: 1849–1858.
4. Lehmann D, Ben-Nun A (1992) Bacterial agents protect against autoimmune disease. I. Mice pre-exposed to Bordetella pertussis or Mycobacterium tuberculosis are highly refractory to induction of experimental autoimmune encephalomyelitis. J Autoimmun 5: 675–690.
5. Tadokoro CE, Vallochi AL, Rios LS, Martins GA, Schlesinger D, et al. (2004) Experimental autoimmune encephalomyelitis can be prevented and cured by infection with Trypanosoma cruzi. J Autoimmun 23: 103–115.
6. Bach JF (2001) Protective role of infections and vaccinations on autoimmune diseases. J Autoimmun 16: 347–353.
7. Korn T, Oukka M, Kuchroo V, Bettelli E (2007) Th17 cells: effector T cells with inflammatory properties. Semin Immunol 19: 362–371.
8. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, et al. (2008) Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. J Immunol 181: 3750–3754.
9. Shevach EM (2009) Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 30: 636–645.
10. Wing K, Sakaguchi S (2010) Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol 11: 7–13.
11. Tang Q, Bluestone JA (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 9: 239–244.
12. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, et al. (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 212: 8–27.
13. O'Connor RA, Anderton SM (2008) Foxp3+ regulatory T cells in the control of experimental CNS autoimmune disease. J Neuroimmunol 193: 1–11.
14. Feger U, Luther C, Poeschel S, Melms A, Tolosa E, et al. (2007) Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. Clin Exp Immunol 147: 412–418.
15. Anderton SM, Liblau RS (2008) Regulatory T cells in the control of inflammatory demyelinating diseases of the central nervous system. Curr Opin Neurol 21: 248–254.
16. McGeachy MJ, Stephens LA, Anderton SM (2005) Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J Immunol 175: 3025–3032.
17. Hisaeda H, Yasutomo K, Himeno K (2005) Malaria: immune evasion by parasites. Int J Biochem Cell Biol 37: 700–706.
18. Finney OC, Nwakanma D, Conway DJ, Walther M, Riley EM (2009) Homeostatic regulation of T effector to Treg ratios in an area of seasonal malaria transmission. Eur J Immunol 39: 1288–1300.
19. Walther M, Jeffries D, Finney OC, Njie M, Ebonyi A, et al. (2009) Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria. PLoS Pathog 5: e1000364.
20. Riley EM, Wahl S, Perkins DJ, Schofield L (2006) Regulating immunity to malaria. Parasite Immunol 28: 35–49.
21. Bueno LL, Morais CG, Araujo FF, Gomes JA, Correa-Oliveira R, et al. Plasmodium vivax: induction of CD4+CD25+FoxP3+ regulatory T cells during infection are directly associated with level of circulating parasites. PLoS ONE 5: e9623.
22. Minigo G, Woodberry T, Piera KA, Salwati E, Tjitra E, et al. (2009) Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. PLoS Pathog 5: e1000402.
23. Walther M, Tongren JE, Andrews L, Korbel D, King E, et al. (2005) Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. Immunity 23: 287–296.
24. Farias AS, de la Hoz C, Castro FR, Oliveira EC, Ribeiro dos Reis JR, et al. (2007) Nitric oxide and TNFalpha effects in experimental autoimmune encephalomyelitis demyelination. Neuroimmunomodulation 14: 32–38.
25. Blanco YC, Farias AS, Goelnitz U, Lopes SC, Arrais-Silva WW, et al. (2008) Hyperbaric oxygen prevents early death caused by experimental cerebral malaria. PLoS ONE 3: e3126.
26. Hafler DA, Slavik JM, Anderson DE, O'Connor KC, De Jager P, et al. (2005) Multiple sclerosis. Immunol Rev 204: 208–231.
27. Omer FM, de Souza JB, Corran PH, Sultan AA, Riley EM (2003) Activation of transforming growth factor beta by malaria parasite-derived metalloproteinases and a thrombospondin-like molecule. J Exp Med 198: 1817–1827.
28. Chen W, Jin W, Hardegen N, Lei KJ, Li L, et al. (2003) Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 198: 1875–1886.
29. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, et al. (2008) A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. Nat Immunol 9: 632–640.
30. Nie CQ, Bernard NJ, Schofield L, Hansen DS (2007) CD4+ CD25+ regulatory T cells suppress CD4+ T-cell function and inhibit the development of Plasmodium berghei-specific TH1 responses involved in cerebral malaria pathogenesis. Infect Immun 75: 2275–2282.
31. Cambos M, Belanger B, Jacques A, Roulet A, Scorza T (2008) Natural regulatory (CD4+CD25+FOXP+) T cells control the production of pro-inflammatory cytokines during Plasmodium chabaudi adami infection and do not contribute to immune evasion. Int J Parasitol 38: 229–238.
32. Scholzen A, Mittag D, Rogerson SJ, Cooke BM, Plebanski M (2009) Plasmodium falciparum-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. PLoS Pathog 5: e1000543.
33. Furtado GC, Olivares-Villagomez D, Curotto de Lafaille MA, Wensky AK, Latkowski JA, et al. (2001) Regulatory T cells in spontaneous autoimmune encephalomyelitis. Immunol Rev 182: 122–134.
34. Olivares-Villagomez D, Wang Y, Lafaille JJ (1998) Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. J Exp Med 188: 1883–1894.
35. Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, et al. (2007) Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 13: 423–431.
36. Bettini M, Vignali DA (2009) Regulatory T cells and inhibitory cytokines in autoimmunity. Curr Opin Immunol 21: 612–618.
37. Strauch UG, Obermeier F, Grunwald N, Gurster S, Dunger N, et al. (2005) Influence of intestinal bacteria on induction of regulatory T cells: lessons from a transfer model of colitis. Gut 54: 1546–1552.
38. Todryk SM, Walther M, Bejon P, Hutchings C, Thompson FM, et al. (2009) Multiple functions of human T cells generated by experimental malaria challenge. Eur J Immunol 39: 3042–3051.
39. Thornton AM, Shevach EM (2000) Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol 164: 183–190.

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Immunogenic properties of a recombinant fusion protein containing the C-terminal 19 kDa of *Plasmodium falciparum* merozoite surface protein-1 and the innate immunity agonist FliC flagellin of *Salmonella* Typhimurium

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ABSTRACT

In a recent study, we demonstrated the immunogenic properties of a new malaria vaccine polypeptide based on a 19 kDa C-terminal fragment of the merozoite surface protein-1 ($MSP1_{19}$) from *Plasmodium vivax* and an innate immunity agonist, the *Salmonella enterica* serovar Typhimurium flagellin (FliC). Herein, we tested whether the same strategy, based on the $MSP1_{19}$ component of the deadly malaria parasite *Plasmodium falciparum*, could also generate a fusion polypeptide with enhanced immunogenicity. The His_6FliC-$MSP1_{19}$ fusion protein was expressed from a recombinant *Escherichia coli* and showed preserved *in vitro* TLR5-binding activity. In contrast to animals injected with His_6MSP1_{19}, mice subcutaneously immunised with the recombinant His_6FliC-$MSP1_{19}$ developed strong $MSP1_{19}$-specific systemic antibody responses with a prevailing IgG1 subclass. Incorporation of other adjuvants, such as CpG ODN 1826, complete and incomplete Freund's adjuvants or Quil-A, improved the IgG responses after the second, but not the third, immunising dose. It also resulted in a more balanced IgG subclass response, as evaluated by the IgG1/IgG2c ratio, and higher cell-mediated immune response, as determined by the detection of antigen-specific interferon-γ secretion by immune spleen cells. $MSP1_{19}$-specific antibodies recognised not only the recombinant protein, but also the native protein expressed on the surface of *P. falciparum* parasites. Finally, sera from rabbits immunised with the fusion protein alone inhibited the *in vitro* growth of three different *P. falciparum* strains. In summary, these results extend our previous observations and further demonstrate that fusion of the innate immunity agonist FliC to *Plasmodium* antigens is a promising alternative to improve their immunogenicity.

1. Introduction

Plasmodium falciparum is estimated to cause around 250 million malaria cases every year, leading to 1 million deaths, mostly of children under 5 years of age [1]. Drug resistance to this parasite has emerged, reducing the efficacy of conventional treatment and often contributing to malaria-related mortality [2]. Therefore, prophylactic alternatives, such as effective vaccines, are urgently needed.

Immunity to malaria is a multi-factorial process that involves various components of the adaptive immune system. Antibody and T-cell mediated mechanisms cooperate to establish resistance to pre- and erythrocytic forms of the parasite. A number of target antigens have been described and are being pursued for the development of a recombinant subunit malaria vaccine, as extensively reviewed [3–6]. Recent phase II clinical trials were performed in African children using a recombinant malaria vaccine that is based on a pre-erythrocytic antigen, the circumsporozoite (CS) protein, in the presence of the adjuvant AS01E or AS02D. Children immunised

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with the vaccine formulations RTS,S/AS01E or RTS,S/AS02D displayed a significant reduction in the incidence of naturally acquired infection, indicating that a certain degree of protective immunity was indeed achieved. In spite of the success, immunity was not ideal because a significant part of the RTS,S/AS01E or RTS,S/AS02D vaccinated children still contracted the infection during the trials [7,8].

Considering the fact that the RTS,S/AS01E and RTS,S/AS02D vaccines did not provide an optimal degree of protective immunity against malaria, a search for new vaccines with improved efficacy is required. One possible approach to achieve this goal is the identification of additional target antigens. Merozoite surface protein 1 (MSP-1) is expressed by the pre- and erythrocytic stages of *P. falciparum* and represents a promising malaria vaccine candidate [9]. The protein is synthesised in a precursor form with a high molecular weight during schizogony and, during the invasion process, a proteolytic cleavage releases most of the molecule from the merozoite membrane, leaving a membrane-anchored 19 kDa fragment ($MSP1_{19}$) on the parasite surface [10]. Genetic modification studies with malaria parasites demonstrated that the essential role of $MSP1_{19}$ in parasite survival during *in vivo* replication is similar among even distantly related *Plasmodium* species [11]. More recently, studies using clonal conditional mutagenesis showed that silencing MSP-1 in sporozoites impaired subsequent merozoite formation in the liver, implicating this molecule in the life cycle of the parasite in the liver as well as in the blood [12].

Over the past 20 years, many studies have been performed that support the use of $MSP1_{19}$ as a component of subunit-based malaria vaccine formulations. Monoclonal antibodies against $MSP1_{19}$ and polyclonal antibodies against $MSP1_{42}$ inhibit the *in vitro* growth of *P. falciparum* [10,13]. In addition, non-human primates injected with recombinant proteins containing the C-terminal region of the *P. falciparum* MSP-1 expressed in baculovirus [14–16], *Saccharomyces cerevisiae* [17], *Escherichia coli* [16,18,19] and mammalian cells [20] are protected against homologous challenge with the parasite. Nonetheless, the lack of an effective malaria vaccine formulation, either based on MSP-1 or other antigens, is often explained by the lack of adequate adjuvants that could be used in humans to promote high and long-lasting antibody responses to the target recombinant proteins.

Recent advances in the field of innate immunity have disclosed the cellular and molecular mechanisms behind the adjuvant effects of pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs in mammalian cells is mediated by innate immune receptors such as TLR5 (specific for bacterial flagellins) and TLR9 (specific for unmethylated CpG DNA), which are expressed by antigen-presenting cells (APC). Following the binding of the specific agonists, the intracellular domain of the TLR receptor activates molecular signalling cascades and promotes the recruitment of adaptor proteins, such as the myeloid differentiation factor 88 (MyD88), and the activation of transcription factors, such as NF-κB and mitogen-activated kinases. These signalling events result in the activation of inflammatory responses and APC maturation, which mediate the activation of T and B cell-dependent adaptive immune responses [21,22].

Flagellins, the structural subunit of flagellar filaments, contribute both to the virulence of bacterial pathogens and to the activation of inflammatory responses in mammalian hosts [21,22]. Bacterial flagellins have been shown to bind extracellular TLR5 as well as intracellular receptors, leading to strong inflammatory responses [23–30]. Flagellins, such as those expressed by *Salmonella* species, have shown strong adjuvant effects when delivered via parenteral or mucosal routes and either admixed or genetically linked to target antigens in mice [31–42] and in non-human primates [43–45]. In a recent work, we generated a recombinant protein consisting of the flagellin FliC of *Salmonella enterica* Typhimurium fused to the $MSP1_{19}$ of *Plasmodium vivax*. Mice immunised with the fusion protein in the absence of adjuvant elicited high and long-lasting antibody titres that recognised the parasite in the blood of infected patients [46]. We also showed that the fusion process did not change the antigenic properties of the malaria antigen or the capacity of flagellin to signals through the innate immunity receptor TLR5.

Here we investigated the immunogenicity of a fusion polypeptide containing the *P. falciparum* $MSP1_{19}$ and an innate immunity agonist, the *Salmonella* Typhimurium FliC flagellin. The immunogenicity of the recombinant fusion protein was assessed by immunisation of mice and rabbits with the recombinant protein alone or in the presence of different adjuvants, such as the TLR9 agonist CpG ODN 1826, Quil-A or complete and incomplete Freund's adjuvants. Additionally, we investigated whether the anti-$MSP1_{19}$ antibodies recognised the malaria parasites and impaired *in vitro* parasite growth. The reported results demonstrate that the incorporation of TLR agonists into $MSP1_{19}$-based formulations represents an alternative for the development of new, simple and inexpensive malaria vaccine candidates.

2. Methods

2.1. Generation of recombinant $MSP1_{19}$-derived proteins

The *S.* Typhimurium FliC and $MSP1_{19}$ gene sequences were obtained by PCR amplification using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen). Template DNA for the amplifications were obtained from *S.* Typhimurium and *P. falciparum* 3D7 blood stages. Specific oligonucleotides for the amplification of the FliC gene, containing EcoRI and HindIII restriction sites (GGGGAATTCATGGCACAAGTCATTAATACA and GGCAAGCTTGACGCAGTAAAGAGAGGAC), and the $MSP1_{19}$ nucleotide sequence, containing HindIII and XhoI restriction sites (GGCAAGCTTGCGGAAAATTCCAAGATATG and GGGCTCGAGTTTAACTGCAGAAAATACCATC), were purchased from Integrated DNA Technologies, Inc. Amplified fragments were cloned in frame in the pET28a vector (Novagen). The recombinant protein was expressed and purified as described previously [47]. Briefly, recombinant *E. coli* BL21 DE3 (Novagen) was cultivated at 37 °C in flasks containing Luria broth (LB) and kanamycin (30 μg/ml). Protein expression was induced at an OD_{600} of 0.6 with 0.1 mM IPTG (Invitrogen) for 4 h. After centrifugation, bacteria were lysed on ice with the aid of an ultrasonic processor (Sonics and Materials INC Vibra Cell VCX 750) in a phosphate buffer with 1.0 mg/ml of lysozyme (Sigma) and 1 mM PMSF (Sigma). Bacterial lysate was centrifuged, and the supernatant was applied to a column with Ni^{2+}–NTA–agarose resin (Qiagen). After several washes, bound proteins were eluted with 0.5 M imidazole (Sigma). The eluted protein was dialysed against 20 mM Tris–HCl (pH 8.0), and the recombinant proteins were purified by ion-exchange chromatography using a Resource Q column (GE Healthcare) coupled to an FPLC system (GE Healthcare). Fractions containing the recombinant proteins with a high degree of purity were pooled and extensively dialysed against phosphate-buffered saline (PBS). Protein concentration was determined with the Bradford assay and by SDS-PAGE analyses.

2.2. FliC purification

Native *S.* Typhimurium FliC was purified from the attenuated *S.* Typhimurium SL3201 strain, which expresses FliC, but not FljB [48]. Briefly, bacteria were grown in LB supplemented with kanamycin (30 μg/ml) overnight at 37 °C under aeration (80 rpm). Cells were washed once with PBS and submitted to mechanical shearing for

four, 2 min cycles in a bench vortex mixer. The cell suspensions were centrifuged to remove the cellular debris, and following acetone precipitation, the flagellar filaments were collected from the supernatant and suspended in PBS. The purity of the preparations was monitored by SDS-PAGE. The recombinant FliC (rFliC) was obtained after cloning the corresponding gene into the pET28a expression vector as previously reported [46]. The recombinant vector was introduced into the *E. coli* BL21 DE3 strain, and the encoded peptide was subsequently purified by affinity chromatography based on standard procedures [46].

2.3. Immunisation regimens

Six- to eight-week-old female C57BL/6 (H-2^b) mice were purchased from the Federal University of São Paulo, Brazil. C57BL/6 TLR4 knock-out mice were kindly provided by Dr. Shizuo Akira at Osaka University, Japan. Experiments were performed in accordance with the guidelines of the Ethics Committee for Animal Handling of the Federal University of São Paulo. Mice were immunised three times, 3 weeks apart, subcutaneously in the two hind footpads, using a final volume of 50 μl in each footpad (first dose) and a final volume of 100 μl at the base of the tail (second and third doses). For each dose, 5 μg of His_6MSP1_{19} or 25 μg of the fusion protein was used. CpG ODN 1826 (TCCATGACGTTCCTGACGTT) was synthesised with a nuclease-resistant phosphorothioate backbone (Coley Pharmaceutical Group); a dose of 10 μg per mouse was admixed with the antigen just before injection. A dose of 2.5 μg of Quil-A (Superfos Biosector a/s) per mouse was alternatively admixed with the antigen just before injection. Complete (CFA) and incomplete (IFA) Freund's adjuvants (Sigma) were emulsified extensively with the proteins (1:1, v/v) prior to injection. For CFA/IFA immunisation regimens, CFA was used for the first dose, and IFA was used for the subsequent doses. Rabbits were immunised four times s.c. in the back skin with 200 μg of His_6FliC-$MSP1_{19}$ or 50 μg of FliC.

2.4. Immunological assays

Serum anti-$MSP1_{19}$ antibodies were detected by ELISA essentially as described previously [47]. The recombinant His_6MSP1_{19} (200 ng/well) antigen was employed as the solid phase bound antigen. A peroxidase conjugated goat anti-mouse IgG (Sigma) or goat anti-rabbit IgG (Sigma) was applied at a final dilution of 1:2000, while the mice or rabbit sera were tested at serial dilutions starting from 1:200. Specific anti-$MSP1_{19}$ titres were determined as the highest dilution yielding an OD_{492} higher than 0.1. Detection of IgG subclass responses was performed as described above, except that the secondary antibody was specific for mouse IgG1, IgG2b and IgG2c (Southern Technologies). The results are presented as mean ± SD.

The amount of IFN-γ secreted into cell culture supernatants was determined with 10^6 spleen cells collected from different immunisation groups and cultivated in flat-bottom 96-well plates in a final volume of 200 μl. The His_6MSP1_{19} protein was added to the culture at a final concentration of 1 or 10 μg/ml. After 120 h, the supernatants were collected for cytokine determination. Cytokine concentration was estimated by capture ELISA using antibodies and recombinant cytokines purchased from Pharmingen (San Diego, CA) as previously described [49]. The cytokine concentration in each sample was determined with standard curves created with known concentrations of recombinant mouse IFN-γ. The detection limit of the assay was 0.2 ng/ml.

Determination of TLR5 bioactivity with native or recombinant flagellins, as well as with His_6MSP1_{19} or His_6FliC-$MSP1_{19}$, was performed with a HEK293 cell line expressing mouse TLR5 (Invivogen). This cell line does not display TLR-expression and is irresponsive to stimulation with TLR agonists unless the receptors are transfected. The cells were maintained in DMEM media supplemented with10% FBS and 10 μg/ml of blasticidin. Non-transfected or TLR5-transfected HEK293 cells (5×10^4 cells/well) were grown overnight in 96-well plates and stimulated with the recombinant proteins for 5 h. The culture supernatants were collected, and the concentration of secreted human HuIL-8 was measured using a Human IL-8 ELISA Kit (BD Biosciences) following the protocol recommended by the manufacturer.

2.5. Cultivation of *P. falciparum*-infected erythrocytes

P. falciparum 3D7, FCR3 [50] and S20 [51] strains were cultured in candle jars as described elsewhere [52]. Briefly, *P. falciparum*-infected erythrocytes were cultivated in fresh type O^+ human erythrocytes (Blood Center - UNICAMP) suspended at a 4% final hematocrit in complete medium (RPMI 1640; Sigma) supplemented with 10% of homologous human plasma and adjusted to pH 7.2. In some experiments, parasites were synchronised (±6 h) by repeated 5% sorbitol treatment as described elsewhere [53].

2.6. *P. falciparum* slide preparation and indirect immunofluorescence assay (IIA)

Assays were performed with 10-well IIA slides containing late stage forms of *P. falciparum* enriched in a Voluven® (Fresenius) gradient as described elsewhere [53]. The infected erythrocytes were diluted 1:1 in fetal bovine serum (FBS), spread on IIA slides (20 μl/well), fixed in acetone for 10 min and air dried. Pooled sera from different immunisation groups were diluted 1:100 in PBS, applied to the slides and kept for 30 min in a humid chamber at 37 °C. The slides were extensively washed with PBS and incubated with 20 μg/ml of Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) and 100 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 30 min in a humid chamber at 37 °C. After several washes with PBS, the slides were sealed with coverslips and viewed under an immunofluorescence microscope. For liquid phase IIA, the infected erythrocytes were fixed in 2% paraformaldehyde and then diluted in FBS. Pooled sera from rabbits were added in a final dilution of 1:20 and kept for 60 min at 37 °C. After two washes with FBS, the cells were incubated with 100 μg/ml of Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) and 200 μg/ml of DAPI (Molecular Probes) for 30 min at 37 °C. The cells were washed twice with FBS and viewed under an immunofluorescence microscope on slides sealed with coverslips.

2.7. Growth inhibition assay (GIA)

The merozoite invasion inhibition assay using the sera from the rabbits was essentially performed as previously described [54]. Briefly, trophozoite synchronised *P. falciparum* 3D7, S20 and FCR3 cultures with a parasitemia of 4.0% and a hematocrit of 4.0% were cultured in micro-plates (50 μl) in the presence of increasing concentrations of rabbit sera at 37 °C for 24–30 h. As positive control, we used a pool of 4 human sera collected from malaria immune adults from Ghana kindly provided by Dr. Marcelo U. Ferreira (University of São Paulo). In order to quantify the parasitemia, blood smears from each well were stained with Giemsa and then analysed by counting the number of rings in at least 500 erythrocytes.

2.8. Statistical analyses

One-way ANOVA, Student's *t*-test, Tukey's HSD test and Kruskal–Wallis one-way analysis of variance were used to compare

the differences between the mean values of the tested immunisation groups.

3. Results

3.1. Production, purification and TLR5 bioactivity of flagellin-related peptides

In the present study, we generated two recombinant proteins: the *P. falciparum* $MSP1_{19}$ peptide linked to a hexa-histidine tag (His_6MSP1_{19}) and a fusion protein consisting of the $MSP1_{19}$ peptide linked to the C-terminal end of FliC (His_6FliC-$MSP1_{19}$). The schematic representation of each of the recombinant polypeptides and purified proteins separated by SDS-PAGE are presented in Fig. 1A and B, respectively. The native FliC protein purified from the monophasic *S.* Typhimurium SL3201 strain was used as control in the immunologic assays.

To determine whether the fusion polypeptide retained the ability to bind to TLR5, HEK293 cells transfected with the mouse TLR5 receptor gene were cultured in the presence of increasing concentrations of the recombinant protein and recombinant or native FliC (positive controls). Exposure to both the native and recombinant *S.* Typhimurium FliC and to the hybrid His_6FliC-$MSP1_{19}$ protein induced the production of HuIL-8 by TLR5-transfected HEK293 cells (Fig. 1C). On a molar basis, all proteins showed similar HuIL-8 induction. In contrast, TLR5-transfected HEK293 cells exposed to His_6MSP1_{19} protein did not secrete significant levels of HuIL-8. We also used as control non-transfected HEK293 cells. After exposure to the recombinant proteins these cells did not produce HuIL-8 (Fig. 1C). These results clearly demonstrate that the $MSP1_{19}$ C-terminal fusion did not impair the TLR5-specific bioactivity of the FliC in the *E. coli* recombinant protein.

3.2. Induction of $MSP1_{19}$-specific antibody responses in mice immunised with $MSP1_{19}$-derived peptides genetically fused to FliC

The serum IgG responses to *P. falciparum* $MSP1_{19}$ were determined in C57BL/6 mice immunised subcutaneously with the purified His_6MSP1_{19} protein (5 μg/dose) emulsified in complete or incomplete Freund's adjuvant (CFA/IFA). Mice parenterally immunised with the recombinant protein in the presence of CFA/IFA developed significantly higher $MSP1_{19}$-specific IgG titres than mice immunised with the His_6MSP1_{19} protein alone ($p < 0.01$). Maximal IgG antibody titres were achieved after the second dose (Fig. 2A). Mice immunised with the recombinant fusion protein His_6FliC-$MSP1_{19}$ alone developed significantly higher $MSP1_{19}$-specific IgG titres than mice immunised with the His_6MSP1_{19} protein alone ($p < 0.01$). Mice immunised twice with the recombinant His_6FliC-$MSP1_{19}$ protein or with His_6MSP1_{19} emulsified in CFA/IFA showed a statistically significant difference in their $MSP1_{19}$-specific antibody titres in favour of the latter group ($p < 0.01$). Nevertheless, after a third immunising dose, no difference was observed between the specific IgG titres from these two mouse groups ($p > 0.05$), indicating that, after three doses, the fusion to FliC conferred the same immunogenicity to the hybrid $MSP1_{19}$ protein as the non-fused antigen emulsified in CFA/IFA, as measured by the serum antigen-specific IgG levels (Fig. 2A). The adjuvant effect of His_6FliC-$MSP1_{19}$ cannot be attributed to contaminating LPS since immunisation of C57BL/6 TLR4 knock-out mice (non-responsive to LPS) with His_6FliC-$MSP1_{19}$ elicited $MSP1_{19}$-specific IgG responses similar to those found in wild type mice (data not shown).

Addition of other adjuvants to His_6FliC-$MSP1_{19}$, specifically CpG ODN 1826, CFA, IFA and Quil-A, increased the antigen-specific antibody responses when compared to mice immunised only with the His_6FliC-$MSP1_{19}$ antigen ($p < 0.05$), as observed by comparison of the specific IgG titres following the two immunising doses. However, after three doses, the presence of adjuvants did not significantly change the magnitude of the $MSP1_{19}$-specific IgG responses (Fig. 2A). These results clearly show that immunisation with the $MSP1_{19}$-peptide fused to FliC was capable of inducing high IgG antibody titres even in the absence of other admixed adjuvants.

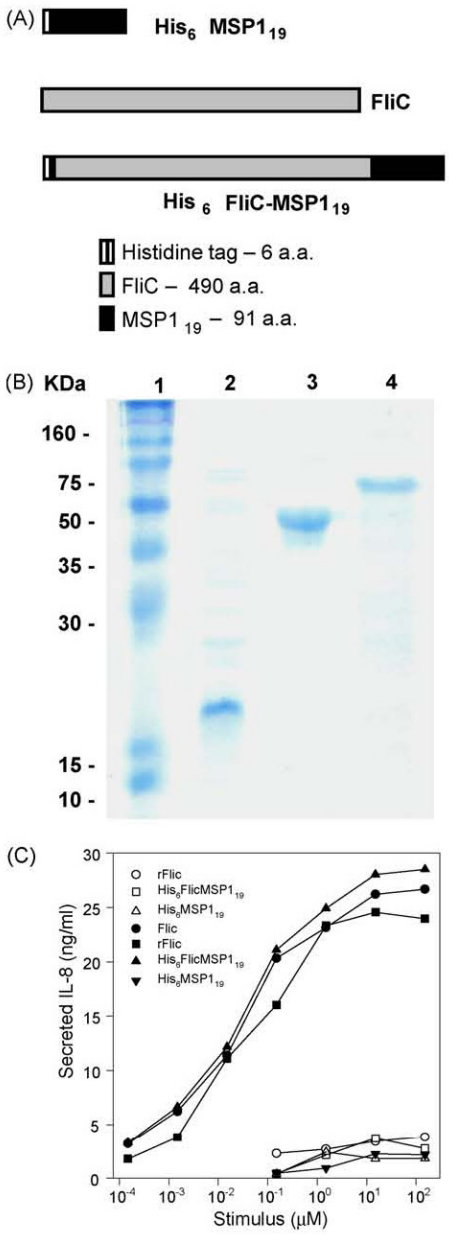
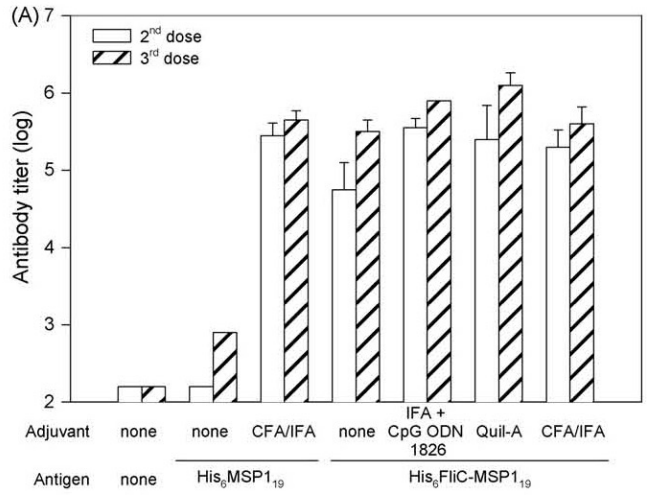


Fig. 1. Generation and characterisation of recombinant *P. falciparum* $MSP1_{19}$-derived peptides. (A) Schematic representation of the recombinant proteins used in the present study. (B) SDS-PAGE analysis of the recombinant proteins. Lanes: 1, molecular weight markers; 2, purified His_6MSP1_{19} protein; 3, purified *S.* Typhimurium FliC flagellin; 4, purified recombinant His_6FliC-$MSP1_{19}$ protein. Each lane was loaded with approximately 1 μg of protein, separated on a 15% polyacrylamide gel and stained with Coomassie Blue. (C) HuIL-8 secretion by TLR5-transfected HEK 293 cells. Non-transfected HEK293 cells (white symbols) and TLR5-transfected HEK293 cells (black symbols) were stimulated for 5 h with different concentrations of native FliC, recombinant FliC (rFliC), His_6MSP1_{19} or His_6FliC-$MSP1_{19}$ antigens as indicated. The amount of secreted HuIL-8 in culture supernatant was determined by capture ELISA. Data are representative of two experiments with similar results.



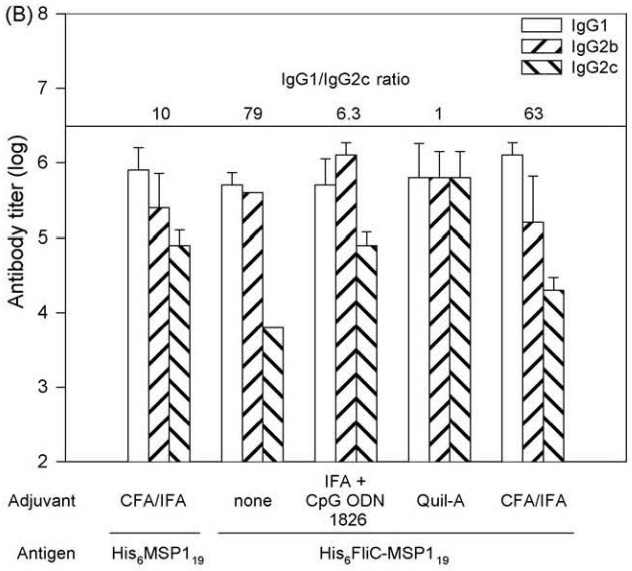


Fig. 2. Induction of total IgG responses to $MSP1_{19}$ and Ig subclass determination in serum samples of mice immunised with the malarial recombinant proteins. Female C57BL/6 mice were immunised three times, either with 5 μg of the recombinant protein His_6MSP1_{19} alone or emulsified in CFA/IFA (1:1, v/v), or with 25 μg of the recombinant fusion protein $His_6FliC\text{-}MSP1_{19}$ alone or admixed with the following adjuvant formulations: (i) 10 μg of CpG ODN 1826 emulsified in IFA (1:1, v/v), (ii) 2.5 μg Quil-A and (iii) CFA/IFA (1:1, v/v). (A) $MSP1_{19}$-specific total IgG titres after the second and third doses. All mice immunised with His_6MSP1_{19} in CFA/IFA or with $His_6FliC\text{-}MSP1_{19}$ had higher IgG titres than the control groups ($p < 0.01$). (B) IgG subclass responses and IgG1/IgG2c ratios in mice submitted to the different immunisation regimens. Data are representative of two experiments using six mice per group.

In order to determine the quality of the humoral immune responses, we measured the IgG subclasses of the $MSP1_{19}$-specific antibody responses elicited in mice parenterally immunised with the recombinant proteins. Mice immunised with His_6MSP1_{19} emulsified in CFA/IFA developed high $MSP1_{19}$-specific IgG1, IgG2b and IgG2c titres, with an IgG1/IgG2c ratio equal to 10 (Fig. 2B). The recombinant fusion protein $His_6FliC\text{-}MSP1_{19}$ alone induced a less balanced subclass response, with low IgG2c and high IgG1 and IgG2b $MSP1_{19}$-specific titres and an IgG1/IgG2c ratio equal to 79 (Fig. 2B). The addition of other adjuvants to the $His_6FliC\text{-}MSP1_{19}$ immunisation regimen was not capable of changing the $MSP1_{19}$-specific IgG1 or IgG2b responses, but addition of IFA plus CpG ODN 1826, or Quil-A increased the $MSP1_{19}$-specific IgG2csubclass

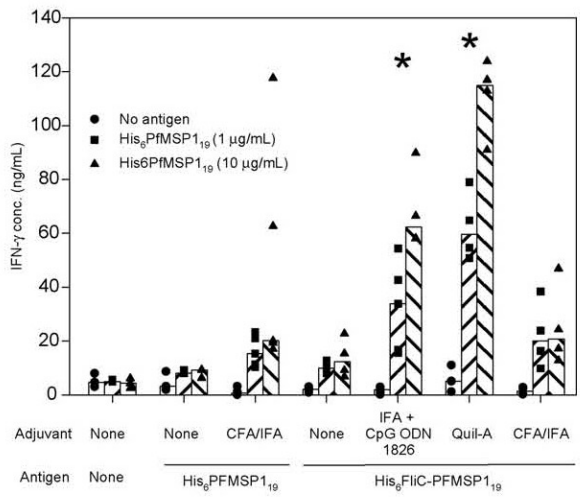


Fig. 3. IFN-γ secretion by *in vitro*-cultured spleen cells harvested from vaccinated mice. Splenocytes collected from different mouse groups immunised as described in Fig. 2 were cultured in medium alone or in the presence of the His_6MSP1_{19} protein (1 or 10 μg/ml) for 120 h. The IFN-γ concentration in the culture supernatants was monitored by ELISA. Results are expressed as individual values for each animal and bars represent the median. Data are representative of two experiments using four or five mice per group. Asterisks denote that values obtained from mice immunised with the recombinant fusion protein $His_6FliC\text{-}MSP1_{19}$ in the presence of adjuvants CpG ODN 1826 emulsified in IFA or Quil-A were significantly higher than the values obtained from mice immunised only with $His_6FliC\text{-}MSP1_{19}$ ($p < 0.01$ for both antigen concentrations, Kruskal–Wallis).

response, leading to a more balanced IgG1/IgG2c ratio (6.3 and 1.0, respectively) (Fig. 2B).

3.3. Incorporation of CpG ODN 1826 and IFA, or Quil-A to the fusion protein induced more IFN-γ secretion by immune spleen cells in response to His_6MSP1_{19} in vitro

To further characterise the cellular-mediated immune responses (CMI) induced by vaccination with the recombinant proteins, we determined the secreted IFN-γ produced by spleen cells of mice immunised with the different vaccine formulations. As shown in Fig. 3, spleen cells from mice immunised with His_6MSP1_{19} secreted IFN-γ in response to the antigen only when administered with the CFA/IFA emulsion. On the other hand, spleen cells from mice immunised with $His_6FliC\text{-}MSP1_{19}$, even when administered in the absence of other adjuvants, secreted modest, but significant, amounts of IFN-γ following incubation with the recombinant protein. Nevertheless, addition of IFA/CpG ODN 1826 or Quil-A to the formulations containing $His_6FliC\text{-}MSP1_{19}$ improved the cellular immunogenicity of the antigen, as measured by the amount of IFN-γ secreted by spleen cells upon *in vitro* stimulation (Fig. 3). Together, these results indicate that the addition of the TLR9 agonist CpG ODN 1826 or Quil-A balanced the immune response pattern and improved the activation of specific cell-dependent immune responses, as evaluated by the IFN-γ secretion from spleen cells.

3.4. Antibodies generated in mice immunised with $His_6FliC\text{-}MSP1_{19}$ recognised in vitro-cultured P. falciparum 3D7 parasites

Sera from mice immunised with $His_6FliC\text{-}MSP1_{19}$ were used in immunofluorescence assays with *P. falciparum* 3D7 parasites cultured under *in vitro* conditions. The $MSP1_{19}$-specific antibodies bound to epitopes exposed on the surface of the parasites, clearly show that the antibody immune responses raised after immunisa-

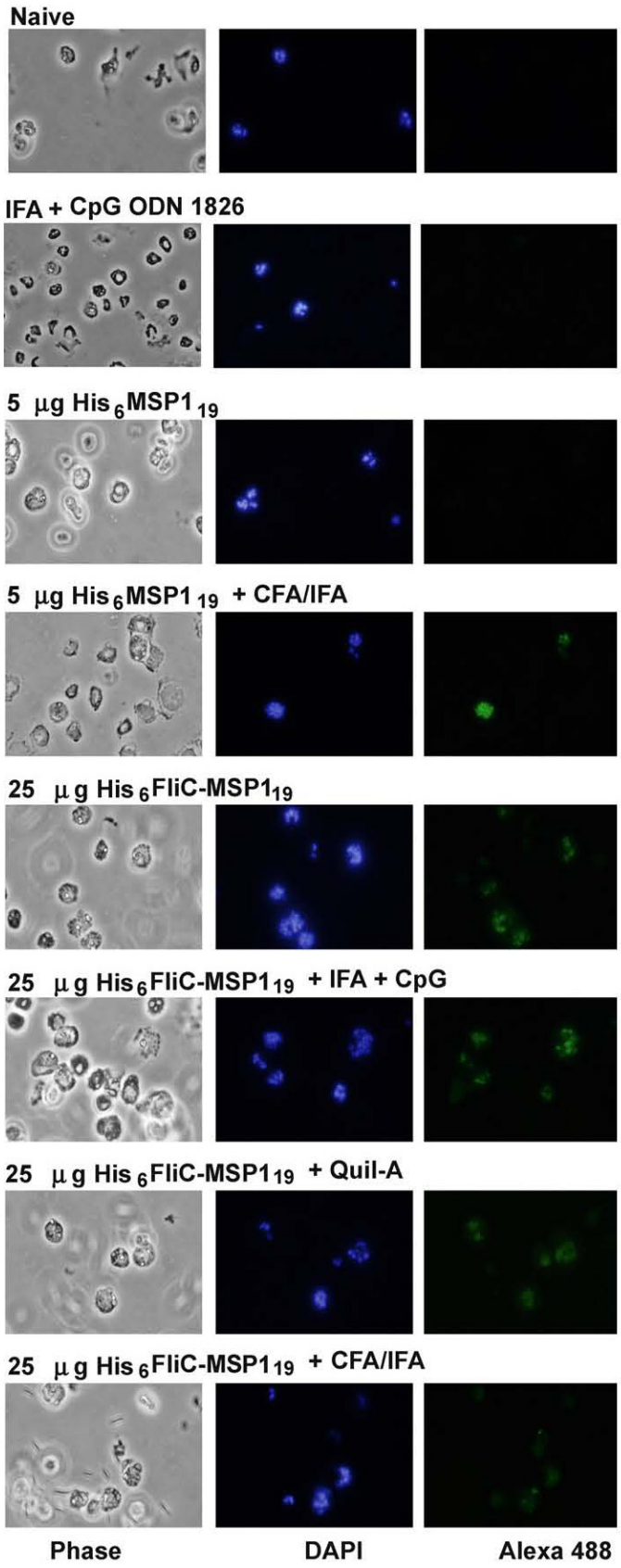


Fig. 4. $MSP1_{19}$-specific antibodies generated in vaccinated mice recognise the native protein expressed by *P. falciparum* 3D7 parasites. Glass slides containing infected cells were incubated with pooled sera, diluted 1:100 in PBS, from mice immunised as indicated. IIA were carried out with bound IgG stained with Alexa Fluor 488. Parasite nuclei were stained with DAPI. Data are representative of two experiments with similar results.

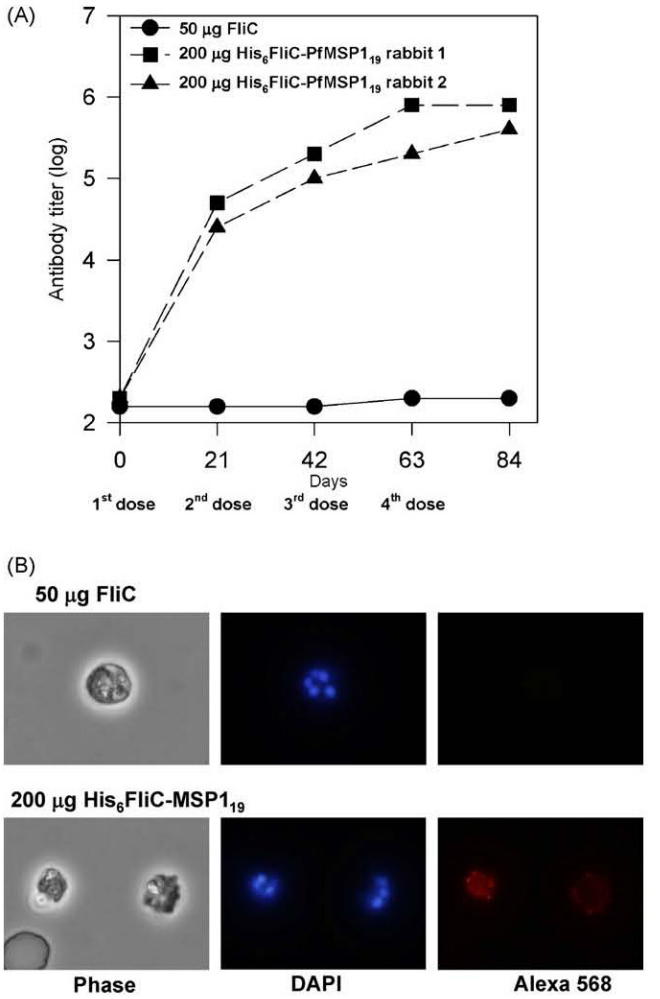


Fig. 5. Rabbits immunised with $His_6FliC\text{-}MSP1_{19}$ induced specific anti-$MSP1_{19}$ antibodies that recognise *P. falciparum* parasites. Two rabbits were immunised with four, 200 μg doses of $His_6FliC\text{-}MSP1_{19}$, and one rabbit received four, 50 μg doses of FliC. (A) $MSP1_{19}$-specific total IgG titres after each dose. The rabbits immunised with $His_6FliC\text{-}MSP1_{19}$ had higher IgG titres than the rabbit immunised with FliC ($p < 0.01$). (B) After four immunising doses, serum samples were tested in liquid phase IIA with *P. falciparum* 3D7 parasites. Surface bound IgG was stained with Alexa Fluor 568, while parasite nuclei were detected with DAPI.

tion with the recombinant proteins are specific against the epitopes that are naturally expressed by the parasite (Fig. 4).

3.5. *Immunisation of rabbits with $His_6FliC\text{-}MSP1_{19}$ induced antibodies that inhibited invasion of parasites of distinct strains in vitro*

Two rabbits were immunised with $His_6FliC\text{-}MSP1_{19}$, and a third rabbit was immunised with FliC as a control. The two rabbits injected subcutaneously with $His_6FliC\text{-}MSP1_{19}$ raised higher specific anti-$MSP1_{19}$ IgG titres as compared to the animal injected with FliC ($p < 0.01$), which had undetectable anti-$MSP1_{19}$ titres (Fig. 5A). The anti-$MSP1_{19}$ IgG titres in the immunised rabbits achieved maximal values following the third dose (Fig. 5A). Additionally, pooled sera from the two rabbits immunised with $His_6FliC\text{-}MSP1_{19}$ labelled surface-exposed epitopes of the *P. falciparum* 3D7 strain, in contrast to the serum harvested from the rabbit immunised with FliC (Fig. 5B).

After four immunising doses, the sera of the three rabbits were used to perform growth inhibition assays (GIAs) with the parasites of three *P. falciparum* strains. The *P. falciparum* 3D7, S20 and

FCR3 strains were grown *in vitro*, and serum samples of the immunised rabbits were added to the cultures at three different dilutions. Serum samples of the two rabbits immunised with His_6FliC-$MSP1_{19}$ efficiently inhibited invasion of erythrocytes by the parasites of the different *P. falciparum* strains (inhibition values ranged from 66% to 89% of normal invasion), whereas serum samples of the rabbit immunised with FliC did not efficiently inhibit parasite invasion (inhibition values ranged from 11 to 21%) (Fig. 6). Analyses of the $MSP1_{19}$ nucleotide sequences of these three *P. falciparum* strains showed identity of at least 97.7% (data not shown). These results clearly show that antibodies raised in rabbits following immunisation with His_6FliC-$MSP1_{19}$, in the absence of admixed adjuvants, can specifically inhibit erythrocyte invasion by different *P. falciparum* strains, an essential step of the parasitic life cycle.

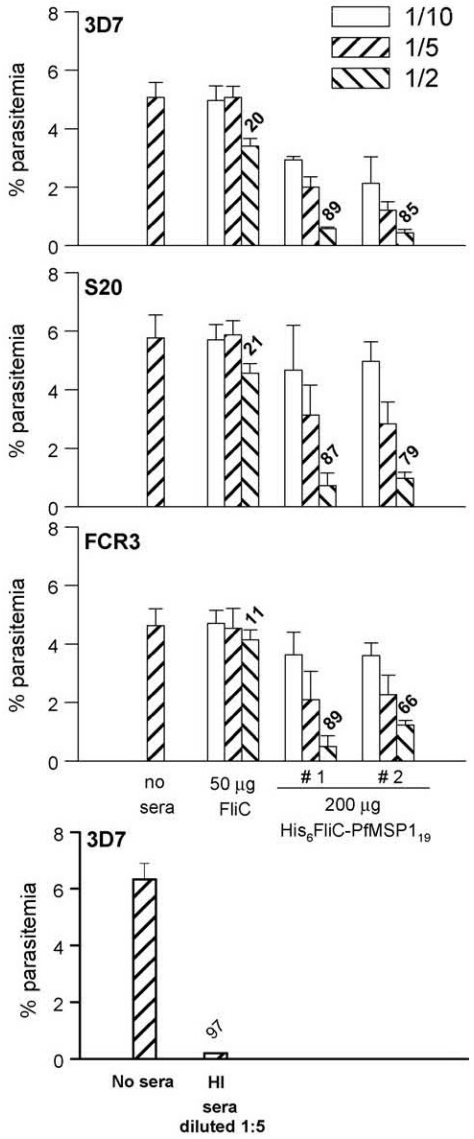


Fig. 6. Sera from rabbits immunised with His_6FliC-$MSP1_{19}$ inhibited erythrocyte invasion by *P. falciparum* 3D7, S20 and FCR3 strains. The parasites were cultured *in vitro* in the presence of sera from rabbits at different dilutions (1:2, 1:5 or 1:10). After 24–30 h of incubation, parasitemia was determined in blood smears by counting the number of rings in at least 500 erythrocytes. Results are shown as mean ± SD of three cultures from each strain and serum sample dilution. Erythrocyte invasion inhibition values were determined in relation to the control, a parasite sample prepared with no added sera. Percentages of erythrocyte invasion mediated by the different tested sera are shown only for the 1:2 serum dilutions. In the lower panel, pooled human serum from malaria immune individuals (HI) was used to inhibit the erythrocyte invasion by *P. falciparum* 3D7 strain. Data are representative of two experiments.

4. Discussion

In the present study, we tested whether the proteic innate immunity activator (*Salmonella* FliC) acts both as an adjuvant for specific humoral and cellular immune responses and an antigen molecular carrier to be used as a simple and inexpensive strategy to improve the immunogenicity of *P. falciparum* $MSP1_{19}$, one of the best studied malaria vaccine candidates. Parenteral immunisation with the fusion protein His_6FliC-$MSP1_{19}$ elicited strong adaptive immune responses in vaccinated mice. The immunogenicity of the malaria vaccine formulation could be further modulated with the use of additional TLR agonists, such as CpG ODN 1826, as well as with commercially available adjuvants, such as Quil-A. In our previous study, we described the adjuvant potential of FliC independent of the molecular carrier effect. The magnitude of the antibody immune response was similar using FliC admixed or genetically fused to *P. vivax* His_6MSP1_{19}. Nevertheless, upon stimulation with *P. vivax* His_6MSP1_{19}, IFN-γ secretion by immune spleen cells from mice immunised with FliC genetically fused to His_6MSP1_{19} was significantly higher than the secretion from cells of mice immunised with His_6MSP1_{19} admixed to FliC [46]. Therefore, we concluded that if there is a carrier effect, it is not related to the antibody production but rather to the expansion of IFN-γ producing cells. We did not consider important to repeat these same experiments with *P. falciparum* $MSP1_{19}$ because we see no reason why it would be significantly different.

The present study reproduces and extends recent reports on the use of *Salmonella* flagellin as an adjuvant and antigen carrier, allowing additional plasticity to the design and production of vaccine antigens endowed with enhanced immunogenicity [31–42]. Indeed, recent studies in non-human primates extend the knowledge obtained for murine hosts, strongly arguing in favour of future clinical trials [43–45].

The protective nature of antibodies targeting the C-terminal domain of the *P. falciparum* MSP-1 protein has been thoroughly documented in a number of *in vitro* and *in vivo* studies. *In vitro*, antibodies directed against *P. falciparum* $MSP1_{19}$, which were obtained from immune individuals, were highly inhibitory for parasite growth [55]. *In vivo*, studies on non-human primates confirmed that protective immunity elicited by vaccination with recombinant proteins correlates with the antibody titres to this specific region of the MSP-1 protein [16,18,19]. In spite of these promising results in some non-human primate models, the observed protective immunity is strain-specific and requires the use of specific adjuvants, some of them endowed with high toxicity and, thus, can not be used in humans [16].

Based on the promising results in the experimental models of malaria infection, a recent phase IIb vaccine trial was performed in Africa [56]. In this clinical trial, children were vaccinated with a formulation containing a recombinant His-tagged fusion protein encompassing the MSP-1 42 kDa C-terminal fragment of the *P. falciparum* 3D7 strain (FMP1) formulated with the adjuvant AS02 [56]. This vaccine formulation was shown to be safe and immunogenic, as demonstrated by detection of specific antibody titres by ELISA. Unfortunately, the trial failed, and no significant reduction in the incidence of malaria infection could be observed in children receiving the FMP1/AS02 formulation [56]. The precise reason why the vaccination failed should be investigated. It might be attributed to the polymorphism of the MSP-1 protein. This fact may be relevant for the interpretation of the results, considering that protective immunity to the C-terminal region of *P. falciparum* MSP-1 can be strain-specific and antibodies targeting this antigen may not show parasite inhibitory activity [16,57]. Furthermore, some of the MSP-1-specific antibodies are endowed with the ability to block the parasite activity of inhibitory antibodies [58]. Although negative, these results do not completely refute that the C-terminal region

of *P. falciparum* MSP-1 could still be part of a subunit malaria vaccine in a new recombinant form or formulation. Currently, the immunogenic properties of distinct recombinant proteins are being compared in experimental animals to select possible candidates for human trials [59]. In that scenario, our approach may help in the search for an ideal malarial vaccine formulation containing blood stage antigens with increased immunogenicity and improved potency, which will generate antibodies that proficiently inhibit the late steps of parasite development.

Although it was described that flagellin is a natural agonist of at least three innate immune receptors, TLR5 [24,30], Ipaf (ICE protease-activating factor) [23,25] and Naip5/Birc1e (Neuronal Apoptosis Inhibitory Protein) [26–29], it is very likely that the mechanism that mediates the adjuvant properties of flagellin involves the activation of TLR5 in antigen-presenting cells. A recent work showed that a conserved region in both human and mouse TLR5 is responsible for the recognition of flagellin, and another recent study showed that the direct stimulation of $TLR5^+$ $CD11c^+$ dendritic cells (myeloid DCs) via TLR5 is necessary for flagellin adjuvant activity [60], which would act mainly by upregulating CD80, CD83, CD86 and MHC class II in these cells. Nevertheless, one laboratory suggests that murine DCs do not express TLR5 [61] and another showed that, in the absence of the TLR5, flagellin is capable of inducing a humoral immune response despite a lack in DC maturation [62]. Additionally, flagellin has been shown to activate APC through inhibition of IL-10 secretion, an immunosuppressive cytokine [63], resulting in a higher adaptive immune response. Therefore, the exact mechanism(s) by which flagellin acts as an adjuvant in these models remains to be elucidated. It is possible that another unknown receptor might be playing a relevant role in this scenario. Despite these unanswered questions, it seems that human myeloid DCs express higher levels of TLR5 than murine DCs [61], which would be an advantage for the development of a vaccine for humans using the strategy of antigen fusion to flagellin.

Acknowledgments

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References

[1] Aregawi M, Cibulskis R, Otten M, Williams R, Dye C. World malaria report 2008. World Health Organization (WHO); 2008.
[2] White NJ. Antimalarial drug resistance. J Clin Invest 2004;113(April (8)):1084–92.
[3] Beeson JG, Osier FH, Engwerda CR. Recent insights into humoral and cellular immune responses against malaria. Trends Parasitol 2008;24(December (12)):578–84.
[4] Yazdani SS, Mukherjee P, Chauhan VS, Chitnis CE. Immune responses to asexual blood-stages of malaria parasites. Curr Mol Med 2006;6(March (2)):187–203.
[5] Doolan DL, Martinez-Alier N. Immune response to pre-erythrocytic stages of malaria parasites. Curr Mol Med 2006;6(March (2)):169–85.
[6] Renia L. Protective immunity against malaria liver stage after vaccination with live parasites. Parasite (Paris, France) 2008;15(September (3)):379–83.
[7] Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, Vekemans J, et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med 2008;359(December (24)):2521–32.
[8] Abdulla S, Oberholzer R, Juma O, Kubhoja S, Machera F, Membi C, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. N Engl J Med 2008;359(December (24)):2533–44.
[9] Diggs CL, Ballou WR, Miller LH. The major merozoite surface protein as a malaria vaccine target. Parasitol Today 1993;9(August (8)):300–2 [Personal ed].
[10] Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J Exp Med 1990;172(July (1)):379–82.
[11] O'Donnell RA, Saul A, Cowman AF, Crabb BS. Functional conservation of the malaria vaccine antigen MSP-119 across distantly related *Plasmodium* species. Nat Med 2000;6(January (1)):91–5.
[12] Combe A, Giovannini D, Carvalho TG, Spath S, Boisson B, Loussert C, et al. Clonal conditional mutagenesis in malaria parasites. Cell Host Microbe 2009;5(April (4)):386–96.
[13] Bergmann-Leitner ES, Duncan EH, Angov E. MSP-1p42-specific antibodies impact growth and development of intra-erythrocytic parasites of *Plasmodium falciparum*. Malar J 2009;8(August (1)):183.
[14] Chang SP, Case SE, Gosnell WL, Hashimoto A, Kramer KJ, Tam LQ, et al. A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects *Aotus* monkeys against malaria. Infect Immun 1996;64(January (1)):253–61.
[15] Stowers AW, Cioce V, Shimp RL, Lawson M, Hui G, Muratova O, et al. Efficacy of two alternate vaccines based on *Plasmodium falciparum* merozoite surface protein 1 in an *Aotus* challenge trial. Infect Immun 2001;69(March (3)):1536–46.
[16] Lyon JA, Angov E, Fay MP, Sullivan JS, Girourd AS, Robinson SJ, et al. Protection induced by *Plasmodium falciparum* MSP1(42) is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. PLoS One 2008;3(7):e2830.
[17] Kumar S, Yadava A, Keister DB, Tian JH, Ohl M, Perdue-Greenfield KA, et al. Immunogenicity and in vivo efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. Mol Med (Cambridge, MA) 1995;1(March (3)):325–32.
[18] Singh S, Miura K, Zhou H, Muratova O, Keegan B, Miles A, et al. Immunity to recombinant *Plasmodium falciparum* merozoite surface protein 1 (MSP1): protection in *Aotus nancymai* monkeys strongly correlates with anti-MSP1 antibody titer and in vitro parasite-inhibitory activity. Infect Immun 2006;74(August (8)):4573–80.
[19] Darko CA, Angov E, Collins WE, Bergmann-Leitner ES, Girouard AS, Hitt SL, et al. The clinical-grade 42-kilodalton fragment of merozoite surface protein 1 of *Plasmodium falciparum* strain FVO expressed in *Escherichia coli* protects *Aotus nancymai* against challenge with homologous erythrocytic-stage parasites. Infect Immun 2005;73(January (1)):287–97.
[20] Stowers AW, Chen Lh LH, Zhang Y, Kennedy MC, Zou L, Lambert L, et al. A recombinant vaccine expressed in the milk of transgenic mice protects *Aotus* monkeys from a lethal challenge with *Plasmodium falciparum*. Proc Natl Acad Sci USA 2002;99(January (1)):339–44.
[21] Janeway Jr CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197–216.
[22] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124(February (4)):783–801.
[23] Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, et al. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nat Immunol 2006;7(June (6)):569–75.
[24] Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 2003;4(December (12)):1247–53.
[25] Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozoren N, Jagirdar R, et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat Immunol 2006;7(June (6)):576–82.
[26] Lamkanfi M, Amer A, Kanneganti TD, Munoz-Planillo R, Chen G, Vandenabeele P, et al. The Nod-like receptor family member Naip5/Birc1e restricts *Legionella pneumophila* growth independently of caspase-1 activation. J Immunol 2007;178(June (12)):8022–7.
[27] Zamboni DS, Kobayashi KS, Kohlsdorf T, Ogura Y, Long EM, Vance RE, et al. The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. Nat Immunol 2006;7(March (3)):318–25.
[28] Vinzing M, Eitel J, Lippmann J, Hocke AC, Zahlten J, Slevogt H, et al. NAIP and Ipaf control *Legionella pneumophila* replication in human cells. J Immunol 2008;180(May (10)):6808–15.
[29] Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2006;2(March (3)):e18.
[30] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410(April (6832)):1099–103.
[31] McDonald WF, Huleatt JW, Foellmer HG, Hewitt D, Tang J, Desai P, et al. A West Nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. J Infect Dis 2007;195(June (11)):1607–17.
[32] Ben-Yedidia T, Tarrab-Hazdai R, Schechtman D, Arnon R. Intranasal administration of synthetic recombinant peptide-based vaccine protects mice from infection by *Schistosoma mansoni*. Infect Immun 1999;67(September (9)):4360–6.
[33] Cuadros C, Lopez-Hernandez FJ, Dominguez AL, McClelland M, Lustgarten J. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. Infect Immun 2004;72(May (5)):2810–6.
[34] Chauhan N, Kumar R, Badhai J, Preet A, Yadava PK. Immunogenicity of cholera toxin B epitope inserted in *Salmonella* flagellin expressed on bacteria and administered as DNA vaccine. Mol Cell Biochem 2005;276(August (1–2)):1–6.
[35] Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands

induces rapid cellular and humoral immunity. Vaccine 2007;25(January (4)): 763–75.
[36] Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 2008;26(January (2)):201–14.
[37] Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, et al. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS One 2008;3(5):e2257.
[38] Adar Y, Singer Y, Levi R, Tzehoval E, Perk S, Banet-Noach C, et al. A universal epitope-based influenza vaccine and its efficacy against H5N1. Vaccine 2009;27(March (15)):2099–107.
[39] Le Moigne V, Robreau G, Mahana W. Flagellin as a good carrier and potent adjuvant for Th1 response: study of mice immune response to the p27 (Rv2108) *Mycobacterium tuberculosis* antigen. Mol Immunol 2008;45(May (9)):2499–507.
[40] Braga CJ, Rittner GM, Munoz Henao JE, Teixeira AF, Massis LM, Sbrogio-Almeida ME, et al. *Paracoccidioides brasiliensis* vaccine formulations based on the gp43-derived P10 sequence and the *Salmonella enterica* FliC flagellin. Infect Immun 2009;77(April (4)):1700–7.
[41] Lee SE, Kim SY, Jeong BC, Kim YR, Bae SJ, Ahn OS, et al. A bacterial flagellin, *Vibrio vulnificus* FlaB, has a strong mucosal adjuvant activity to induce protective immunity. Infect Immun 2006;74(January (1)):694–702.
[42] Pino O, Martin M, Michalek SM. Cellular mechanisms of the adjuvant activity of the flagellin component FljB of *Salmonella enterica* serovar Typhimurium to potentiate mucosal and systemic responses. Infect Immun 2005;73(October (10)):6763–70.
[43] Honko AN, Sriranganathan N, Lees CJ, Mizel SB. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis*. Infect Immun 2006;74(February (2)):1113–20.
[44] Mizel SB, Graff AH, Sriranganathan N, Ervin S, Lees CJ, Lively MO, et al. Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates. Clin Vaccine Immunol 2009 Jan;16(1):21–8.
[45] Weimer ET, Lu H, Kock ND, Wozniak DJ, Mizel SB. A fusion protein vaccine containing OprF epitope 8, OprI, and type A and B flagellins promotes enhanced clearance of nonmucoid *Pseudomonas aeruginosa*. Infect Immun 2009;77(June (6)):2356–66.
[46] Bargieri DY, Rosa DS, Braga CJ, Carvalho BO, Costa FT, Espindola NM, et al. New malaria vaccine candidates based on the *Plasmodium vivax* merozoite surface protein-1 and the TLR-5 agonist *Salmonella* Typhimurium FliC flagellin. Vaccine 2008;26(November (48)):6132–42.
[47] Cunha MG, Rodrigues MM, Soares IS. Comparison of the immunogenic properties of recombinant proteins representing the *Plasmodium vivax* vaccine candidate MSP1(19) expressed in distinct bacterial vectors. Vaccine 2001;20(November (3–4)):385–96.
[48] Schmitt CK, Darnell SC, O'Brien AD. The attenuated phenotype of a *Salmonella typhimurium* flgM mutant is related to expression of FliC flagellin. J Bacteriol 1996;178(May (10)):2911–5.
[49] Rodrigues MM, Ribeirao M, Pereira-Chioccola V, Renia L, Costa F. Predominance of CD4 Th1 and CD8 Tc1 cells revealed by characterization of the cellular immune response generated by immunization with a DNA vaccine containing a *Trypanosoma cruzi* gene. Infect Immun 1999;67(August (8)): 3855–63.
[50] Lekana Douki JB, Traore B, Costa FT, Fusai T, Pouvelle B, Sterkers Y, et al. Sequestration of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A, a receptor for maternal malaria: monoclonal antibodies against the native parasite ligand reveal pan-reactive epitopes in placental isolates. Blood 2002;100(August (4)):1478–83.
[51] di Santi SM, Boulos M, Vasconcelos MA, Oliveira S, Couto A, Rosario VE. Characterization of *Plasmodium falciparum* strains of the State of Rondonia, Brazil, using microtests of sensitivity to antimalarials, enzyme typing and monoclonal antibodies. Rev Inst Med Trop Sao Paulo 1987;29(May–June (3)):142–7.
[52] Trager W, Jensen JB. Human malaria parasites in continuous culture. Science (New York, NY) 1976;193(August (4254)):673–5.
[53] Ljungström I, Perlmann H, Schlichtherle M, Scherf A, Wahlgren M. Methods in malaria research. 4th ed; 2004 [Manassas, Virginia, USA: MR4/ATCC].
[54] Kulane A, Ekre HP, Perlmann P, Rombo L, Wahlgren M, Wahlin B. Effect of different fractions of heparin on *Plasmodium falciparum* merozoite invasion of red blood cells in vitro. Am J Trop Med Hygiene 1992;46(May (5)):589–94.
[55] O'Donnell RA, de Koning-Ward TF, Burt RA, Bockarie M, Reeder JC, Cowman AF, et al. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J Exp Med 2001;193(June (12)):1403–12.
[56] Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, et al. Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. PLoS One 2009;4(3):e4708.
[57] Guevara Patino JA, Holder AA, McBride JS, Blackman MJ. Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. J Exp Med 1997;186(November (10)):1689–99.
[58] Holder AA. The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. Parasitology 2009;136(October (12)):1445–56.
[59] Reed ZH, Kieny MP, Engers H, Friede M, Chang S, Longacre S, et al. Comparison of immunogenicity of five MSP1-based malaria vaccine candidate antigens in rabbits. Vaccine 2009;27(March (10)):1651–60.
[60] Bates JT, Uematsu S, Akira S, Mizel SB. Direct stimulation of tlr5+/+ CD11c+ cells is necessary for the adjuvant activity of flagellin. J Immunol 2009;182(June (12)):7539–47.
[61] Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol 2003;170(May (10)):5165–75.
[62] Sanders CJ, Franchi L, Yarovinsky F, Uematsu S, Akira S, Nunez G, et al. Induction of adaptive immunity by flagellin does not require robust activation of innate immunity. Eur J Immunol 2009;39(February (2)):359–71.
[63] Vicente-Suarez I, Takahashi Y, Cheng F, Horna P, Wang HW, Wang HG, et al. Identification of a novel negative role of flagellin in regulating IL-10 production. Eur J Immunol 2007;37(November (11)):3164–75.

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Hyperbaric Oxygen Prevents Early Death Caused by Experimental Cerebral Malaria

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Abstract

Background: Cerebral malaria (CM) is a syndrome characterized by neurological signs, seizures and coma. Despite the fact that CM presents similarities with cerebral stroke, few studies have focused on new supportive therapies for the disease. Hyperbaric oxygen (HBO) therapy has been successfully used in patients with numerous brain disorders such as stroke, migraine and atherosclerosis.

Methodology/Principal Findings: C57BL/6 mice infected with *Plasmodium berghei* ANKA (PbA) were exposed to daily doses of HBO (100% O_2, 3.0 ATA, 1–2 h per day) in conditions well-tolerated by humans and animals, before or after parasite establishment. Cumulative survival analyses demonstrated that HBO therapy protected 50% of PbA-infected mice and delayed CM-specific neurological signs when administrated after patent parasitemia. Pressurized oxygen therapy reduced peripheral parasitemia, expression of TNF-α, IFN-γ and IL-10 mRNA levels and percentage of $\gamma\delta$ and $\alpha\beta$ $CD4^+$ and $CD8^+$ T lymphocytes sequestered in mice brains, thus resulting in a reduction of blood-brain barrier (BBB) dysfunction and hypothermia.

Conclusions/Significance: The data presented here is the first indication that HBO treatment could be used as supportive therapy, perhaps in association with neuroprotective drugs, to prevent CM clinical outcomes, including death.

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Introduction

Cerebral malaria (CM) causes 1–2 million deaths annually; mainly in sub-Saharan African children aged 2–6. It is estimated that 250,000 children that do not succumb to CM will develop neurocognitive impairments per year [1] and most CM patients die before the beneficial effects of drug treatment are observed [2]; thus indicating the need to explore new supportive therapies.

CM is a multi-factorial syndrome characterized by neurological signs, seizures and coma, which can, in turn, lead to death. This syndrome can be associated with a loss of cerebrospinal fluid spaces and ischemia [3], alterations in cerebral blood flow velocity [4], a decrease in cerebral oxygen consumption in CM comatose patients [5] and an increase in the lactate levels of the cerebrospinal fluid [6] which decreases after patients recover consciousness [7]. Recent imaging and postmortem analyses have revealed the presence of Durck granulomas, blood-brain barrier (BBB) dysfunction and diffuse cerebral edema with multiple petechial hemorrhages and ischemic changes in the brain of adults with CM [8,9].

Although the CM pathogenic process is controversial and still not fully understood, evidence suggests that the host's immune system plays a major role in expressing certain cytokines, e.g. TNF-α and IFN-γ, and activating immunocompetent cells [10–15]. In fact, recent immunological analyses have shown that, unlike individuals with mild and severe non-cerebral malaria, CM patients present elevated levels of a specific cluster of cytokines, which include TGF-β, TNF-α, IL-1β and IL-10 [16].

Hyperbaric oxygen therapy (HBO; $pO_2 = 760$ mmHg) has been successfully used against bacterial and fungal infections and as an adjunct therapy in surgeries [17–19]. In addition, reports have recently shown that HBO therapy transiently suppresses the inflammatory process of ischemic wounding and trauma [20,21]. Indeed, immunological analyses have revealed that HBO therapy significantly decreases the levels of TNF-α and IL-1β secreted by monocytes and macrophage collected from rats or from human peripheral blood after stimulation with LPS [22,23]. In an experimental model for ischemia, HBO reduces immunocompetent cell sequestration and the synthesis of TNF-α [24]; probably by decreasing ICAM-1 expression levels [25]. Moreover, HBO

reduces the expression of the cyclooxygenase-2 (COX-2) mRNA, an enzyme involved in inflammation, and the hypoxia-inducible factor-1α (HIF-1α), a transcriptional factor associated with low oxygen concentrations [26,27]. HBO therapy has been used in patients with numerous brain disorders such as stroke, migraine and atherosclerosis, due to its capacity to decrease cerebral edema and brain infarction while maintaining BBB integrity, reducing neuronal death and improving blood flow in damaged areas of the brain [28]. Nevertheless, depending on the protocol used for treatment, HBO therapy has potential side effects associated to ear and sinus barotraumas, myopia and convulsion [29].

In an early study, HBO was observed to alter the parasitemia levels of mice infected with a non-cerebral line of *Plasmodium berghei* [30]. However, the HBO effect on the entire curve of parasitemia, on the clinical symptoms and on the mechanisms of the illness were not further investigated. Moreover, although the pathological process involved in CM displays some features in common with brain stroke, the effect of HBO on CM, to our knowledge, has never been assessed. Here we show that in conditions also suitable for human use, HBO therapy prevents CM clinical symptoms in C57BL/6 mice infected with *P. berghei* ANKA, a model widely used for experimental cerebral malaria (ECM) [31].

Methods

Mice and parasites

C57BL/6 mice (7–10 weeks old) were purchased from the University of São Paulo (São Paulo, SP, Brazil) and maintained in our specific pathogen-free animal facility. All experiments and procedures were approved by the UNICAMP Committee for Ethics in Animal Research (Protocol No. 857-1).

Two different strains of *P. berghei* were used: the cloned line of *P. berghei* ANKA (PbA) and *P. berghei* NK-65 (PbNK-65), respectively an ECM- and non-ECM-causing strain; kindly provided by Dr. Laurent Rénia (Singapore Immunology Network, Agency for Science, Technology and Research, Biopolis, Singapore) and Dr. Nobuko Yoshida (Federal University of São Paulo, São Paulo, SP, Brazil), respectively. The blood stage forms of both parasites were stored in liquid nitrogen after *in vivo* passages in C57BL/6 mice according to the protocol described elsewhere [31]. Mice were infected intraperitoneally (i.p.) with 10^6 infected red blood cells (iRBC) and parasitemia and the neurological signs for CM were monitored daily.

Hyperbaric oxygen treatment

Groups of 8–10 PbA-infected mice were exposed daily to 100% oxygen at a pressure of 3.0 atmospheres (ATA) for 1 h per day in a hyperbaric animal research chamber (Research Chamber, model HB 1300B, Sechrist, USA) from day 0 to 10 post-infection (11-day exposure), or for 2 h from day 4–7 post-infection (4-day exposure). The chamber was pressurized and decompressed at a rate of 0.5 ATA/min as described elsewhere [32]. For the 11-day exposure protocol, mice were previously exposed to HBO for 1 h before PbA infection, whereas for the 4-day exposure protocol, PbA-infected mice were randomly selected and placed in the hyperbaric chamber. To determine the effect of 100% oxygen (hyperoxia), regardless of pressurization, PbA-infected mice were submitted to the 11-day exposure protocol, but at 1.0 ATA (normobaric) instead of 3.0 ATA. Infected mice in the control group (non-exposed) were left in an airy room. The temperature inside the hyperbaric chamber was 21°C, the same as in the room, and was measured with the aid of a high-pressure resistant thermometer (model TB-0261, Instrucamp, Brazil). For the direct HBO effect assays, normal red blood cells (nRBC) or iRBC were collected from a naïve mouse or a PbA-infected animal on day 6 post-infection (12% parasitemia), and then diluted in an RPMI 1640 medium (Sigma, USA) supplemented with 10% of fetal bovine serum (Hyclone, USA). One mL of nRBC or iRBC (10^7/mL) were plated in five replicates on a 24 well-plate and exposed to HBO (100% O_2, 3.0 ATA) in a hyperbaric chamber for up to 6 hours.

Parasitemia, temperature and red blood cell density assessment

The percentage of parasitemia was determined by counting the number of iRBC in at least 1,000 erythrocytes in Giemsa-stained blood smears. The mice's corporal temperature and the density of red blood cells (DRBC/mL$\times 10^9$) were evaluated daily, starting on day −1 post-infection (p.i.), by rectal introduction of a precision digital thermometer (model TE-300, Instrucamp, Brazil), and with the aid of a Neubauer chamber, respectively. In the *in vitro* assays, DRBC were counted from 0 hour. The percentage of RBC density relative to day −1 p.i. or to 0 hour was calculated with the following formula: [(DRBC per mL$\times 10^9$ of a determined day p.i. or hour/DRBC per mL$\times 10^9$ on day −1 p.i. or at 0 h)$\times$100].

Measuring cytokine gene expression in the brain

The expression of several cytokine genes was evaluated by real-time quantitative reserve transcription-PCR (RT-qPCR) in the brain of PbA-infected animals removed on day 7 p.i.. Mice brains were frozen with crushed liquid nitrogen placed in the Trizol™ reagent (Invitrogen, USA) according to the protocol described by the manufacturer. Shortly, after the addition of 1 mL of Trizol™ (Invitrogen, USA) in 40 mg of the brain powder, 0.2 mL of chloroform was added and the lysate was vigorously mixed. The sample was centrifuged at 12,000× g for 15 min and the aqueous phase was transferred to a new tube. The RNA was precipitated by adding 0.5 mL of isopropanol followed by a centrifugation at 12,000× g, then washed with 1 mL of 75% ethanol and resuspended in RNAse free water. RNA was then treated with Deoxyribonuclease I (Fermentas, Canada) in order to degrade contaminating genomic DNA. The cDNA was synthesized using approximately 2 µg of the total RNA with the aid of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the protocol provided by the manufacturer. The polymerase chain reaction was performed with an ABI Prism 7500 (Applied Biosystems, USA) and the reactions were carried out in 25 µL volume and in the presence of the TaqMan PCR Master Mix™ (Applied Biosystems, USA) and different sets of oligonucleotides and probes for the amplification of the β-actin, IFN-γ, TNF-α, IL-1β, IL-6 and IL-10 genes. These corresponded (respectively) to the following reference numbers (Applied Biosystems, USA): Rn00667869_m1, Mm00443258_m1, Mm00443285_m1, Mm00434228_m1, Mm00446190_m1 and Mm00439616_m1. Expression levels of cytokine genes in PbA-infected animals were represented as a relative copy numbers by using the delta threshold cycle method ($2^{-\Delta Ct}$) [33].

Purification of brain-sequestered T cells (BST)

Adherent leukocytes were isolated from mice brains as described elsewhere [14]. Briefly, on day 7 p.i., PbA-infected mice were perfused intracardially with PBS to remove both circulating and non-adherent RBC and leukocytes. Brains were collected and crushed in an RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and gentamycin. The cellular suspension was collected and centrifuged at 15,000× g for 5 min. The pellet was resuspended with 10 mL of an HEPES buffer (Sigma, USA) and supplemented with collagenase (Roche,

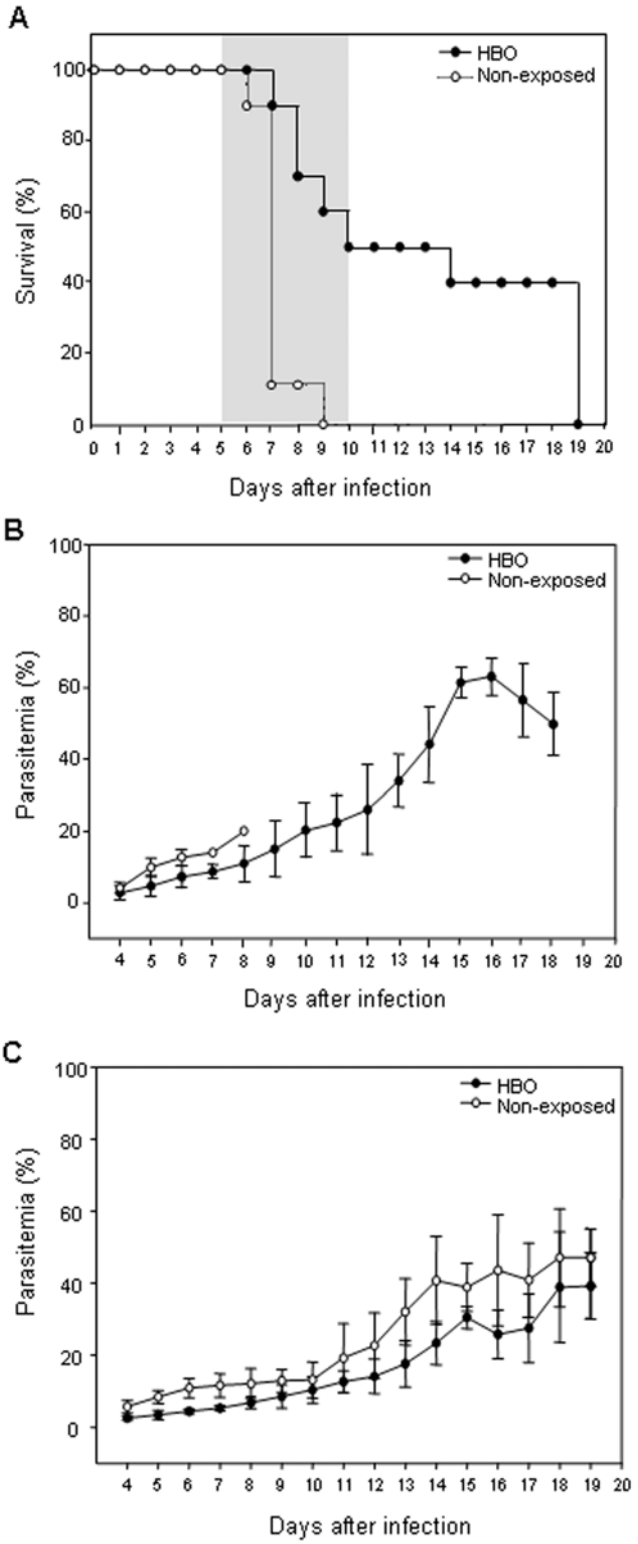

Figure 1. HBO's effect on the survival and the parasite development in *P. berghei*-infected mice. (**A**) Groups of 10 mice infected i.p. with 10^6 iRBC were exposed or not to HBO (100% O_2, 3.0 ATA) for 1 h from day 0 to 10. Pressurized oxygen significantly protected mice against CM neurological symptoms ($P<0.0005$). Neurological signs of CM appeared on days 5–10 with death occurring approximately 24 h after onset (shaded area). Parasitemia levels were assessed daily in mice infected with (**B**) *P. berghei* ANKA (PbA; cerebral line) or (**C**) *P. berghei* NK-65 (PbNK-65; non-cerebral line) regardless of exposure to HBO. HBO significantly ($P<0.05$) reduced the parasite burden on days 4–6 and 4–13 p.i., respectively in PbA- and PbNK-65-infected mice when compared to non-exposed animals.
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USA) and DNase I (Roche, Germany). The mixture was stirred at room temperature for 30 min. The tissue extract was passed through sterile gauze and centrifuged at 5,000× g for 30 s to remove debris. The supernatant was deposited on a 30% Percoll™ (GE Healthcare, Sweden) gradient and centrifuged at 3,000× g for 10 min. The pellet was collected and residual RBC were removed by an ACK lysis buffer. BST were resuspended in PBS containing 5% FBS and counted.

Immunolabeling and flow cytometry analysis of BST

Cells were stained with appropriate dilutions of the following fluorochrome-labeled monoclonal antibodies (mAbs): FITC/anti-CD4 (clone H129-19), FITC/anti-CD8 (clone 53-6.7), PE/anti-TCR γδ (clone GL3) and APC/anti TCR αβ (clone H57-597) and then washed with PBS, fixed and analyzed by flow cytometry in a FACSCanto™ device (Becton Dickinson, USA). All these reagents were purchased from Pharmingen/Becton-Dickinson (USA). Analyses were performed after recording 10,000 events for each sample using Diva™ software. BST were identified by their size (forward light scatter) and granulosity (side light scatter) as previously described [34].

Evaluating Blood-brain barrier dysfunction

Blood-brain barrier (BBB) integrity was assessed in PbA-infected mice on day 7 p.i. by i.v. injection of Evans Blue (1% in saline) in the retro-orbital plexus as previously described [35]. One hour after injection, mice brains were extracted and photographed using a digital camera (Nikon, USA). Brain staining was quantified by measuring the brightness intensity using the red channel in a delimited circular area of 12,294 pixels^2 with the aid of the ImageJ™ software (http://rsb.info.nih.gov/ij). The brightness intensity of mice brain was inversely proportional to the levels of Evans Blue staining.

Statistical analysis

The statistical significance between control and experimental groups were determined with the Log-Rank test for the cumulative survival experiments. The Mann-Whitney *U* test was used to compare parasitemia levels, the drop in relative temperature, the relative RBC density, BBB integrity and parasite and cytokine gene expression among brains collected from both naïve animals and infected mice. Calculations were performed using BioEstat™ version 3.0 (CNPq, Brazil) and Prism™ version 3.02 (Graphpad, USA) software. Values were considered significant when $P<0.05$.

Results

HBO effects on ECM associated mortality and on parasite development

To evaluate the neuroprotective effect of pressurized oxygen, two groups of 10 mice each were infected with PbA. One of these groups was submitted daily to HBO conditions (100% O_2, 3.0 ATA, 1 hour) during 11 consecutive days. As shown on Figure 1A, 100% of PbA-infected mice not exposed to HBO exhibited CM-specific neurological signs within 5 to 8 days after infection and died of fatal cerebral malaria in the following 24 hours; most (80%) died on day 7 p.i.. All animals from this group were dead by day 9 p.i.. In contrast to the non-exposed animals, 50% of the

mice from the HBO group did not develop CM symptoms and survived. In the HBO group, CM neurological signs began to appear later and the mortality rate increased slowly throughout days 7–10, representing 10, 20, 10 and 10%, respectively, on days 7–10. Of note, 1 animal (10%) died on day 14 and 4 (40%) on day 19 post-infection. Cumulative survival statistical analyses clearly demonstrated that HBO therapy had a significant ($P<0.0005$) neuroprotective effect against ECM. As expected, in the mice that did not develop CM, parasite burden progressed and mice died as a result of hyperparasitemia (Figure 1B).

As previously reported, HBO therapy inhibits the development of *Leishmania amazonensis* and of a non-cerebral line of *P. berghei* [30,32,36]. To further explore the effects of HBO, we monitored the parasitemia levels of infected mice exposed daily, or not, to HBO (11-day exposure protocol) for up to 19 days. We observed that HBO significantly ($P<0.05$) reduced the parasite burden of PbA-infected mice on days 4, 5 and 6 p.i., when compared to non-exposed animals (Figure 1B). However, since 100% of non-exposed PbA-infected mice died, we decided to evaluate whether the reduction on parasitemia levels in HBO exposed animals could be sustained over longer periods. Mice infected with *P. berghei* NK-65, a non-cerebral strain that displays similar parasitemia levels, were submitted to pressurized oxygen sessions as in the 11-day exposure protocol (Figure 1C). As observed in PbA-infected animals submitted to pressurized oxygen, a significant ($P<0.05$) decrease in PbNK-65 development was observed on day 4–13 p.i.. Nevertheless, no correlation was found between mice that presented a reduction of parasitemia levels with protection or attenuation of the neurological symptoms (Table S1).

Because we observed that HBO had a significant effect on the parasite burden in the infections of PbA and PbNK-65, we addressed the question as to whether pressurized oxygen therapy could damage normal red blood cells (nRBC) or inhibit parasite development directly. For this purpose, normal RBC (nRBC) collected from a naïve mouse were exposed to pressurized oxygen (100% O_2, 3 ATA) during 4 or 6 hours. The relative percentage of nRBC density was not significantly altered ($P>0.05$) after direct exposure to HBO for up to 6 hours (data not shown), demonstrating that HBO therapy was not toxic to healthy erythrocytes in these conditions. Next, to evaluate HBO's effect directly on parasite development, infected RBC (iRBC) from a PbA-infected mouse were collected and exposed to HBO (100% O_2, 3 ATA). Figure 2A shows a significant reduction ($P<0.05$) on parasite development after 4 and 6 hours in comparison to 0 hour, regardless of exposure to pressurized oxygen. However, when we compared the reduction on parasitemia levels of iRBC left in room air or exposed to HBO, we noticed a significant ($P=0.01$) and more pronounced reduction of the non-exposed iRBC than of the infected cells directly exposed to HBO up to 6 hours. Inhibition of parasite development was also observed after 4 hours of exposure; however, no statistical difference was found ($P>0.05$). Then, to assess whether these iRBC were still able to induce CM neurological signs, we collected 10^6 iRBC exposed directly to HBO or left outside the hyperbaric chamber for 6 hours and injected them in susceptible mice. As shown on Figure 2B, mice infected with iRBC directly exposed to HBO or with the cells left outside the chamber did not present significant differences ($P>0.05$) when the survival curves were compared. Taken together, these data suggest that 6 hours of HBO exposure do not directly affect PbA-infected erythrocytes nor alter their ability to induce CM clinical symptoms.

Next, to investigate whether pressurized oxygen could have an effect when parasitemia was already patent (4%), we randomly selected half of the PbA-infected mice on day 4 p.i. and exposed them to daily HBO sessions (100% O_2, 3.0 ATA, 2 hours per day) until day 7 (Figure 3A). As expected, non-treated mice started to display CM clinical features early on day 5 and 6 and began dying within 20–24 hours on days 5 (10%) and 6 (10%), though the majority (80%) died on day 7 p.i.. All mice were dead by day 7. Notably, hyperbaric oxygen significantly delayed ($P<0.01$) CM specific mortality by up to two days, when compared to non-exposed animals, and reduced the rate of mortality on day 7 from 80% to 40% (Figure 3A). Moreover, two HBO-exposed mice (20%) only exhibited CM neurological signs on days 8 and 9, dying within 24 hours on days 9 and 10. This shows that HBO is capable of interfering significantly with the manifestation of the CM clinical symptoms, including death, even when administrated after parasite establishment. As observed in the 11-day exposure protocol, the administration of pressurized oxygen starting on day 4 p.i. (4-day-exposure) in PbA-infected mice reduced the parasitemia levels ($P<0.01$) significantly on days 4–6 (data not shown).

To confirm that only pressurized oxygen had neuroprotective effects, PbA-infected mice were submitted to the 11-day exposure protocol, but using 1.0 ATA as the atmospheric air pressure (Figure 3B). In this assay, no significant difference ($P>0.05$) was observed after cumulative survival analyses between infected animals exposed to HBO-1.0 ATA and the control mice. Of note, most of the non-exposed mice began to present CM symptoms and died earlier than the HBO-1.0 ATA treated animals. Although a minimal beneficial effect was observed after the administration of 100% oxygen (hyperoxia) under normobaric conditions, this was not enough to protect or even delay CM neurological symptoms, thus demonstrating that HBO's neuroprotective effect does not rely solely on the administration of 100% oxygen.

The effect of HBO on cytokine expression levels and adherent T cells in the brain

Based on the anti-inflammatory features of the HBO treatment reported in ischemic models [21,26] and since the up-regulation of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-1β) [10–12] and the participation of $CD4^+$ and $CD8^+$ T lymphocytes [14,37] is essential for CM pathology to occur, we examined the mRNA levels of different cytokines in the brain of PbA-infected mice scarified on day 7 p.i.. According to Figure 4, after RT-qPCR analysis the mRNA levels of IFN-γ ($P<0.05$), TNF-α ($P<0.01$) and IL-10 ($P<0.05$) significantly decreased in the brain of mice submitted to the 11-day exposure HBO protocol in comparison to non-exposed animals. No significant difference ($P>0.05$) was noted in the mRNA levels of IL-1β and IL-6. RT-negative controls did not generate a detectable amplification product. All cDNA samples resulted in a product when the β-actin set of oligonucleotides and specific probe were present. Regardless of exposure to HBO, animals that presented an increase in the expression of IFN-γ mRNA also presented elevated levels of TNF-α and IL-10.

Next, we asked whether the neuroprotective effect of the pressurized oxygen therapy could be associated to the percentage of $\gamma\delta$ and $\alpha\beta$ T lymphocytes sequestered in mice brains collected on day 7 p.i. (Figure 5). As compared with brains of non-exposed animals, HBO treatment reduced about 1.6 fold the percentage of both $\gamma\delta$ (1.9 vs. 1.2%) and $\alpha\beta$ (7.0 vs. 4.2%) $CD4^+$ T cells between the pools of mice of these two groups (Figure 5A–B). However, a more pronounced decline, about 2.5 fold, was observed on the percentage of both $\gamma\delta$ (7.1 vs. 2.8%) and $\alpha\beta$ (43.1 vs. 17.7%) $CD8^+$ T lymphocytes in the mice exposed to HBO in contrast to the non-exposed animals (Figure 5C–D). Taken together, our data demonstrate that HBO's neuroprotective effect is related to the reduction of the T cells sequestered in mice brains; and

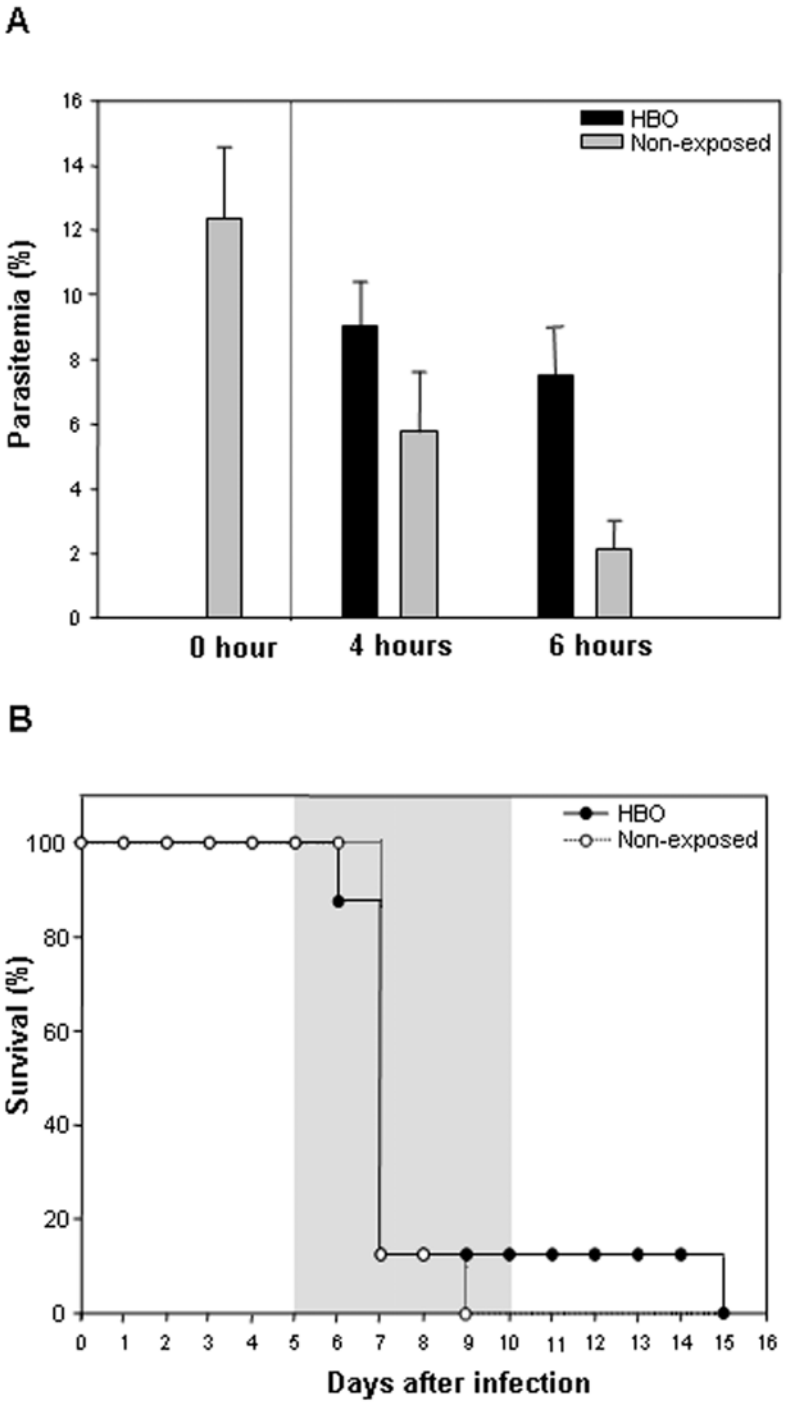

Figure 2. The direct effect of HBO therapy on RBC infected, or not, by PbA. 10^6 iRBC/mL of PbA in a 24-well-plate were directly exposed or not to HBO (100% O_2, 3 ATA). (**A**) Parasitemia levels were evaluated four or six hours after direct iRBC exposure to pressurized oxygen conditions. The parasite burden decreased significantly ($P<0.05$) after 4 or 6 hours in comparison to 0 hour. The reduction of parasitemia levels were more pronounced in infected cells left in normal room air than iRBC submitted directly to HBO after 4 ($P>0.05$) or 6 hour-exposure ($P=0.01$). Results are expressed as the mean of quadruplicates ± standard deviation. (**B**) Mice (n = 8 each group) were infected with 10^6 iRBC of PbA collected after either six hours of direct exposure or no exposure to HBO. No statistical difference was noted when survival curves were compared ($P>0.05$).
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corroborate with existing literature, in which T lymphocytes, mainly αβ $CD8^+$ T cells, are implicated in CM pathology [14,37] No immunolabeling was detected on T lymphocytes in the absence of mAbs (data not shown).

HBO effects on severe ECM symptoms

Severe hypothermia and dysfunction of the BBB are common features in ECM [35]. To investigate whether HBO therapy could improve poor ECM outcomes, we measured the corporal

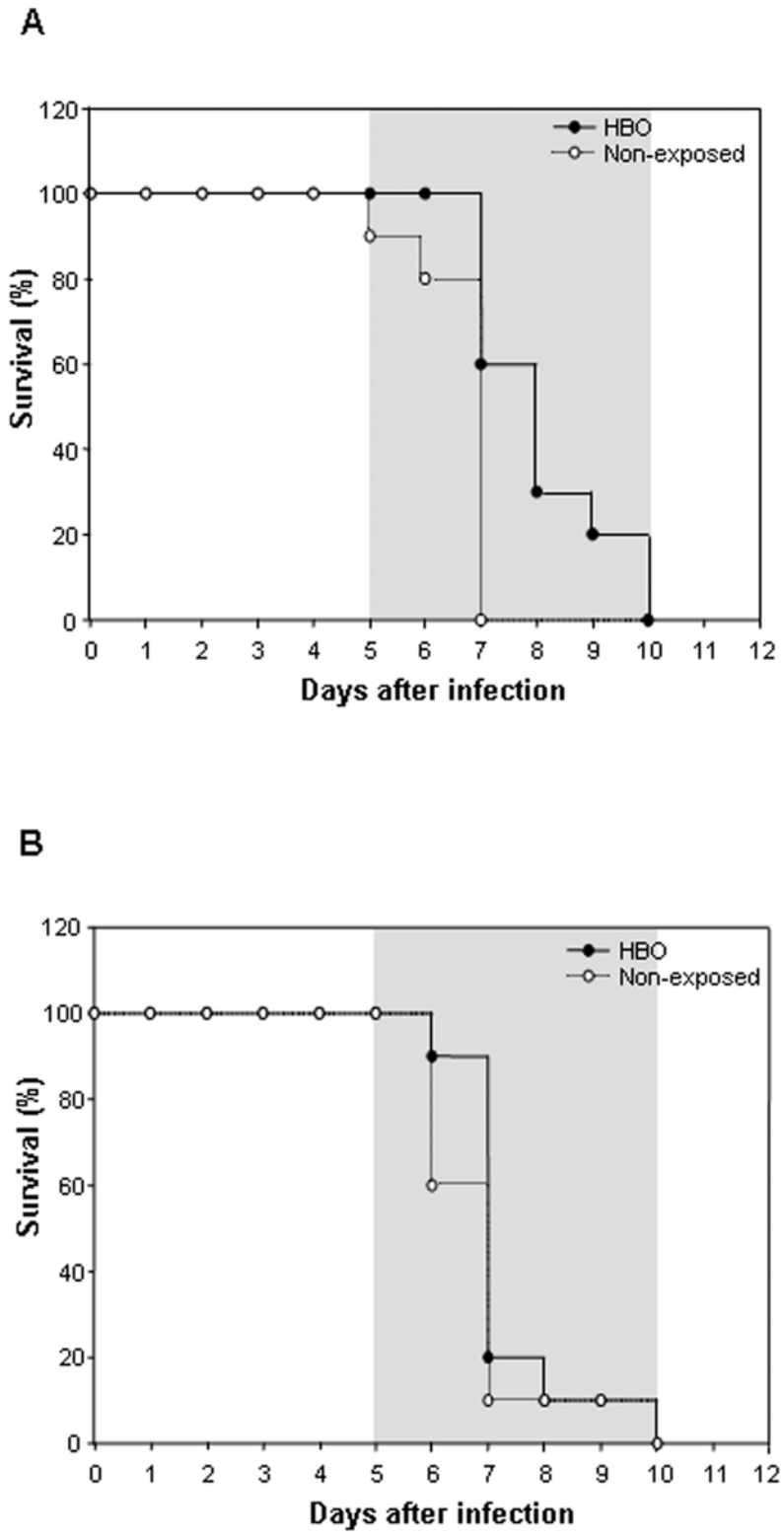

Figure 3. Evaluation of HBO's neuroprotective effect after parasite establishment and the role of pressure in mice survival. (**A**) Twenty mice were injected i.p. with 10^6 iRBC; on day 4 p.i. (parasitemia of 4%) 10 animals, randomly selected, were daily exposed to HBO therapy (100% O_2, 3.0 ATA) for 2 hours from days 4–7 after parasite inoculation. The survival curves of both groups demonstrated that HBO significantly delayed mice mortality ($P<0.01$). (**B**) Groups of 10 PbA-infected mice were exposed daily or not exposed to HBO (100% O_2, 1 hour per day) at 1.0 ATA until all the animals died. Survival curves of the one hundred percent normobaric oxygen exposed mice and animals exposed to normal air did not differ significantly ($P>0.05$).
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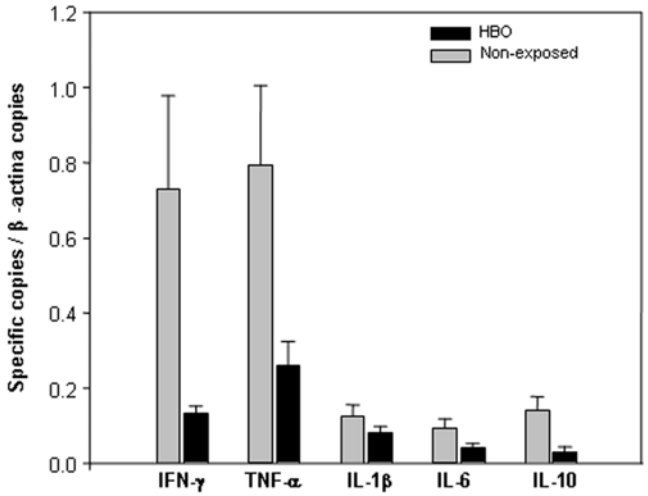

Figure 4. Cytokine gene expression is altered in the brains of PbA-infected mice exposed to HBO. Groups of 6–7 PbA-infected mice were either submitted or not to pressurized oxygen therapy (100% O_2, 3.0 ATA, 1 hour per day) and on day 7 p.i. brains were collected for real-time quantitative reserve transcription-PCR analysis. HBO significantly reduced IFN-γ ($P<0.05$), TNF-α ($P<0.01$) and IL-10 ($P<0.05$), but did not alter IL-1β and IL-6 mRNA expression levels in contrast to non-exposed mice. Values are expressed as the mean of specific cytokine genes copies relative to β–actin copies of six-seven mice±standard deviation.
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temperature of PbA-infected mice daily regardless of exposure to pressurized oxygen in the same conditions as the 11-day exposure protocol. Unlike in the case of non-exposed mice, HBO therapy significantly prevented ($P<0.001$) hypothermia in mice from day 6 p.i., when severe neurological signs were evident in most of the animals (data not shown). Then, by injecting Evans Blue solution, we analyzed and quantified the BBB integrity in HBO exposed and non-exposed animals and in naïve animals early on day 7 p.i.. One hour after Evans Blue injection, mice brains were collected and photographed. As seen in Figure 6A, brains collected from non-exposed mice were darker than those of HBO treated animals due to a high incorporation of Evans Blue in the brain tissue as a consequence of BBB destruction [26]. As expected, we did not observe any staining in naïve mice brains. To quantify the Evans Blue staining and, in turn the BBB integrity, we measured the light intensity in naïve animals and infected mice brains submitted or not to pressurized oxygen. According to Figure 6B, HBO therapy significantly reduced ($P<0.005$) the brain staining in treated mice. Moreover, when we compared the Evans Blue staining in naïve and PbA-infected animals that received HBO treatment, no significant difference was observed ($P>0.05$). As expected, a statistical difference in light intensity levels was observed between naïve mice and non-exposed infected animals ($P<0.005$). Collectively, these data clearly demonstrate that HBO prevents temperature drops and BBB dysfunction.

Discussion

In the present study, we show that HBO therapy (100% O_2, 3.0 ATA) is capable of partially protecting PbA-infected mice against CM and delaying CM-specific neurological signs (Figures 1 and 3). These observations demonstrate for the first time that pressurized oxygen therapy under hyperbaric conditions well-tolerated in humans and animals can prevent CM clinical outcomes, including death.

In an experimental rat model of brain trauma, recent studies have shown that HBO has a neuroprotective effect against focal cerebral ischemia, especially when initiated within the first 6 hours [38]. HBO was thus found to reduce BBB damage, prevent

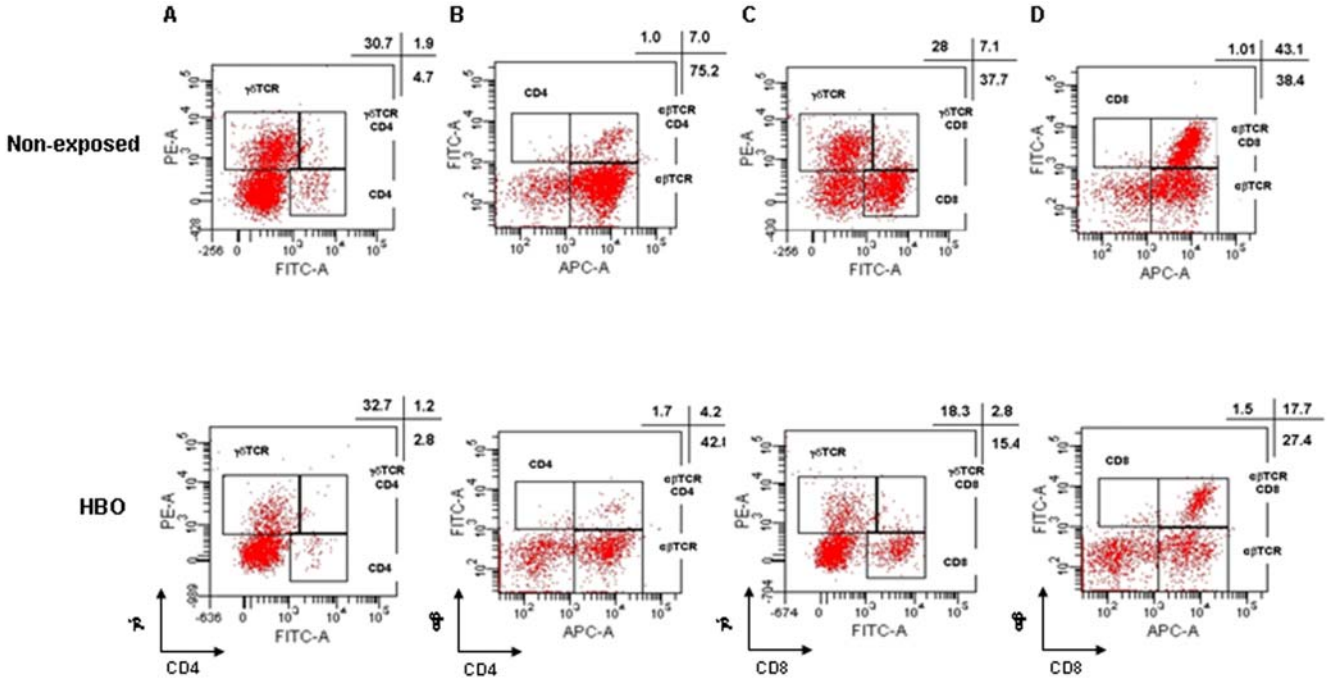

Figure 5. Reduced brain-sequestered T lymphocytes in PbA-infected mice exposed to HBO treatment. Flow cytometric analyses were done on γδ and αβ $CD4^+$ and $CD8^+$ T cells sequestered in mice brains (a pool of 4–5 mice per group) collected on day 7 after PbA infection between the groups regardless of exposure to HBO conditions. Pressurized oxygen therapy reduced the percentage of all cellular subsets, but mainly αβ $CD8^+$ T cells. Representative dot blots of (**A**) γδ and CD4, (**B**) αβ and CD4, (**C**) γδ and CD8, (**D**) αβ and CD8 double staining.
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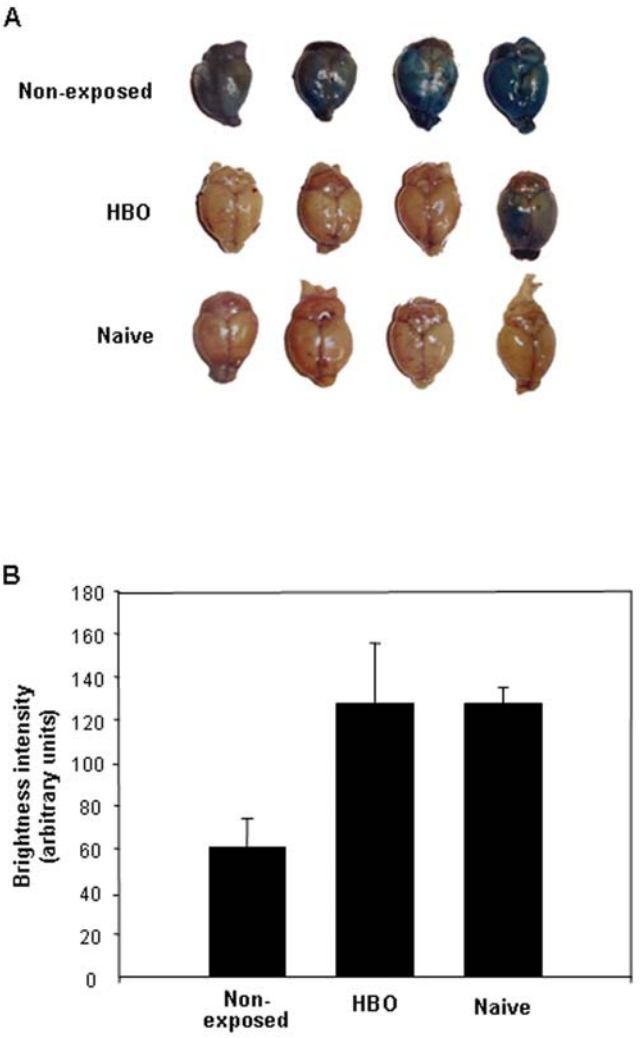

Figure 6. HBO preserves integrity of the blood-brain barrier in PbA-infected mice. Four PbA-infected mice, representative of each group (n = 8) exposed or not to HBO treatment (100% O_2, 3.0 ATA, 1 hour per day), received i.v. injections of 1% Evans Blue solution early on day 7 p.i.. **(A)** One hour after Evans Blue injection, brains of naïve animals, PbA-infected mice and HBO-treated PbA-infected mice were collected and photographed (n = 4 of each group). **(B)** The BBB dysfunction of naïve mice or PbA-infected animals, regardless of submission to hyperbaric conditions, was determined by brain staining quantification with the aid of the ImageJ™ software (n = 4 of each group). HBO significantly reduced ($P<0.005$) the staining in the brains of infected-mice in comparison to non-treated animals. No statistical difference ($P>0.05$) was noticed between naïve and HBO-treated infected mice and brains collected from non-treated infected mice were significantly ($P<0.005$) darker than naïve animals. Results are expressed as the mean of brightness intensity of each delimited brain area of six mice±standard deviation.
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apoptosis and maintain lipid oxidation levels stable [39–42]. HBO's neuroprotection was also observed in neonatal rats after the induction of the ischemic process [43]. Rabbits exposed to pressurized oxygen for 90 min during 3 consecutive days presented a significant reduction in the edema area of the brain and cerebral necrosis [44]. In addition, the preservation of BBB, the reduction in HIF-1α levels, and decreased apoptosis and neuronal damage were observed in a rat model for subarachnoid hemorrhage after exposure to HBO [45]. In humans, exposure of thirty-seven brain-injured patients to sixty minutes of HBO treatment every 24 hours increased the cerebral metabolic oxygen rate and reduced cerebrospinal lactate levels [46]. In another study, 10 out of 22 patients with cerebral infarction presented an amelioration of their motor function, while 7 of these patients experienced improved revascularization after pressurized oxygen sessions [47].

When comparing exposed animals with non-exposed animals, we noticed a significant reduction on the parasitemia levels of PbA-infected mice exposed to HBO (11-day exposure protocol) during infection (4–6 p.i.; Figure 1B). PbNK-65-infected mice exposed to HBO in the same conditions also presented a significant reduction of their parasite burden on day 4–13 p.i. (Figure 1C). These findings are in line with a recent study in which daily sessions of 100% pressurized oxygen at 2.5 ATA significantly reduced the size of *Leishmania amazonensis* induced lesions and the parasite development in infected mice [36]. Nevertheless, as in ECM parasites in the brain are necessary, but not sufficient, to neurological symptoms appearing [15], the lack of correlation between survival and the reduction of parasitemia levels, measured daily until the death of PbA-infected animals exposed to HBO, might be related to the fact that parasitemia levels probably do not determine the parasite load in the brain. Indeed, methods aimed at inducing protection against ECM often do not reduce parasitemia levels [48].

Also, direct exposure to HBO for up to 6 hours observed in our *in vitro* analyses was not harmful to normal or PbA-infected erythrocytes (data not shown and Figure 2), differing from previous studies where direct exposure of *L. amazonensis* promastigotes to HBO for up to 6 hours significantly decreased parasite viability [32]. However, as it is assumed that HBO increases the levels of reactive oxygen intermediates (ROI) [49], we believe that the disparity of these two protozoan parasites in terms of HBO susceptibility might be linked to differential killing mediated by reactive oxygen intermediates (ROI). In fact, it has been shown that *Leishmania* parasite killing is sensitive to ROI, whereas PbA-infected erythrocytes are resistant to killing by ROI, even at supraphysiological doses, and ROI are not essential for controlling *Plasmodium* sp. parasitemia [50–52].

We have also shown that the neuroprotective effects of daily hyperbaric sessions rely on the combination of hyperoxia and pressure at 3.0 ATA (Figure 1A), as ECM-specific mortality of PbA-infected mice submitted to 100% oxygen pressurized at 1.0 ATA did not differ significantly from the non-exposed animals (Figure 3B). In an experimental model for cerebral ischemia, HBO neuroprotection was not achieved in animals submitted to pure oxygen at only 1.0 ATA [39,40], and human stimulated monocyte-macrophages cultured in hyperoxia did not present changes in their cytokine expression levels [23]. More importantly, in a study of 12 CM comatose patients who breathed 95% oxygen, no improvement in the consciousness levels were observed in any of the individuals [7].

Brain macrophages from adults and children who died of CM had higher levels of immunological markers that are normally not upregulated [9], such as IFN-γ, IL-1β, IL-10 and TNF-α [10,11,16] neuroprotection in ECM is often associated with the reduction of IFN-γ, and TNF-α levels [53–55]. IL-10 is higher in severe malaria patients from different regions despite the fact that CM individuals presented lower levels of IL-10 in comparison to the non-cerebral malaria group [16,56] Furthermore, $CD8^+$ αβ T cells migrating to the brain have been implicated in cytotoxicity and BBB disruption, thus contributing to ECM mortality [14,15]. Here, we showed that HBO therapy reduced IFN-γ, TNF-α and IL-10 mRNA expression levels in the brain and the percentage of

brain-sequestered $CD4^+$ and $CD8^+$ γδ and αβ T lymphocytes (Figures 4–5). Moreover, the reduction in the IL-10 levels in PbA-infected mice exposed to HBO might be associated with the decrease in expression of IFN-γ and TNF-α. These data are in line with the fact that pressurized oxygen is able to inhibit synthesis of cytokines, such as TNF-α and IFN-γ, T lymphocyte proliferation, decrease the migration of immunocompetent cells and improve tissue transplantation by down-regulating lymphoid system functions [19,22,23,28,57,58].

Finally, when we assessed the HBO effects on cerebral outcomes, we noticed a significant reduction in hypothermia (data not shown) and in the BBB breakdown (Figure 6) in mice exposed to pressurized oxygen. This corroborates previous findings where HBO (100% O_2, 2.8–3.0 ATA) prevented BBB permeability and functionality in animals submitted to a brain injury [31,36]. Based on these observations, it is plausible to assume that HBO prevents BBB breakdown and then avoids vascular leakage by down-regulating the inflammatory immune response in ECM, but mainly, by reducing the percentage of brain-sequestered $CD8^+$ T lymphocytes [10]. Therefore, we cannot rule out that other mechanisms are also involved in HBO neuroprotective effects in ECM, as HBO also inhibits ICAM-1 expression and neuronal apoptosis and upregulates the expression of vascular endothelial growth factor (VEGF), which is involved in angiogenesis in human endothelial cells [22,23,25,28,59]. Also, HBO led to an increase in the brain levels of nitric oxide (NO) [60], a molecule that contributes to protection against ECM [61].

In summary, we have presented evidence of the beneficial effects induced by HBO therapy against ECM. We also demonstrated that the administration of pressurized oxygen down-regulates IFN-γ, TNF-α and IL-10 cytokine expression and the migration to the brain of T lymphocytes, preventing BBB breakdown and severe mice hypothermia without directly affecting iRBC viability and infectivity. Since complementary therapies such as steroids, sodium bicarbonate and heparin are deleterious in CM, and treatment with an anti-TNF-α monoclonal can worsen neurological symptoms [62]. The data presented here create promising perspectives for further investigation of additional HBO's neuroprotective mechanisms and to consider it as a new supportive therapy that could act alone or in association with conventional treatment or with recently discovered neuroprotective or anti-inflammatory molecules to improve poor CM outcomes [63,64].

Supporting Information

Table S1

Found at: doi:10.1371/journal.pone.0003126.s001 (0.01 MB PDF)

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Author Contributions

Conceived and designed the experiments: YCB UG WWAS FTMC. Performed the experiments: YCB ASF UG SCPL BOC. Analyzed the data: YCB ASF UG SCPL WWAS RA GW LMBS SG FTMC. Contributed reagents/materials/analysis tools: RA GW LMBS SG FTMC. Wrote the paper: FTMC.

References

1. Carter JA, Ross AJ, Neville BG, Obiero E, Katana K, et al. (2005) Developmental impairments following severe falciparum malaria in children. Trop Med Int Health 10: 3–10.
2. Newton CRJC, Krishna S (1998) Severe Falciparum malaria in children: current understanding of pathophysiology and supportive treatment. Pharmacol Ther 79: 1–53.
3. Newton CR, Peshu N, Kendall B, Kirkham FJ, Sowunmi A, et al. (1994) Brain swelling and ischaemia in Kenyans with cerebral malaria. Arch Dis Child 70: 281–287.
4. Newton CR, Marsh K, Peshu N, Kirkham FJ (1996) Perturbations of cerebral hemodynamics in Kenyans with cerebral malaria. Pediatr Neurol 15: 41–49.
5. Pongponratn E, Riganti M, Punpoowong B, Aikawa M (1991) Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. Am J Trop Med Hyg 44: 168–175.
6. White NJ, Warrell DA, Looareesuwan S, Chanthavanich P, Phillips RE, et al. (1985) Pathophysiological and prognostic significance of cerebrospinal-fluid lactate in cerebral malaria. Lancet 1: 776–778.
7. Warrell DA, White NJ, Veall N, Looareesuwan S, Chanthavanich P, et al. (1988) Cerebral anaerobic glycolysis and reduced cerebral oxygen transport in human cerebral malaria. Lancet 2: 534–538.
8. Patankar TF, Karnad DR, Shetty PG, Desai AP, Prasad SR (2002) Adult cerebral malaria: prognostic importance of imaging findings and correlation with postmortem findings. Radiology 224: 811–816.
9. Medana IM, Turner GD (2006) Human cerebral malaria and the blood-brain barrier. Int J Parasitol 36: 555–568.
10. Hunt NH, Grau GE (2003) Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol 24: 491–499.
11. Taylor TE, Fu WJ, Carr RA, Whitten RO, Mueller JS, et al. (2004) Differentiating the pathologies of cerebral malaria by postmortem parasite counts. Nat Med 10: 143–145.
12. Rudin W, Favre N, Bordmann G, Ryffel B (1997) Interferon-γ is essential for the development of cerebral malaria. Eur J Immunol 27: 810–815.
13. Hermsen CC, Crommert JV, Fredix H, Sauerwein RW, Eling WM (1997) Circulating tumour necrosis factor alpha is not involved in the development of cerebral malaria in Plasmodium berghei-infected C57BL mice. Parasite Immunol 19: 571–577.
14. Belnoue E, Kayibanda M, Vigario AM, Deschemin JC, van Rooijen N, et al. (2002) On the pathogenic role of brain-sequestered αβ $CD8^+$ T cells in experimental cerebral malaria. J Immunol 169: 6369–6375.
15. Nitcheu J, Bonduelle O, Combadiere C, Tefit M, Seilhean D, et al. (2003) Perforin-dependent brain-infiltrating cytotoxic $CD8^+$ T lymphocytes mediate experimental cerebral malaria pathogenesis. J Immunol 170: 2221–2228.
16. Prakash D, Fesel C, Jain R, Cazenave PA, Mishra GC, et al. (2006) Clusters of cytokines determine malaria severity in Plasmodium falciparum-infected patients from endemic areas of Central India. J Infect Dis 194: 198–207.
17. Gudewicz TM, Mader JT, Davis CP (1987) Combined effects of hyperbaric oxygen and antifungal agents on the growth of Candida albicans. Aviat Space Environ Med 58: 673–678.
18. Park MK, Myers RA, Marzella L (1992) Oxygen tensions and infections: modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. Clin Infect Dis 14: 720–740.
19. Kaide CG, Khandelwal S (2008) Hyperbaric oxygen: applications in infectious disease. Emerg Med Clin North Am 26: 571–595.
20. Al-Waili NS, Butler GJ (2006) Effects of hyperbaric oxygen on inflammatory response to wound and trauma: possible mechanism of action. The Scientific World Journal 6: 425–441.
21. Zhang Q, Chang Q, Cox RA, Gong X, Gould LJ (2008) Hyperbaric oxygen attenuates apoptosis and decreases inflammation in an ischemic wound model. J Invest Dermatol 128: 2102–2112.
22. Lahat N, Bitterman H, Yaniv N, Kinarty A, Bitterman N (1995) Exposure to hyperbaric oxygen induces tumour necrosis factor-alpha (TNF-α) secretion from rat macrophages. Clin Exp Immum 102: 655–659.
23. Benson RM, Minter LM, Osborne BA, Granowitz EV (2003) Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. Clin Exp Immunol 134: 57–62.
24. Yang ZJ, Bosco G, Montante A, Ou XI, Camporesi EM (2001) Hyperbaric O_2 reduces intestinal ischemia-reperfusion-induced TNF-α production and lung neutrophils sequestration. Eur J Appl Physiol 85: 96–103.
25. Buras JA, Stahl GL, Svoboda KK, Reenstra WR (2000) Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS. Am J Physiol Cell Physiol 278: 292–302.
26. Yin W, Badr AE, Mychaskiw G, Zhang JH (2002) Down regulation of COX-2 is involved in hyperbaric oxygen treatment in a rat transient focal cerebral ischemia model. Brain Res 926: 165–171.
27. Li Y, Zhou C, Calvert JW, Colohan AR, Zhang JH (2005) Multiple effects of hyperbaric oxygen on the expression of HIF-1 alpha and apoptotic genes in a global ischemia-hypotension rat model. Exp Neurol 191: 198–210.

28. Al-Waili N, Butler GJ, Beale J, Abdullah MS, Hamilton RWB, et al. (2005) Hyperbaric oxygen in treatment of patients with cerebral stroke, brain trauma, and neurological disease. Adv Therapy 22: 659–678.
29. Thackham JA, McElwain DL, Long RJ (2008) The use of hyperbaric oxygen therapy to treat chronic wounds: A review. Wound Repair Regen 16: 321–330.
30. Rencricca NJ, Coleman RM, Altschule MD, Faletra PP, Gray AD, et al. (1981) Quantification of hyperbaric oxygen-induced toxicity utilizing a malarial system. Aviat Space Environ Med 52: 85–87.
31. Engwerda C, Belnoue E, Grüner AC, Renia L (2005) Experimental models of cerebral malaria. Curr Top Microbiol Immunol 297: 103–143.
32. Arrais-Silva WW, Collhone MC, Ayres DC, de Souza Souto PC, Giorgio S (2005) Effects of hyperbaric oxygen on Leishmania amazonensis promastigotes and amastigotes. Parasitol Int 54: 1–7.
33. Overbergh L, Giulietti A, Valckx D, Decallonne R, Bouillon R, Mathieu C (2003) The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. J Biomol Tech 14: 33–43.
34. Irani DN, Griffin DE (1991) Isolation of brain parenchymal lymphocytes for flow cytometric analysis. Application to acute viral encephalitis. J Immunol Methods 139: 223–231.
35. Piguet PF, Kan CD, Vesin C, Rochat A, Donati Y, et al. (2001) Role of CD40-CVD40L in mouse severe malaria. Am J Pathol 159: 733–742.
36. Arrais-Silva WW, Pinto EF, Rossi-Bergmann B, Giorgio S (2006) Hyperbaric oxygen therapy reduces the size of Leishmania amazonensis-induced soft tissue lesions in mice. Acta Trop 98: 130–136.
37. Renia L, Potter SM, Mauduit M, Rosa DS, Kayibanda M, et al. (2006) Pathogenic T cells in cerebral malaria. Int J Parasitol 36: 547–554.
38. Lou M, Eschenfelder CC, Herdegen T, Brecht S, Deuschl G (2004) Therapeutic window for use of hyperbaric oxygenation in focal transient ischemia in rats. Stroke 35: 578–583.
39. Veltkamp R, Siebing DA, Heiland S, Schoenffeldt-Varas P, Veltkamp C, et al. (2005a) Hyperbaric oxygen induces rapid protection against focal cerebral ischemia. Brain Res 1037: 134–138.
40. Veltkamp R, Siebing DA, Sun L, Heiland S, Bieber K, et al. (2005b) Hyperbaric oxygen reduces blood-brain barrier damage and edema after transient focal cerebral ischemia. Stroke 36: 1679–1683.
41. Schabitz WR, Schade H, Heiland S, Kollmar R, Bardutzky J, et al. (2004) Neuroprotection by hyperbaric oxygenation after experimental focal cerebral ischemia monitored by MRI. Stroke 35: 1175–1179.
42. Yin D, Zhou C, Kusaka I, Calvert JW, Parent AD, et al. (2003) Inhibition of apoptosis by hyperbaric oxygen in a rat focal cerebral ischemic model. J Cereb Blood Flow Metab 23: 855–864.
43. Calvert JW, Yin W, Patel M, Badr A, Mychaskiw G, et al. (2002) Hyperbaric oxygenation prevented brain injury induced by hypoxia-ischemia in a neonatal rat model. Brain Res 951: 1–8.
44. Niklas A, Brock D, Schober R, Schulz A, Schneider D (2004) Continuous measurements of cerebral tissue oxygen pressure during hyperbaric oxygenation-HBO effects on brain edema and necrosis after severe brain trauma in rabbits. J Neurol Sci 219: 77–82.
45. Ostrowski RP, Colohan ART, Zhang JH (2005) Mechanisms of therapeutic of oxygen-induces neuroprotection in a rat model of subarachnoid hemorrhage. J Cereb Blood Flow Metab 25: 554–571.
46. Rockswold SB, Rockswold GL, Vargo JM, Erickson CA, Sutton RL, et al. (2001) Effects of hyperbaric oxygenation therapy on cerebral metabolism and intracranial pressure in severely brain injured patients. J Neurosurg 94: 403–411.
47. Kapp JP (1981) Neurological response to hyperbaric oxygen a criterion for cerebral revascularization. Surg Neurol 15: 43–46.
48. Jennings VM, Lal AA, Hunter RL (1998) Evidence for multiple pathologic and protective mechanisms of murine cerebral malaria. Infect Immun 66: 5972–5979.
49. Hink J, Jansen E (2001) Are superoxide and/or hydrogen peroxide responsible for some of the beneficial effects of hyperbaric oxygen therapy? Med Hypotheses 57: 764–769.
50. Reiner SL, Zheng S, Wang ZE, Stowring L, Locksley RM (1994) Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. J Exp Med 179: 447–456.
51. Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde H (2005) Plasmodium berghei resists killing by reactive oxygen species. Infect Immun 73: 6704–6710.
52. Gillman BM, Batchelder J, Flaherty P, Weidanz WP (2004) Suppression of Plasmodium chabaudi parasitemia is independent of the action of reactive oxygen intermediates and/or nitric oxide. Infect Immun 72: 6359–6366.
53. Medana IM, Hunt NH, Chaudhri G (1997) Tumor necrosis factor-alpha expression in the brain during fatal murine cerebral malaria: evidence for production by microglia and astrocytes. Am J Pathol 150: 1473–1486.
54. De Kossodo S, Grau GE (1993) Profiles of cytokine production in relation with susceptibility to cerebral malaria. J Immunol 151: 4811–4820.
55. Jennings VM, Actor JK, Lal AA, Hunter RL (1997) Cytokine profile suggesting that murine cerebral malaria is an encephalitis. Infect Immun 65: 4883–4887.
56. Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, et al. (1999) The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. J Infect Dis 180: 1288–1297.
57. Gadd MA, McClellan DS, Neuman TS, Hansbrough JF (1990) Effect of hyperbaric oxygen on murine neutrophil and T-lymphocyte functions. Crit Care Med 18: 974–979.
58. Granowitz EV, Skulsky EJ, Benson RM, Wright J, Garb JL, et al. (2002) Exposure to increased pressure or hyperbaric oxygen suppresses interferon-gamma secretion in whole blood cultures of healthy humans. Undersea Hyperb Med 29: 216–225.
59. Lee CC, Chen SC, Tsai SC, Wang BW, Liu YC, et al. (2006) Hyperbaric oxygen induces VEGF expression through ERK, JNK and c-Jun/AP-1 activation in human umbilical vein endothelial cells. J Biomed Sci 13: 143–156.
60. Ohgami Y, Chung E, Shirachi DY, Quock RM (2008) The effect of hyperbaric oxygen on regional brain and spinal cord levels of nitric oxide metabolites in rat. Brain Res Bull 75: 668–73.
61. Gramaglia I, Sobolewski P, Meays D, Contreras R, Nolan JP, et al. (2006) Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. Nat Med 12: 1417–1422.
62. Idro R, Jenkins NE, Newton CRJ (2005) Pathogenesis, clinical features, and neurological outcomes of cerebral malaria. The Lancet 4: 827–840.
63. Kaiser K, Texier A, Ferrandiz J, Buguet A, Meiller A, et al. (2006) Recombinant human erythropoietin prevents the death of mice during cerebral malaria. J Infect Dis 193: 987–995.
64. Wassmer SC, Cianciolo GJ, Combes V, Grau GE (2005) Inhibition of endothelial activation: a new way to treat cerebral malaria? PloS Med 2: 245e.