MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ

Doutorado em Programa de Pós-graduação em Biologia Parasitária

IDENTIFICAÇÃO E AVALIAÇÃO DA IMUNOGENICIDADE DE EPÍTOPOS DE CÉLULAS B DE PROTEÍNAS DE ESPOROZOÍTOS (PVCELTOS) E MEROZOÍTOS (PVMSP-9) DE *PLASMODIUM VIVAX*

RODRIGO NUNES RODRIGUES DA SILVA

Rio de Janeiro
Dezembro de 2017

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ
Programa de Pós-Graduação em Biologia Parasitária

RODRIGO NUNES RODRIGUES DA SILVA

IDENTIFICAÇÃO E AVALIAÇÃO DA IMUNOGENICIDADE DE EPÍTOPOS DE CÉLULAS B DE PROTEÍNAS DE ESPOROZOÍTOS (PVCELTOS) E MEROZOÍTOS (PVMSP-9) DE *PLASMODIUM VIVAX*

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária

Orientador (es): Prof. Dr. Josué da Costa Lima Junior

RIO DE JANEIRO
Dezembro de 2017

Silva, Rodrigo Nunes Rodrigues da.

IDENTIFICAÇÃO E AVALIAÇÃO DA IMUNOGENICIDADE DE EPÍTOPOS DE CÉLULAS B DE PROTEÍNAS DE ESPOROZOÍTOS (PVCELTOS) E MEROZOÍTOS (PVMSP-9) DE PLASMODIUM VIVAX / Rodrigo Nunes Rodrigues da Silva. - Rio de janeiro, 2017.
189 f.; il.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Parasitária, 2017.

Orientador: Josué da Cota Limea-Junior.

Bibliografia: Inclui Bibliografias.

1. Malária. 2. Resposta imune humoral. 3. Plasmodium vivax. 4. Identificação in silico de epítopos. 5. Resposta imune celular. I. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da Biblioteca de Manguinhos/ICICT com os dados fornecidos pelo(a) autor(a).

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Programa de Pós-Graduação em Biologia Parasitária

AUTOR: RODRIGO NUNES RODRIGUES DA SILVA

IDENTIFICAÇÃO E AVALIAÇÃO DA IMUNOGENICIDADE DE EPÍTOPOS DE CÉLULAS B DE PROTEÍNAS DE ESPOROZOÍTOS (PVCELTOS) E MEROZOÍTOS (PVMSP-9) DE *PLASMODIUM VIVAX*

ORIENTADOR (ES): Prof. Dr. Josué da Costa Lima Junior

Aprovada em: 15 / 12 / 2017

EXAMINADORES:

Prof. Dr. Renato Porrozzi de Almeida **- Presidente -** (Fiocruz-RJ)
Prof. Dra. Flávia Lima Ribeiro-Gomes **-** (Fiocruz-RJ)
Prof. Dra. Lilian Lacerda bueno - (UFMG)
Prof. Dr. Leonardo José de Moura Carvalho - (Fiocruz-RJ)
Prof. Dra. Joanna Reis Santos de Oliveira - (IFRJ)

Rio de Janeiro, 15 de Dezembro de 2017

Ministério da Saúde

Fundação Oswaldo Cruz
Instituto Oswaldo Cruz

Ata da defesa de tese de doutorado em Biologia Parasitária de **Rodrigo Nunes Rodrigues da Silva**, sob orientação do Dr. Josué da Costa Lima Junior. Ao décimo quinto dia do mês de dezembro de dois mil e dezessete, realizou-se às quatorze horas, na Sala 6 do Pavilhão Cardoso Fontes, o exame da tese de doutorado intitulada: **"IDENTIFICAÇÃO E AVALIAÇÃO DA IMUNOGENICIDADE DE EPÍTOPOS DE CÉLULAS B DE PROTEÍNAS DE ESPOROZOÍTOS (PVCELTOS) E MEROZOÍTOS (PVMSP-9) DE PLASMODIUM VIVAX"** No programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutor em Ciências - área de concentração: Imunologia e Patogenia, na linha de pesquisa: Imunologia de Doenças Infecciosas e Parasitárias. A banca examinadora foi constituída pelos Professores: Dr. Renato Porrozzi de Almeida - IOC/FIOCRUZ (Presidente), Drª. Lilian Lacerda Bueno - UFMG/MG, Drª. Flávia Lima Ribeiro Gomes - IOC/FIOCRUZ e como suplentes: Dr. Leonardo José de Moura Carvalho – IOC/FIOCRUZ e Drª. Joanna Reis Santos de Oliveira – IFRJ/RJ. Após arguir o candidato e considerando que o mesmo demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela APROVAÇÃO da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutor em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 15 de dezembro de 2017.

Dr. Renato Porrozzi de Almeida (Presidente da Banca):

Drª. Lilian Lacerda Bueno (Membro da Banca):

Drª. Flávia Lima Ribeiro Gomes (Membro da Banca): Flávia Lima Ribeiro Gomes

Dr. Rafael Maciel de Freitas (Coordenador do Programa):

Av. Brasil, 4365 Manguinhos Rio de Janeiro RJ Brasil **CEP:** 21040-360
Contatos: (21) 2562-1201 / 2562-1299 **E-mail:** atendimentoseac@ioc.fiocruz.br **Site:** www.fiocruz.br/iocensino

À Josué, cujas broncas, amizade e confiança, foram determinantes para existência deste trabalho.

À Priscilla, por toda paciência, amor e suporte, vitais para chegada até aqui.

À Milena, minha filha, pela
inesgotável fonte de inspiração.

À minha mãe e irmão por
todo apoio incondicional.

AGRADECIMENTOS

Sempre tive grande dificuldade de enumerar agradecimentos. Contudo, imaginando que poucas pessoas lerão realmente este tópico, não me incomodarei em fazê-lo de modo bem informal e possivelmente quebrar alguns padrões usais.

Deste modo, antes de listar nominalmente pessoas vitais pela existência deste trabalho e, possivelmente vitais a minha vida, gostaria de agradecer a Fundação Oswaldo Cruz. Sim, a instituição como um todo. Faço isso, pois certamente seria impossível listar aqui todas as pessoas, que fazem parte da mesma, e que de algum modo contribuíram para minha formação. Assim, através deste parágrafo deixo meus sinceros agradecimentos a todos aqueles que de algum modo me permitiram chegar até aqui e que, infelizmente, acabaram esquecidos ou não mencionados.

Agradeço, primeiramente, ao Doutor, professor, orientador e mais do que tudo, amigo Josué da Costa Lima Junior, por sempre se fazer presente e mesmo diante de todas as dificuldades e incontáveis broncas, ter sido capaz de me permitir concluir com êxito não apenas essa etapa, mas a maior parte de minha formação acadêmica. Obrigado pelo imensurável aprendizado, pelas incontáveis horas e conselhos, mesmo os furados. Obrigado pelos “poucos” esporros, com ou sem razão, pelos debates construtivos, pelas risadas sem fim e alegrias compartilhadas. Deixo em meio agradecimento a certeza de que criamos um elo que certamente, queira você ou não, se propagará por toda vida.

Agradeço a Milena Nunes Rodrigues, a pequena criança que possivelmente fará parte do seleto grupo de pessoas que jamais chegará a ler isso, mas a quem devo mais do que um dia poderia pagar. Obrigado pelos sorrisos que sempre trouxeram luz e alegria aos momentos mais tenebrosos. Obrigado por ser sempre a criança doce que desde antes de nos vermos pela primeira vez me dá razão para viver e evoluir, buscando ser melhor a cada dia, para quem sabe em algum momento, retribuir ao menos em parte a felicidade que é tê-la em minha vida.

Agradeço a Priscilla Fernanda dos Santos, pelo companheirismo, por mais conturbado que seja. Obrigado, não apenas por estar disposta a estar do meu lado mesmo nas horas ruins, mas por sempre me ajudar a sair delas. Obrigado por me ensinar a acreditar que as coisas podem se acertar e que as fases ruins e complicadas passam. Espero que de algum modo, eu possa retribuir tudo que faz

por mim, e ser, por muito tempo, tão importante para você quanto você tem sido para mim nos últimos anos.

Agradeço a Danielly Correa, por além de me tornar um fumante passivo e possivelmente diminuir minha expectativa de vida, ser uma companheira de bancada e de vida. Obrigado, Dany pelo sangue frio e absoluta ausência de coração durante os experimentos, e mais do que isso, pelo imenso coração que sempre demonstrou fora deles. Obrigado pela paciência de escutar, pelos conselhos, quase sempre sábios, pelas histórias engraçadas e risadas subsequentes. Mais do que isso, obrigado por me alimentar tantas vezes nos últimos anos. Seu arroz a piamontese sempre terá espaço no meu estômago e coração.

Agradeço a Rafaela Silva Pessanha da Veiga, por não surtar com meu inesgotável repertório de piadas ruins e por sempre ter disposição o bastante para ajudar com tudo, independente do quanto fosse reclamar ao fazê-lo. Obrigado Rafa, de verdade, por ser a pessoa que é, tenha certeza de que muito de todo esse trabalho passa também por você. Obrigado por ter se tornado uma amiga tão importante nestes quase 10 anos de convivência.

Não poderia deixar de modo algum de agradecer a Isabela Ferreira Soares. Obrigado Isa, não só por ter sido uma IC boa, facilitado e muito minha árdua tarefa de tentar te ensinar algo, mas também por ter evoluído tanto em tão pouco tempo. Espero que tenha conseguido lhe passar algo além do fardo de ser a menina prodígio do grupo, em quem grandes esperanças estão agora depositadas. Esteja ciente, que junto a esse pesado fardo, deixo também minhas costas, pernas e braços para ajudá-la a carregar e construir tudo que aquilo que vislumbrar.

Agradeço ainda, aos amigos que estiveram do meu lado no LIP ao longo de muito tempo. Obrigado, Virgínia Araújo, pela doçura inesgotável e pelo “inesquecível” bombom de cereja que por toda vida perturbará meu paladar. Obrigado Juanito (Juan Camilo) por me ensinar tanta coisa e pela inesgotável disposição em ajudar. Obrigado também a Alinne Renzetti, pelas várias risadas, pelo compartilhamento de aflições e por tantas vezes tentar ajudar.

Obviamente, a lista de amigos não termina aqui. Mais óbvio que isso é a certeza que acabarei não mencionando muitos nomes. Por conta disso, agradeço ainda a todos os amigos que me acompanharam de perto ao longo dos anos de

mestrado e doutorado, servindo de pilar não apenas nos estudos mas também em pequenas caminhadas “embriagado”. Obrigado Maria Augusta Dario, não apenas pelo formidável whisky, mas pelo companheirismo, pelas conversas sempre divertidas, pelas inesgotáveis ajudas e risadas, que nos acompanham desde que nos conhecemos. Esteja ciente que aqui, em “vics”, em “Glaskow”, ou onde quer que seja, sempre poderá contar comigo para o que precisar. Obrigado ainda, ao Edson, pelas perturbadoras indicações de filmes, pelos papos cabeça, quase sempre acompanhados de cerveja e pela amizade. Saiba que mesmo eu não sendo lá muito útil, sempre estarei pronto para ajuda-lo com o que precisar. Obrigado a Joana, pelas conversas divertidas, pela paciência, por também cooperar com a diminuição de minha expectativa de vida em função do fumo passivo, e mais que tudo, por me ensinar tanto. Obrigado, Diogo Gama, pelos memes infinitos e cada vez melhores, Oscar, Larissa, Kate e todos que de algum modo ajudaram nessa conturbada caminhada.

Obrigado especial ainda a Bruno da Cunha Cabral, por ter se tornado um amigo tão valioso. Obrigado mesmo por todo companheirismo que nos acompanha já há tantos anos. Agradeço por tabela a sua mãe, pai, irmã e todos, que me abraçaram como parte da família e tem sempre estado ao meu lado.

Agradeço também as novas integrantes do “grupo Josué”, Ada Matos e Lana Bittencourt, pela ajuda em experimentos, risadas e conversas. Em especial, obrigado, Ada pelo café, sempre revigorante.

Obrigado a Dra. Dalma Maria Banic, por ter aberto as portas da Fiocruz para mim há muitos anos atrás e por tudo que, direta ou indiretamente, fez por mim desde então. Esteja certa que se de algum modo eu puder retribuir e ajudar, estarei sempre disposto a fazê-lo.

Obrigado a Doutora Joseli Oliveira Ferreira, carinhosamente Lila, por ter aberto espaço para mim há muito tempo atrás. Obrigado por todo aprendizado, toda ajuda e todo lado humano que sempre lhe acompanhou durante muito tempo. Espero ter de algum modo ajudado e me ponho a disposição para continuar ajudando, pois certamente sempre estarei em débito por tudo que um dia fez por mim.

Obrigado à Dra. Paula Mello de Luca, não apenas pelo conturbado trabalho com a citometria, mas também por toda ajuda e conselhos de vida.

Obrigado a Dra. Andrea Ponce, bem como todos os envolvidos nas plataformas de citometria por se empenharem com uma das etapas mais complexas deste projeto.

Propositalmente deixados ao final, obrigado à minha família, Zilda Nunes, minha mãe, Diego, meu irmão, os quais independente de distância ou qualquer outro obstáculo, sempre estiveram ao meu lado, me apoiando e oferecendo o suporte necessário para que aqui pudesse chegar. Obrigado ainda a meu pai, Jorge, que mesmo não estando mais conosco, deixou um legado de aprendizado que certamente me ajudou a chegar mais longe.

Por fim, mas não menos importante, agradeço a todos os voluntários envolvidos neste estudo, que gentilmente aceitaram participar do projeto. Sem eles nada seria possível. Espero que de algum modo, todos os esforços aqui empreendidos um dia retornem a vocês, melhorando suas vidas, sendo essa a única e melhor retribuição que posso lhes oferecer.

“Quando penso que já vi de tudo, sou lembrado de quão pouco sei de verdade”
(Paul Jenkins)

INSTITUTO OSWALDO CRUZ

IDENTIFICAÇÃO E AVALIAÇÃO DA IMUNOGENICIDADE DE EPÍTOPOS DE CÉLULAS B DE PROTEÍNAS DE ESPOROZOÍTOS (PVCELTOS) E MEROZOÍTOS (PVMSP-9) DE *PLASMODIUM VIVAX*

RESUMO
TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Rodrigo Nunes Rodrigues da Silva

Dentre as espécies causadoras de malária humana, o *Plasmodium vivax* se destaca como a espécie mais dispersa geograficamente no mundo, resultando em um significativo impacto socioeconômico. Todavia, a falta de um sistema in vitro para o cultivo contínuo, aliado ao equívoco generalizado de que a malária causada pelo *P. vivax* é branda, têm tornado o desenvolvimento de vacinas candidatas contra *P. vivax* uma tarefa ainda mais complexa. Por outro lado, a vacinologia reversa surge como uma alternativa promissora, pois através de diferentes ferramentas de bioinformática, é possível identificar potenciais candidatos vacinais com menor custo, tempo e sem a necessidade de uma grande estrutura laboratorial. Desse modo, a determinação de regiões imunogênicas e específicas dentro de candidatas vacinais já estabelecidas também pode ser utilizada na geração de antígenos quiméricos, contendo múltiplos epítopos contra diferentes alvos, levando a um avanço no desenvolvimento de vacinas eficazes. Baseados nesse conceito, nos propomos a identificar *in silico* epítopos de célula B em proteínas candidatas vacinas de esporozoítos (PvCelTOS) e merozoítos (PvMSP-9) de *P. vivax*, confirmar sua imunogenicidade natural em populações expostas a malária e avaliar sua antigenicidade e imunogenicidade em modelos animais. **(Artigo 1)** Primeiramente em relação a proteína PvCelTOS, utilizando a combinação de 3 algoritmos de predição, identificamos 4 potenciais epítopos lineares de célula B nesta proteína. Para confirmar os potenciais epítopos e validar a estratégia utilizada utilizamos o plasma de 528 indivíduos naturalmente expostos a malária. Inicialmente observamos que 17% destes indivíduos apresentavam anticorpos IgG contra a PvCelTOS recombinante com prevalência de anticorpos IgG1. Adicionalmente, utilizando o plasma dos indivíduos respondedores frente a um peptide array composto de 32 peptídeos representando toda a PvCelTOS, confirmamos a presença de 50% dos epítopos indicados pela predição. Estes dados confirmam o êxito de nossa estratégia e corroboram o uso da vacinologia reversa para identificação de epítopos lineares em malária. **(Artigo 2)** Baseados no estudo anterior, realizamos a análise *in silico* da PvMSP9, uma candidata vacinal de estágio eritrocítico, identificando a sequência EAAPENAEPVHENA ($PvMSP9_{E795\text{-}A808}$) como um potencial epítopo célula B, que se repete 5 vezes ininterruptas em tandem e corresponde a 29% dos blocos de repetição da proteína, a região mais imunogênica da PvMSP9. A imunogenicidade deste epítopo foi confirmada, pois 56% dos respondedores para a proteína recombinante representando os blocos de repetição da PvMSP9, reconheciam o peptídeo $PvMSP9_{E795\text{-}A808}$. Este epítopo, foi considerado um bom alvo vacinal, pois a reatividade de anticorpos específicos contra o peptídeo $PvMSP9_{E795\text{-}A808}$ foi diretamente correlacionada com o tempo passado desde o último episódio de malária e inversamente associado com o número de episódios recentes da doença. **(Artigo 3)** Por fim, visando avaliar a imunogenicidade do epítopo $PvMSP9_{E795\text{-}A808}$ em modelos animais, duas repetições desta sequência foram sintetizadas como peptídeos lineares em sua forma simples (RII) e conjugado a epítopos T-helper descritos na PvMSP9 (pLRII) e na toxina-tetânica (TTRII); emulsificados em Montanide ISA-51 e utilizados na imunização de camundongos BALB/c. Todos os peptídeos induziram anticorpos específicos contra o epítopo $PvMSP9_{(E795\text{-}V808)2}$, confirmando sua imunogenicidade. Todavia, os peptídeos conjugados pLRII e TTRII, contendo epítopos T-helper fusionados a sequência RII, induziram maiores títulos de anticorpos que o peptídeo simples, sendo este o primeiro trabalho evidenciando potencial do epítopo pL como epítopo T-helper em camundongos. Considerando que anticorpos anti-PvMSP9 se mostraram capazes de inibir a invasão de merozoiítas *in vitro* em estudos anteriores, nossos dados corroboram um papel protetor dos anticorpos anti-$PvMSP9_{(E795\text{-}A808)2}$, uma vez que estes reconheceram tanto a proteína recombinante representando os blocos de repetição da PvMSP9, quanto a proteína nativa do parasito. Acerca da resposta celular, apenas o peptídeo TTRII foi capaz de induzir produção de IFN-γ, em níveis comparáveis aos induzidos pela PvMSP9 recombinante. Concluindo, nosso estudo reforça o uso da vacinologia reversa como uma alternativa viável para identificar e explorar candidatos vacinais contra o *P. vivax*, caracterizando seus epítopos e permitindo a elaboração de novas e melhores construções vacinais, baseadas em multi-epítopos.

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

IDENTIFICATION AND EVALUATION OF IMMUNOGENICITY OF B-CELL EPITOPES OF *PLASMODIUM VIVAX* MEROZOITES AND SPOROZOITES

ABSTRACT
PHD THESIS IN PARASITIC BIOLOGY
Rodrigo Nunes Rodrigues da Silva

Among the species that cause human malaria, *Plasmodium vivax* is the most geographically dispersed species in the world, resulting in a significant socioeconomic impact. However, the lack of an *in vitro* system for continuous culture, associated with the widespread misconception that *P. vivax* malaria is a mild disease, has made the development of candidate vaccines against *P. vivax* an even more complex task. On the other hand, reverse vaccinology emerge as a promising alternative, because through different bioinformatics tools, it is possible to identify potential vaccine candidates with low cost, time and without the need of a large laboratory structure. Thus, the determination of immunogenic and specific regions within already established vaccine candidates can also be used in the generation of chimeric antigens, containing multiple epitopes against different targets, leading to an advance in the development of effective vaccines. Based on this concept, we propose to identify *in silico* B-cell epitopes in sporozoite (PvCelTOS) and merozoite (PvMSP-9) vaccine candidates against *P. vivax*, confirm their natural immunogenicity in populations exposed to malaria, and assess their antigenicity and immunogenicity in animal models. **Article 1:** First, in relation to PvCelTOS protein, using the combination of three prediction algorithms, we identified 4 potential linear B cell epitopes in this protein. To confirm the potential epitopes and validate the strategy used we used the plasma of 528 individuals naturally exposed to malaria. Initially, we observed that 17% of these individuals had IgG antibodies against recombinant PvCelTOS with prevalence of IgG1 antibodies. Additionally, using the plasma of responders against a peptide array composed of 32 peptides representing the entire PvCelTOS, we confirmed the presence of 50% of the epitopes indicated by the prediction. These data confirm the success of our strategy and corroborate the use of reverse vaccination for the identification of linear epitopes in malaria. **Article 2:** Based on the previous study, we performed the *in silico* analysis of PvMSP9, an erythrocytic stage vaccine candidate, identifying the sequence EAAPENAEPVHENA (PVMSP9$_{E795-A808}$) as a potential B-cell epitope, which is presented as 5 uninterrupted tandem repeats corresponds to 29% of the repetitive region, the most immunogenic region of PvMSP9. The immunogenicity of this epitope was confirmed by ELISA, as 56% of the responders to the recombinant protein representing the replicate blocks of PvMSP9 recognized the peptide PVMSP9$_{E795-A808}$. This epitope was considered a good vaccine target because the reactivity of specific antibodies against the peptide PvMSP9E795-A808 was directly correlated with the time since the last malaria episode and inversely associated with the number of recent episodes of the disease. **Article 3**: In order to evaluate the immunogenicity of the PVMSP9$_{E795-A808}$ epitope in animal models, two replicates of this sequence were synthesized as linear peptides in their simple form (RII) and conjugated to T-helper epitopes described in PvMSP9 (pLRII) and in tetanus toxin (TTRII); emulsified in Montanide ISA-51 and used in the immunization of BALB / c mice. All peptides induced specific antibodies against the epitope PvMSP9 (E795-V808)2, confirming its immunogenicity. However, the pLRII and TTRII conjugated peptides, containing T-helper epitopes fused to the RII sequence, induced higher antibody titers than the simple peptide, being this work the first evidence of the potential of pL epitope as T-helper epitope in mice. Considering that anti-PvMSP9 antibodies were shown to inhibit merozoite invasion in vitro in previous studies, our data corroborate a protective role of anti-PvMSP9 (E795-A808) antibodies, since they recognized both the recombinant protein representing the PvMSP9 repeat blocks and the parasite's native protein in Immunofluorescence. Regarding the cellular response, only the TTRII peptide was able to induce IFN-γ production, at levels comparable to those induced by recombinant PvMSP9. In conclusion, our study reinforces the use of reverse vaccinology as a viable alternative to identify and explore vaccine candidates against *P. vivax*, characterizing its epitopes and allowing the development of new and/or better multi-epitope-based vaccine constructs.

ÍNDICE

ÍNDICE DE FIGURAS

LISTA DE SIGLAS

ADCC	Citotoxicidade celular dependente de anticorpos
ADCI	Inibição celular dependente de anticorpos
AMA-1	Apical Membrane Antigen 1
CCR7	Receptor de quimiocina do tipo 7
CD19	Cluster Differentiation 19
CD27	Cluster Differentiation 27
CD 3	Cluster Differentiation 3
CD4	Cluster Differentiation 4
CD44	Cluster Differentiation 44
CD8	Cluster Differentiation 8
CelTOS	Cell-Traversal Protein for Ookinetes and Sporozoites
CSP	Circumsporozoite surface protein
CTL	Linfócito T citotóxico
CXCL	Ligante de Quimiocina
ELISA	Enzyme Linked ImmunonoSorbent Assay
HLA	Human leukocite Antigen (Antígeno leucocitário humano)
IFN-γ	Interferon gamma (Interferon gama)
IgD	Imunoglobulina D
IgG	Imunoglobulina G
IgG1	Imunoglobulina G 1
IgG2	Imunoglobulina G 2
IgG3	Imunoglobulina G 3
IgG4	Imunoglobulina G 4
Irf	Interferon regulatory factor
Mavs	Mitochondrial antiviral signaling protein
Mda5	Melanoma differentiation-associated gene 5
MyD88	Myeloid differentiation factor 88
MHC	Major Histocompatibility Complex
MSP-9	Merozoíte Surface Protein 9
NK	Célula Natural killer
PAHO	Pan American Health Organization
PBS	Solução salina tamponada
PvDBP	*P. vivax* Duffy Binding protein

RTS,S/AS01	Candidato vacinal (Sequências da proteína Circumsporozoíta e do vírus da hepatite B + o adjuvante AS01)
SFC	Spots forming cells
SIVEP	Sistema de Vigilância Epidemiológica
SVS	Sistema de Vigilância em Saúde
TRIF	“TIR-domain-containing adapter-inducing interferon-β”
TLR	Receptor do tipo Toll
WHO	World Health Organization

INTRODUÇÃO

1.1 Malária: um breve histórico

Também conhecida como impaludismo, febre palustre, febre intermitente, febre terçã benigna, febre terçã maligna, febre quartã, febre palúdica, maleita, sezão, tremedeira, batedeira ou mãe das febres, o termo malária provém do século XVIII, em virtude do nome italiano de "mal aire", que significa mau ar ou ar insalubre, já que à época acreditava-se que era causada pelas emanações e miasmas provenientes dos pântanos. Identificada em escritos chineses, datados de 3000 a.C., a doença é considerada em diversas referências a febres sazonais e intermitentes em textos religiosos e médicos antigos, entre os assírios, chineses e indianos, que a relacionavam à punição de deuses e presença de maus espíritos. No século V a.C., na Grécia, Hipócrates realizou uma detalhada descrição da doença, relacionando-a às estações do ano ou aos locais frequentados pelos doentes, além de descrever o quadro clínico da malária e algumas de suas complicações (Hippocrates 1985). Posteriormente, no século II d.C., diversos médicos gregos e romanos deixaram várias referências sobre a doença, conhecida como "Febre Romana", que ocorria em epidemias cíclicas na Grécia, Itália e diversas partes da Europa (Ruppert& Barnes 1996; Ruppert et al. 2005).

Recentemente, análises imunológicas e genéticas confirmaram a antiguidade das infecções maláricas, evidenciando sua presença em múmias egípcias (Bianucci et al. 2008; M. et al. 2000; Miller et al. 1994; Nerlich et al. 2008), além de casos na Roma antiga (Fornaciari et al. 2010a; Frias et al. 2013) e Europa renascentista (Fornaciari et al. 2010b). Entretanto, apesar de todo impacto histórico da malária na história da humanidade, somente em 1880, Charles Alphonse Laveran, médico do exército francês, a associou a parasitos observados no interior de hemácias humanas (Tan& Ahana 2009). Em 1897, Ronald Ross elucidou o ciclo completo do parasito em mosquitos culicíneos e aves infectadas por *Plasmodium relictum*. A partir deste estudo, em 1898, os malariologistas italianos Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava demonstraram conclusivamente que a malária humana também era transmitida por mosquitos, neste caso do gênero *Anopheles* (Cox 2010).

Atualmente, sabe-se que a malária é uma doença infectocontagiosa de transmissão vetorial, que apresenta elevada prevalência, letalidade e morbidade, causada por parasitos do gênero *Plasmodium*, o qual comporta cerca de 120 espécies capazes de infectar naturalmente aves, répteis e mamíferos, dentre os quais morcegos, roedores, humanos e primatas não-humanos (Rey 1991).

Neste contexto, sete espécies plasmodiais representam ameaça para a saúde dos seres humanos: *P. falciparum*, associada a elevada mortalidade e maioria dos casos graves da doença; *P. vivax*, espécie mais dispersa no mundo, para qual acredita-se que número de mortes seja subestimado (Naing et al. 2014; Rahimi et al. 2014); *P. malariae*, endêmico na África tropical, sudeste asiático, e Américas do Sul e Central, é capaz de infectar primatas não humanos e apresenta formas sanguíneas que podem permanecer por longos períodos no corpo do hospedeiro, gerando quadros de recrudescência (Collins& Jeffery 2007; Collins et al. 1989); *P. ovale curtisi* e *P. ovale wallikeri*, duas espécies não-recombinantes simpátricas na África e na Ásia (Sutherland et al. 2010), que compartilham além de semelhanças morfológicas, a habilidade de formar hipnozoítas (formas latentes que permanecem no fígado e levam a casos de recaída, longos períodos após a infecção inicial) (Chin& Coatney 1971; Marty et al. 1987), também observada no *P. vivax*. Adicionalmente, há ainda espécies plasmodiais vinculadas a casos de zoonoses, que agravam ainda mais o risco epidemiológico da doença. Dentre as espécies simianas, *P. knowlesi* é considerado uma local, mas importante, causa de malária (incluindo casos graves) na Malásia e outras áreas do sudeste asiático, onde é predominantemente uma zoonose, sem evidências de transmissão primária de homem para homem (Ahmed& Cox-Singh 2015). Adicionalmente, Patrícia Brasil e colaboradores demonstraram recentemente a transmissão zoonótica do *P. simium*, 50 anos após o único relato prévio desta espécie em humanos, sugerindo que esta espécie também possa ter sido responsável por casos de malária no estado Rio de Janeiro e regiões de mata Atlântica brasileira, tendo sido ao longo do tempo diagnosticado como *P. vivax*, devido à falta de um diagnóstico molecular adequado (Brasil et al. 2017). Além destas duas espécies, das 25 espécies plasmodiais que infectam primatas não humanos, outras 8 são descritas como capazes de infectar experimentalmente o homem (Collins 1974).

Por fim, os vetores do *Plasmodium* são as fêmeas de mosquitos do gênero *Anopheles*, que agrupa cerca de 400 espécies, das quais cerca de 60 são descritas como transmissoras naturais da malária (Tuteja 2007), sendo *Anopheles gambiae* e

A. darlingi considerados os principais vetores no mundo e no Brasil, respectivamente (Blandin et al. 2004; Mirabello& Conn 2006).

1.2 Malária no mundo

Segundo a organização mundial da saúde (OMS) aproximadamente metade da população mundial se encontra em risco de adquirir malária, uma vez que 91 países ainda apresentam transmissão contínua da doença (Figura 1). A despeito dos investimentos globais no controle e eliminação da malária, que geraram uma redução de 21% no número de casos entre 2000 e 2015, a doença persiste como um dos maiores problemas de saúde pública no mundo. Em 2015, foram estimados cerca de 212 milhões de casos de malária no mundo, resultando em mais de 429 mil mortes, especialmente na África subsaariana, onde crianças menores de 5 anos representam o maior percentual de óbitos (W.H.O 2015).

Acerca da distribuição global dos casos de malária, o continente africano comportou cerca de 88% dos casos mundiais de malária em 2015 e aproximadamente 90% das mortes associadas à doença no mesmo ano. Fora do continente africano, o sudeste asiático e a região do mediterrâneo foram as mais afetadas pela doença, correspondendo a 9% e 1,3% dos casos, respectivamente (W.H.O 2015).

Dentre as espécies causadoras de malária no ser humano duas se destacam: *P. falciparum*, responsável por 90% dos casos no continente africano, onde é relacionado a maioria dos casos graves/letais de malária, e *P. vivax*, espécie geograficamente mais dispersa no mundo (Mueller et al. 2009), responsável mais de 70% dos casos de malária das Américas e Ásia (W.H.O 2015). Esta espécie ocasiona quadros clínicos que, embora apresentem baixa letalidade em comparação ao *P. falciparum*, podem ser debilitantes e resultar em graves impactos econômicos e de saúde sobre as populações afetadas (Adams& Mueller 2017).

Nas Américas, apesar da redução no número de casos na última década, 21 países ainda são endêmicos para malária, estando a maioria dos casos concentrados na região amazônica. Em 2015, dos 660 mil casos registrados, 83% se distribuíram entre 4 países: Venezuela (30%), Brasil (24%), Peru (19%) e Colômbia (10%) (PAHO 2016; W.H.O. 2016). Dentre as espécies causadoras de malária, o *P. vivax* é a espécie prevalente nas Américas, correspondendo a 72% dos

casos em 2015, seguido por casos de *P. falciparum*, espécie prevalente no Haiti e República Dominicana, e *P. malariae*, que corresponde a cerca de 0,1% dos casos (W.H.O. 2016).

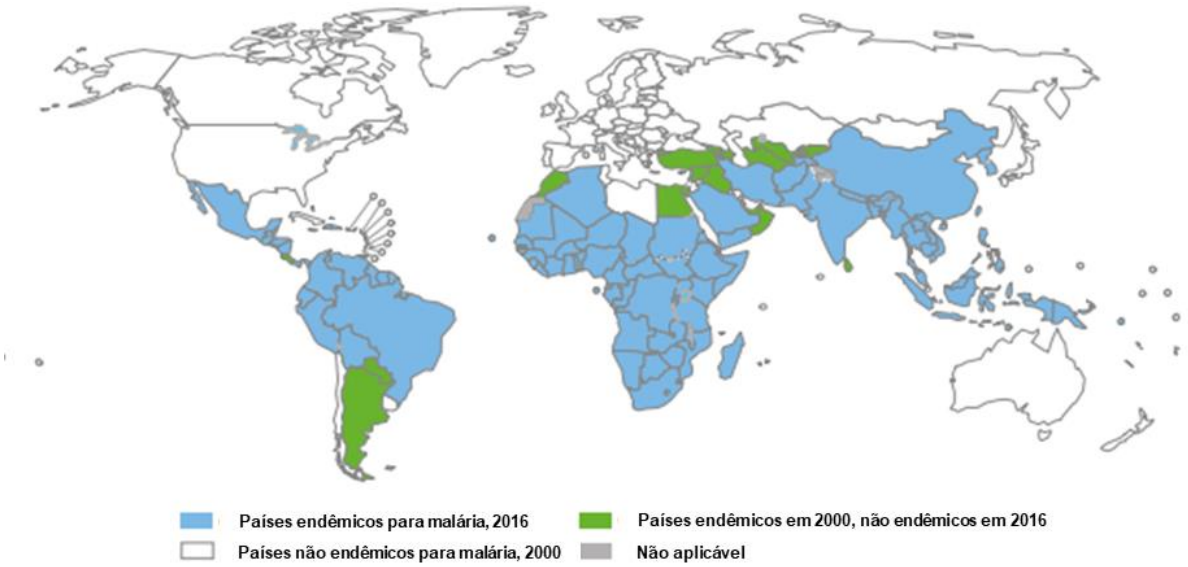


Figura 1: Países endêmicos para malária em 2000 e 2016, W.H.O. (2016)

1.3 Malária no Brasil

No Brasil, a transmissão de malária é classificada como instável e de baixa ou média intensidade (Recht et al. 2017). Nos últimos anos, em virtude dos investimentos e políticas de controle, o número de casos registrados no país tem diminuído drasticamente (Figura 2). Enquanto em 2005, o país registrou mais de 600 mil casos de malária, os dados mais recentes indicam o registro de 128.745 casos de malária em 2016, sendo *P. vivax* a espécie prevalente, correspondendo a 88% dos casos, seguido por *P. falciparum* (11,2% dos casos) e *P. malariae* (menos de 0,1% dos casos) (SIVEP 2017).

Embora o Brasil seja um país de dimensões continentais, a malária se concentra quase exclusivamente na região da Amazônia legal, composta pelos estados do Acre, Amazonas, Amapá, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins (Oliveira-Ferreira et al. 2010), onde apresenta uma distribuição heterogênea (Figura 3). Em 2013, entre os 808 municípios amazônicos, 37 municípios comunicaram 80,37% dos casos, sendo que 21,48% do total de casos na

Amazônia se concentraram em 3 municípios [Cruzeiro do Sul (AC), Porto Velho (RO), Itaituba (PA)] (de Pina-Costa et al. 2014).

Por outro lado, cabe ressaltar, que no Brasil, 3 perfis de transmissão de malária podem ser observados: (1) casos autóctones, que ocorrem no interior da região amazônica e correspondem a grande maioria de casos no país; (2) casos importados, adquiridos fora do país ou da região onde a pessoa vive. Estes casos, muitas vezes em virtude da demora na identificação da doença, podem gerar quadros graves e óbitos, e em 2016 representaram aproximadamente 4% do total casos de malária notificados no país, sendo a maioria dos casos notificados fora da região amazônica. (3) Casos autóctones de malária reportados em áreas de mata Atlântica. Estes casos correspondem a apenas 0.05% do total de casos no país, mas possuem grande importância por poderem representar casos zoonóticos da doença, pela transmissão de *P. simium* e transmitidos por *Anopheles (Kerteszia) cruzii* (Brasil et al. 2017; de Pina-Costa et al. 2014).

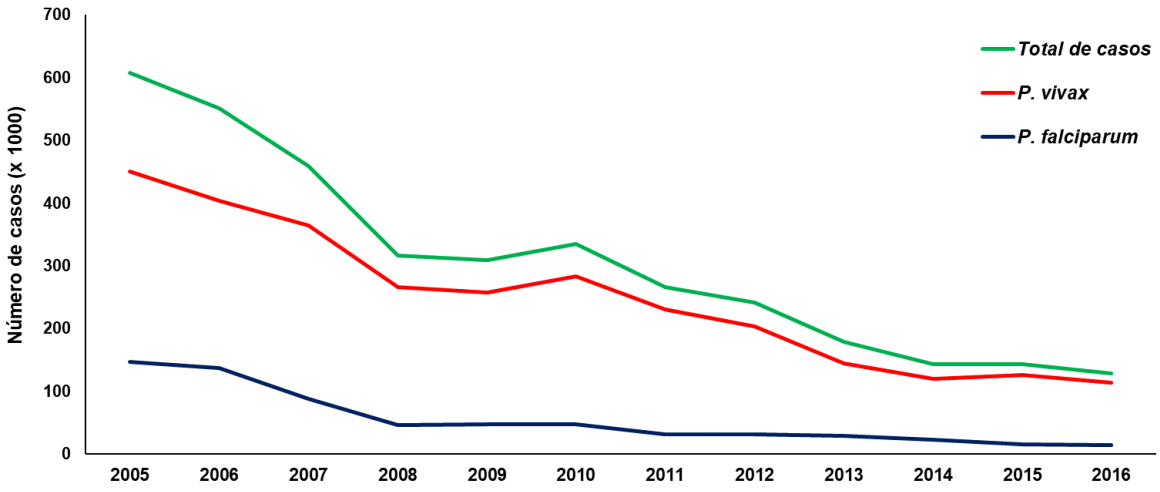


Figura 2: Série histórica do número de casos no Brasil, 2005 a 2016
Fonte: (SIVEP 2017)

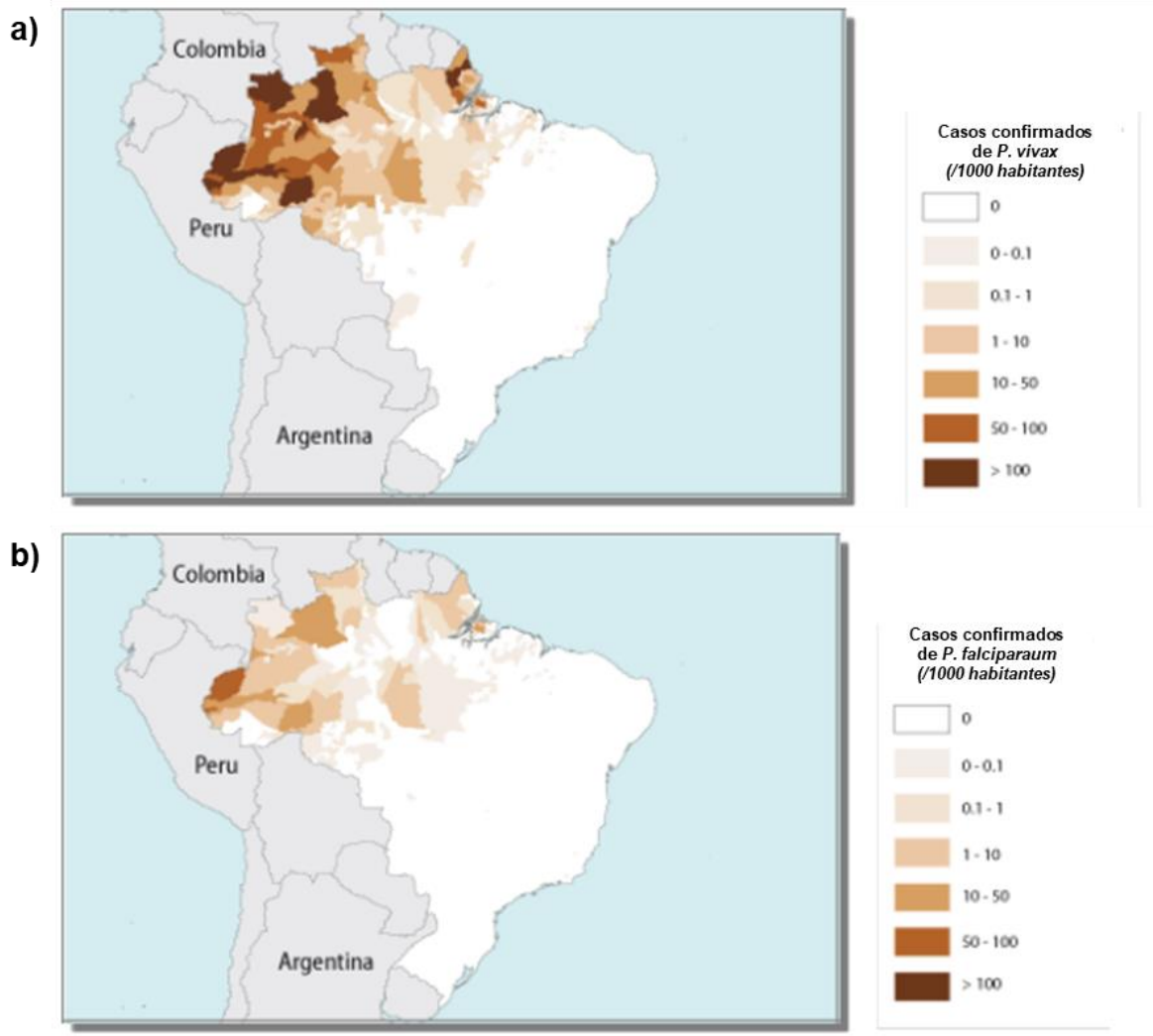


Figura 3: Distribuição de casos confirmados de malária por (a) *P. vivax* e por (b) *P. falciparum*
Fonte: (W.H.O 2015)

1.4 Ciclo biológico do *Plasmodium* sp.: aspectos gerais

O ciclo dos plasmódios é complexo, apresentando duas fases no hospedeiro vertebrado, fases exo-eritrocítica e eritrocítica, além de uma fase sexuada (esporogônica) no hospedeiro invertebrado (Figura 4).

No hospedeiro vertebrado a infecção se inicia quando, durante o repasto sanguíneo, fêmeas anofelinas infectadas inoculam esporozoítas (formas infectantes do parasito) na pele do hospedeiro, onde podem permanecer por horas até encontrarem vasos sanguíneos ou serem drenados por vasos linfáticos (Amino et al. 2006a; Gueirard et al. 2010).

Os esporozoítos que atingem a circulação sanguínea são transportados passivamente até o fígado, dando início ao estágio hepático ou exo-eritrocítico, no

qual se aderem as células endoteliais dos sinusóides hepáticos e iniciam um processo de movimentação denominado de *gliding*, “deslizando” até atravessarem a barreira sinusoidal. Uma vez no parênquima hepático, estes parasitos migram ativamente, atravessando o citosol de vários hepatócitos, até se aderirem a um e iniciar a formação do vacúolo parasitóforo. Os fatores que tornam um hepatócito adequado para o desenvolvimento do esporozoíta ainda não estão esclarecidos (Holz et al. 2016; Silvie et al. 2008), no entanto, a intensa travessia por diversos hepatócitos parece ser um processo essencial do ciclo de vida do *Plasmodium*, obrigatório para torna-los capazes de estabelecer a infecção nesta célula-alvo (Mota et al. 2002).

Dentro do vacúolo parasitóforo, os esporozoítas se convertem em trofozoítas hepáticos e iniciam um processo de maturação e divisão celular (esquizogonia extra-eritrocítica), resultando no desenvolvimento de um sincício multinucleado, conhecido como esquizonte hepático, repleto de merozoítas em seu interior (Lindner et al. 2012). Durante esta etapa do ciclo a infecção é assintomática (Silvie et al. 2008; Stevenson& Riley 2004). A duração deste estágio varia de acordo com a espécie plasmodial, durando de 6 a 8 dias em infecções por *P. vivax*, de 5 a 7 dias em infecções por *P. falciparum* e de 14 a 16 dias em infecções por *P. malariae* (McKenzie et al. 2002). Após a maturação destes esquizontes, milhares de merozoítas são liberados na corrente sanguínea através de merossomas ou pela ruptura dos hepatócitos infectados, iniciando o estágio eritrocítico da infecção (Sturm et al. 2006).

Uma vez na corrente sanguínea, os merozoítas invadem de forma ativa eritrócitos, se diferenciando em trofozoítos jovens (forma de anel) e posteriormente em trofozoítos maduros, os quais iniciam um estágio de reprodução assexuada formando esquizontes sanguíneos, repletos de merozoítas. No final desse estágio os merozoítas recém-formados são liberados e invadem outros eritrócitos, dando continuidade ao ciclo eritrocítico, resultando nos sintomas clínicos da doença. Após algumas gerações de merozoítos sanguíneos, uma pequena parte destes parasitos se diferencia em estágios sexuados, os gametócitos femininos e masculinos, que, ao serem ingeridos pelo mosquito do gênero *Anopheles*, durante o repasto sanguíneo, dão início à fase sexuada do ciclo.

Por fim, no intestino médio do anofelino vetor, pelo processo de exoflagelação, os gametócitos se diferenciam em gametas, ocorre a fecundação e formação do zigoto. Este se diferencia no oocineto, uma forma móvel e invasiva, que atravessa a

membrana peritrófica e o epitélio do intestino médio, se alojando na lâmina basal do intestino médio (Baton& Ranford-Cartwright 2005b; Vlachou et al. 2006), onde perde a motilidade e adquiri uma nova forma, denominada oocisto. Neste estágio, inicia-se uma intensa multiplicação assexuada (esporogonia), levando a formação de milhares de esporozoítos (Baton& Ranford-Cartwright 2005b), os quais, após o rompimento do oocisto maduro, são liberados na hemolinfa do inseto, podendo migrar ativamente até as glândulas salivares do inseto, completando o ciclo evolutivo do *Plasmodium*, podendo ser liberados na pele de um novo hospedeiro vertebrado durante o repasto sanguíneo (Angrisano et al. 2012).

Curiosamente, estudos recentes utilizando roedores demonstraram que a maioria dos esporozoítas é injetada na derme, permanecendo no local por mais de 6 horas, com alguma migração para os vasos linfáticos proximais e linfonodos drenantes cutâneos, ondem podem sobreviver por pelo menos 24 horas após a infecção. Nos linfonodos, a maioria dos esporozoítas é degradada, atuando como fontes de antígenos para o sistema imune (Douglas et al. 2015; Vanderberg 2014; Voza et al. 2012; Wilson et al. 2016), mas alguns podem se desenvolver parcialmente em formas exo-eritrocíticas (Amino et al. 2006b). Curiosamente, os esporozoítas podem também se desenvolver em merozoítas na derme, epiderme e nos folículos pilosos, onde podem sobreviver por dias ou semanas até serem degradados (Amino et al. 2006b; Douradinha& Doolan 2011; Menard et al. 2013).

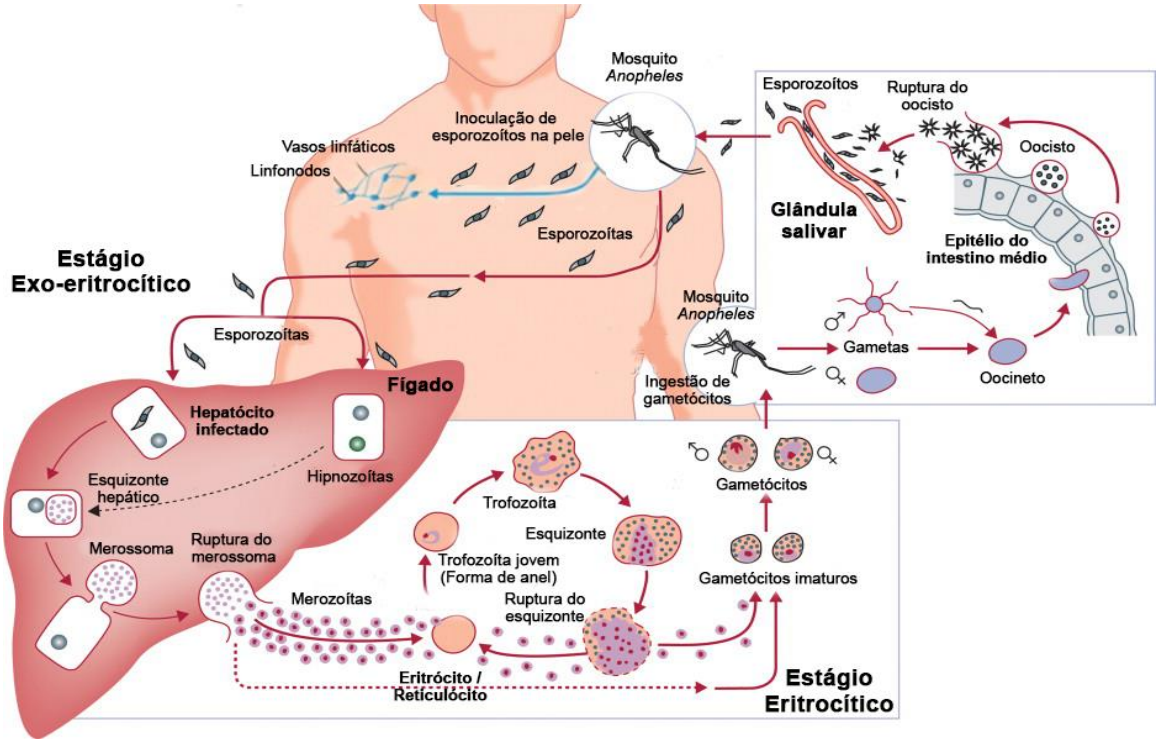


Figura 4: Ciclo biológico do *Plasmodium* sp.
Adaptado de (Garcia-Basteiro et al. 2012)

1.5 Particularidades do *Plasmodium vivax*

Embora o ciclo biológico do gênero *Plasmodium* já seja complexo por si só, uma série de particularidades podem ser observadas quando comparamos a biologia das duas principais espécies causadoras da malária humana, *P. vivax* e *P. falciparum*. Inicialmente, *P. vivax* é capaz de desenvolver formas latentes no fígado, denominadas hipnozoítas, responsáveis por episódios de recaídas, longos períodos após o contato inicial com o parasito, permitindo sua persistência no hospedeiro humano mesmo durante os períodos de reprodução do mosquito, no qual sua transmissão seria baixa (Krotoski 1985, 1989; Richter et al. 2010).

Com relação ao estágio eritrocítico do ciclo do *Plasmodium*, *P. vivax* infecta preferencialmente reticulócitos (eritrócitos jovens), que representam menos de 1% da população de eritrócitos sanguíneos, enquanto *P. falciparum* infecta eritrócitos em todos os estágios de desenvolvimento (Handayani et al. 2009; Mueller et al. 2009; Suwanarusk et al. 2004). Essa diferença na disponibilidade de células em diferentes estágios de maturação dos eritrócitos-alvos pode estar relacionada com a maior parasitemia normalmente observada em infecções por *P. falciparum* (Bassat et al. 2016; Mueller et al. 2009).

Acerca do estágio sexuado do ciclo, na infecção por *P. vivax*, os gametócitos são gerados mais cedo, de 2 a 3 dias após o início do estágio eritrocítico, e com densidades parasitárias mais baixas que nas infecções por *P. falciparum*, quando são gerados cerca de 10 dias após o início do estágio eritrocítico (Bennink et al. 2016; Josling& Llinas 2015; Sinden& Gilles 2002). Além disso, o ciclo no mosquito completa-se mais rápido e em temperaturas mais baixas, nas infecções por *P. vivax*, ampliando a chance de transmissão e permitindo sua existência em uma maior gama de condições climáticas (Bassat et al. 2016; Mueller et al. 2009).

Em virtude de sua tendência a recidivas após a cura da infecção primária, associadas a formação de hipnozoítas, e ao surgimento prévio de gametócitos, muitas vezes antes do início dos quadros sintomáticos e subsequente tratamento, facilitando a dispersão da doença, considera-se o *P. vivax* a espécie causadora de malária mais difícil de se controlar e eliminar. Os fatores que controlam as recaídas e determinam sua periodicidade ainda são desconhecidos. Cabe ainda ressaltar que ocorrem recidivas, ou recaídas, em infecções causadas por *P. ovale* e em várias espécies símias, como *P. cynomolgi*, espécie filogeneticamente próxima de *P. vivax* e por conta disso, utilizado como modelo animal de malária *vivax* (White 2011).

1.6 A resposta imune do hospedeiro na malária

A aquisição de uma imunidade natural na infecção malárica é um processo complexo, lento e extremamente delicado, influenciado pelo perfil de transmissão da infecção nas áreas endêmicas e pela idade do indivíduo (Ladeia-Andrade et al. 2009; Langhorne et al. 2008).

Como mencionado anteriormente, durante seu desenvolvimento o *Plasmodium* infecta diferentes células e sofre uma série de mudanças morfológicas, exibindo em cada estágio do seu desenvolvimento um vasto repertório de antígenos responsáveis pelo estímulo de diferentes respostas imunes estágio-específicas. Ao longo dos diferentes estágios do ciclo do *Plasmodium* diversos mecanismos imunológicos são requeridos em diferentes níveis (Figura 5), desempenhando papéis importantes e determinantes no desfecho da infecção.

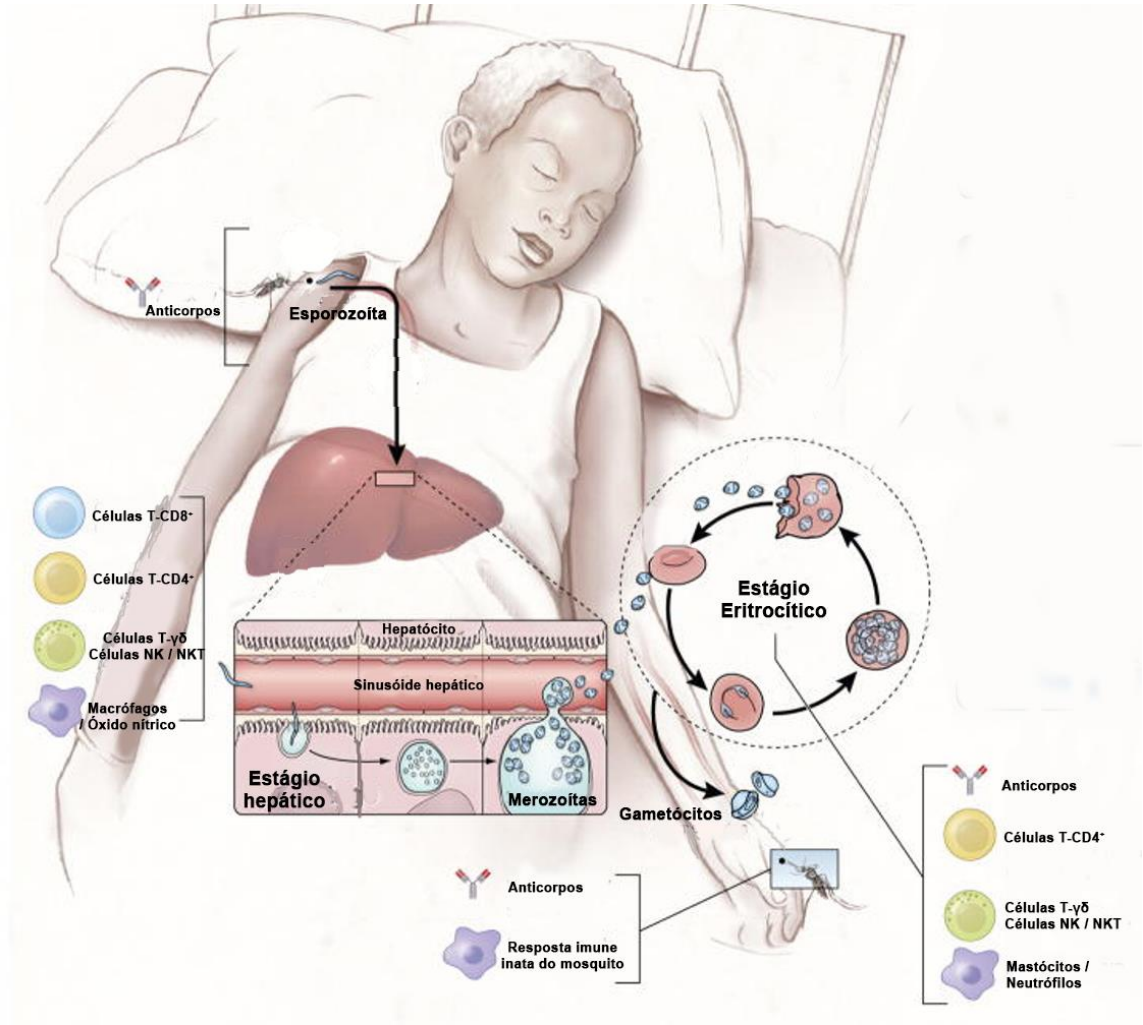


Figura 5: Mecanismos imunes requeridos ao longo do ciclo do *Plasmodium*
Adaptado e traduzido de (Crompton et al. 2014)

1.6.1 Resposta imune contra o estágio pré-eritrocítico do *Plasmodium*

O estágio pré-eritrocítico dura cerca de uma semana em humanos, com variações entre as espécies plasmodiais, e pode ser dividido em duas fases, a fase de esporozoíta, que é curto, podendo durar de minutos a horas, e vai desde sua introdução na pele durante o repasto sanguíneo do anofelino infectado até atingir o fígado via corrente sanguínea e infectar hepatócitos; e a fase hepática, que representa a maior parte do tempo de duração do estágio pré-eritrocítico, culminando na formação e liberação de merossomos repletos de merozoítas (Figura 4) (Holz et al. 2016). Interessantemente, diferentes mecanismos imunes são requeridos durante o estágio pré-eritrocítico quando direcionados à esporozoítas e a formas hepáticas do parasito.

Estudos em áreas endêmicas de malária na África e na Ásia, demonstraram que populações naturalmente expostas à infecção desenvolvem resposta contra o estágio pré-eritrocítico, detectando anticorpos anti-esporozoítas com maior frequência em indivíduos com mais de 50 anos e apenas em uma minoria de crianças menores de 5 anos (Druilhe et al. 1986; Nardin et al. 1979; Tapchaisri et al. 1983). Mais do que isso, o soro de indivíduos de áreas endêmicas, respondedores contra esporozoítas, se mostrou capaz de inibir *in vitro* a invasão de hepatócitos por este estágio parasitário (Hoffman et al. 1986; Hollingdale et al. 1989; Hollingdale et al. 1984; Mellouk et al. 1986). Ainda nesse contexto, Baird e colaboradores (1998), demonstraram que a prevalência e a densidade da parasitemia em indivíduos residentes nas áreas endêmicas de malária diminuem gradativamente ao longo dos anos, enquanto os títulos de anticorpos anti-esporozoítas e a aquisição de imunidade contra a doença aumentam. Esse conjunto de dados corroborou a ideia de que a imunidade contra malária seja desenvolvida lentamente num cenário de infecções contínuas, além de evidenciarem o efeito protetor de anticorpos direcionados contra os esporozoítas.

Sabe-se que os anticorpos anti-esporozoítas podem auxiliar na destruição *in vivo* dos parasitos, facilitando sua fagocitose por macrófagos (Yoshida et al. 1980), reconhecendo antígenos do parasito presentes na superfície dos hepatócitos infectados e mediando o processo de citotoxicidade celular dependente de anticorpos (ADCC), especialmente por células Natural Killers (NK) (Hoffman& Doolan 2000). Tais anticorpos reconhecem um vasto repertório de antígenos, muitos dos quais parecem estar associados ao processo de invasão dos hepatócitos (Gruner et al. 2007). Dentre tais antígenos, a proteína de circumsporozoíta (CSP) foi

o primeiro antígeno identificado em esporozoítas de roedores e humanos (Nussenzweig& Nussenzweig 1989) e atualmente é o alvo da principal formulação vacinal contra malária, a vacina RTS,S (PATH 2017; Vandoolaeghe& Schuerman 2016).

Após infectar hepatócitos, os esporozoítas dão início a fase hepática do estágio pré-eritrocítico, a qual é tradicionalmente vista como silenciosa, em virtude da falta de sintomas clínicos. Apesar disso, dados recentes sugerem que uma complexa série de eventos ocorre para iniciar a resposta imune (Holz et al. 2016). Recentemente, estudos com camundongos infectados com *P. berghei* e *P. yoelii* demonstraram que, diferente de vírus ou bactérias, estes parasitos podem desencadear a produção de IFN tipo I nas células infectadas na ausência de TLR (TLR3 e TLR4) e suas proteínas de sinalização (MyD88 e TRIF) (Liehl et al. 2014). Em vez disso, o *Plasmodium* é detectado nos hepatócitos infectados através da proteína do gene 5 associada a diferenciação do melanoma (Mda5), sinalizado pela proteína de sinalização mitocondrial antiviral (Mavs), que ativa os fatores de transcrição Irf3 e Irf7 e resulta no recrutamento de células linfóides para o fígado. Essas células foram freqüentemente encontradas em torno de hepatócitos infectados, mas não estavam presentes na ausência dessa via indicando que a fase hepática não é inerte (Liehl et al. 2014).

Acerca do papel das células T na imunidade durante o estágio pré-eritrocítico, em 1977, Chen e seus colaboradores já haviam demonstrado que uma resposta imune protetora, independente de anticorpos, é induzida por esporozoítas irradiados em camundongos depletados de linfócitos B (Chen et al. 1977). Diversos estudos tem demonstrado que a resposta imune celular no estágio hepático é mediada principalmente por células T $CD8^+$, induzidas contra antígenos expressos na superfície dos hepatócitos e associados às moléculas do MHC de classe I (Doolan& Hoffman 2000; Good& Doolan 1999; Hoffman& Doolan 2000; Prato et al. 2005; Romero et al. 1992). Tais células poderiam agir de duas maneiras: (i) destruindo os hepatócitos infectados diretamente pela ação dos linfócitos T citotóxicos (CTL) através da liberação de perforinas e granzima B, e (ii) induzindo o processo de apoptose nos hepatócitos infectados, através do *cross-linking* da molécula de Fas ligante (CD95L) na superfície das células $CD8^+$ CTL, com a molécula de Fas (CD95) na membrana plasmática dos hepatócitos (Carvalho et al. 2002a; Crispe 2003).

Adicionalmente, outras populações celulares também parecem desempenhar um importante papel na imunidade celular contra o estágio hepático da doença,

dentre as quais: Células Natural-Killer (NK), que estão aumentadas no fígado de camundongos após a infecção por *P. yoelii*, e podem inibir o desenvolvimento do parasito nos hepatócitos *in vitro* (Roland et al. 2006); células T $CD4^+$, as quais parecem modular a expansão e completo desenvolvimento da resposta T $CD8^+$ (Carvalho et al. 2002a; Overstreet et al. 2011), além de células T- γδ e NKT que também parecem atuar diretamente eliminando hepatócitos infectados (Kuk 2007; Langhorne et al. 2008; Rodrigues et al. 1993; Roland et al. 2006; Tewari et al. 2010; Todryk et al. 2008)

Interessantemente, é mais difícil detectar uma resposta robusta de células T contra antígenos de fase pré-eritrocítica em indivíduos naturalmente expostos a doença em áreas endêmicas do que em voluntários imunizados com esporozoítas irradiados (Seder et al. 2013) ou vacinas de subunidades (Ewer et al. 2013). Este dado sugere que a exposição natural ao parasito pode não ser suficiente para induzir uma robusta resposta de células T. Apesar disso, os promissores resultados de ensaios vacinais e modelos animais, suportam o uso de antígenos de fase pré-eritrocítica no desenvolvimento vacinal contra malária.

1.6.2 Resposta imune contra o estágio eritrocítico assexuado do *Plasmodium*

Os estágios assexuados do ciclo eritrocítico tem sido alvo de numerosos estudos por estarem acessíveis no sangue de pessoas infectadas e serem responsáveis pela patologia associada a doença (Long& Zavala 2017). Uma vez que os eritrócitos não apresentam moléculas de MHC classe I ou classe II, que permitiriam o reconhecimento das células infectadas por células do sistema imune, a imunidade contra o estágio sanguíneo da infecção é amplamente mediada por anticorpos (Beeson et al. 2016).

É sabido que indivíduos que vivem em áreas endêmicas de malária, expostos a altas taxas de transmissão da doença, desenvolvem progressivamente uma imunidade adquirida que leva a tolerância da infecção ou diminuição do risco de formas da grave da doença por *P. falciparum* (Beeson et al. 2008; Langhorne et al. 2008) e por *P. vivax* (Lin et al. 2010; Michon et al. 2007). Estudos utilizando a transferência de soros de aves (Manwell& Goldstein 1940), camundongos (Parashar et al. 1977), primatas não-humanos (Coggeshall& Kumm 1937) e humanos com imunidade naturalmente adquirida (Cohen et al. 1961; McGregor 1964) para animais ou humanos não infectados os protegeu contra malária clínica ou atenuou a

gravidade da malária, corroborando o papel fundamental dos anticorpos na proteção da malária clínica.

Os merozoítas, por serem as únicas formas extracelulares do parasito durante o estágio eritrocítico do ciclo, são os alvos mais acessíveis para a resposta humoral. Nesse contexto, ao longo do estágio eritrocítico, os anticorpos podem atuar: (i) bloqueando proteínas da região apical dos merozoítas, responsáveis pelas interações iniciais com a superfície dos eritrócitos, impedindo-os, assim, de invadirem esses eritrócitos (Hodder et al. 2001; Mitchell et al. 2004); (ii) aglutinando-os no momento da ruptura dos eritrócitos contendo os esquizontes maduros, prevenindo a sua liberação e dispersão na corrente sangüínea (Long 1993); (iii) inibindo eventos bioquímicos, como a liberação de proteases pelo parasito, necessárias ao processo de reconhecimento, invasão e ruptura dos eritrócitos (Blackman 2000; Ersmark et al. 2004); e (iv) opsonizando os merozoítas, através dos anticorpos citofílicos IgG1 e IgG3, facilitando o seu reconhecimento e fagocitose pelos macrófagos, ou mediando o processo de ADCC, levando a remoção e morte desses merozoítas (Carvalho et al. 2002b; Lusingu et al. 2005; Soe et al. 2004).

Além de atuar sobre os merozoítas, os anticorpos podem agir: (i) na neutralização direta das toxinas liberadas pelo parasito após a ruptura dos esquizontes, diminuindo as manifestações clínicas da doença associadas a elas (Arevalo-Herrera& Herrera 2001), (ii) na maturação intraeritrocitária dos trofozoítas e dos esquizontes sanguíneos, através da interiorização desses anticorpos durante o processo de invasão dos eritrócitos pelos merozoítas, ou por difusão através do ducto parasitóforo, atingindo o vacúolo parasitóforo e impedindo o processo de degradação e digestão da hemoglobina pelos parasitos, sua principal fonte de nutrientes (Banerjee et al. 2002; Pouvelle et al. 1994; Pouvelle et al. 1991) e reconhecendo moléculas induzidas pelo parasito na superfície dos eritrócitos infectados (Bull et al. 1998)

Por outro lado, apesar do foco dado à resposta humoral, durante o estágio eritrocítico da infecção malárica, o sistema imune do hospedeiro desenvolve uma potente resposta contra o parasito, causando alterações em quase todos os componentes desse sistema. A ativação e a mobilização de elementos celulares requeridos tanto na resposta imune inespecífica, quanto na específica, resultam de interações complexas entre diferentes mecanismos efetores de imunidade, como a produção de citocinas. Nesse estágio, uma grande quantidade de citocinas é liberada na circulação sanguínea, contribuindo direta ou indiretamente para a

eliminação do parasito, ou então, apresentando efeitos totalmente contrários, podendo influenciar no desenvolvimento dos sintomas clínicos e das complicações associadas à infecção (Ramharter et al. 2003).

Nesse contexto, cabe destacar importantes trabalhos em modelos animais, que atribuem a participação de células do sistema imune inato - incluindo mastócitos (Guermonprez et al. 2013), neutrófilos (Porcherie et al. 2011) e células NK, NKT e Tγδ (Hansen et al. 2007) - na detecção da infecção inicial no estágio sanguíneo, promovendo inflamação, inibindo o crescimento de parasitos e moldando respostas imunes adaptativas (Smith et al. 2002; Stevenson& Riley 2004; Taylor-Robinson 1999). Além disso, Horowitz e colaboradores demonstraram que células NK humanas são uma fonte inicial particularmente importante de IFN-γ em resposta a eritrócitos infectados *in vitro*. Esta citocina desempenha um papel central na resposta imune a malária, promovendo a destruição das células infectadas pela ativação de macrófagos. No mesmo trabalho, foi demonstrado que células Tγδ contribuem, num grau menor, para a produção inicial desta citocina, enquanto as células Tαβ dominam a produção tardia de IFN-γ (Horowitz et al. 2010).

Por fim, apesar da clara evidência de função protetora atribuída a células B e anticorpos, as contribuições das células T-CD4$^+$ e T-CD8$^+$ na proteção contra o estágio eritrocítico ainda não foram completamente esclarecidas, sendo os principais dados acerca deste tema oriundos de modelos murinos (Long& Zavala 2017). Com relação a proteção, as células T-CD8$^+$ parecem reconhecer o MHC-I de eritroblastos infectados (Imai et al. 2013) e estar envolvidas no clearence parasitário (Safeukui et al. 2015), por outro lado, a apresentação cruzada de antígenos parasitários, por células endoteliais de microcapilares cerebrais, para as células T-CD8$^+$ é uma característica da malária cerebral experimental (Howland et al. 2015; Swanson et al. 2016). Combinado a uma restrição no efluxo de sangue venoso devido ao sequestro de células (Nacer et al. 2014), os processos mediados por células T-CD8$^+$ são considerados fundamentais na degradação vascular e condição fatal (Draheim et al. 2017).

Do mesmo modo, as células T-CD4$^+$, sendo os produtoras de citocinas inflamatórias e regulatórias, são fundamentais no equilíbrio entre proteção e patologia (Draheim et al. 2017). Por um lado, estas células contribuem para o controle parasitário, uma vez que células T-CD4$^+$ de memória efetora Th1 conferem proteção parcial no modelo de malária crônica por *P. chabaudi chabaudi* (Stephens& Langhorne 2010) e a perda do fator de transcrição T-bet, um importante regulador

da diferenciação Th1, prejudica o controle da parasitemia no modelo de *P. berghei ANKA* (Oakley et al. 2013). Além disso, uma subpopulação de células T-CD4$^+$, denominadas células T helper foliculares, libera IL-21, uma citocina que auxilia as células B (Perez-Mazliah et al. 2015) e é fundamental para promover a formação do centro germinativo e a imunidade humoral anti-parasito (Sebina et al. 2016; Zander et al. 2016). Ao mesmo tempo, a produção de citocinas reguladoras, como a IL-10 (Couper et al. 2008; Freitas do Rosario et al. 2012; Freitas do Rosario& Langhorne 2012; Villegas-Mendez et al. 2016) e IL-27 (Kimura et al. 2016), pelas células T-CD4$^+$ é fundamental para determinação da patologia em modelos murinos. Em contraponto, a secreção de IFN-γ por células T-CD4$^+$ no início da infecção (Belnoue et al. 2002; Villegas-Mendez et al. 2012) promove o acúmulo de células T-CD8$^+$ no cérebro, através de mecanismos dependentes CXCL9 e CXCL10 e mediados por CXCR3 (Campanella et al. 2008; Van den Steen et al. 2008). Do mesmo modo, camundongos deficientes para o fator de transcrição T-bet (Oakley et al. 2013) e deficientes para IL-12-Rβ2 (Fauconnier et al. 2012) são menos suscetíveis para o desenvolvimento de malária cerebral experimental. Por fim, apesar dos controversos estudos acerca do papel das células T-CD4$^+$ na malária, seus antígenos cognatos, bem como as células apresentadoras de antígeno que controlam sua diferenciação, ainda são pouco conhecidos.

1.6.3 Respostas imunes contra o estágio sexuado do *Plasmodium*

Embora as respostas imunes direcionadas aos estágios pré-eritrocítico e eritrocítico assexuado sejam as mais pesquisadas e, aparentemente, mais importantes na defesa contra malária, as respostas contra os gametócitos podem representar um importante mecanismo na diminuição da transmissão da doença.

Os primeiros estudos avaliando a resposta contra estágios sexuados do parasito foram conduzido em modelos de malária aviária e demonstraram que aves imunizadas com sangue contendo gametócitos mortos tornavam-se menos infectivas para mosquitos (Huff et al. 1958). Quase duas décadas depois, foi demonstrado que galinhas imunizadas com gametócitos inativados de *P. gallinaceum* produziram uma atividade bloqueadora de transmissão em seu soro (Carter& Chen 1976; Gwadz 1976), que não afetava diretamente o gametócito, mas sim o desenvolvimento do parasito no mosquito. Posteriormente, esta imunidade bloqueadora de transmissão foi demonstrada em camundongos (Mendis& Targett 1979) e primatas não humanos (Gwadz& Green 1978).

Os anticorpos contra as formas sexuadas do parasito podem induzir a eliminação dos gametócitos no sangue do hospedeiro vertebrado, (Margos et al. 2001; Read et al. 1994). Além disso, no mosquito podem prevenir a fusão dos gametas (Blagborough& Sinden 2009), induzir via-complemento a destruição dos gametas ou oocineto (Volohonsky et al. 2010) e prevenir a motilidade do oocineto, impedindo sua penetração na parede do intestino médio e formação do oocisto (Baton& Ranford-Cartwright 2005a; Duffy et al. 1993; Ranawaka et al. 1994; Sieber et al. 1991).

Conjuntamente com anticorpos do hospedeiro vertebrado, diferentes mecanismos no organismo do mosquito são descritos como capazes de controlar a infecção pelo *Plasmodium* (Smith& Barillas-Mury 2016). No interior do mosquito a eliminação do parasito é multimodal, com duas fases da imunidade inata do mosquito agindo nos estágios e oocineto e oocisto de plasmódios humanos e murinos (Gupta et al. 2009; Smith et al. 2015; Smith et al. 2014).

Inicialmente, o epitélio do intestino médio do mosquito funciona como uma barreira para a chegada do parasito na hemocele, uma vez que cerca de 80% dos oocinetos invasores são destruídos pelo mosquito (Shiao et al. 2006), através de espécies reativas de oxigênio e nitrogênio (Han et al. 2000) e pelo sistema “tipo-complemento” do mosquito, que interage com a superfície do oocineto, modificada pela nitração no intestino médio (Garver et al. 2013; Kumar et al. 2004; Oliveira Gde et al. 2012; Ramphul et al. 2015). Adicionalmente, trabalhos recentes evidenciaram que oocistos de *P. berghei* e *P. falciparum* são suscetíveis a efeitos do organismo do hospedeiro invertebrado em laboratório (Goulielmaki et al. 2014; Smith et al. 2015) e em estudos de campo (Awono-Ambene& Robert 1998). Embora o modo como o sistema imune do mosquito afeta o desenvolvimento dos oocistos ainda não esteja completamente esclarecido, a diferenciação de hemócitos após a infecção pelo *Plasmodium*, resulta no aumento das proporções de oenocitóides e granulócitos circulantes (Ramirez et al. 2014; Smith et al. 2015), que é capaz de conferir imunidade protetora em experimentos de desafio (Rodrigues et al. 2010).

1.7 Desenvolvimento de vacinas anti-maláricas

Diversas estratégias têm sido utilizadas visando a diminuição dos casos de malária no mundo, dentre os quais combate aos vetores e rápido diagnóstico associado ao adequado tratamento do paciente. Infelizmente, a ineficácia dos

inseticidas como controle em diversas áreas endêmicas e a resistência dos parasitos às drogas antimaláricas, tornam o desenvolvimento de uma vacina segura, eficaz e acessível, uma prioridade da saúde pública global (Polhemus et al. 2009).

Um vasto repertório de evidências corrobora que seja possível produzir uma vacina anti-malárica eficaz. Em 1967, Nussenzweig e seus colaboradores demonstraram que a imunização de camundongos com esporozoítos irradiados foi capaz de gerar proteção contra o desafio com *P. berghei* (Nussenzweig et al. 1967). Posteriormente, o mesmo foi demonstrado para primatas não humanos. Entre os anos de 1989 e 1999, humanos imunizados com picadas de mosquitos irradiados carregando esporozoítos de *P. falciparum* apresentaram proteção de 100% contra desafios feitos 9 semanas após a última imunização (Hoffman et al. 2002). Adicionalmente, vem sendo demonstrado que indivíduos naturalmente expostos a malária adquirem certo grau de imunidade, razão pela qual os casos graves da doença acometem principalmente crianças e infantes (Langhorne et al. 2008; Richie& Saul 2002; Tetteh& Polley 2007). Entretanto, esta imunidade adquirida não parece ser duradoura, uma vez que indivíduos imunes parecem se tornar novamente suscetíveis a malária grave quando retornam a áreas endêmicas após longos períodos fora de áreas endêmicas para doença (Struik& Riley 2004).

Em contraponto, a imunidade provida por um complexo de antígenos como esporozoítos irradiados e infecções naturais tem sido difícil de se replicar usando antígenos purificados. Acredita-se que as diferentes composições antigênicas apresentadas pelo *Plasmodium* nos diferentes estágios de seu ciclo biológico, aliada a variabilidade antigênica dentre as espécies e cepas dos parasitos, representam um dos maiores obstáculos ao desenvolvimento vacinal (Rappuoli& Aderem 2011), justificando assim a necessidade do desenvolvimento de uma vacina que contenha vários antígenos de uma única fase ou uma combinação de antígenos de diferentes fases do parasito (Miller& Hoffman 1998).

Apesar disso, a maioria das vacinas pensadas para combater a malária tem como alvo um único estágio de vida do parasito, sendo classificadas com base nesse critério em: vacinas pré-eritrocíticas, vacinas de estágio sanguíneo, ou vacinas bloqueadoras de transmissão (Arama& Troye-Blomberg 2014).

1.7.1 Vacinas pré-eritrocíticas

Possuem o objetivo de bloquear a doença, impedindo a invasão dos hepatócitos pelos esporozoítos. Atualmente, em função do baixo número de

esporozoítos liberados durante o repasto sanguíneo, este estágio representa o principal alvo vacinal contra *Plasmodium* (Kebaier et al. 2009; Yamauchi et al. 2007). Contudo, uma vacina pré-eritrocítica precisa ser 100% efetiva, uma vez que, em virtude de sua elevada taxa de replicação, um pequeno número de esporozoíta é o suficiente para que a infecção atinja o estágio eritrocítico (March et al. 2013; Okie 2005; Risco-Castillo et al. 2015).

São exemplos desta estratégia, as vacinas que utilizam esporozoítos irradiados ou atenuados (Haeberlein et al. 2017; Hansen et al. 1979; Nussenzweig et al. 1969; Nussenzweig et al. 1967). Tais vacinas representam um interessante alvo, uma vez que quando funcionais impedem o estabelecimento da infecção inicial no fígado, inibindo assim a formação hipnozoítas e eventos subsequentes de recaídas, no caso de infecções por *P. vivax* ou *P. ovale*, além dos sintomas, frutos do ciclo eritrocítico do *Plasmodium*, e a transmissão da doença, uma vez que os parasitos não atingiriam o estágio de gametócitos (Arama& Troye-Blomberg 2014).

Dentre as 19 formulações vacinais contra alvos deste estágio em ensaios clínicos ou pré-clínicos (WHO 2017), a vacina RTS,S, que utiliza a proteína Circunsporozoíto (CSP) de *P. falciparum*, proteína imunodominante na superfície do esporozoíto, ligada ao antígeno de superfície da hepatite B, se destaca como a principal formulação vacinal anti-malária. Tal formulação começou a ser testada em 1980, apresentou sucesso parcial na imunização de crianças em ensaios de clínicos de fase 3 (Olotu et al. 2016; Rosenthal 2015; Rts 2015; Targett 2015), e será implementada a partir de 2018 em áreas selecionadas do Gana, Quênia e Malawi, segundo a Organização Mundial de Saúde (PATH 2017; Vandoolaeghe& Schuerman 2016). Apesar disso, a limitada taxa de proteção induzida pela vacina RTS,S reforça a ideia de construção vacinais contendo combinações de diferentes antígenos.

Nesse contexto, destacamos a proteína CelTOS (Cell-Traversal Protein for Ookinetes and Sporozoites) como uma nova e promissora candidata vacinal. Recentemente, Jimah e colaboradores demonstraram que esta proteína é capaz de romper os lipossomas compostos de ácido fosfatídico através da formação de poros, sendo importante no processo de travessia celular desempenhado pelos esporozoítos nos hepatócitos, atuando principalmente na saída do parasito da célula hospedeira (Jimah et al. 2016). A CelTOS é secretada durante os estágios de esporozíta e oocineto do parasito (Kariu et al. 2006) e é altamente conservada entre parasitos do filo Apicomplexa, apresentando semelhança estrutural com a

glicoproteína gp41 de HIV-1 e com uma toxina formadora de poro de *Mycobacterium tuberculosis* (Jimah et al. 2016). Seu potencial como candidata vacinal está vinculado a estudos que apontam que a interrupção de genes da CelTOS em *P. berghei* reduz a infectividade do parasito tanto no mosquito quanto no fígado de camundongos (Kariu et al. 2006). Além disso, a imunização com a proteína CelTOS de *P. falciparum* (PfCelTOS) foi capaz de induzir proteção cruzada contra *P. berghei* em modelos murinos (Bergmann-Leitner et al. 2010). No entanto, embora a PfCelTOS seja vista como um antígeno vacinal promissor, estando inclusive em ensaios clínicos de pesquisa (WHO 2017), a proteína homóloga de *P. vivax* (PvCelTOS) é pouco explorado. Dentre os poucos trabalhos que avaliam seu potencial como candidata vacinal, destacamos o estudo de Longley e colaboradores, que demonstra que a proteína é naturalmente imunogênica em indivíduos expostos à malária da Tailândia, induzindo anticorpos que apresentam longevidade superior a um ano (Longley et al. 2015). Recentemente nosso grupo demonstrou que a PvCelTOS é uma proteína altamente conservada entre isolados de diferentes regiões geográficas, uma característica que dá suporte a seu potencial como candidata vacinal (Bitencourt Chaves et al. 2017). Apesar disso, os alvos antigênicos desta proteína, bem como seu papel protetor permanecem desconhecidos.

1.7.2 Vacinas de estágio sanguíneo

Também chamadas de vacinas eritrocíticas, são projetadas para desenvolver anticorpos que inibam a invasão dos eritrócitos pelos merozoítas (Moorthy et al. 2004). Embora esta estratégia vacinal não induza uma imunidade estéril, uma vez que é incapaz de inibir o estágio hepático da infecção, ao bloquear a invasão dos eritrócitos, pode impedir a evolução da doença, gerando imunidade clínica e/ou diminuindo a morbidade e a gravidade dos quadros clínicos (Arama& Troye-Blomberg 2014).

Em função de sua alta expressão na superfície dos merozoítas, diversos antígenos contra o estágio sanguíneo se encontram em teste nas 10 formulações vacinais hoje em ensaio clínico (WHO 2017), dentre os quais podemos destacar: A proteína-rica em glutamato (GLURP) e a proteína-3 de superfície de merozoíta (MSP3), componentes da formulação GMZ2 (Milligan et al. 2017; Sirima et al. 2016); o antígeno-1 de membrana apical (AMA)1 (Sirima et al. 2017); o antígeno-5 de repetição de serina (SERA5), componente da vacina SE36 (Palacpac et al. 2013); a proteína homóloga-5 de ligação de reticulócitos, componente das vacinas ChAd63

RH5 e MVA RH5 (Payne et al. 2017b); e a proteína de ligação Duffy de *P. vivax* (PvDBP) (Payne et al. 2017a), única candidata vacinal contra esta espécie plasmodial em ensaios clínicos atualmente (WHO 2017).

Além destas, as proteínas de superfície de merozoitas (MSP) têm sido consideradas um promissor grupo de proteínas alvo, uma vez que são expressas durante a esquizogonia e se tornam associadas a superfície dos merozoítos ao longo do desenvolvimento do esquizonte (Barnwell et al. 1999). Além disso, em função de sua repetida exposição ao sistema imune do hospedeiro, diversas MSPs tem tido seu potencial imunológico investigado (Cheng et al. 2014; Osier et al. 2014; Stanisic et al. 2013).

Dentre estas proteínas, a proteína-9 de superfície de merozoítas de *P. vivax* (PvMSP9) é considerada uma promissora candidata vacinal em função de diversos fatores dentre os quais: (1) sua localização na superfície do merozoíto; (2) a homologia com plasmódios simianos e *P. falciparum*; (3) a habilidade de IgG específicas em inibir a invasão dos merozoítas; (4) sua alta imunogenicidade em ensaios realizados em modelos animais (Barnwell et al. 1999; Galinski et al. 1999; Oliveira-Ferreira et al. 2004b). Embora a MSP9 seja uma proteína identificada em diversas espécies de *Plasmodium*, sua análise filogenética demonstra que *P. vivax* é estritamente relacionado a espécies que infectam primatas não-humanos [34]. Estruturalmente, a MSP9 apresenta um longo domínio N-terminal, não repetitivo e conservado e uma região C-terminal contendo dois blocos espécie-específicos de repetição em tandem [28,35]. Acerca da imunogenicidade da PvMSP9, sua região N-terminal contém 5 epítopos promíscuos de célula T (pE, pJ, pK, pH e pL), que interagem com um amplo espectro de moléculas de HLA de classe II [36,37]; é imunogênica em camundongos [36] e naturalmente imunogênica em adultos [26] e crianças [31]. Por outro lado, os blocos de repetição situados na região C-terminal da proteína são apontados como a região mais imunogênica da PvMSP9 [26], e anticorpos direcionados a seus blocos de repetição se mostraram correlacionados a exposição à malária [26,35].

Apesar do considerável número de candidatos vacinais contra este estágio, ao menos para *P. falciparum*, um considerável número de estudos aponta resultados desanimadoras acerca da proteção induzida por muitos destes antígenos (Duncan et al. 2011; Hermsen et al. 2007; Sagara et al. 2009). Acredita-se que a extensa diversidade genética do parasito e a pressão seletiva exercida pela resposta imune do hospedeiro sejam importantes fatores a serem considerados no desenvolvimento

de vacinas (Takala& Plowe 2009). Nesse sentido, além do uso de formulações vacinais combinando diferentes antígenos, atualmente, se sugere o foco em antígenos conservados, capazes de induzir reatividade cruzada, capaz de cobrir a ampla diversidade genética dos parasitos do gênero *Plasmodium*.

1.7.3 Vacinas bloqueadoras de transmissão

Também chamadas de vacinas altruístas, estas construções têm como alvo antígenos presentes durante os estágios de gametócito, gametas, zigoto e/ou oocineto do *Plasmodium*. Embora tais vacinas não geram imunidade estéril, nem tão pouco imunidade clínica, são capazes de evitar o desenvolvimento do parasito no intestino médio do vetor, impedindo a transição de oocineto para oocisto e consequentemente a geração de esporozoítas e dispersão da doença (Carter et al. 2000). Dentre os principais alvos direcionados a esta estratégia vacinal hoje em ensaios clínicos ou pré-clínicos, podem ser destacadas antígenos de *P. falciparum* presentes na superfície de gametócitos e gametas, como Pfs230 (Farrance et al. 2011) e Pfs48/45 (Outchkourov et al. 2008) , e antígenos expressos nos estágios de zigoto e oocineto, como a Pfs25 (Goodman et al. 2011).

Cabe ressaltar que, embora o uso desta estratégia vacinal possa representar uma importante ferramenta para programas de eliminação da malária, atualmente, não há nenhuma formulação vacinal deste tipo direcionada ao *P. vivax* em ensaios clínicos ou pré-clínicos, espécie mais dispersa no planeta.

1.8 Desenvolvimento de vacinas contra o *P. vivax*

Acerca do atual estágio das vacinas anti-maláricas, a “WHO Initiative for Vaccine Research” mantém uma lista atualizada de candidatas vacinais com base em dados inseridos pelas principais agências de fomento. Atualmente, além da vacina RTS,S, há cerca de 23 formulações vacinais em ensaios clínicos e 17 em ensaios pré-clínicos contra o *P. falciparum*, em contraponto, quando tratamos de pesquisas direcionadas ao *P. vivax*, o número de formulações em ensaios clínicos e pré-clínicos é drasticamente reduzido a 3 formulações: (1) **ChAd63/MAV PvDBP**, que tem como alvo a proteína que se liga ao antígeno Duff de *P. vivax*, e é hoje a única formulação vacinal contra esta espécie em ensaios clínicos; (2) **VMP002**, que contém a PvCSP expressa em *Escherichia coli*; e (3) **PvSPZ**, composta por esporozoítas atenuados (WHO 2017).

Este evidente viés na busca por vacinas contra o *P. falciparum* e o *P. vivax*, pode ser visto como resultado da reconhecida importância clínica atribuída ao *P. falciparum*, em função da alta letalidade e número de casos graves associados a esta espécie, que direcionou esforços e investimentos em estudos envolvendo esta espécie ao longo do tempo. Além disso, cabe destacar que a existência de um cultivo *in vitro* contínuo bem estabelecido de *P. falciparum*, facilita o uso de diversas metodologias da vacinologia convencional, acelerando o desenvolvimento de pesquisas.

Contudo, os registros de casos de malária grave e letal por *P. vivax* (Geleta& Ketema 2016; Gupta et al. 2015; Mitra et al. 2015), a emergência de cepas resistentes à cloroquina (de Santana Filho et al. 2007; Karunajeewa et al. 2008; Teka et al. 2008) e à primaquina (Arias& Corredor 1989; Kristensen& Dragsted 2014; Nayar et al. 1997), além de sua ampla dispersão e enorme impacto socioeconômico causado por esta espécie em áreas endêmicas, tornam o desenvolvimento de uma vacina eficaz contra o *P. vivax* essencial para as estratégias de controle e eliminação da malária no mundo. Infelizmente, a falta de um cultivo *in vitro* de longa duração dificulta os esforços nesse sentido, impossibilitando o uso importantes abordagens da vacinologia convencional para identificação de alvos potenciais (Patarroyo et al. 2012; Thomson-Luque et al. 2017).

1.9 Vacinologia reversa: uma abordagem promissora contra *P. vivax*

A vacinologia reversa utiliza análises *in silico* de dados do genoma, transcriptoma ou proteômica do parasito para identificar características que podem distinguir potenciais candidatos vacinais (Rappuoli 2000, 2001). Esta abordagem reduz o tempo e o custo para identificação de alvos vacinais, sendo uma promissora alternativa nos estudos de parasitos não cultiváveis, como o *P. vivax* (Mora et al. 2003).

Desde o completo sequenciamento dos genomas de *P. falciparum* (Gardner et al. 2002) e *P. vivax* (Carlton et al. 2008; Dharia et al. 2010), uma quantidade substancial de informações sobre estes parasitos ficaram disponíveis. Estudos comparativos evidenciaram que essas espécies plasmodiais têm o potencial de expressar aproximadamente 5000 proteínas hipotéticas, sendo cerca de 75% destas ortólogas com outras espécies. Nos últimos anos, estudos baseados no transcriptoma e proteoma de *P. vivax*, identificaram possíveis candidatos vacinais

(Acharya et al. 2009; Bozdech et al. 2008; Westenberger et al. 2010), incluindo variantes de antígenos de superfície da família de multigênica *Plasmodium interspersed repeat* (pir), a maior família de genes identificada até agora, compartilhada por *P. vivax* e espécies plasmodiais de primatas não humanos, como *P. knwolesi*, e de roedores, como *P. chabaudi*, *P. yoelii* e *P. berghei* (Cunningham et al. 2010; Janssen et al. 2004).

Por outro lado, a vacinologia reversa também permite, através de diferentes ferramentas de bioinformática, a identificação de epítopos alvos dentro de proteínas sabidamente antigênicas/imunogênicas. Desse modo, a determinação de regiões imunogênicas curtas e específicas dentro de candidatas vacinais, pode ser utilizada na geração de peptídeos quiméricos, contendo múltiplos epítopos contra diferentes alvos, e levar a um avanço no desenvolvimento de vacinas eficazes. Baseados nesse conceito, Bueno e seus colaboradores, utilizando a combinação de diferentes algoritmos de predição, identificaram um epítopo linear de célula B, altamente antigênico dentro do domínio II, a região mais imunogênica da candidata vacinal AMA-1 de *P. vivax* (PvAMA-1) (Bueno et al. 2011).

Atualmente, o mapeamento por predição *in silico* e a posterior confirmação imuno-molecular dos epítopos de células B e T de proteínas candidatas vacinais vem sendo utilizada em diversos patógenos como o HIV e *Influenza* (Soria-Guerra et al. 2015). Além do possível uso em peptídeos quiméricos, a identificação de epítopos alvos dentro de candidatas vacinais conhecidas pode representar uma interessante estratégia para contornar mecanismos de escape do parasito, dentre os quais: a diversidade antigênica, uma vez que podem ser selecionados epítopos conservados entre diferentes cepas do parasito, e o mecanismo de “cortina- de fumaça” (Anders 1986), através da seleção de epítopos protetores específicos do candidato vacinal explorado, diminuindo o efeito de imunodominância de proteínas que não induzem imunidade protetora (Renia& Goh 2016).

Em contraponto, apesar dos esforços empreendidos nas últimas décadas no desenvolvimento de vacinas contra malária, as candidatas vacinais hoje conhecidas não representam mais de 1% do proteoma predito para *P. vivax* (Valencia et al. 2011).

1.10 Parasitos quiméricos: solução alternativa para um “velho” problema.

Apesar dos avanços na identificação de alvos vacinais contra *P. vivax*, a ausência de um cultivo *in vitro* persiste sendo um obstáculo para avaliação do potencial protetor de novos antígenos. Nesse contexto, “parasitos quiméricos” surgem como uma alternativa viável e promissora para avaliação do potencial protetor de vacinas de subunidades (Cockburn 2013; Mlambo& Kumar 2008; Mlambo et al. 2008; Salman et al. 2015) e novas drogas terapêuticas (Blume et al. 2011; Tewari et al. 2014) contra espécies plasmodiais que infectam humanos. Em suma, através desta abordagem são desenvolvidos parasitos murinos transgênicos, expressando proteínas alvos de *P. vivax* ou *P. falciparum* em lugar de suas homólogas na espécie original (Othman et al. 2017).

Deste modo, novos ensaios vacinais contra *P. vivax* têm sido desenvolvidos, através da imunização de camundongos com candidatos vacinais e subsequente desafio com *Plasmodium* murino transgênico expressando o alvo vacinal (Blagborough et al. 2016; Gimenez et al. 2017; Mizutani et al. 2016; Salman et al. 2017). Nesse cenário, a combinação de “parasitos e antígenos quiméricos” pode representar um novo caminho para o desenvolvimento de vacinas eficazes contra o *P. vivax*.

1.11 Justificativa

O desenvolvimento de vacinas candidatas contra *P. vivax* tem sido difícil principalmente pela falta de um sistema *in vitro* para o cultivo contínuo e o equívoco generalizado de que a malária causada pelo *P. vivax* é branda. Contudo, a elevada dispersão e prevalência do *P. vivax* no mundo, somados ao crescente número de relatos de malária grave por esta espécie e ao surgimento e dispersão de cepas resistentes ao tratamento na Ásia, Brasil e África, tornam o desenvolvimento de vacinas para esta espécie plasmodial um passo fundamental para o controle da malária no Brasil e no mundo.

Apesar da reconhecida importância epidemiológica da malária causada pelo *P. vivax* em várias partes do mundo, inclusive no Brasil, a busca de vacinas contra esta espécie plasmodial é pouco explorada e o número de proteínas antigenicamente relevantes ainda é restrito. Atualmente, enquanto existem 17 formulações vacinais em ensaio pré-clínico e 24 em ensaios clínicos direcionadas ao *P. falciparum*, estes

números são drasticamente reduzidos a 2 e 1, respectivamente, quando se tratando de formulações direcionadas ao *P. vivax* (WHO 2017). Em meio a este cenário, a vacinologia reversa surge como uma alternativa promissora para identificação de alvos vacinais contra o *P. vivax*. Atualmente, o mapeamento por predição *in silico* e a posterior confirmação imuno-molecular dos epítopos de células B e T de proteínas candidatas vacinais vem sendo utilizada em diversos patógenos como o HIV e *Influenza* (Soria-Guerra et al. 2015), fornecendo constantemente subsídios para o desenvolvimento de vacinas e novos testes de diagnósticos eficazes. Na malária, apesar do considerável número de proteínas candidatas vacinais citados anteriormente, estudos que utilizem ferramentas de bioinformática aliados a confirmação experimental com antígenos de *Plasmodium* são escassos.

Nesse contexto, a identificação de epítopos de células B e T em promissoras candidatas vacinais, bem como sua utilização em ensaios de imunização, pode representar uma importante contribuição no campo das pesquisas em vacinas contra o *P. vivax*, uma vez que fornecerá informações relevantes sobre a validação e identificação de candidatos vacinais, bem como sua antigenicidade e imunogenicidade. Para tanto, a construção de quimeras lineares sintéticas contendo epítopos identificado *in* silico em proteínas candidatas vacinais tem se mostrado uma alternativa viável, de baixo custo e com um relativo sucesso reportado em plasmódios murinos (Caro-Aguilar et al. 2005; Singh et al. 2012).

OBJETIVOS

2.1 Objetivo Geral

Identificar e validar epítopos de célula B de diferentes proteínas candidatas vacinais de *Plasmodium vivax*, bem como avaliar seus potenciais antigênicos e imunogênicos, quando inseridos em peptídeos lineares sintéticos.

2.2 Objetivos Específicos

- Avaliar a resposta imune naturalmente adquirida contra a proteína de fase pré-eritrocítica PvCelTOS em indivíduos da Amazônia brasileira (Artigo 1);
- Identificar *in silico* epítopos lineares de célula B na PvCelTOS (Artigo 1);
- Realizar o mapeamento de epítopos da PvCelTOS, identificando suas regiões imunogênicas em indivíduos naturalmente expostos a malária (Artigo 1)
- Validar os métodos de predição *in silico* através da sobreposição dos dados de predição e dados de mapeamento antigênico da PvCelTOS (Artigo 1);
- Avaliar impactos da diversidade genética da PvCelTOS na estrutura da proteína e potencial imunogênico de epítopos identificados *in silico* (Artigo 2)
- Identificar *in silico* epítopos lineares de célula B na proteína de fase eritrocítica PvMSP9 (Artigo 3);
- Confirmar a antigenicidade do epítopo predito em indivíduos naturalmente expostos a malária (Artigo 3);
- Determinar a existência de correlações entre dados epidemiológicos e a resposta específica para o epítopo identificado (Artigo 3);
- Sintetizar peptídeos lineares representando epítopos de célula T e B da PvMSP9 (Artigo 4);
- Avaliar a resposta imune celular e humoral induzidas pelos peptídeos sintéticos, formulados em adjuvante, em camundongos BALB/c (Artigo 4);
- Averiguar o reconhecimento da proteína recombinante PvMSP9-RIRII pelos anticorpos induzidos pelos peptídeos sintéticos (Artigo 4);
- Verificar o reconhecimento da proteína nativa no parasito por anticorpos gerados em camundongos imunizados com peptídeos sintéticos (Artigo 4).

RESULTADOS

3.1 Artigo 1 - *Plasmodium vivax* Cell-traversal protein for ookinetes and sporozoites (PvCelTOS): Resposta humoral naturalmente adquirida e mapeamento de epítopos de célula B em indivíduos da Amazônia brasileira.

3.1.1 Objetivos específicos:

- Avaliar a resposta imune naturalmente adquirida contra a proteína de fase pré-eritrocítica PvCelTOS em indivíduos da Amazônia brasileira (Artigo 1);
- Identificar *in silico* epítopos lineares de célula B na PvCelTOS (Artigo 1);
- Realizar o mapeamento de epítopos da PvCelTOS, identificando suas regiões imunogênicas em indivíduos naturalmente expostos a malária (Artigo 1)
- Validar os métodos de predição *in silico* através da sobreposição dos dados de predição e dados de mapeamento antigênico da PvCelTOS (Artigo 1);

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 February 2017
doi: 10.3389/fimmu.2017.00077

Plasmodium vivax Cell-Traversal Protein for Ookinetes and Sporozoites: Naturally Acquired Humoral Immune Response and B-Cell Epitope Mapping in Brazilian Amazon Inhabitants

Rodrigo Nunes Rodrigues-da-Silva[1], Isabela Ferreira Soares[1], Cesar Lopez-Camacho[2], João Hermínio Martins da Silva[3], Daiana de Souza Perce-da-Silva[4], Antônio Têva[5], Antônia Maria Ramos Franco[6], Francimeire Gomes Pinheiro[6], Lana Bitencourt Chaves[1], Lilian Rose Pratt-Riccio[7], Arturo Reyes-Sandoval[2], Dalma Maria Banic[4] and Josué da Costa Lima-Junior[1]*

[1] Laboratory of Immunoparasitology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil, [2] Nuffield Department of Medicine, The Jenner Institute, The Henry Wellcome Building for Molecular Physiology, University of Oxford, Oxford, UK, [3] Computational Modeling Group, Fiocruz, Fortaleza, Brazil, [4] Laboratory of Clinical Immunology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil, [5] Laboratory of Immunodiagnostics, Department of Biological Sciences, National School of Public Health, Fiocruz, Rio de Janeiro, Brazil, [6] Laboratory of Leishmaniasis and Chagas Disease, National Institute of Amazonian Research, Manaus, Brazil, [7] Laboratory of Malaria Research, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil

OPEN ACCESS

Edited by:
Clarisa B. Palatnik-de-Sousa, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Manuel Alfonso Patarroyo, Fundación Instituto de Inmunología de Colombia (FIDIC), Colombia
Daniel Youssef Bargieri, University of Sao Paulo, Brazil

***Correspondence:**
Josué da Costa Lima-Junior
josue@ioc.fiocruz.br

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 10 November 2016
Accepted: 17 January 2017
Published: 07 February 2017

Citation:
Rodrigues-da-Silva RN, Soares IF, Lopez-Camacho C, Martins da Silva JH, Perce-da-Silva DS, Têva A, Ramos Franco AM, Pinheiro FG, Chaves LB, Pratt-Riccio LR, Reyes-Sandoval A, Banic DM and Lima-Junior JC (2017) Plasmodium vivax Cell-Traversal Protein for Ookinetes and Sporozoites: Naturally Acquired Humoral Immune Response and B-Cell Epitope Mapping in Brazilian Amazon Inhabitants. Front. Immunol. 8:77. doi: 10.3389/fimmu.2017.00077

The cell-traversal protein for ookinetes and sporozoites (CelTOS), a highly conserved antigen involved in sporozoite motility, plays an important role in the traversal of host cells during the preerythrocytic stage of *Plasmodium* species. Recently, it has been considered an alternative target when designing novel antimalarial vaccines against *Plasmodium falciparum*. However, the potential of *Plasmodium vivax* CelTOS as a vaccine target is yet to be explored. This study evaluated the naturally acquired immune response against a recombinant *P. vivax* CelTOS (PvCelTOS) (IgG and IgG subclass) in 528 individuals from Brazilian Amazon, as well as the screening of B-cell epitopes *in silico* and peptide assays to associate the breadth of antibody responses of those individuals with exposition and/or protection correlates. We show that PvCelTOS is naturally immunogenic in Amazon inhabitants with 94 individuals (17.8%) showing specific IgG antibodies against the recombinant protein. Among responders, the IgG reactivity indexes (RIs) presented a direct correlation with the number of previous malaria episodes ($p = 0.003$; $r = 0.315$) and inverse correlation with the time elapsed from the last malaria episode ($p = 0.031$; $r = -0.258$). Interestingly, high responders to PvCelTOS (RI > 2) presented higher number of previous malaria episodes, frequency of recent malaria episodes, and ratio of cytophilic/non-cytophilic antibodies than low responders (RI < 2) and non-responders (RI < 1). Moreover, a high prevalence of the cytophilic antibody IgG1 over all other IgG subclasses ($p < 0.0001$) was observed. B-cell epitope mapping revealed five immunogenic regions in PvCelTOS, but no associations between the specific IgG response to peptides and

exposure/protection parameters were found. However, the epitope ($PvCelTOS_{I136-E143}$) was validated as a main linear B-cell epitope, as 92% of IgG responders to PvCelTOS were also responders to this peptide sequence. This study describes for the first time the natural immunogenicity of PvCelTOS in Amazon individuals and identifies immunogenic regions in a full-length protein. The IgG magnitude was mainly composed of cytophilic antibodies (IgG1) and associated with recent malaria episodes. The data presented in this paper add further evidence to consider PvCelTOS as a vaccine candidate.

Keywords: PvCelTOS, *P. vivax*, vaccines, epitope mapping, epitope prediction, malaria vaccines, malaria

INTRODUCTION

Malaria remains a major public health problem worldwide. It is caused by protozoan parasites of the genus *Plasmodium,* being responsible for nearly 438,000 deaths and 150–300 million new infections in 2015 (1) and the reason of enormous socioeconomic impact in endemic settings (2). Among the *Plasmodium* species able to infect humans, *Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent malaria parasites. *P. falciparum* is extremely prevalent in Africa and is responsible for the majority of cases and deaths worldwide, while *P. vivax* is the most prevalent species outside Africa (3). Despite the reduction in the number of malaria cases and deaths over the past decade (1), the emergence of drug resistance and the significant ongoing burden of morbidity and mortality emphasize the need for an effective malaria vaccine. Unfortunately, potential *P. vivax* vaccine candidates lag far behind those for *P. falciparum* (4). Currently, besides the RTS, S vaccine, there are 30 candidate vaccine formulations in clinical trials against *P. falciparum*, while there is only one against *P. vivax* (5). These data allied to the impact caused by the high *P. vivax* prevalence (2), the severity of the disease (6–11), and the emergence of strains resistant to chloroquine (12–14) and primaquine (15–17), reiterate the importance of identifying and exploring the potential of vaccine candidates against *P. vivax* as an essential step in the development of a safe and affordable vaccine.

Malaria liver-stage vaccines are one of the leading strategies and the only approach that has demonstrated complete, sterile protection in clinical trials. Therefore, vaccines targeting sporozoite and liver-stage parasites, when parasite numbers are low, can lead to the elimination of the parasite before it advances to the symptomatic stage of the disease (18). Corroborating this idea, the sterile protection against *P. falciparum* by immunization with radiation-attenuated sporozoites was demonstrated in several studies (19–21) and the protection lasted for at least 10 months and extended to heterologous strain parasites (22). Based on these findings, sporozoite surface antigens are one of the most promising vaccine targets against malaria, to protect and prevent the symptoms and block its transmission. To date, RTS,S, the subunit vaccine consisting of a portion of *P. falciparum* circumsporozoite protein (CSP), conferred partial protection in Phase III trials and fell short of community-established vaccine efficacy goals (23–26). Conversely, Gruner and collaborators have demonstrated that the sterile protection against sporozoites can be obtained in the absence of specific immune responses to CSP (27). In addition, a recent study found 77 parasite proteins associated with sterile protection against irradiated sporozoites (28). Collectively, these data reinforce the concept that a multivalent anti-sporozoite vaccine targeting several surface-exposed antigens would induce a higher protection efficacy.

In this scenario, cell-traversal protein of *Plasmodium* ookinetes and sporozoites, a highly conserved protein among *Plasmodium* species, emerged as a novel target in the development of a vaccine against *Plasmodium* parasites (29). This secretory microneme protein is translocated to the sporozoites and ookinetes surface, being necessary for sporozoites and ookinetes to break through cellular barriers and establish infection in the new host, having a crucial role on cell-transversal ability in both stages (29, 30). The disruption of the genes encoding CelTOS in *Plasmodium berghei* reduces the infectivity in the mosquito host and also the infectivity of the sporozoite in the liver, almost eliminating their ability to cell pass (29). Interestingly, *P. falciparum* CelTOS (PfCelTOS) was naturally recognized by acquired antibodies in exposed populations (31), able to induce cross-reactive immunity against *P. berghei* and inhibit sporozoite motility and invasion of hepatocytes *in vitro* (32). However, the knowledge about *P. vivax* CelTOS (PvCelTOS) has remained limited. Only recently, a study reported PvCelTOS as naturally immunogenic in infected individuals from Western Thailand. Our group, investigating the genetic diversity of genes encoding PvCelTOS in field isolates from five different regions of the Amazon forest, reveals a high-conserved profile. Together, both findings support the potential of PvCelTOS as an interesting target on *P. vivax* sporozoite surface, but further studies are still necessary to consolidate this protein as an alternative in future multitarget vaccines. Therefore, the present study aimed at evaluating the naturally acquired humoral immune response against PvCelTOS in exposed populations from Brazilian Amazon, determining the antibody subclass profile, identifying its B-cell epitopes and verifying the existence of associations between the specific IgG and subclass response against PvCelTOS and epidemiological data that can reflect the exposition and/or protection degree.

PARTICIPANTS AND METHODS

Study Area and Volunteers

A cross-sectional cohort study was conducted involving 528 individuals from Rio Preto da Eva (2°50′50″S/59°56′28″W), located north of the Amazon River and 80 km distant from Manaus, the capital of Amazon state. This city has an area of 6,000 km²

and a population of about 22,000 people, who live in rural areas inside the forests. Transmission of malaria in the Amazon occurs throughout the whole year, with seasonal fluctuations with maximum transmission occurring during the dry season from May to October and prevalence of infections by *P. vivax*, responsible for more than 85% of reported malaria cases.

Samples and survey data were collected from November 2013 to March 2015. In addition, we also included, as control subjects, 10 naive individuals living in Manaus, and with no reported previous malaria episodes. Written informed consent was obtained from all adult donors or from parents of donors in the case of children. The study was reviewed and approved by the Fundação Oswaldo Cruz Ethical Committee and the National Ethical Committee of Brazil.

Epidemiological Survey

In order to evaluate the possible influence of epidemiological factors on humoral immunity against PvCelTOS, all donors were interviewed upon informed consent prior to blood collection. The survey included questions related to personal exposure to malaria, such as years of residence in the endemic area, recorded individual and family previous malaria episodes, use of malaria prophylaxis, presence/absence of symptoms, and personal knowledge of malaria transmission. All epidemiological data were stored in Epi-Info for subsequent analysis (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Malaria Diagnosis and Blood Sampling

Venous peripheral blood was drawn into heparinized tubes and plasma collected after centrifugation (350 × *g*, 10 min). Plasma samples were stored at −20°C and transported to our laboratory. Thin and thick blood smears of all donors were examined for malaria parasites. Parasitological evaluations were done by examination of 200 fields at 1,000× magnification under oil-immersion and a research expert in malaria diagnosis examined all slides. Donors positive for *P. vivax* and/or *P. falciparum* at the time of blood collection were subsequently treated using the chemotherapeutic regimen recommended by the Brazilian Ministry of Health.

Recombinant PvCelTOS Expression in HEK-293T Cells

As previously described (33), the *P. vivax* sequence for CelTOS (Salvador I; Uniprot accession number Q53UB7) was cloned in the expression vector pHLsec, which is flanked by the chicken β-actin/rabbit β-globin hybrid promoter with a signal secretion sequence and a Lys-His6 tag. The protein was expressed upon transient transfection in HEK-293T cells with endotoxin-free plasmids in roller bottles (2,125 cm^2). The secreted protein was purified from the supernatant by immobilized Ni Sepharose affinity chromatography. The presence of proteins in the elution samples was confirmed using 6xHis epitope tag antibody [horseradish peroxidase (HRP) conjugate] in a Western blot. The sample was concentrated using an Amicon Ultra centrifugal filter system (Life Technologies) until reaching 10 ml of final volume. Contaminant proteins and salts were removed from the concentrate by size exclusion purification (SEC) using Superdex medium in the column. Protein concentration after recovery was tested using a Bradford protein assay, and purity was assessed by silver staining and by Western blotting.

Antibody Assays

Anti-PvCelTOS specific antibodies were evaluated on plasma samples from 528 exposed individuals from Brazilian Amazon and 10 healthy individuals, who had no reported malaria episodes, using enzyme-linked immunosorbent assay (ELISA), essentially as previously described (33, 34). Briefly, MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) were coated with PBS containing 1.5 μg/ml of recombinant protein. After overnight incubation at 4°C, the plates were washed and blocked for 1 h at 37°C. Individual plasma samples diluted 1:100 in PBS-Tween containing 5% non-fat dry milk (PBS-Tween-M) were added in duplicate wells. After 1 h at 37°C and three washings with PBS-Tween, bound antibodies were detected with peroxidase-conjugated goat antihuman IgG (Sigma, St. Louis) and followed by addition of *o*-phenylenediamine and hydrogen peroxide. Optical density was identified at 492 nm using a SpectraMax 250 ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The results for total IgG were expressed as reactivity indexes (RIs), which were calculated by the mean optical density of an individual's tested sample divided by the mean optical density of 10 non-exposed control individuals' samples plus 3 standard deviations. Subjects were scored as responders to PvCelTOS if the RI of IgG against the recombinant protein was higher than 1. Additionally, the RIs of IgG subclasses were evaluated on responders individuals by a similar method, using peroxidase-conjugated goat antihuman IgG1, IgG2, IgG3, and IgG4 (Sigma, St. Louis).

B Cell Epitope Prediction on PvCelTOS

The prediction of linear B-cell epitopes was carried out using the program BepiPred (35), which is based on hidden Markov model profiles of known antigens, and also incorporates hydrophilicity and secondary structure prediction. For each input FASTA sequence, the server outputs a prediction score for each amino acid. The recommended cutoff of 0.35 was used to determine potential B-cell linear epitopes, ensuring sensibility of 49% and specificity of 75% to this approach. Linear B-cell epitopes are predicted to be located at the residues with the highest scores. In this study, BepiPred was used to predict B-cell linear epitopes and to evaluate the prediction value of peptides containing short amino acid sequences of PvCelTOS.

The Emini surface accessibility (ESA) was used to evaluate the probability of predicted linear B-cell epitopes to be exposed on the surface of the protein. This approach calculates the surface accessibility of hexapeptides and values greater than 1.0 indicate an increased probability of being found on the surface (36). Sequences with BepiPred score above 0.35 and ESA score above 1.0 were considered potential linear B-cell epitopes in regions that could be accessed by naturally acquired antibodies.

B-Cell Epitope Mapping of PvCelTOS

A peptide library of 32 PvCelTOS synthetic 15-mer peptides overlapping by nine amino acids (GenOne Biotechnologies; purity 95% based on HPLC) was synthesized. To evaluate the specific

response to each peptide, the peptide array was performed using MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) coated with PBS containing 5 μg/ml of each peptide in duplicates. After overnight incubation at 4°C, the plates were washed with PBS and blocked with PBS-Tween containing 5% non-fat dry milk (PBS-Tween-M) for 1 h at 37°C. Individual plasma samples were diluted 1:100 with PBS-Tween-M and added in duplicate wells for each sequence and the plates incubated at room temperature for 1 h. After three washings with PBS-Tween, bound antibodies were detected with peroxidase-conjugated goat antihuman IgG (Sigma, St. Louis) followed by addition of *o*-phenylenediamine and hydrogen peroxide. The absorbance was read at 492 nm using an ELISA reader (Spectramax 250, Molecular Devices, Sunnyvale, CA, USA). The results for total IgG were expressed as RIs, which were calculated by the mean optical density of the tested samples plus 3 standard deviations of pools of control individuals. Subjects were scored as positive if the RI was higher than 1.

Root Mean Square Fluctuation (RMSF) and Electrostatic Potential Surface Calculation

Molecular dynamics simulations were carried out using GROMACS 5.1.2 (37) software package. Gromos53a6 (38) force field was used. Simple point charge water model (39) was used to solvate the system. Charges were neutralized using Na+ and Cl− ions. Steepest descent method was used for energy minimization. Further, 100 ps temperature equilibration was carried out at a temperature of 300 K in the presence of position restraints of 1,000 KJ/mol and the pressure coupling of 1,000 ps at 1 bar of atmospheric pressure. After equilibration, the simulation of 200,000 ps (200 ns) without position restraints was carried out. All simulations were run three times, and consistent results were recorded. RMSF was analyzed from simulation trajectory using GROMACS utilities. The Electrostatic potential surface for the PvCelTOS was calculated using APBS (40) and visualized in PyMOL (Pymol LLC) and the electrostatic potential surfaces for the contours from −3kT/e (red) to +3kT/e (blue) were visualized. The figures were rendered using PyMol.

Statistical Analysis

All statistical analyzes were carried out using Prism 5.0 for Windows (GraphPad Software, Inc.). The one-sample Kolmogorov–Smirnoff test was used to determine whether a variable was normally distributed. The Mann–Whitney test was used to compare RIs of IgG against recombinant PvCelTOS between studied groups. Differences in proportions of the RI of IgG subclasses and epidemiological parameters were evaluated by Fisher's exact test and associations between antibody responses and epidemiological data were determined by Spearman rank test. A two-sided p value <0.05 was considered significant.

RESULTS

Epidemiological Profile of Studied Individuals

Most studied individuals were adults and naturally exposed to malaria infection throughout the years (**Table 1**). Age ranged from 10 to 89 years with an average of 36.9. The proportion of men was significantly higher (53.8%) than for women (46.2%; $\chi^2 = 5.761$, $p < 0.0164$). Regarding the previous personal history of malaria, only seven individuals reported no malaria episode (1.3%). Among those who remembered the *Plasmodium* species, the majority (29.9%) reported infections by *P. falciparum* and *P. vivax*. The number of past malaria episodes also varied greatly among donors, ranging from 0 to 50 (mean = 7.74 ± 16.5). Finally, the time elapsed since the last malaria episode ranged from 0 to 480 months (mean = 71.7 ± 77.9). Interestingly, a correlation trend was observed between the time of residence in the endemic area and the number of previous malaria infections ($p = 0.0003$; $r = 0.153$). Collectively, the epidemiological inquiry indicated that the studied population had different degrees of exposure and/or immunity.

TABLE 1 | Summary of the epidemiological data of the studied population.

	Overall	PvCelTOS IgG responders	PvCelTOS IgG non-responders (NRs)
Gender—*N* (%)			
Male	284 (53.8%)	55 (58.5%)	229 (52.8%)
Female	244 (46.2%)	39 (41.5%)	205 (47.2%)
Total	528	94	434
Malaria exposure—median (IR)			
Age (years)	36 (25–50)	38 (21–55.5)	36 (21–50)
Time of residence in endemic area (years)	33 (19–49)	35 (21–55)	33 (19–48)
Number of previous malaria episodes (*n*)	4 (2–10)	4.5 (2–10)	4 (2–10)
Time since the last malaria episode (months)	51 (24–91)	60 (13.7–89.2)	51 (24–90.5)
Frequency of recent malaria episodes (%)	12.7%	16.0%	13.1%
Previous malaria species contracted—*N* (%)			
Never infected	7 (1.3%)	0 (0%)	7 (1.6%)
Plasmodium falciparum	32 (6.1%)	5 (5.3%)	27 (6.2%)
Plasmodium vivax	125 (23.7%)	25 (26.6%)	100 (23%)
Both species	158 (29.9%)	31 (33%)	127 (29.3)
Not reported/remember	206 (39%)	33 (35.1%)	173 (39.9%)

Values of age, time of residence in endemic areas, number of previous malaria episodes, and time elapsed from the last malaria episode represent the median (interquartile range), while the parameter "frequency of recent malaria episodes" represents the percentage of individuals who reported malaria episode in the last year. The frequency of individuals who present recent malaria episodes was compared by Fisher's test, and other epidemiological parameters were compared by Mann–Whitney test. No statistical difference was observed between epidemiological parameters of responders and NR individuals.

PvCelTOS Is Naturally Immunogenic with the Prevalence of Cytophilic Antibodies in Brazilian Amazon Individuals

To test if the PvCelTOS is a target for naturally acquired antibodies in Amazon individuals, we first assessed the IgG reactivity profile against the recombinant protein. The plasma samples collected from the 528 individuals living in the endemic area reveal a low frequency of responders to PvCelTOS, since only 17.8% of the studied population (94 individuals) presented

specific IgG antibodies against the protein. Interestingly, the epidemiological data were similar between responders and NRs against this protein (**Table 1**). On both groups, responders and NRs, the age, time of residence in endemic area, the number of previous malaria episodes, the number of recent malaria episodes, the frequency of individuals with recent malaria episodes, and months elapsed from the last malaria episode were similar ($p > 0.05$).

Among the group of responders to PvCelTOS, the RI ranged from 1.01 to 19.93 (median = 1.205; interquartile range = 1.082; 1.552), reflecting a wide spectrum in magnitude of naturally acquired IgG response. The IgG subclass profile was marked by IgG1, the most prevalent subclass, present in 65.96% of responders, and with major RI (median = 1.15; interquartile range = 0.86–1.68) compared to IgG2 (median = 0.9; interquartile range = 0.66–1.2), IgG3 (median = 0.88; interquartile range = 0.72–1.06), and IgG4 (median = 0.62; interquartile range = 0.51–0.76) (**Figure 1**). Moreover, IgG3 RIs were directly correlated to the number of recent malaria episodes ($p = 0.003$; $r = 0.315$; Figure S1A in Supplementary Material) and inversely associated with the time elapsed from the last malaria episode ($p = 0.031$; $r = -0.258$; Figure S1B in Supplementary Material).

High IgG RIs against PvCelTOS Are Driven by Cytophilic Antibodies and Associated with Recent Infections

In order to identify possible factors that could be associated with this large spectrum of reactivity against PvCelTOS in IgG-positive individuals, we explored epidemiological data among responders. Initially, we observed that the RI against PvCelTOS was directly correlated with the number of previous malaria episodes ($p = 0.047$; $r = 0.227$; Figure S1C in Supplementary Material) and inversely correlated with the time elapsed from the last malaria episode ($p = 0.045$; $r = -0.24$; Figure S1D in Supplementary Material). Based on these findings, responder individuals were divided into two subgroups: high responders (HRs; individuals who had RI of IgG against PvCelTOS higher than 2) and low responders (LRs; individuals who had RI of IgG against PvCelTOS between 1 and 2). **Figure 2A** illustrates the means of epidemiological parameters of HRs, LRs, and NRs to PvCelTOS. Interestingly, while NRs and LRs presented a very similar profile of epidemiological parameters, HRs presented a statistically higher number of previous malaria episodes in comparison to NR and LR ($p = 0.0058$; $p = 0.0051$, respectively). Moreover, despite no statistical differences could be observed on the time elapsed from the last malaria episode ($p = 0.15$ in ANOVA test), the frequency of individuals who reported recent episodes of malaria was higher in HR (41.6%) than LR (12%, $p = 0.02$) and NR (13.1%, $p = 0.016$). Moreover, the proportion of RIs of cytophilic over non-cytophilic antibodies (IgG1 + IgG3/IgG2 + IgG4) presented direct correlation with RI of IgG of responder individuals ($p = 0.0016$; $r = 0.32$), suggesting that higher RI could be associated with a cytophilic profile of humoral response against PvCelTOS. Interestingly, although the proportion of individuals with cytophilic profile was similar in both groups, HR and LR (83% and 78%, respectively), the ratio of (cytophilic/non-cytophilic) antibodies was significantly higher in HR than LR ($p = 0.0076$) (**Figure 2B**).

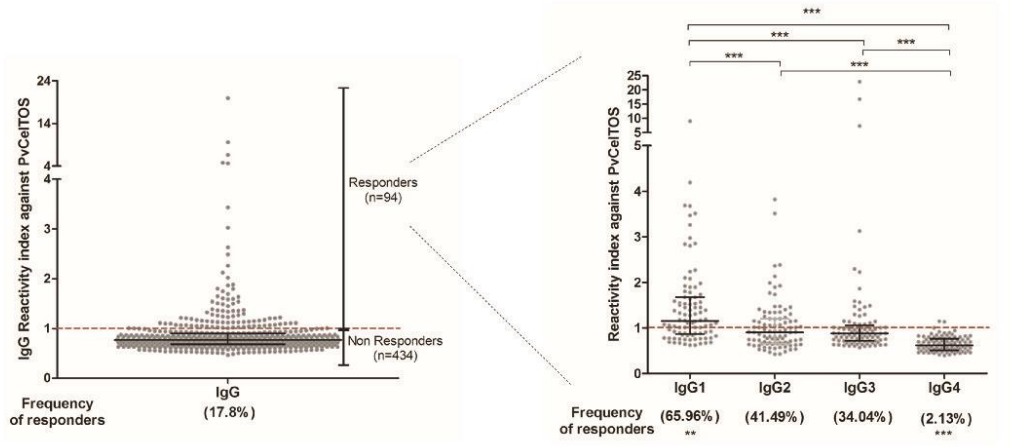


FIGURE 1 | Reactivity index (RI) of IgG and subclass against PvCelTOS. On both graphs, each point represents an individual RI against PvCelTOS and the red traced line represents the cutoff. Ninety-four individuals presented RI against PvCelTOS higher than 1 and were considered responders to this protein. Among the responders, IgG1 was the prevalent subclass in comparison to IgG2 ($p = 0.0012$), IgG3 ($p < 0.0001$), and IgG4 ($p < 0.0001$). Additionally, the RI of IgG1 was higher than the RI of all other subclasses ($p < 0.0001$), and the RI of IgG4 was statistically lower than all other subclasses ($p < 0.0001$).

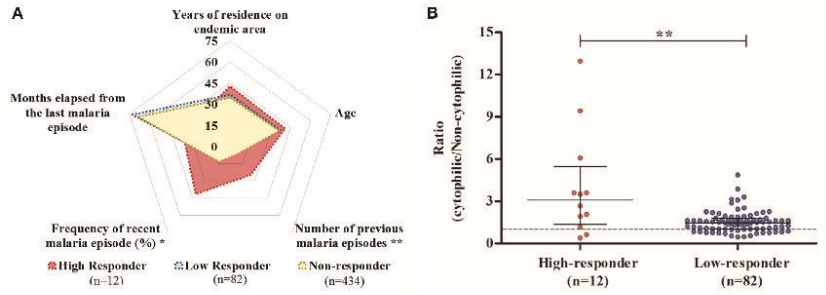


FIGURE 2 | Comparison of profiles of high responders (HRs), low responders (LRs), and non-responders (NRs) to PvCelTOS. (A) Diagram of epidemiological data. The mean values of epidemiological parameters of HRs, LRs, and NRs to PvCelTOS are represented by red, blue, and yellow areas, respectively. Age and time of residence in endemic areas are expressed in years, while time elapsed from the last malaria episode is expressed in months and frequency of recent malaria episodes indicates the percentage of individuals who reported malaria episodes in the last year. HRs presented a higher number of previous malaria episodes and higher frequency of recent malaria episodes than LRs ($p = 0.0051$ and $p = 0.02$, respectively) **(B)** Comparison of ratio (cytophilic/non-cytophilic) antibodies. Red and blue points represent the HR and LR, respectively. Points above the value of 1 represent individuals with a cytophilic profile of IgG (IgG1 + IgG3, higher than IgG2 + IgG4).

Five Immunogenic Regions Identified in PvCelTOS and Two Linear B-Cell Epitopes Broadly Recognized by Naturally Acquired IgG Antibodies

Four B-cell linear epitopes were predicted *in silico* in the entire sequence of PvCelTOS ($PvCelTOS_{K6\text{-}N13}$; $PvCelTOS_{G38\text{-}R57}$; $PvCelTOS_{I136\text{-}E143}$; $PvCelTOS_{K166\text{-}S191}$).

In order to validate the prediction data and identify possible non-predicted immunogenic regions of PvCelTOS, plasma from IgG responders to PvCelTOS was tested against 32 overlapping peptides corresponding to the complete amino acid sequence. First, 10 peptides (N13-L27; S19-V33; E73-I87; L79-K93; S97-A111; P127-V141; I133-G147; P139-V153; L181-L195; E182-D196) were broadly recognized by responders to PvCelTOS (**Figure 3**). Two of the predicted epitopes ($PvCelTOS_{I136\text{-}E143}$ and $PvCelTOS_{K166\text{-}S191}$) were present (partially or entirely) in peptides confirmed as naturally immunogenic. Interestingly, peptides I133-G147 and E182-D196 were recognized by IgG specific antibodies of responders to PvCelTOS in frequencies higher than 50% (92% and 54%, respectively) and presented median of RI higher than 1 (1.79 and 1.14, respectively). In addition, peptides P127-V141, P139-V153, and L181-L195 were located besides the most immunogenic peptides and presented overlapped sequences, which were also recognized by IgG antibodies in moderate frequencies. Peptide I133-G147 (ASTIKPPRVSEDAYF) presented the highest IgG RI ($p < 0.0001$ by ANOVA test) and the highest frequency of recognition (92%) compared to all other peptides. While it contains the entire sequence of predicted epitope $PvCelTOS_{136\text{-}143}$, peptides P127-V141 and P139-V153, which contain only the partial sequence of the predicted epitope, presented minor frequencies of recognition (38% and 39%, respectively; $p < 0.0001$ on Fisher's exact test). The peptides L181-L195 and 186-196 were both partially inserted in the predicted linear epitope $PvCelTOS_{166\text{-}191}$ and could be the immune dominant sequence of this longer predicted epitope. These data supported the prediction of linear B-cell epitopes $PvCelTOS_{I136\text{-}E146}$ and $PvCelTOSK_{166\text{-}S191}$. Conversely, peptides N13-L27, S19-V33, and S97-A111 also presented frequency of recognition about 40% (38, 40, and 36%, respectively). After the confirmation of five immunogenic regions and two immunodominant epitopes in PvCelTOS, we also compared the RI and frequencies between HR and LR for PvCelTOS. However, no differences were found.

Main B Cell Epitopes Are Present on PvCelTOS Surface

Peptides that presented overlapped amino acids and were recognized by more than 20% of responders to PvCelTOS (**Figure 3**) were grouped as immunogenic regions. All peptides inserted in identified immunogenic regions are listed in **Table 2** with their respective frequencies of recognition, BepiPred and ESA scores. In this context, we identified five immunogenic regions $PvCelTOS_{N13\text{-}V33}$, $PvCelTOS_{E73\text{-}K93}$, $PvCelTOS_{S97\text{-}A111}$, $PvCelTOS_{P127\text{-}V153}$, and $PvCelTOS_{L181\text{-}D196}$, in which B-cell epitopes could be inserted. Interestingly, the peptides with higher frequency of specific responders (I133-G147, L181-L195, and 182-186) presented a good combination of BepiPred and ESA score. The molecular dynamics and electrostatic potential surface of PvCelTOS indicate regions P127-V153, N13-V33, and L181-D186 as more flexible than E73-K93 and S97-A111 (**Figure 4A**). Regarding solvent exposure, all immunogenic regions were exposed and accessible in solution. Interestingly, the immunogenic regions L181-D196 and E73-K93 are part of a very negatively charged region, while N13-V33 and P127-V153 are in a mostly neutral-positive region (**Figure 4B**).

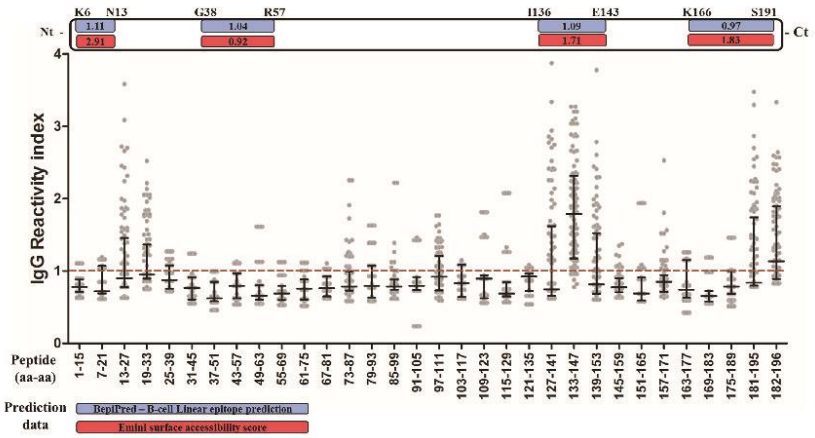


FIGURE 3 | Mapping of B-cell epitopes in PvCelTOS. Each column represents a peptide, the numbers indicate the first and last amino acid (aa-aa) of the peptide. The points represent the value of IgG reactivity index (RI) specific for each peptide of one responder to PvCelTOS and the red traced line represents the cutoff value. The black lines indicate median and interquartile range. If the RI for one peptide was higher than 1, the individual was considered positive to this peptide. The white bar on top represents the linear structure of the protein, in which the blue boxes indicate the BepiPred prediction score and red boxes indicate the Emini surface accessibility score of predicted linear epitopes.

DISCUSSION

Despite significant advances in the understanding of the biology of *Plasmodium* parasites and the immune response elicited by these pathogens, there is not yet a subunit vaccine capable of providing long-lasting protection. The cell-traversal protein for ookinetes and sporozoites (CelTOS) has been considered a potential novel alternative for a vaccine against malaria (29, 32, 41), but the knowledge on *P. vivax* CelTOS potential remains scarce. Unfortunately, many conventional vaccinology strategies applied to *P. falciparum* are especially difficult when dealing with non-cultivable microorganisms such as *P. vivax*. Consequently, seroepidemiological studies have played a significant role in the identification and validation of *P. vivax* vaccine candidates (42–48). Therefore, we confirmed the naturally acquired humoral response against PvCelTOS (IgG and IgG subclass) and identified five B-cell epitopes along the entire PvCelTOS amino acid sequence, which were recognized by IgG antibodies from malaria-exposed populations from Brazilian Amazon.

Plasma samples were collected in three cross-sectional studies with Brazilian Amazon communities between 2013 and 2015. The profile of the studied individuals shows that our population included rainforest region natives and migrants from non-endemic areas of Brazil who had lived in the area for more than 10 years. The majority of individuals reported a prior experience with *P. vivax* and/or *P. falciparum* malaria. Concerning malaria history, the highly variable range of number of previous infections, time of residence in endemic areas, and time since the last infection suggests differences in exposure and immunity, since it is well known that the acquisition of clinical immunity mediated by antibodies depends on continued exposure to the parasite (49–51). The correlation between time of residence in endemic areas and months since the last infection observed in our study also indicates that this phenomenon could be occurring in low/medium endemic areas like the Brazilian Amazon. Therefore, the selection of these individuals was ideal to detect the presence of antibodies against the new recombinant antigen and distinguish whether the alterations found were related to malaria exposure and/or indicatives of protection.

First, we found 94 individuals presenting specific antibodies to PvCelTOS and confirmed the natural immunogenicity of PvCelTOS among exposed individuals from Brazilian Amazon. Recently, Longley and collaborators also reported the first evidence of naturally induced IgG responses to *Pv*CelTOS in human volunteers from Western Thailand (33). Interestingly, the frequency of responders to PvCelTOS observed in our studied population (17.8%) was similar to the frequency observed by Longley on uninfected and clinical malaria individuals (33). Moreover, the low humoral reactivity against PvCelTOS is commonly found in other *Plasmodium* preerythrocytic antigens (48, 52, 53). The short life of specific antibodies, host genetic factors, and/or epidemiological parameters could be possible reasons for the low frequency of responders against PvCelTOS in endemic areas. The short life of specific PvCelTOS humoral response hypothesis does not seem to occur since Longley et al. verified that IgG positivity and magnitude of response were present over the 1-year period in the absence of *P. vivax* infections (33). Our study also describes anti-PvCelTOS antibodies in individuals who reported no malaria in the last 10 years or more. However, in both cases, the contact between human host and sporozoite antigens in transmission areas was not evaluated. In relation to host genetic factors, there is a significant body of

TABLE 2 | Identification of immunogenic regions in PvCelTOS.

Immunogenic region	Sequence	Reactivity index (RI) Mean (CI 95%)	Responders (%)	Position	Peptide sequence	Specific responders (%)	BepiPred score	ESA score
$PvCelTOS_{N13-V33}$	NKVNRV**SIICAFLAL**FGFVNV	1.17 (1.07–1.27)	45%	13–27	NKVNRV**SIICAFLAL**	38%	−1.52	0.35
				19–33	**SIICAFLAL**FGFVNV	40%	−2.37	0.07
$PvCelTOS_{E73-K93}$	EVIGNE**LADNIANEI**VSSLQK	0.93 (0.88–0.99)	30%	73–87	EVIGNELADNIANEI	22%	0.01	0.70
				79–93	LADNIANEIVSSLQK	20%	0.14	0.56
$PvCelTOS_{S97-K111}$	SFLQSGFDVKTQLKA	0.96 (0.89–1.01)	38%	97–111	SFLQSGFDVKTQLKA	38%	0.12	0.91
$PvCelTOS_{P127-V163}$	PTEKIVAST**IKPPRVSEDAYFLLG**PVV	1.39 (1.27–1.50)	69%	127–141	PTEKIVASTIKPPRV	38%	0.69	0.89
				133–147	IKPPRVSEDAYFLLG	92%	0.52	1.14
				139–153	PRVSEDAYFLLGPVV	39%	−0.08	0.75
$PvCelTOS_{L181-D196}$	L**EEEEAEDEFSDELL**D	1.32 (1.21–1.44)	53%	181–195	LEEEEAEDEFSDELL	43%	0.95	2.15
				182–196	EEEEAEDEFSDELLD	54%	0.84	2.08

Peptides with overlapping and recognized by more than 20% of responders to PvCelTOS were grouped in immunogenic regions. The RI of an immunogenic region represents the mean of RI of all peptides inserted in that immunogenic region with a confident interval of 95%. The frequency of recognition of immunogenic regions was defined based on the number of individuals whith RI to immunogenic region higher than 1. The peptides combined in an immunogenic region were listed with their respective frequencies of recognition, BepiPred score, and Emini surface accessibility (ESA) score. Overlapped mers were signalized by underlined bold typeface on immunogenic region sequence.

evidences of its influence in malaria outcomes and the capacity to mount a humoral immune response (54–57). To date, associations of HLA class II on humoral immune response to malaria antigens were reported in individuals living in malaria-endemic areas from Brazilian Amazon (58, 59) and in human vaccine trials (60–62). In *P. vivax* preerythrocytic targets, the presence of HLA-DRB1*03 and DR5 was associated with the absence of antibody response to the CSP amino-terminal region (48) and HLA-DRB1*07 was related to the absence of specific antibodies for CSP repeats of VK210 (52). Moreover, Chaves and collaborators reported that PvCelTOS gene sequence is highly conserved among isolates from different Brazilian geographic regions (unpublished data), suggesting a low selective pressure by immune response against PvCelTOS. In our view, the influence of immunogenetic factors in PvCelTOS-specific humoral response are feasible, but more studies are still necessary to confirm this hypothesis.

Regarding the influence of epidemiological factors, we initially tried to investigate the associations between exposition to malaria and the frequency of IgG responders to PvCelTOS. Surprisingly, although the association of epidemiological data with specific response against *Plasmodium* antigens was well characterized on several studies (63–65), we observed a similar epidemiological profile between responders and NRs to PvCelTOS. Therefore, we focused on the search of distinct epidemiological and IgG subclass profiles among PvCelTOS responder individuals. The knowledge about the antibody subclass profile is critical to suggest functional antimalarial immunity and to evaluate potential vaccine candidates. Cytophilic antibodies (IgG1 and IgG3) are frequently prevalent on immune serum from high-transmission areas (66–69) and often correlate with protection from disease (70–72). In our study, IgG1 presented higher frequencies of responders and median RI than all other subclasses. Moreover, IgG3 RIs were directly associated with the number of malaria episodes over the last 12 months and inversely correlated with the time elapsed from the last malaria episode, suggesting that recent *P. vivax* infections can raise the levels of anti-PvCelTOS specific IgG3. The sterile protective immunity to malaria was recently associated with a panel of antigens (28), and the relationship of cytophilic antibodies and reduced risk of symptoms are a common finding in high endemic areas (70–74). However, in our study, concerning the higher levels of IgG1 for PvCelTOS and the association of IgG3 levels with recent infections, we cannot confirm or discard its role as part of protective humoral response until more conclusive studies, such as sporozoite inhibition by anti-PvCelTOS specific antibodies, are conducted. In the same way, among responders, IgG RIs were directly correlated with the number of previous malaria episodes and inversely correlated with the time elapsed from the last malaria episodes, suggesting that antibody levels for PvCelTOS could be associated with recent infections.

The influence of epidemiological parameters on immunity to malaria was previously observed in studies from Brazilian Amazon population. Based on previous studies that associated high levels of antibodies with multiple preerythrocytic antigens with reduced risk of clinical malaria in children (75) and decreased risk of infection in adults (68), we also aimed to investigate if

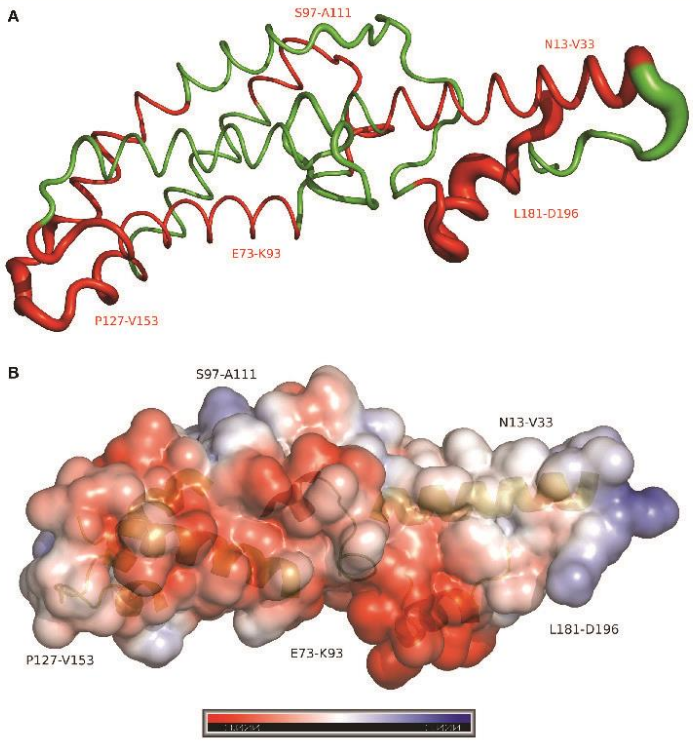


FIGURE 4 | Molecular dynamics and electrostatic potential surface for the PvCelTOS. (A) Sausage plot of the PvCelTOS. The red color identifies the immunogenic regions of PvCelTOS. Thickness depicts relative fluctuation as calculated during molecular dynamics. The thinnest segments represent the most stable regions of the protein. **(B)** The surface model shows the electrostatic potential surface of the PvCelTOS, representing the positive (blue) and negative (red) charges. The secondary structure in the background represents the immunogenic region.

the epidemiological parameters could reveal new findings about the role of exposition on PvCelTOS immunogenicity. Therefore, we subdivided the large spectrum IgG RIs among PvCelTOS responders into HRs (RI > 2) and LRs (RI < 2). Although LRs and NRs to PvCelTOS presented similar exposition factors to malaria, interestingly, HR individuals presented a remarkable higher number of previous malaria episodes, frequency of recent malaria episodes, and a higher ratio of cytophilic/non-cytophilic antibodies than LRs. This observation suggested that higher level of exposition to malaria induced a more intense and improved humoral response against PvCelTOS. Unfortunately, the cross-sectional design of our study limited the investigation to retrospective malaria histories, and the best approximation of an individual's protection was the estimated amount of time that had passed since their last malaria episode, which presented no significant association with IgG response against PvCelTOS. Prospective studies on humoral immune responses and studies addressing the ability of these antibodies to interfere the motility/invasion of sporozoites (76, 77) will provide more evidences of the protective role of anti-PvCelTOS antibodies.

Information at the amino acid level about the epitopes of proteins recognized by antibodies is important for their use as biological tools and for understanding general molecular recognition events (78). In this context, epitope prediction programs have been widely used in malaria research (4, 79–81). Nevertheless, the use of chemically prepared arrays of short peptides is a more powerful tool to identify and characterize epitopes recognized by antibodies (46, 82, 83). It is also important to mention that in order to raise antibodies for a peptide, a minimum length of six amino acids is required, and peptides of >10 amino acids are generally required for the induction of antibodies that may bind to the native protein (84). In this context, the synthesis of 15 amino acid peptides, with 9 overlapping, has allowed the identification of PvCelTOS B-cell epitopes encompassed in sequences ranging from 15 to 27 amino acids in length. Therefore, after the confirmation of PvCelTOS as naturally immunogenic in exposed populations, the present

paper describes for the first time the fine B cell epitope mapping of a full-length protein. Initially, 10 peptides were specifically recognized by naturally acquired antibodies from PvCelTOS responders. After a combination of *in silico* approaches and recognition of overlapped peptides, five immunogenic regions were confirmed ($PvCelTOS_{13-33}$, $PvCelTOS_{73-93}$, $PvCelTOS_{97-111}$, $PvCelTOS_{127-153}$, and $PvCelTOS_{181-196}$) in different frequencies and RIs. Moreover, the main linear epitope (ASTIKPPRVSEDAYF) presented highest IgG RI and frequency compared to all other naturally recognized peptides, suggesting that the majority of naturally acquired antibodies against PvCelTOS are directed to the C-terminal region. Moreover, T cell responses to PvCelTOS may also help to determine the immunodominant repertoire in individuals living in malaria-endemic regions, which could also supply information for the development of a vaccine for PvCelTOS. In humans, PfCelTOS derivate peptides elicited proliferative and IFN-γ responses in *ex vivo* ELISPOT assays using peripheral blood mononuclear cells from naturally exposed individuals living in Ghana (30).

Recently, CelTOS was demonstrated as highly conserved protein across several large groups of apicomplexan parasites including *Plasmodium* spp., *Cytauxzoon*, *Theileria*, and *Babesia* and considered essential to cell infection, traversal, and membrane disruption (85). Despite the genetical differences between PfCelTOS and PvCelTOS, it is important to mention that Bergmann-Leitner and colleagues immunized mice and rabbits with recombinant PfCelTOS and also observed specific antibodies for linear B-cell epitopes at C-terminal (82). These observations suggested that CelTOS could present a similar conformation among species, with similar regions targeted by antibodies. We considered that the exposition of linear epitopes is a critical step to their recognition by circulating antibodies; therefore, the combination of ESA, molecular dynamics, and electrostatic potential surface was used as a complementary approach to predict the exposition of epitope sequences on protein surface. All immunogenic regions identified were exposed and accessible to antibodies. This finding could be important in a future subunit vaccine composition based on these identified regions. However, the potential of these specific antibodies directed main PvCelTOS epitopes in the inhibition of sporozoite motility, invasion, and/or traversal remains to be investigated.

AUTHOR CONTRIBUTIONS

JL-J did study designing, performed experiments, data analysis, manuscript preparation, and manuscript review. RR-d-S did study designing, performed experiments, data analysis, and manuscript preparation. IS performed experiments. CL-C did recombinant protein expression and manuscript review. JM did molecular dynamics and bioinformatics and manuscript review. DP-d-S performed collection of blood and epidemiological data. AF did fieldwork support. AT performed collection of blood and epidemiological data and diagnosis. FP did fieldwork support. LC performed experiments. LP-R did data analysis and manuscript review. AR-S did recombinant protein expression, data analysis, and manuscript review. DB did study designing, fieldwork support, manuscript review, and data analysis.

ACKNOWLEDGMENTS

We are grateful to all volunteers who made this study possible.

FUNDING

This study was supported by the Brazilian National Research Council – CNPq/PAPES. JL-J is recipient of FAPERJ APQ1 (E-26/210.653/2015), Jovem Cientista do Nosso Estado (E26/203.255/2016), and CPNq-Universal research grants (445150/2014-9). AR-S is supported by a Wellcome Trust Career Development Fellowship 097395/Z/11/Z.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00077/full#supplementary-material.

FIGURE S1 | Associations of humoral response and exposition parameters in responders to PvCelTOS. (A) Spearman correlation between IgG3 reactivity index (RI) and number of recent malaria episodes; **(B)** Spearman correlation between IgG3 and months elapsed since the last malaria episode; **(C)** Spearman correlation between anti-PvCelTOS IgG reactivity index and number of previous malaria episodes; and **(D)** Spearman correlation between anti-PvCelTOS IgG RI and months elapsed since the last malaria episode.

REFERENCES

1. W.H.O. *World Malaria Report*. Geneva: WHO (2015).
2. Richie TL, Saul A. Progress and challenges for malaria vaccines. *Nature* (2002) 415(6872):694–701. doi:10.1038/415694a
3. W.H.O. *World Malaria Report 2014*. Geneva: WHO (2014).
4. Rodrigues-da-Silva RN, Martins da Silva JH, Singh B, Jiang J, Meyer EV, Santos F, et al. In silico identification and validation of a linear and naturally immunogenic B-cell epitope of the *Plasmodium vivax* malaria vaccine candidate merozoite surface protein-9. *PLoS One* (2016) 11(1):e0146951. doi:10.1371/journal.pone.0146951
5. WHO. *Tables of Malaria Vaccine Projects Globally ("Rainbow Tables")*. (2015). Available from: http://www.who.int/immunization/research/development/Rainbow_tables/en/
6. 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(7):449–54. doi:10.1016/S1473-3099(08)70153-1
7. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM. Vivax malaria: neglected and not benign. *Am J Trop Med Hyg* (2007) 77(6 Suppl):79–87.
8. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. *Malar J* (2014) 13:481. doi:10.1186/1475-2875-13-481
9. O'Brien AT, Ramirez JF, Martinez SP. A descriptive study of 16 severe *Plasmodium vivax* cases from three municipalities of Colombia between 2009 and 2013. *Malar J* (2014) 13:404. doi:10.1186/1475-2875-13-404
10. Gougoutsi A, Karageorgopoulos DE, Dimitriadou A, Melas N, Kranidiotis G, Voutsinas D, et al. Severe *Plasmodium vivax* malaria complicated with acute

respiratory distress syndrome: a case associated with focal autochthonous transmission in Greece. *Vector Borne Zoonotic Dis* (2014) 14(5):378–81. doi:10.1089/vbz.2012.1192
11. Zubairi AB, Nizami S, Raza A, Mehraj V, Rasheed AF, Ghanchi NK, et al. Severe *Plasmodium vivax* malaria in Pakistan. *Emerg Infect Dis* (2013) 19(11):1851–4. doi:10.3201/eid1911.130495
12. Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ. Global extent of chloroquine-resistant *Plasmodium vivax*: a systematic review and meta-analysis. *Lancet Infect Dis* (2014) 14(10):982–91. doi:10.1016/S1473-3099(14)70855-2
13. de Santana Filho FS, Arcanjo AR, Chehuan YM, Costa MR, Martinez-Espinosa FE, Vieira JL, et al. Chloroquine-resistant *Plasmodium vivax*, Brazilian Amazon. *Emerg Infect Dis* (2007) 13(7):1125–6. doi:10.3201/eid1307.061386
14. Ruebush TK II, Zegarra J, Cairo J, Andersen EM, Green M, Pillai DR, et al. Chloroquine-resistant *Plasmodium vivax* malaria in Peru. *Am J Trop Med Hyg* (2003) 69(5):548–52.
15. Nayar JK, Baker RH, Knight JW, Sullivan JS, Morris CL, Richardson BB, et al. Studies on a primaquine-tolerant strain of *Plasmodium vivax* from Brazil in Aotus and Saimiri monkeys. *J Parasitol* (1997) 83(4):739–45. doi:10.2307/3284254
16. Kristensen KL, Dragsted UB. Recurrent *Plasmodium vivax* malaria due to dose-dependent primaquine resistance: a case report. *Scand J Infect Dis* (2014) 46(1):63–5. doi:10.3109/00365548.2013.822093
17. Arias AE, Corredor A. Low response of Colombian strains of *Plasmodium vivax* to classical antimalarial therapy. *Trop Med Parasitol* (1989) 40(1):21–3.
18. Swearingen KE, Lindner SE, Shi L, Shears MJ, Harupa A, Hopp CS, et al. Interrogating the Plasmodium Sporozoite Surface: Identification of Surface-Exposed Proteins and Demonstration of Glycosylation on CSP and TRAP by Mass Spectrometry-Based Proteomics. *PLoS Pathog* (2016) 12(4):e1005606. doi:10.1371/journal.ppat.1005606
19. Clyde DF, McCarthy VC, Miller RM, Hornick RB. Specificity of protection of man immunized against sporozoite-induced falciparum malaria. *Am J Med Sci* (1973) 266(6):398–403. doi:10.1097/00000441-197309000-00002
20. Rieckmann KH, Carson PE, Beaudoin RL, Cassells JS, Sell KW. Letter: sporozoite induced immunity in man against an Ethiopian strain of *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg* (1974) 68(3):258–9. doi:10.1016/0035-9203(74)90129-1
21. Egan JE, Hoffman SL, Haynes JD, Sadoff JC, Schneider I, Grau GE, et al. Humoral immune responses in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. *Am J Trop Med Hyg* (1993) 49(2):166–73.
22. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* (2002) 185(8):1155–64. doi:10.1086/339409
23. Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, et al. Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. *N Engl J Med* (2013) 368(12):1111–20. doi:10.1056/NEJMoa1207564
24. Rts SCTP, Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N Engl J Med* (2012) 367(24):2284–95. doi:10.1056/NEJMoa1208394
25. Bejon P, Cook J, Bergmann-Leitner E, Olotu A, Lusingu J, Mwacharo J, et al. Effect of the pre-erythrocytic candidate malaria vaccine RTS,S/AS01E on blood stage immunity in young children. *J Infect Dis* (2011) 204(1):9–18. doi:10.1093/infdis/jir222
26. Olotu A, Lusingu J, Leach A, Lievens M, Vekemans J, Msham S, et al. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. *Lancet Infect Dis* (2011) 11(2):102–9. doi:10.1016/S1473-3099(10)70262-0
27. Gruner AC, Mauduit M, Tewari R, Romero JF, Depinay N, Kayibanda M, et al. Sterile protection against malaria is independent of immune responses to the circumsporozoite protein. *PLoS One* (2007) 2(12):e1371. doi:10.1371/journal.pone.0001371
28. Trieu A, Kayala MA, Burk C, Molina DM, Freilich DA, Richie TL, et al. Sterile protective immunity to malaria is associated with a panel of novel *P. falciparum* antigens. *Mol Cell Proteomics* (2011) 10(9):M111007948. doi:10.1074/mcp.M111.007948
29. Kariu T, Ishino T, Yano K, Chinzei Y, Yuda M. CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Mol Microbiol* (2006) 59(5):1369–79. doi:10.1111/j.1365-2958.2005.05024.x
30. Anum D, Kusi KA, Ganeshan H, Hollingdale MR, Ofori MF, Koram KA, et al. Measuring naturally acquired ex vivo IFN-gamma responses to *Plasmodium falciparum* cell-traversal protein for ookinetes and sporozoites (CelTOS) in Ghanaian adults. *Malar J* (2015) 14:20. doi:10.1186/s12936-014-0539-5
31. Kusi KA, Bosomprah S, Dodoo D, Kyei-Baafour E, Dickson EK, Mensah D, et al. Anti-sporozoite antibodies as alternative markers for malaria transmission intensity estimation. *Malar J* (2014) 13:103. doi:10.1186/1475-2875-13-103
32. Bergmann-Leitner ES, Mease RM, De La Vega P, Savranskaya T, Polhemus M, Ockenhouse C, et al. Immunization with pre-erythrocytic antigen CelTOS from *Plasmodium falciparum* elicits cross-species protection against heterologous challenge with *Plasmodium berghei*. *PLoS One* (2010) 5(8):e12294. doi:10.1371/journal.pone.0012294
33. Longley RJ, Reyes-Sandoval A, Montoya-Diaz E, Dunachie S, Kumpitak C, Nguitragool W, et al. Acquisition and longevity of antibodies to *Plasmodium vivax* preerythrocytic antigens in Western Thailand. *Clin Vaccine Immunol* (2016) 23(2):117–24. doi:10.1128/CVI.00501-15
34. Stanisic DI, Fowkes FJ, Koinari M, Javati S, Lin E, Kiniboro B, et al. Acquisition of antibodies against *Plasmodium falciparum* merozoites and malaria immunity in young children and the influence of age, force of infection, and magnitude of response. *Infect Immun* (2015) 83(2):646–60. doi:10.1128/IAI.02398-14
35. Larsen JE, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. *Immunome Res* (2006) 2:2. doi:10.1186/1745-7580-2-2
36. Emini EA, Hughes JV, Perlow DS, Boger J. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J Virol* (1985) 55(3):836–9.
37. Berendsen HJC, van der Spoel D, van Drunen R. GROMACS: a message-passing parallel molecular dynamics implementation. *Comput Phys Commun* (1995) 91(1–3):13. doi:10.1016/0010-4655(95)00042-E
38. Oostenbrink C, Villa A, Mark AE, van Gunsteren WF. A biomolecular force field based on the free enthalpy of hydration and solvation: the GROMOS force-field parameter sets 53A5 and 53A6. *J Comput Chem* (2004) 25(13):1656–76. doi:10.1002/jcc.20090
39. William LJ, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J Chem Phys* (1983) 79:926–35. doi:10.1063/1.445869
40. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A* (2001) 98(18):10037–41. doi:10.1073/pnas.181342398
41. Bergmann-Leitner ES, Legler PM, Savranskaya T, Ockenhouse CF, Angov E. Cellular and humoral immune effector mechanisms required for sterile protection against sporozoite challenge induced with the novel malaria vaccine candidate CelTOS. *Vaccine* (2011) 29(35):5940–9. doi:10.1016/j.vaccine.2011.06.053
42. Bueno LL, Morais CG, Soares IS, Bouillet LE, Bruna-Romero O, Fontes CJ, et al. *Plasmodium vivax* recombinant vaccine candidate AMA-1 plays an important role in adaptive immune response eliciting differentiation of dendritic cells. *Vaccine* (2009) 27(41):5581–8. doi:10.1016/j.vaccine.2009.07.031
43. Amarasinghe S, Kathriarachchi H, Udagama P. Conserved regions of *Plasmodium vivax* potential vaccine candidate antigens in Sri Lanka: consious in silico analysis of prospective conformational epitope regions. *Asian Pac J Trop Med* (2014) 7(10):832–40. doi:10.1016/S1995-7645(14)60146-2
44. Xia H, Fang Q, Jangpatarapongsa K, Zhiyong T, Cui L, Li B, et al. A comparative study of natural immune responses against *Plasmodium vivax* C-terminal merozoite surface protein-1 (PvMSP-1) and apical membrane antigen-1 (PvAMA-1) in two endemic settings. *EXCLI J* (2015) 14:926–34. doi:10.17179/excli2015-388
45. Lima-Junior JC, Tran TM, Meyer EV, Singh B, De-Simone SG, Santos F, et al. Naturally acquired humoral and cellular immune responses to *Plasmodium vivax* merozoite surface protein 9 in Northwestern Amazon individuals. *Vaccine* (2008) 26(51):6645–54. doi:10.1016/j.vaccine.2008.09.029
46. Lima-Junior JC, Jiang J, Rodrigues-da-Silva RN, Banic DM, Tran TM, Ribeiro RY, et al. B cell epitope mapping and characterization of naturally acquired antibodies to the *Plasmodium vivax* merozoite surface protein-3alpha

(PvMSP-3alpha) in malaria exposed individuals from Brazilian Amazon. *Vaccine* (2011) 29(9):1801–11. doi:10.1016/j.vaccine.2010.12.099

47. Ladeia-Andrade S, Ferreira MU, Scopel KK, Braga EM, Bastos Mda S, Wunderlich G, et al. Naturally acquired antibodies to merozoite surface protein (MSP)-1(19) and cumulative exposure to *Plasmodium falciparum* and *Plasmodium vivax* in remote populations of the Amazon Basin of Brazil. *Mem Inst Oswaldo Cruz* (2007) 102(8):943–51. doi:10.1590/S0074-02762007000800009
48. Storti-Melo LM, da Costa DR, Souza-Neiras WC, Cassiano GC, Couto VS, Povoa MM, et al. Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with *Plasmodium vivax*. *Acta Trop* (2012) 121(2):152–5. doi:10.1016/j.actatropica.2011.10.009
49. Braga EM, Barros RM, Reis TA, Fontes CJ, Morais CG, Martins MS, et al. Association of the IgG response to *Plasmodium falciparum* merozoite protein (C-terminal 19 kD) with clinical immunity to malaria in the Brazilian Amazon region. *Am J Trop Med Hyg* (2002) 66(5):461–6.
50. Baird JK. Age-dependent characteristics of protection v. susceptibility to *Plasmodium falciparum*. *Ann Trop Med Parasitol* (1998) 92(4):367–90. doi:10.1080/00034989859366
51. Soe S, Theisen M, Roussilhon C, Aye KS, Druilhe P. Association between protection against clinical malaria and antibodies to merozoite surface antigens in an area of hyperendemicity in Myanmar: complementarity between responses to merozoite surface protein 3 and the 220-kilodalton glutamate-rich protein. *Infect Immun* (2004) 72(1):247–52. doi:10.1128/IAI.72.1.247-252.2004
52. Oliveira-Ferreira J, Pratt-Riccio LR, Arruda M, Santos F, Daniel Ribeiro CT, Goldberg AC, et al. HLA class II and antibody responses to circumsporozoite protein repeats of *P. vivax* (VK210, VK247 and *P. vivax*-like) in individuals naturally exposed to malaria. *Acta Trop* (2004) 92(1):63–9. doi:10.1016/j.actatropica.2004.02.011
53. Yildiz Zeyrek F, Palacpac N, Yuksel F, Yagi M, Honjo K, Fujita Y, et al. Serologic markers in relation to parasite exposure history help to estimate transmission dynamics of *Plasmodium vivax*. *PLoS One* (2011) 6(11):e28126. doi:10.1371/journal.pone.0028126
54. Modiano D, Petrarca V, Sirima BS, Luoni G, Nebie I, Diallo DA, et al. Different response to *Plasmodium falciparum* in west African sympatric ethnic groups: possible implications for malaria control strategies. *Parassitologia* (1999) 41(1–3):193–7.
55. Modiano D, Chiucchiuini A, Petrarca V, Sirima BS, Luoni G, Roggero MA, et al. Interethnic differences in the humoral response to non-repetitive regions of the *Plasmodium falciparum* circumsporozoite protein. *Am J Trop Med Hyg* (1999) 61(4):663–7.
56. Brisebarre A, Kumulungui B, Sawadogo S, Afridi S, Fumoux F, Rihet P. Genome-wide significant linkage to IgG subclass responses against *Plasmodium falciparum* antigens on chromosomes 8p22-p21, 9q34 and 20q13. *Genes Immun* (2015) 16(3):187–92. doi:10.1038/gene.2014.66
57. Afridi S, Atkinson A, Garnier S, Fumoux F, Rihet P. Malaria resistance genes are associated with the levels of IgG subclasses directed against *Plasmodium falciparum* blood-stage antigens in Burkina Faso. *Malar J* (2012) 11:308. doi:10.1186/1475-2875-11-308
58. Beck HP, Felger I, Barker M, Bugawan T, Genton B, Alexander N, et al. Evidence of HLA class II association with antibody response against the malaria vaccine SPF66 in a naturally exposed population. *Am J Trop Med Hyg* (1995) 53(3):284–8.
59. Banic DM, Goldberg AC, Pratt-Riccio LR, De Oliveira-Ferreira J, Santos F, Gras-Masse H, et al. Human leukocyte antigen class II control of the immune response to p126-derived amino terminal peptide from *Plasmodium falciparum*. *Am J Trop Med Hyg* (2002) 66(5):509–15.
60. Nardin EH, Oliveira GA, Calvo-Calle JM, Castro ZR, Nussenzweig RS, Schmeckpeper B, et al. Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes. *J Infect Dis* (2000) 182(5):1486–96. doi:10.1086/315871
61. Murillo LA, Rocha CL, Mora AL, Kalil J, Goldenberg AK, Patarroyo ME. Molecular analysis of HLA DR4-beta 1 gene in malaria vaccinees. Typing and subtyping by PCR technique and oligonucleotides. *Parasite Immunol* (1991) 13(2):201–10. doi:10.1111/j.1365-3024.1991.tb00275.x
62. Stephens HA, Brown AE, Chandanayingyong D, Webster HK, Sirikong M, Longta P, et al. The presence of the HLA class II allele DPB1*0501 in ethnic Thais correlates with an enhanced vaccine-induced antibody response to a malaria sporozoite antigen. *Eur J Immunol* (1995) 25(11):3142–7. doi:10.1002/eji.1830251123
63. Maitland K, Williams TN, Bennett S, Newbold CI, Peto TE, Viji J, et al. The interaction between *Plasmodium falciparum* and *P. vivax* in children on Espiritu Santo island, Vanuatu. *Trans R Soc Trop Med Hyg* (1996) 90(6):614–20. doi:10.1016/S0035-9203(96)90406-X
64. Luxemburger C, Thwai KL, White NJ, Webster HK, Kyle DE, Maelankirri L, et al. The epidemiology of malaria in a Karen population on the western border of Thailand. *Trans R Soc Trop Med Hyg* (1996) 90(2):105–11. doi:10.1016/S0035-9203(96)90102-9
65. Kaneko A, Chaves LF, Taleo G, Kalkoa M, Isozumi R, Wickremasinghe R, et al. Characteristic age distribution of *Plasmodium vivax* infections after malaria elimination on Aneityum Island, Vanuatu. *Infect Immun* (2014) 82(1):243–52. doi:10.1128/IAI.00931-13
66. Bouharoun-Tayoun H, Druilhe P. *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect Immun* (1992) 60(4):1473–81.
67. Chelimo K, Ofulla AV, Narum DL, Kazura JW, Lanar DE, John CC. Antibodies to *Plasmodium falciparum* antigens vary by age and antigen in children in a malaria-holoendemic area of Kenya. *Pediatr Infect Dis J* (2005) 24(8):680–4. doi:10.1097/01.inf.0000172151.28851.fd
68. John CC, Moormann AM, Pregibon DC, Sumba PO, McHugh MM, Narum DL, et al. Correlation of high levels of antibodies to multiple pre-erythrocytic *Plasmodium falciparum* antigens and protection from infection. *Am J Trop Med Hyg* (2005) 73(1):222–8.
69. Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, et al. Immunoglobulin G subclass-specific responses against *Plasmodium falciparum* merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infect Immun* (2009) 77(3):1165–74. doi:10.1128/IAI.01129-08
70. Aribot G, Rogier C, Sarthou JL, Trape JF, Balde AT, Druilhe P, et al. Pattern of immunoglobulin isotype response to *Plasmodium falciparum* blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). *Am J Trop Med Hyg* (1996) 54(5):449–57.
71. Metzger WG, Okenu DM, Cavanagh DR, Robinson JV, Bojang KA, Weiss HA, et al. Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. *Parasite Immunol* (2003) 25(6):307–12. doi:10.1046/j.1365-3024.2003.00636.x
72. Nebie I, Diarra A, Ouedraogo A, Soulama I, Bougouma EC, Tiono AB, et al. Humoral responses to *Plasmodium falciparum* blood-stage antigens and association with incidence of clinical malaria in children living in an area of seasonal malaria transmission in Burkina Faso, West Africa. *Infect Immun* (2008) 76(2):759–66. doi:10.1128/IAI.01147-07
73. Shi YP, Sayed U, Qari SH, Roberts JM, Udhayakumar V, Oloo AJ, et al. Natural immune response to the C-terminal 19-kilodalton domain of *Plasmodium falciparum* merozoite surface protein 1. *Infect Immun* (1996) 64(7):2716–23.
74. Roussilhon C, Oeuvray C, Muller-Graf C, Tall A, Rogier C, Trape JF, et al. Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. *PLoS Med* (2007) 4(11):e320. doi:10.1371/journal.pmed.0040320
75. John CC, Tande AJ, Moormann AM, Sumba PO, Lanar DE, Min XM, et al. Antibodies to pre-erythrocytic *Plasmodium falciparum* antigens and risk of clinical malaria in Kenyan children. *J Infect Dis* (2008) 197(4):519–26. doi:10.1086/526787
76. Mishra S, Nussenzweig RS, Nussenzweig V. Antibodies to *Plasmodium* circumsporozoite protein (CSP) inhibit sporozoite's cell traversal activity. *J Immunol Methods* (2012) 377(1–2):47–52. doi:10.1016/j.jim.2012.01.009
77. Stewart MJ, Nawrot RJ, Schulman S, Vanderberg JP. *Plasmodium berghei* sporozoite invasion is blocked in vitro by sporozoite-immobilizing antibodies. *Infect Immun* (1986) 51(3):859–64.
78. Reineke U, Sabat R. Antibody epitope mapping using SPOT peptide arrays. *Methods Mol Biol* (2009) 524:145–67. doi:10.1007/978-1-59745-450-6_11
79. Lima-Junior JC, Banic DM, Tran TM, Meyer VS, De-Simone SG, Santos F, et al. Promiscuous T-cell epitopes of *Plasmodium* merozoite surface protein 9 (PvMSP9) induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon. *Vaccine* (2010) 28(18):3185–91. doi:10.1016/j.vaccine.2010.02.046

80. Lin HH, Zhang GL, Tongchusak S, Reinherz EL, Brusic V. Evaluation of MHC-II peptide binding prediction servers: applications for vaccine research. *BMC Bioinformatics* (2008) 9(Suppl 12):S22. doi:10.1186/1471-2105-9-S12-S22
81. Bueno LL, Lobo FP, Morais CG, Mourao LC, de Avila RA, Soares IS, et al. Identification of a highly antigenic linear B cell epitope within *Plasmodium vivax* apical membrane antigen 1 (AMA-1). *PLoS One* (2011) 6(6):e21289. doi:10.1371/journal.pone.0021289
82. Bergmann-Leitner ES, Chaudhury S, Steers NJ, Sabato M, Delvecchio V, Wallqvist AS, et al. Computational and experimental validation of B and T-cell epitopes of the in vivo immune response to a novel malarial antigen. *PLoS One* (2013) 8(8):e71610. doi:10.1371/journal.pone.0071610
83. Lin M, McRae H, Dan H, Tangorra E, Laverdiere A, Pasick J. High-resolution epitope mapping for monoclonal antibodies to the structural protein Erns of classical swine fever virus using peptide array and random peptide phage display approaches. *J Gen Virol* (2010) 91(Pt 12):2928–40. doi:10.1099/vir.0.023259-0
84. Dyrberg T, Oldstone MB. Peptides as antigens. Importance of orientation. *J Exp Med* (1986) 164(4):1344–9. doi:10.1084/jem.164.4.1344
85. Jimah JR, Salinas ND, Sala-Rabanal M, Jones NG, Sibley LD, Nichols CG, et al. Malaria parasite CelTOS targets the inner leaflet of cell membranes for pore-dependent disruption. *Elife* (2016) 5:e20621. doi:10.7554/eLife.20621

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

Copyright © 2017 Rodrigues-da-Silva, Soares, Lopez-Camacho, Martins da Silva, Perce-da-Silva, Têva, Ramos Franco, Pinheiro, Chaves, Pratt-Riccio, Reyes-Sandoval, Banic and Lima-Junior. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

3.2 Artigo 2: “Sequência gênica e epítopos potenciais da PvCelTOS (*Plasmodium vivax* Cell-traversal protein for ookinetes and sporozoites) são altamente conservados entre isolados de diferentes regiões da Amazônia brasileira. ”

3.2.1 Objetivos Específicos

- Caracterizar a PvCelTOS em isolados brasileiros de diferentes áreas endêmicas de malária;
- Avaliar o polimorfismo genético da PvCelTOS nos isolados;
- Realizar a predição *in silico* de epítopos lineares potencialmente reconhecimentos por células B nas diferentes sequências encontradas;

Avaliar impactos da diversidade genética da PvCelTOS na estrutura da proteína e potencial imunogênico de epítopos identificados *in silico*.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Plasmodium vivax Cell Traversal Protein for Ookinetes and Sporozoites (PvCelTOS) gene sequence and potential epitopes are highly conserved among isolates from different regions of Brazilian Amazon

Lana Bitencourt Chaves[1], Daiana de Souza Perce-da-Silva[2], Rodrigo Nunes Rodrigues-da-Silva[1], João Hermínio Martins da Silva[3], Gustavo Capatti Cassiano[4], Ricardo Luiz Dantas Machado[5], Lilian Rose Pratt-Riccio[6], Dalma Maria Banic[2], Josué da Costa Lima-Junior[1]*

1 Laboratory of Immunoparasitology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Rio de Janeiro, Brazil, **2** Laboratory of Clinical Immunology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Rio de Janeiro, Brazil, **3** Computational Modeling Group - FIOCRUZ - CE, Fortaleza, Brazil, **4** Laboratory of Tropical Diseases - Prof. Luiz Jacintho da Silva, Department of Genetics, Evolution and Bioagents, University of Campinas, Campinas, São Paulo, Brazil, **5** Malaria Immunogenetic Laboratory, Instituto Evandro Chagas, Ananindeua, Pará, Brazil, **6** Laboratory of Malaria Research, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Rio de Janeiro, Brazil

* josue@ioc.fiocruz.br

Check for updates

OPEN ACCESS

Citation: Bitencourt Chaves L, Perce-da-Silva DdS, Rodrigues-da-Silva RN, Martins da Silva JH, Cassiano GC, Machado RLD, et al. (2017) *Plasmodium vivax* Cell Traversal Protein for Ookinetes and Sporozoites (PvCelTOS) gene sequence and potential epitopes are highly conserved among isolates from different regions of Brazilian Amazon. PLoS Negl Trop Dis 11(2): e0005344. doi:10.1371/journal.pntd.0005344

Editor: Nicholas P. Day, Mahidol University, THAILAND

Received: August 23, 2016

Accepted: January 21, 2017

Published: February 3, 2017

Copyright: © 2017 Bitencourt Chaves et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by Brazilian National Research Council–CNPq/PAPES, (Conselho Nacional de Desenvolvimento Científico e Tecnológico/Programa de Apoio e Pesquisa Estratégica em Saúde) Fiocruz. JdCLJ is recipient

Abstract

The *Plasmodium vivax* Cell-traversal protein for ookinetes and sporozoites (PvCelTOS) plays an important role in the traversal of host cells. Although essential to PvCelTOS progress as a vaccine candidate, its genetic diversity remains uncharted. Therefore, we investigated the PvCelTOS genetic polymorphism in 119 field isolates from five different regions of Brazilian Amazon (Manaus, Novo Repartimento, Porto Velho, Plácido de Castro and Oiapoque). Moreover, we also evaluated the potential impact of non-synonymous mutations found in the predicted structure and epitopes of PvCelTOS. The field isolates showed high similarity (99.3% of bp) with the reference Sal-1 strain, presenting only four Single-Nucleotide Polymorphisms (SNP) at positions 24A, 28A, 109A and 352C. The frequency of synonymous C109A (82%) was higher than all others ($p<0.0001$). However, the non-synonymous G28A and G352C were observed in 9.2% and 11.7% isolates. The great majority of the isolates (79.8%) revealed complete amino acid sequence homology with Sal-1, 10.9% presented complete homology with Brazil I and two undescribed PvCelTOS sequences were observed in 9.2% field isolates. Concerning the prediction analysis, the N-terminal substitution (Gly10Ser) was predicted to be within a B-cell epitope (PvCelTOS Accession Nos. AB194053.1) and exposed at the protein surface, while the Val118Leu substitution was not a predicted epitope. Therefore, our data suggest that although G28A SNP might interfere in potential B-cell epitopes at PvCelTOS N-terminal region the gene sequence is highly conserved among the isolates from different geographic regions, which

of a FAPERJ APQ1 (E-26/210.653/2015), Jovem Cientista do Nosso Estado (E26/203.255/2016). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

is an important feature to be taken into account when evaluating its potential as a vaccine candidate.

Author summary

Cell-traversal protein for ookinetes and sporozoites (CelTOS) presents a pivotal role in the cell traversal of host cells in mosquito and vertebrate hosts. For this reason, it has been considered a potential novel alternative for a vaccine against malaria caused by *P. falciparum*. However, little is known about its orthologous *P. vivax* CelTOS. Although the genetic diversity of this protein could be a limiting factor for acquisition of immunity and present implications for an effective vaccine development, it has never been explored. Thus, considering that the epidemiology of malaria in Brazil presents variable transmission rates and the knowledge on the genetic polymorphism of PvCelTOS remains unknown, we aimed to identify the *pvceltos* gene in isolates from five different regions of the Brazilian Amazon and to study the potential impacts of the genetic diversity of PvCelTOS in protein structures and predicted epitopes. Our findings indicate that PvCelTOS is an extremely conserved protein, presenting only four SNPs in the entire sequences of field isolates from Brazilian Amazon. The two non-synonymous mutations found in our field isolates presented no significant effect on the protein structure and a very low impact on potential T and B-cell epitopes indicated by our epitope prediction. Collectively, our data suggest that the small need to avoid the immune recognition by the human host and its importance on the parasite's survival and transmission reflects a very conservative profile of *pvceltos* gene in field samples from Brazil and other endemic areas worldwide.

Introduction

Malaria is an infectious parasitic disease with high prevalence and morbidity. Globally, it is estimated that 3.2 billion people in 95 countries and territories are at risk of being infected and develop the disease. In 2015, malaria caused an estimate of 438,000 deaths, mostly in African children [1]. Among the protozoa species causative of human malaria, *Plasmodium vivax*, although less prevalent than *P. falciparum* in absolute numbers, presents the world's largest spread, an increasing morbidity [2] and became the main cause of malaria outside Africa. In Brazil, although there are three species of *Plasmodium* that cause malaria (*P. falciparum*, *P. vivax* and *P. malariae*), approximately 87% of the 142,000 cases reported in 2015 were caused by *P. vivax* [3]. Thus, it is extremely important to develop new methods and intervention strategies to block or reduce this transmission.

Significant effort and progress on *P. vivax* control have occurred over the last years, but the understanding of *P. vivax* biology is still crucial to develop potential vaccines and to achieve the goal of eliminating malaria. The ability of the *Plasmodium* to recognize, and then invade hepatocytes or red blood cells, is central to the life cycle and also to the disease process. During the pre-erythrocytic stage, it is well established that *Plasmodium* sporozoites migrate through Kupffer cells and several hepatocytes before finally infecting a hepatocyte. Therefore, antigens located on the surface of the parasite or specifically in apical organelles of the parasite during this stage have been suggested as a target for a better understanding of *Plasmodium* lifecycle and, consequently possibly used as vaccine [4]. In this context, the Cell-Traversal protein for Ookinetes and Sporozoites (CelTOS) has been considered a new alternative for vaccine

development [5,6]. This protein, secreted by micronemes, is important to the success of cell crossing by sporozoites and ookinetes, and also hepatocyte invasion carried out by sporozoites. Studies have shown that the disruption of the CelTOS gene encoding, in *P. berghei*, reduces the infectivity in the mosquito host and also the infectivity of the sporozoite in the liver, almost eliminating their ability to cell pass [7]. In addition, the CelTOS is necessary for the motility of the parasite in both the mosquito vector and the human host, being determinant for the success of malaria infections [8]. Recently, studies from Jimah et al. suggested that the CelTOS is responsible for breaking the cell membranes from the inside of infected human and mosquito cells to enable the parasites to exit and complete the traversal process (Jimah et al 2016). In relation to its potential as a vaccine candidate, antibodies against PfCelTOS were able to inhibit sporozoite traversal of hepatocytes [9], and induce protection in animals [10]. In humans, PfCelTOS derivative peptides elicited proliferative and IFN-γ responses in *ex vivo* ELISPOT assays using peripheral blood mononuclear cells (PBMCs) from irradiated sporozoite-immunized volunteers [8] and recombinant PfCelTOS were recognized by naturally acquired antibodies from exposed populations living in highly endemic areas from Africa [11]. However, all those previous studies used CelTOS protein of *P. falciparum* and/or *P. berghei*. Despite the antigenic and immunogenic properties of PfCelTOS, there is only one recent finding concerning the antigenic potential of its counterpart in *P. vivax*, the PvCelTOS, whose naturally acquired antibodies were able to recognize the recombinant protein [12].

Although essential to the development of its potential as a vaccine candidate, there is no available published data on the identification of *pvceltos* gene in field isolates and the evaluation of its genetic diversity in endemic areas. In fact, the extensive genetic diversity in natural parasite populations is a major obstacle for the development of an effective vaccine against the human malaria parasite, since antigenic diversity limits the efficacy of acquired protective immunity to malaria [13]. Despite the genetic diversity, which is one of the most prominent features of *P. vivax* infections, there is also a paucity of information on *celtos* gene polymorphism. Such data have importance in documenting the parasite genetic diversity changes and contribute to malaria control interventions in the future. Therefore, we proposed to identify *pvceltos* gene isolates from different regions of Brazilian Amazon and to study the potential impacts of the genetic diversity of PvCelTOS in protein structures and potential epitopes through bioinformatics tools.

Methods

Study sites and blood sample collection

Most cases of malaria in Brazil are concentrated in the Amazon Region, an endemic area for the disease [14]. Therefore, the study was carried out in five different regions of Brazilian Amazon (Fig 1). A subset of 81 patients was analyzed out of 312 individuals previously evaluated by Cavasini *et al* (2007) [15] (21 individuals from Plácido de Castro, 9 individuals from Oiapoque, 25 individuals from Novo Repartimento and 26 individuals from Porto Velho) and, additionally, blood samples were collected from 38 *P. vivax* infected individuals from Manaus. Thus, a total of 119 blood samples were used in this study.

Plácido de Castro (PLC), is a city 90 km far from the capital of the State of Acre, located in Western Brazilian Amazon, with a population of 17,334 thousand inhabitants (16% aged above 18 years, at 153 meters above sea level, with a territorial area of 2,047,000 km^2, latitude of -09° 58' 29" and longitude of 67° 48' 36", where the main economic activities are cattle breeding, rubber agriculture and farming. Active malaria transmission takes place during all periods of the year.

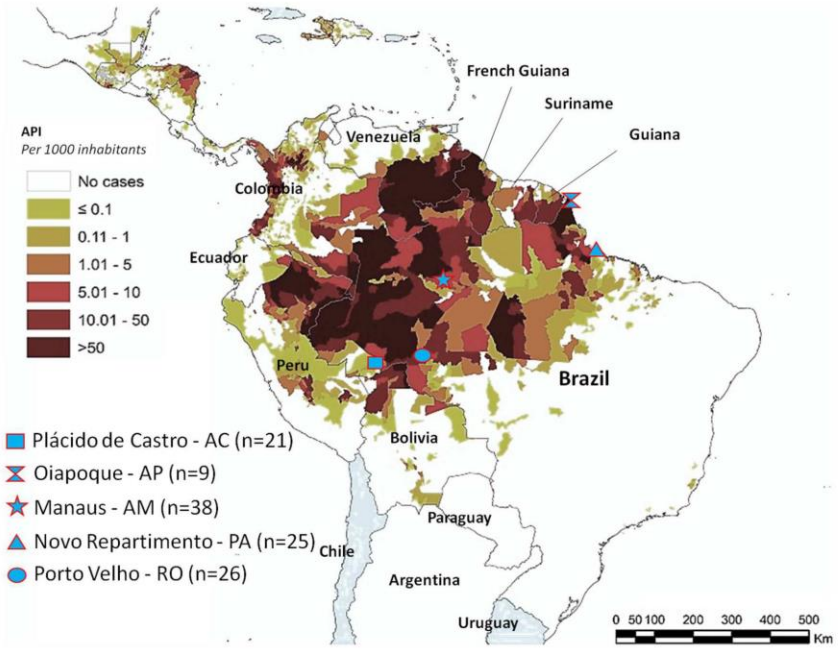


Fig 1. Geographical map showing the five study sites and the respective Annual Parasitic Incidence (API) (SIVEP-Malaria).

doi:10.1371/journal.pntd.0005344.g001

Oiapoque (OIP), Amapá State, located in the Brazilian Eastern Amazon, a mining gold area, with 17,423 a thousand inhabitants, presenting latitude of 03˚ 49' 58" and longitude of 51˚ 49' 51".

Manaus (MAO), the capital of Amazonas State, located in the Northern Region of Brazil, with a population of more than 2 million people. It is the most populous city of Amazonas state, presenting latitude of -03˚ 06' 07" and longitude of -60˚ 01' 3".

Novo Repartimento (NR), is a city 600 km far from Belém, capital of the State of Pará, located in Brazilian Eastern Amazon, with 47,197 thousand inhabitants, at 460 meters above sea level, with a territorial area of 11,407 km^2, presenting latitude of 04˚ 19' 5" and longitude of 49˚ 47' 47", whose main economic activities are cattle breeding, commerce of manufactured products and farming. It presents active malaria transmission from January to December, with around 2,000 heterochthonous and autochthonous cases.

Porto Velho (PVL), capital of the Rondônia State, located in Western Brazilian Amazon, with a population of 360,068 thousand inhabitants (16% aged above 18 years), at 85 meters above sea level, with a territorial area of 34,082 km^2, latitude of -08˚ 45' 43" and longitude of 63˚ 45' 43", where the main economic activities are cattle breeding, rubber agriculture, wood exploration and farming. Active malaria transmission takes place during all periods of the year. The distances between the study sites are shown in Table 1.

Table 1. Distance in km between the five study sites.

	Distance (km)			
Locality	**OIP**	**MAO**	**NR**	**PVL**
OIP	-	-	-	-
MAO	1,196	-	-	-
NR	938	1,143	-	-
PVL	1,939	760	1,635	-
PLC	2,313	1,119	2,024	393

OIP: Oiapoque, AP; MAO: Manaus, AM; NR: Novo Repartimento, PA; PVL: Porto Velho, RO; PLC: Plácido de Castro, AC.

doi:10.1371/journal.pntd.0005344.t001

All *P. vivax* participants were enrolled according to the following criteria: sought medical assistance for clinical malaria symptoms, presented uncomplicated malaria symptoms, were > 18 years of age, and had a positive *P. vivax* malaria diagnosis. Pregnant women, patients < 18 years of age, and *P. vivax*- and *P. falciparum*-infected individuals were excluded from the study. Thin and thick blood smears were examined for the identification of the malaria parasite by a technician experienced in malaria diagnosis from the Brazilian Malaria Health Services. Thick blood smears from all of the subjects were stained with Giemsa, and a total of 200 microscopic fields were examined under a 1,000-fold magnification. Thin blood smears of the positive samples were examined for species identification. To increase the sensitivity of parasite detection, molecular analyses using specific primers for genus (*Plasmodium* sp) and species (*P. falciparum* and *P. vivax*) were performed in all of the samples as previously described. Donors positive for *P. vivax* and/or *P. falciparum* at the time of blood collection were subsequently treated by the chemotherapeutic regimen recommended by the Brazilian Ministry of Health.

Ethical considerations

The study protocol was approved by the Research Ethics Committee of each locality, which included obtaining the following patients' written consents for research use of their blood samples: Belém (Novo Repartimento/PA): 68473–970; Porto Velho (CEPEN): 76812–329; Rio Branco (Hospital Geral de Plácido de Castro/AC): 69928–000; Oiapoque (Hospital Municipal do Oiapoque/AP): 68980–000; Manaus (CEP-FIOCRUZ): 346–613. Written informed consents were obtained from all adult donors or from the parents of donors in the case of children. All the procedures adopted in this study fully complied with specific federal permits issued by the Brazilian Ministry of Health.

Genomic DNA extraction

The DNA was extracted from blood samples using the QIAamp DNA blood midi kit (QIAgen) according to the manufacturer's instructions and stored at -20˚C until amplification.

Design of PvCelTOS specific primers

The *pvceltos* gene is conserved among different species of *Plasmodium* and to obtain that of *P. vivax*, specific primers were designed using standard gene sequences of *P. vivax* Salvador-1 strain from NCBI database with Accession Nos. AB194053.1. All oligonucleotides were checked for specificity by using the Primer-BLAST tool provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The forward

primer (5'-CCCCCAAAGGCAAAATGAACAA-3') corresponded position 20 to 41 of the *pvceltos* gene sequence and the reverse primer (5'-AACTCATCTTCAGCTTCTTCCTC-3') corresponded to position 569 to 547. The specific primers were chemically synthesized to perform PCR reaction and DNA sequencing.

PCR amplification of *pvceltos* gene

The *pvceltos* gene was amplified in a conventional PCR method using the pair of primers PvCelTOS 5'—CCCCCAAAGGCAAAATGAACAA—3' (forward) and PvCelTOS 5'—AACT CATCTTCAGCTTCTTCCTC—3' (reverse). Amplification of the *pvceltos* gene was conducted in a reaction volume of 25 μL using 1 μL of DNA, 10 pmol/μL of each primer and the Master Mix kit (Promega) containing Taq DNA polymerase, PCR buffer and 10 nmol of each deoxynucleotide triphosphate (dNTP, Promega, Madison, WI USA). The conventional PCR reactions were carried out using a GeneAmp PCR system 9700 (Applied Biosystem) and the cycling conditions were as follows: one step at 95˚C for 2 min.; 30 cycles at 95˚C for 1 min., 57˚C for 1 min. and 72˚C for 1 min.; and a last step at 72˚C for 1 min. In all reactions two negative controls were used (one without DNA and other with DNA extracted from *in vitro* culture of *P. falciparum* PSS1 strain) and a positive control (*P. vivax*-infected sample). To confirm the presence of DNA from the *in vitro* culture of *P. falciparum* and that the lack of amplification was due the specificity of the primers for PvCelTOS, we performed the amplification of the *P. falciparum* P126 gene fragment and electrophoresis as previously described [16]. Moreover, three *P. vivax*-infected samples from our study sites were randomly chosen. Five μL of PCR product were submitted to electrophoresis in 2% agarose gel (Sigma) in 1x TAE buffer (0.04 M TRIS-acetate, 1 mM EDTA) in the presence of 10x GelRed nucleic acid stain (Biotium) and afterwards the products were visualized by ultraviolet (UV) illumination. Sizing of products was performed using a GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific). Then, PCR fragments were purified using the GE Healthcare Lifesciences kit according to the manufacturer's protocol and sequenced.

DNA sequencing and polymorphism analysis

The specificity of the assay was confirmed by sequencing the PCR products from all positive samples using a Big Dye terminator sequencing kit (Applied Biosystems) following the manufacturer's instructions. The DNA sequencing was carried out on the 3730xl DNA analyzer (Applied Biosystems) and the results were analyzed using DNASTAR's sequence alignment software to identify polymorphism relative to the Sal-1 reference sequence from NCBI.

3D model and electrostatic analysis of PvCelTOS

The 3D structure of PvCelTOS was predicted using the Robetta algorithm [17]. The amino acid sequence was retrieved from NCBI under Accession Nos. AB194053.1. The Robetta is an automated algorithm for predictions of the 3D structure of proteins through *ab initio* and comparative modeling. The first step is the search for structural homologs using BLAST [18] or PSI-BLAST [19]. In the protein sequence, the target primary structure is broken down into separated domains, or independently folding units of proteins, by comparing the sequence to structural families in the Pfam database [20]. Domains with homolog structures follow a template-based modeling protocol. The final five structures are selected by taking the lowest energy models as determined by the Rosetta energy function. The electrostatic surface was calculated with the Adaptive Poisson-Boltzmann Solver (APBS) software [21] integrated with Pymol. The APBS software solves the Poisson-Boltzmann equation in order to describe

electrostatic interactions between solute in aqueous solution. Continuous electrostatics plays a very important role in determining ligand-protein and protein-protein binding kinetics.

Prediction of linear B-cell and T-cell epitopes

The prediction of linear B-cell epitopes was carried out using the program BepiPred [22]. This software takes a single sequence in FASTA format input and each amino acid receives a prediction score based on Hidden Markov Model profiles of known antigens and incorporates propensity scale methods based on hydrophilicity and secondary structure prediction. For each input sequence the server outputs a prediction score. The positions of the linear B-cell epitopes are predicted to be located at the residues with the highest scores. In order to consider a given region as a valid linear B-cell epitope for PvCelTOS, the cut-off value of 0.35 was used to warrant similar values of specificity (0.75) and sensitivity (0.49). Therefore, the epitope score represents the average of the scores of at least eight consecutive amino acids above the cut-off, and the sequences with higher mean values were chosen as potential linear epitopes.

The differential binding of T-cell epitopes spanning the full PvCelTOS sequence were made on 4/18/2016 using the IEDB analysis resource Consensus tool [23] which combines predictions from ANN aka NetMHC (3.4) [24,25], SMM [26] and Comblib [27]. Considering lengths of 9 mers, the prediction score of each length was evaluated against 26 of the most frequent HLA alleles (HLA-A*01:01; HLA-A*02:01; HLA-A*11:01; HLA-A*23:01; HLA-A*25:01; HLA-A*26:01; HLA-A*30:01; HLA-A*31:01; HLA-A*32:01; HLA-A*68:01; HLA-B*08:01; HLA-B*15:01; HLA-B*18:01; HLA-B*35:01; HLA-B*38:01; HLA-B*39:01; HLA-B*40:01; HLA-B*46:01; HLA-B*48:01; HLA-B*51:01; HLA-B*53:01; HLA-B*57:01; HLA-B*58:01; HLA-C*04:01; HLA-C*05:01; HLA-C*07:01). Peptides with median consensus percentile rank 20.0 as predicted binders and at least 60% of HLA binding frequency was considered potential T-cell epitopes.

Statistical analysis

The one-sample Kolmogorov-Smirnoff test was used to determine whether a variable was normally distributed. Differences in proportions of haplotypes frequencies between studied localities were evaluated by the Fisher's exact test using Prism 5.0 for Windows (GraphPad Software, Inc.). A two-sided P value < 0.05 was considered significant. Sequences were aligned using CLUSTAL X2 and the number of segregation sites (S), number of haplotypes, nucleotide diversity (π) and haplotype diversity were computed using DnaSP v5 [28]. The Tajima's D test [29] for determining departure from the predictions of the neutral theory of evolution was also estimated with DnaSP v5. The genetic differentiation between populations was investigated evaluating the rate of fixation (F_{ST}) by analysis of molecular variance (AMOVA) implemented in ARLEQUIN v3.5.2.2 [30] and significances were estimated using 10,000 permutations. The significance level was adjusted by Bonferroni correction for multiple tests.

Results

Standardization and molecular characterization of PvCelTOS in the studied regions

In order to identify the gene encoding the PvCelTOS in isolates from Brazilian endemic areas, 119 blood samples from infected individuals living in the cities of Porto Velho, Plácido de Castro, Manaus, Novo Repartimento and Oiapoque had the DNA extracted and subjected to molecular diagnosis by PCR. The primers designed from the Primer-BLAST program and PCR analysis by agarose gel revealed the amplification in 100% samples. All field isolates

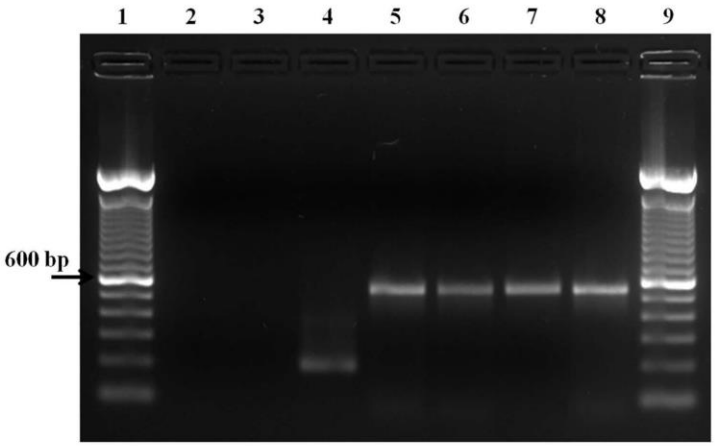


Fig 2. PCR amplification of the *pvceltos* gene. Fig 2 shows in **Lane 1**: 100 bp Molecular Marker; **Lane 2**: Negative control (water); **Lane 3**: *In vitro* culture of *P. falciparum* (amplification with PvCelTOS primers); **Lane 4**: *In vitro* culture of *P. falciparum* (amplification with p126 primers); **Lane 5**: PCR positive control (*P. vivax*-infected sample); **Lanes 6, 7 and 8**: samples; **Lane 9**: 100 bp Molecular Marker.

doi:10.1371/journal.pntd.0005344.g002

presented only one type of fragment corresponding to 550 base pair (bp). In addition to these samples, *P. falciparum* specimens were also tested, but proved negative for PCR amplification of the *pvceltos* gene (Fig 2). Therefore, the 119 samples from individuals infected with *P. vivax* amplified by PCR were subjected to sequencing reactions in order to screen the possible single nucleotide polymorphisms of the gene encoding the PvCelTOS.

Pvceltos gene is highly conserved among field isolates, but presents few synonymous and non-synonymous mutations at specific positions

Standard gene sequences of *P. vivax* Salvador-1 (Sal-1) encoding PvCelTOS were aligned to sequences from different regions of Brazilian Amazon isolates. Identification of variants and novel haplotypes was done and our interpretations were confirmed with available standard gene sequence of the *P. vivax* CelTOS in PubMed database. The polymorphism identification in the gene encoding the PvCelTOS from our studied regions revealed that all isolates had a high degree of similarity in relation to base pair alignments with the reference strain (99.3%). However, from the 550 bp sequenced and aligned, four nucleotide bases (0.7%) presented mutations in specific bp positions (24, 28, 109 and 352), shown in Table 2. Interestingly, we did not detect point mutations in a single field or geographic area and all SNPs were present in at least two isolates and two sampling localities. Even with the high conservation degree of *pvceltos* gene sequence, 85% of the studied isolates presented at least one SNP in relation to the reference strain. As shown in Fig 3a, the synonymous mutation C109A was present in 82% field isolates and was significantly higher than all other 3 mutations ($p<0.0001$), while the other synonymous mutation C24A was the least frequent mutation. Two non-synonymous mutations, G28A and G352C, which represent the substitution of Glycine for Serine and Valine for Leucine, respectively, were also detected in frequencies of 9.2% and 11.7%, respectively. In addition, regarding the endemic areas studied, the higher frequency of C109A was

Table 2. Mutations and corresponding amino acid substitutions in *pvceltos* gene.

Sequences	Nucleotide position			
	22 to 24	28 to 30	109 to 111	352 to 354
Wild Type	CCC	GGC	CGG	GTG
Mutants	- - A	A - -	A - -	C - -
	Amino acid position			
	8	10	37	118
Wild Type	Pro	Gly	Arg	Val
Mutants	-	Ser	-	Leu

Nucleotide and amino acid positions

doi:10.1371/journal.pntd.0005344.t002

maintained in all localities. Manaus presented the highest diversity, since we detected all four mutations among the 38 samples, while Porto Velho presented the lowest diversity, with only the synonymous mutation C109A. Lastly, in field isolates from Plácido de Castro, the non-synonymous SNP G352C was also significantly higher than C24A (p = 0.0086) and G28A (p = 0.0480), while in all other localities this predominance did not occur (Fig 3b).

PvCelTOS haplotype frequency in field isolates

Only 18 isolates (15%) maintained their sequences identical to the reference strain in positions 24, 28, 109 and 352 (H1 = CGCG). Furthermore, the mutations resulted in nine different haplotypes (H2 = AGCG; H3 = CACG; H4 = CGAG; H5 = CAAG; H6 = CGAC; H7 = AGAG, H8 = CAAC; H9 = AAAG), whose frequencies are shown in Table 3. Among all field isolates studied the haplotype H4 presented the highest frequency and was significantly higher when compared to the reference H1 (p<0.0001). On the other hand H2 (p<0.0001), H3 (p = 0.0002), H5 (0.0328), H7 (p = 0.0028), H8 (p<0.0001) and H9 (p<0.0001) presented significantly lower frequencies when compared to H1. However, regarding these haplotypes obtained from human isolates from the Amazon regions, we could not determine a genetic

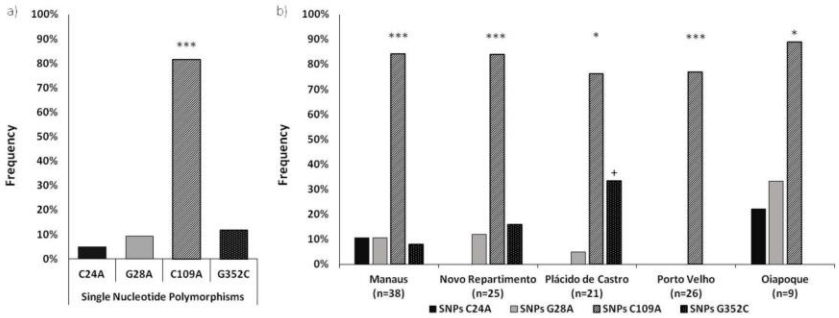


Fig 3. Analysis of genetic diversity of PvCelTOS in *Plasmodium vivax* isolates. (A) Fig A represents four mutations in specific bp positions (24, 28, 109 and 352). (B) The graphic represents the frequency of mutations in isolates from each studied locality. The black bar indicates the synonymous mutation C24A; the gray bar, the non-synonymous mutations G28A; the striped bar, the synonymous mutation C109A and the white dotted bar represents the non-synonymous G352C. (*) Indicates that the differences between the frequency of SNP C109A was higher than that of other mutations by exact test and (+) indicates that the frequency of SNP G352C was higher than the frequency of SNPs C24A and G28A by Fisher's. (*): p<0.05; (**): p<0.01; (***): p<0.0001.

doi:10.1371/journal.pntd.0005344.g003

Table 3. Distribution of PvCelTOS haplotypes among five studied localities of Brazilian Amazon.

	Haplotypes PvCelTOS—n (%) (Nucleotide positions 24, 28, 109 and 352—CGCG)								
Locality	**Ref. strain—H1 (CGCG)**	**H2 (AGCG)**	**H3 (CACG)**	**H4 (CGAG)**	**H5 (CAAG)**	**H6 (CGAC)**	**H7 (AGAG)**	**H8 (CAAC)**	**H9 (AAAG)**
Manaus (n = 38)	**5 (13.1%)**	1 (2.6%)	-	22 (57.8%) ***	4 (10.5%)	3 (7.9%)	3 (7.9%)	-	-
Novo Repartimento (n = 25)	**3 (12%)**	-	1 (4%)	16 (64%) ***	1 (4%)	3 (12%)	-	1 (4%)	-
Plácido de Castro (n = 21)	**3 (14.3%)**	-	1 (4.8%)	10 (47.6%)*	-	7 (33.3%)	-	-	-
Porto Velho (n = 26)	**6 (23.1%)**	-	-	20 (76.9%) ***	-	-	-	-	-
Oiapoque (n = 9)	**1 (11.1%)**	-	-	4 (44.4%)	2 (22.2%)	-	1 (11.1%)	-	1 (11.1%)
Total (n = 119)	**18 (15.1%)**	1 (0.8%)***	2 (1.7%) ***	72 (60.5%) ***	7 (5.9%) *	13 (10.9%)	4 (3.4%) **	1 (0.8%) ***	1 (0.8%) ***

The values represent the number and frequency (%) of found haplotypes on each studied locality. H4 represents the consensus sequence. (*) Indicates that the difference between the frequencies of mutate haplotype and reference strain (H1) was significant by Fisher's exact test.

(*): p<0.05;

(**): p<0.01;

(***): p<0.0001.

doi:10.1371/journal.pntd.0005344.t003

structure based on the localities. Therefore, we observed that H1 and H4 were present in all studied localities while H2, H8 and H9 were detected in only a single locality (Manaus, Novo Repartimento and Oiapoque respectively). Even though the haplotypes could not be segregated according to their geographic origin, Manaus and Novo Repartimento presented the highest diversity of field isolates with six different haplotypes, while Porto Velho presented the lowest diversity, with only two haplotypes, which were common to all localities (H1 and H4). Interestingly, despite the difference in number of field isolates, Oiapoque presented a high diversity of *pvceltos* gene sequence with five haplotypes while only four different haplotypes were detected in Plácido de Castro (Table 3). Due to the very high similarity among sequences from different geographic origins and the consequent lack of phylogenetic signal, it was not possible to analyze the haplotypes in reliable clades.

Population genetic analysis

We sequenced *pvceltos* gene (positions 19–569) of 119 samples collected from five regions of Brazilian Amazon. From the alignment with reference strain (Sal-1), four distinct SNPs were identified. Two SNPs were synonymous (C24A and 109A) and two were non-synonymous (G28A and G352C). The nucleotide diversity (π) for *pvceltos* of 119 sequences analyzed was 0.00141 ± 0.00014. The highest nucleotide diversity was observed in the Oiapoque group (0.00202 ± 0.00044), followed by the Plácido de Castro group (0.00161 ± 0.00029). Among all 5 populations, Porto Velho sequences displayed the lowest nucleotide diversity (0.00067 ± 0.00017) as expected, since only one SNP was detected in this group (Table 4). Similarly, parasites from Oiapoque presented the highest estimate of haplotype diversity (H_d) (0.806 ± 0.014) whereas parasites from Porto Velho showed the lowest H_d (0.369 ± 0.091). Haplotype diversity was similar among the other studied areas (Table 4). The Tajima's D test was performed to asses if there is selective pressure on the *pvceltos* gene. Although the Tajima's D values ranged between -0.279 and 0.699, tests showed no significant departures from neutrality in all studied areas, indicating no significant selection in the *pvceltos* gene (Table 4).

Pairwise comparisons between each parasite population were performed using the F_{ST} statistics to check whether there was indicative of genetic differentiation between parasite

Table 4. Comparison of genetic diversity among isolates from Brazil.

	No. of segregating sites (S)	No. of haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)	Tajima's test
Novo Repartimento	3	6	0.58	0.00142	-0.045^{ns}
Manaus	4	6	0.66	0.00153	-0.279^{ns}
Oiapoque	3	5	0.81	0.00202	0.025^{ns}
Porto Velho	1	2	0.37	0.00067	0.699^{ns}
Plácido de Castro	3	4	0.67	0.00161	0.163^{ns}
All samples	4	9	0.61	0.00141	0.077^{ns}

The extent of *pvceltos* gene sequence corresponds to nucleotides 19–569 (reference clone Sal-1).

^{ns}: not significant ($p > 0.10$).

doi:10.1371/journal.pntd.0005344.t004

populations, but all F_{ST} values were non-significant, suggesting lack of genetic differentiation between the studied populations (Table 5).

Non-synonymous mutations reveal low diversity of PvCelTOS protein sequence in relation to genome sequences available worldwide

The detected non-synonymous mutations characterized the specific amino acid substitutions in positions 10 (Glycine for Serine) and 118 (Valine for Leucine). As observed in the protein sequence alignments, PvCelTOS also presented high amino acid sequence conservation degree, since only 24 isolates (19.2%) presented non-synonymous mutations and had different sequences in comparison with the reference Sal-1 strain, whose frequency was significantly higher than all other protein sequences found in our field isolates (79.8%; $p<0.0001$). Therefore, we subsequently aligned the protein sequence of these mutant field isolates in relation to other three hypothetical CelTOS protein derivatives from *P. vivax* genome data available in PubMed protein database (Fig 4a). Only 13 isolates (10.9%) presented sequences identical to Brazil I strain and none of our field isolates presented complete homology with North Korean and India VII strains, however both Asian strains also presented mutations in C terminal region at position 178 (Lysine for Threonine) that was not detected in our Amazon isolates. Interestingly, the N-terminal mutation at position 10 (Gly10Ser) was never detected in available sequences, but it was present in 9.2% of our field samples. Regarding the five regions studied, all isolates from Porto Velho presented full homology with Sal-1 amino acid sequence, while in other regions the frequencies of mutant sequences ranged from 21% to 44% (Fig 4b). Noting the diversity identified following the *pvceltos* gene, our data indicate that it is limited in isolates from different regions of the Brazilian Amazon. However, these two non-synonymous mutations found may have an impact on the protein folding and also influence its potential as an epitope.

Table 5. Genetic differentiation between samples from Brazil, measured by pairwise F_{ST} values.

	Novo Repartimento	Manaus	Oiapoque	Porto Velho
Novo Repartimento	-	-	-	-
Manaus	-0.015	-	-	-
Oiapoque	0.056	0.010	-	-
Porto Velho	0.036	0.015	0.196	-
Plácido de Castro	-0.004	0.048	0.143	0.123

The F_{ST} values are not significant after Bonferroni correction ($p > 0.05$).

doi:10.1371/journal.pntd.0005344.t005

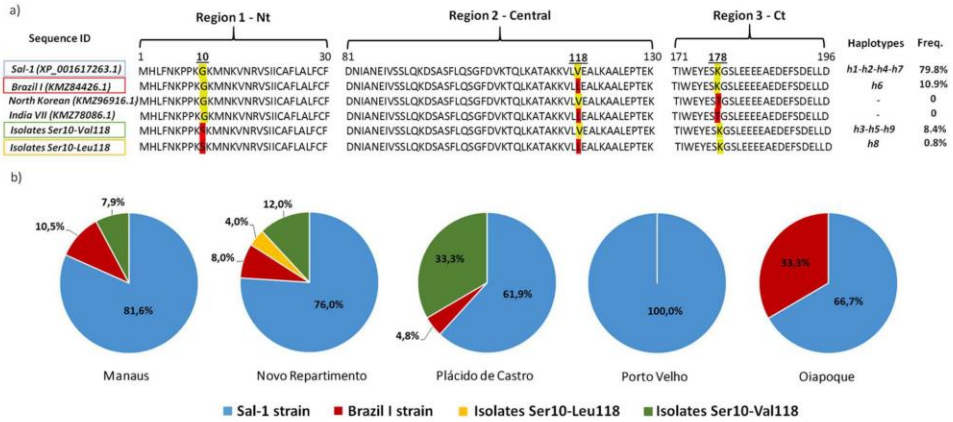


Fig 4. Alignment of protein sequences and frequency of mutations on field isolates. (A) CelTOS protein derivatives from *P. vivax* genome. The yellow mers represent the reference strain and the red mers the described mutate amino acid. (B) Frequencies of mutations in isolates from different Brazilian Amazon regions, where the colors blue, red, yellow and green represent genome identical to strains Sal-1; Brazil I and mutant isolates Ser10-Leu118 and Ser10-Val118, respectively.

doi:10.1371/journal.pntd.0005344.g004

Non-synonymous mutations could modify the potential of predicted B-cell epitopes but not for T_{CD8+} epitopes.

Fig 5a depicts the electrostatic potential around the mutations. The region encompassing Arg37 shows a strong negatively charged surface. The Lys178 position showed the same negative pattern, while Val118 and Gly10 are positively charged. Pro8 region is mostly neutral. Arg37 and Val118 are part of a stable alpha helix structure, whereas Pro8, Gly10, and Lys178 belong to flexible loop structures. Also, all residues are exposed to the surface, except Arg37 which is hidden inside the negative pocket. As shown in Fig 5b four high scored potential linear epitopes with at least eight amino acids were identified in the entire protein sequence (Lys6-Asn13; Gly38-Arg57; Ile136-Glu143 and Lys166-Ser191). The prediction scores ranged from 0.97 to 1.17 and no immunodominant epitopes could be identified by this approach. Considering that two of non-synonymous mutations were inserted in predicted B-cell linear epitopes (Gly10Ser and Lys178Thr), we analyzed the prediction scores of mutate epitopes. Interestingly, the C-terminal mutation Lys178Thr, observed only in Asian strains, North Korean (KMZ96916.1) and Indian VII (KMZ78086.1), resulted in a slight increase of the predicted score; while the N-terminal mutation Gly10Ser, observed in our Brazilian isolates Ser10-Val118 and Ser10-Leu118, resulted in a decrease of the predicted score for an epitope (Fig 5b). On the other hand, the predicted T_{CD8+} epitopes were conserved among all known strains and isolates, once non-synonymous mutations were not observed inside these epitopes. Analyzing the full sequence of PvCelTOS, six T_{CD8+} predicted epitopes presented consensus score smaller than 20 and were predicted to be recognized by more than 60% of analyzed HLA (Fig 5b). Among these epitopes, the sequence RVSEDAYFL ($PvCelTOS_{I83-L91}$) was considered a potential promiscuous T_{CD8+} epitope, since it was predicted as bonded by 81% of evaluated HLAs and presented a mean consensus score of 11.81. However, the potential of predicted epitopes as target of immune response and the effects of mutations on immune response against PvCelTOS remain unexplored.

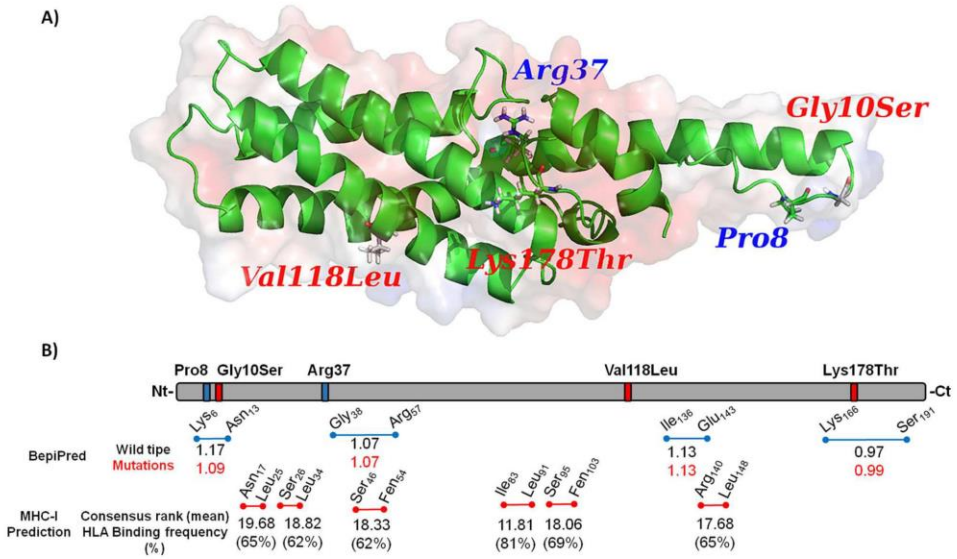


Fig 5. Modeling structure and *In silico* analysis of PvCelTOS. (A) Synonymous and non-synonymous mutations were illustrated by blue title and red title, respectively, on 3D structure of PvCelTOS. The red and blue clouds represent the negative and positive surface, respectively. (B) Synonymous and non-synonymous mutations found in our population and other described mutations are illustrated by blue bars and red bars, respectively, on PvCelTOS structure. The blue lines represent predicted linear B-cell epitopes and the red lines represent predicted T_{CD8+} epitopes. On both (B and T predicted epitopes) the letter and number of each epitope indicate the C-terminal and N-terminal amino acid. The BepiPred values represent the predicted score of linear B-cell epitope in wild type haplotype (H1) and mutate strain (red number). The IEDB MHC-I indicates the mean binding prediction score of T_{CD8+} epitopes and respective HLA binding frequency among 27 evaluated HLA. No differences of prediction T-cell epitopes are observed between wild types or mutate PvCelTOS.

doi:10.1371/journal.pntd.0005344.g005

Discussion

Cell-traversal protein for ookinetes and sporozoites (CelTOS) has been considered a potential novel alternative for a vaccine against malaria. Although the biological function is not completely elucidated, its pivotal role in the cell traversal of host cells in mosquito and vertebrate host is important to a successful hepatocyte traversal and infection. Immunologic studies have demonstrated that CelTOS is target of naturally acquired cellular [8] and humoral response in exposed individuals [9]. However, one of the major obstacles to malaria vaccine development is still the low efficiency in inducing protection, which, in part, can be explained by genetic polymorphisms encoding different proteins used as immunogens [31]. In this context, the genome sequence of various organisms and the advances in bioinformatics have revolutionized the field of vaccinology, allowing the identification of vaccine candidates presenting low antigenic variation. Actually, several studies concerning the genetic diversity of *Plasmodium* spp. have described *P. vivax* and the gene coding for antigenic determinants such as circumsporozoite surface protein (CSP) [32], Merozoite Surface Proteins (MSP) [33], Duffy Binding Protein (DBP) [34] and Apical Membrane Antigen-1 (AMA-1) [35]. (Reviewed by [36]). In fact, the genetic diversity of these proteins in hyperendemic areas has been described as a limiting factor for the rapid acquisition of protective immunity, and as a consequence for the development of an effective vaccine. Moreover, the antigenic polymorphism of *P. vivax*

vaccine candidates has been little discussed in unstable transmission areas such as the Brazilian endemic areas. Thus, considering that the epidemiology of malaria in Brazil presents unstable transmission and the knowledge about the genetic polymorphism of PvCelTOS remains unknown, we aimed to identify the *pvceltos* gene in isolates from different regions of the Brazilian Amazon and to study the potential impacts of the genetic diversity of PvCelTOS in protein structures and predicted epitopes.

The identification and evaluation of the genetic diversity of *pvceltos* gene in isolates from different geographic regions has not been previously studied and this was the first report. Despite the large distance among the studied localities and the possible existence of a gene flow of *Plasmodium vivax* genome among the studied populations which, associated with migration of people, could promote the gene flow of the parasite [37], our first results showed that *pvceltos* gene is highly conserved, presenting only 4 SNPs along its entire sequence, 2 synonymous and 2 non-synonymous mutations. This high conservation degree was expected, once it has been shown that CelTOS amino acid sequence is partially conserved even among three different *Plasmodium* species (*P. vivax*, *P. falciparum* and *P. berghei*) [7]. In relation to specific *P. vivax celtos* gene, there is a paucity of information available. In fact, it was described for only four different strains used in complete genome studies: Sal-1, Brazil I, North Korean and India VII. Therefore, even with the high conservation degree of *pvceltos* gene sequence in relation to the reference strain Sal-1, all these strains also presented at least one SNP. In our studied isolates, the synonymous mutation C109A was predominant and significantly higher than all other 3 mutations found, while the other synonymous mutation C24A was the least frequent mutation. It is important to mention that this predominant mutation (C109A) is also present in human P01 strain, a new reference genome for *P. vivax* from an Indonesian clinical isolate [38]. Classically, synonymous changes were thought to have no effect on the protein and were called silent, however, recent studies show that even synonymous nucleotide changes can affect protein folding and function [39–41] (Reviewed by [42]). Indeed, in most of the gene encoding proteins, the rate of synonymous substitutions is higher than the rate of non-synonymous substitutions, a condition known as purifying selection, and this has been demonstrated in other *Plasmodium* proteins, such as PfAMA-1 [43]. Interestingly, in relation to *pvceltos* we observed a perfect balance of synonymous and non-synonymous substitutions in the few polymorphisms found in all gene sequences among geographically distinct isolates.

This balance and the low diversity observed could raise at least two hypothesis: firstly, a possible low selective pressure of the immune system against this antigen, which can be corroborated by recent findings from Longley and colleagues that demonstrated a low frequency of naturally acquired antibodies against PvCelTOS in comparison with other sporozoite antigens such as CSP [44]; secondly, the high importance of this protein in sporozoite and ookinetes traversal process could be a consequence of this high conserved profile observed in the sequences of our study. Therefore, aiming to evaluate the degree of diversity of PvCelTOS in different field isolates from Brazilian Amazon, we also compared the amino acid sequence of each field isolate with the reference strain (Sal-1) and the three other hypothetical CelTOS protein derivatives from *P. vivax* genome (Brazil I, North Korean and India VII). Curiously, our isolates presented higher similarity in relation to the reference strain than to Brazil I strain which presented identical sequences in only 13 isolates. Additionally, none of our field isolates presented complete homology with North Korean and India VII strains, both Asian strains presented a mutation in C terminal region at position 178 that was not detected in our Amazon isolates. Moreover, we observed an N-terminal mutation at position 10 (Gly10Ser), which had never been detected in available sequences, but was present in 9.2% of our field samples, as isolates Ser10-Leu118 and Ser10-Val118. This mutation was present in three distant study localities (Manaus, Novo Repartimento and Plácido de Castro) and it was more frequent than

the sequence of Brazil I strain in Novo Repartimento and Plácido de Castro. Interestingly, although the distance from Novo Repartimento, Plácido de Castro and Manaus to Oiapoque could difficult the gene flow and thus explain the absence of this mutation in Oiapoque population, the low frequency of gene flow promoted by the distance would not be the reason for the absence of this mutation in populations; since Porto Velho, which is closer to Plácido Castro (the locality with the highest frequency of this mutation), did not present this mutation.

Unfortunately, due to this high similarity degree we could not determine a genetic structure based on the localities, and the sequences and haplotypes could not be eligible to construct a phylogenetic tree. However, it was possible to identify 9 different haplotypes of *pvceltos* among the 119 *P. vivax* field isolates from the Amazon regions that were analyzed. Regarding the *pvceltos* sequences, we observed that haplotype H1 and H4 were present in all studied localities, however haplotype H4 presented the highest frequency and was significantly higher when compared to the reference H1. These findings suggest a global distribution of parasites containing similar *pvceltos* genotypes. Moreover, the existence of the same haplotypes in different malaria endemic areas will be important for the rationale of malaria vaccine designs.

Like other antigens of pre-erythrocyte stage, the immunity focused on CelTOS depends on humoral and cellular immune responses [10]. Antibodies induced by immunization with *P. berghei* CelTOS were able to recognize live as well as fixed *P. berghei* sporozoites [10] and immunization with *P. falciparum* CelTOS elicits cross-species protection against heterologous challenge with *P. berghei* [9]. Despite this cross-species reactivity, the low degree of similarity between the *P. falciparum* and *P. vivax* CelTOS (63%), and the knowledge that the protection can be reduced by depleting T-cell subsets in immunized animals prior to the sporozoite challenge thus eliminating the contribution of cellular components in protection [10], make crucial the evaluation of both arms of the adaptive response against PvCelTOS to validate it as a vaccine candidate. Additionally, studies based on the genetic diversity of *P. falciparum* merozoite surface proteins, have demonstrated that non-synonymous SNPs contribute to the variability of the parasite and provide escape from host immunity [45]. Thus, to assess the targets of immune response in PvCelTOS and evaluate the potential effects of non-synonymous mutations on immune response against PvCelTOS, we used *in silico* approaches to determine differences on predicted T_{CD8+} epitopes and linear B-cell epitopes among the reference strain (Salvador-1) and mutant PvCelTOS. Firstly, four epitopes were predicted as linear B-cell epitopes on full sequence of PvCelTOS. Interestingly, non-synonymous mutations could modify the potential of these predicted epitopes, once the N-terminal and C-terminal described non-synonymous mutations (Gly10Ser and Lys178Thr) were inserted in predicted linear B-cell epitopes and affected its prediction score. We hypothesize that this finding could not justify the low frequency of responders observed in the unique work that evaluated the natural immune response against PvCelTOS on exposed individuals from Western Thailand [44], but it could indicate the genetic diversity of *Plasmodium vivax* and therefore, its possible effects on immune response can be considered in future studies. Moreover, it has been demonstrated that few amino acid changes can prejudice the binding of peptides to MHC molecules, reduce recognition by T cells or generate antagonistic peptides that inhibit activation of specific T cells by the MHC-peptide complex (Reviewed by [42]). Therefore, in relation to potential T-cell epitopes, six T_{CD8+} epitopes were predicted as hypothetical promiscuous epitopes, presenting an HLA binding frequency higher than 60% and a mean consensus rank smaller than 20. Curiously, PvCelTOS has conserved T_{CD8+} epitopes among all different strains and isolates; once there are not non-synonymous mutations inserted on any predicted T-cell epitope. This finding allied to the showed cellular response to *Plasmodium falciparum* CelTOS in exposed individuals [8] supports the necessity to identify and validate PvCelTOS T-cell epitopes that could be interesting on new vaccine approaches.

P. vivax displays almost twice as much genetic diversity as *P. falciparum* in terms of SNP diversity and gene family variability. This implies that the global population of *P. vivax* may have a capacity for greater functional variation, mainly in gene families associated with immune evasion and erythrocyte invasion. In summary, our findings in PvCelTOS indicate that the very low variations in gene sequences could suggest that this conservative profile is important to the parasite's survival and transmission. Moreover, although some studies have shown the influence of positive natural selection on genetic variability of other *P. vivax* vaccine candidates such as PvAMA-1, PvDBP and PvTRAP [46–48], our epitope prediction results indicate that the few CelTOS polymorphism in *P. vivax* is not maintained by balancing selection related to avoidance of immune recognition by the human host. However, future investigations aiming the naturally acquired cellular and humoral immune response against PvCelTOS derived antigens are still needed to corroborate the potential of PvCelTOS as a vaccine candidate.

Genes and protein sequences used

Plasmodium vivax pvCelTOS mRNA for Pv cell-traversal protein, complete CDs. Accession number: AB194053.1; S4 [*Plasmodium vivax* Sal-1] Accession number: XP_001617263.1; Hypothetical protein PVBG_00206 [Plasmodium vivax Brazil I] Accession number: KMZ84426.1; Hypothetical protein PVNG_01740 [Plasmodium vivax North Korean] Accession number: KMZ 96916.1; Hypothetical protein PVIIG_00773 [Plasmodium vivax India VII] Accession number: KMZ 78086.1

Acknowledgments

We are grateful to all individuals who participated in this study, for their cooperation and generous donation of blood, which made this study possible. To the sequencing platform of the Program for Technological Development of Health Products (PDTIS-Fiocruz) for use of their facilities.

Author Contributions

Conceptualization: JdCLJ DMB LRPR LBC.

Formal analysis: LBC GCC JdCLJ RNRdS JHMdS.

Investigation: LBC DdSPdS RNRdS JHMdS.

Methodology: LBC RNRdS DdSPdS GCC JHMdS.

Resources: DMB RLDM JdCLJ.

Writing – original draft: LBC RNRdS JdCLJ.

Writing – review & editing: DdSPdS LRPR RLDM GCC DMB JHMdS.

References

1. W.H.O. (2015) World Malaria Report.
2. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, et al. (2010) The international limits and population at risk of Plasmodium vivax transmission in 2009. PLoS Negl Trop Dis 4: e774. doi: 10.1371/journal.pntd.0000774 PMID: 20689816
3. Sanitária S-SdV (2015) DATASUS—SIVEP -MALARIA. http://200.214.130.44/sivep_malaria/. pp. Epidemiological report of malaria cases in Brazil on period of 01/01/2015 to 2031/2012/2015.

4. Mueller I, Taime J, Ibam E, Kundi J, Lagog M, et al. (2002) Complex patterns of malaria epidemiology in the highlands region of Papua New Guinea. P N G Med J 45: 200–205. PMID: 12968790
5. Ferraro B, Talbott KT, Balakrishnan A, Cisper N, Morrow MP, et al. (2013) Inducing humoral and cellular responses to multiple sporozoite and liver-stage malaria antigens using exogenous plasmid DNA. Infect Immun 81: 3709–3720. doi: 10.1128/IAI.00180-13 PMID: 23897618
6. Draper SJ, Angov E, Horii T, Miller LH, Srinivasan P, et al. (2015) Recent advances in recombinant protein-based malaria vaccines. Vaccine 33: 7433–7443. doi: 10.1016/j.vaccine.2015.09.093 PMID: 26458807
7. Kariu T, Ishino T, Yano K, Chinzei Y, Yuda M (2006) CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. Mol Microbiol 59: 1369–1379. doi: 10.1111/j.1365-2958.2005.05024.x PMID: 16468982
8. Anum D, Kusi KA, Ganeshan H, Hollingdale MR, Ofori MF, et al. (2015) Measuring naturally acquired ex vivo IFN-gamma responses to Plasmodium falciparum cell-traversal protein for ookinetes and sporozoites (CelTOS) in Ghanaian adults. Malar J 14: 20. doi: 10.1186/s12936-014-0539-5 PMID: 25604473
9. Bergmann-Leitner ES, Mease RM, De La Vega P, Savranskaya T, Polhemus M, et al. (2010) Immunization with pre-erythrocytic antigen CelTOS from Plasmodium falciparum elicits cross-species protection against heterologous challenge with Plasmodium berghei. PLoS One 5: e12294. doi: 10.1371/journal.pone.0012294 PMID: 20808868
10. Bergmann-Leitner ES, Legler PM, Savranskaya T, Ockenhouse CF, Angov E (2011) Cellular and humoral immune effector mechanisms required for sterile protection against sporozoite challenge induced with the novel malaria vaccine candidate CelTOS. Vaccine 29: 5940–5949. doi: 10.1016/j.vaccine.2011.06.053 PMID: 21722682
11. Kusi KA, Bosomprah S, Dodoo D, Kyei-Baafour E, Dickson EK, et al. (2014) Anti-sporozoite antibodies as alternative markers for malaria transmission intensity estimation. Malar J 13: 103. doi: 10.1186/1475-2875-13-103 PMID: 24635830
12. Longley RJ, Reyes-Sandoval A, Montoya-Diaz E, Dunachie S, Kumpitak C, et al. (2015) Acquisition and Longevity of Antibodies to Plasmodium vivax Preerythrocytic Antigens in Western Thailand. Clin Vaccine Immunol 23: 117–124. doi: 10.1128/CVI.00501-15 PMID: 26656115
13. Healer J, Murphy V, Hodder AN, Masciantonio R, Gemmill AW, et al. (2004) Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in Plasmodium falciparum. Mol Microbiol 52: 159–168. doi: 10.1111/j.1365-2958.2003.03974.x PMID: 15049818
14. Oliveira-Ferreira J, Lacerda MV, Brasil P, Ladislau JL, Tauil PL, et al. (2010) Malaria in Brazil: an overview. Malar J 9: 115. doi: 10.1186/1475-2875-9-115 PMID: 20433744
15. Cavasini CE, de Mattos LC, Couto AA, Couto VS, Gollino Y, et al. (2007) Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malar J 6: 167. doi: 10.1186/1475-2875-6-167 PMID: 18093292
16. Pratt-Riccio LR, Sallenave-Sales S, de Oliveira-Ferreira J, da Silva BT, Guimaraes ML, et al. (2008) Evaluation of the genetic polymorphism of Plasmodium falciparum P126 protein (SERA or SERP) and its influence on naturally acquired specific antibody responses in malaria-infected individuals living in the Brazilian Amazon. Malar J 7: 144. doi: 10.1186/1475-2875-7-144 PMID: 18667071
17. Raman S, Vernon R, Thompson J, Tyka M, Sadreyev R, et al. (2009) Structure prediction for CASP8 with all-atom refinement using Rosetta. Proteins 77 Suppl 9: 89–99.
18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410. doi: 10.1016/S0022-2836(05)80360-2 PMID: 2231712
19. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402. PMID: 9254694
20. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, et al. (2014) Pfam: the protein families database. Nucleic Acids Res 42: D222–230. doi: 10.1093/nar/gkt1223 PMID: 24288371
21. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. Proc Natl Acad Sci U S A 98: 10037–10041. doi: 10.1073/pnas.181342398 PMID: 11517324
22. Larsen JE, Lund O, Nielsen M (2006) Improved method for predicting linear B-cell epitopes. Immunome Res 2: 2. doi: 10.1186/1745-7580-2-2 PMID: 16635264
23. Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, et al. (2012) Immune epitope database analysis resource. Nucleic Acids Res 40: W525–530. doi: 10.1093/nar/gks438 PMID: 22610854
24. Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, et al. (2003) Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci 12: 1007–1017. doi: 10.1110/ps.0239403 PMID: 12717023

25. Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, et al. (2008) NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. Nucleic Acids Res 36: W509–512. doi: 10.1093/nar/gkn202 PMID: 18463140

26. Peters B, Sette A (2005) Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. BMC Bioinformatics 6: 132. doi: 10.1186/1471-2105-6-132 PMID: 15927070

27. Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, et al. (2008) Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. Immunome Res 4: 2. doi: 10.1186/1745-7580-4-2 PMID: 18221540

28. Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497. PMID: 14668244

29. Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595. PMID: 2513255

30. Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10: 564–567. doi: 10.1111/j.1755-0998.2010.02847.x PMID: 21565059

31. Miller LH, Roberts T, Shahabuddin M, McCutchan TF (1993) Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). Mol Biochem Parasitol 59: 1–14. PMID: 8515771

32. Mann VH, Huang T, Cheng Q, Saul A (1994) Sequence variation in the circumsporozoite protein gene of Plasmodium vivax appears to be regionally biased. Mol Biochem Parasitol 68: 45–52. PMID: 7891747

33. Putaporntip C, Jongwutiwes S, Tanabe K, Thaithong S (1997) Interallelic recombination in the merozoite surface protein 1 (MSP-1) gene of Plasmodium vivax from Thai isolates. Mol Biochem Parasitol 84: 49–56. PMID: 9041520

34. Sousa TN, Ceravolo IP, Fernandes Fontes CJ, Couto A, Carvalho LH, et al. (2006) The pattern of major polymorphisms in the Duffy binding protein ligand domain among Plasmodium vivax isolates from the Brazilian Amazon area. Mol Biochem Parasitol 146: 251–254. doi: 10.1016/j.molbiopara.2005.11.006 PMID: 16384615

35. Figtree M, Pasay CJ, Slade R, Cheng Q, Cloonan N, et al. (2000) Plasmodium vivax synonymous substitution frequencies, evolution and population structure deduced from diversity in AMA 1 and MSP 1 genes. Mol Biochem Parasitol 108: 53–66. PMID: 10802318

36. de Souza-Neiras WC, de Melo LM, Machado RL (2007) The genetic diversity of Plasmodium vivax—a review. Mem Inst Oswaldo Cruz 102: 245–254. PMID: 17568928

37. Zakeri S, Safi N, Afsharpad M, Butt W, Ghasemi F, et al. (2010) Genetic structure of Plasmodium vivax isolates from two malaria endemic areas in Afghanistan. Acta Trop 113: 12–19. doi: 10.1016/j.actatropica.2009.08.025 PMID: 19716798

38. Auburn S, Bohme U, Steinbiss S, Trimarsanto H, Hostetler J, et al. (2016) A new Plasmodium vivax reference sequence with improved assembly of the subtelomeres reveals an abundance of pir genes. Wellcome Open Res 1: 4. doi: 10.12688/wellcomeopenres.9876.1 PMID: 28008421

39. Chamary JV, Parmley JL, Hurst LD (2006) Hearing silence: non-neutral evolution at synonymous sites in mammals. Nat Rev Genet 7: 98–108. doi: 10.1038/nrg1770 PMID: 16418745

40. Plotkin JB, Kudla G (2011) Synonymous but not the same: the causes and consequences of codon bias. Nat Rev Genet 12: 32–42. doi: 10.1038/nrg2899 PMID: 21102527

41. Schattner P, Diekhans M (2006) Regions of extreme synonymous codon selection in mammalian genes. Nucleic Acids Res 34: 1700–1710. doi: 10.1093/nar/gkl095 PMID: 16556911

42. Sauna ZE, Kimchi-Sarfaty C (2011) Understanding the contribution of synonymous mutations to human disease. Nat Rev Genet 12: 683–691. doi: 10.1038/nrg3051 PMID: 21878961

43. Verra F, Hughes AL (2000) Evidence for ancient balanced polymorphism at the Apical Membrane Antigen-1 (AMA-1) locus of Plasmodium falciparum. Mol Biochem Parasitol 105: 149–153. PMID: 10613707

44. Longley RJ, Reyes-Sandoval A, Montoya-Diaz E, Dunachie S, Kumpitak C, et al. (2016) Acquisition and Longevity of Antibodies to Plasmodium vivax Preerythrocytic Antigens in Western Thailand. Clin Vaccine Immunol 23: 117–124.

45. Kiwanuka GN (2009) Genetic diversity in Plasmodium falciparum merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997–2007. J Vector Borne Dis 46: 1–12. PMID: 19326702

46. Kang JM, Lee J, Cho PY, Moon SU, Ju HL, et al. (2015) Population genetic structure and natural selection of apical membrane antigen-1 in Plasmodium vivax Korean isolates. Malar J 14: 455. doi: 10.1186/s12936-015-0942-6 PMID: 26572984

47. Valizadeh V, Zakeri S, Mehrizi AA, Djadid ND (2014) Population genetics and natural selection in the gene encoding the Duffy binding protein II in Iranian Plasmodium vivax wild isolates. Infect Genet Evol 21: 424–435. doi: 10.1016/j.meegid.2013.12.012 PMID: 24384095

48. Kosuwin R, Putaporntip C, Tachibana H, Jongwutiwes S (2014) Spatial variation in genetic diversity and natural selection on the thrombospondin-related adhesive protein locus of Plasmodium vivax (PvTRAP). PLoS One 9: e110463. doi: 10.1371/journal.pone.0110463 PMID: 25333779

3.3 Artigo 3: “Identificação *in silico* e validação de um epítopo linear de célula B e naturalmente imunogênico na Proteína-9 de superfície de merozoíta, uma candidata vacinal de *Plasmodium vivax*”

3.3.1 Objetivos Específicos

- Identificar *in silico* epítopos lineares de célula B na proteína de fase eritrocítica PvMSP9;
- Confirmar a antigenicidade do principal epítopo predito em indivíduos naturalmente expostos a malária;
- Determinar a existência de correlações entre dados epidemiológicos e a resposta específica para o epítopo identificado.

PLOS | ONE

RESEARCH ARTICLE

In silico Identification and Validation of a Linear and Naturally Immunogenic B-Cell Epitope of the *Plasmodium vivax* Malaria Vaccine Candidate Merozoite Surface Protein-9

Rodrigo Nunes Rodrigues-da-Silva[1], João Hermínio Martins da Silva[2], Balwan Singh[3], Jianlin Jiang[3], Esmeralda V. S. Meyer[4], Fátima Santos[5], Dalma Maria Banic[6], Alberto Moreno[3,7], Mary R. Galinski[3,7], Joseli Oliveira-Ferreira[1]*, Josué da Costa Lima-Junior[1]*

1 Laboratório de Imunoparasitologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, Brazil, **2** Computational Modeling Group—FIOCRUZ-CE, Fortaleza, Brazil, **3** Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, United States of America, **4** Environmental Health and Safety Office, Emory University, Atlanta, GA, United States of America, **5** National Health Foundation, Department of Entomology, Central Laboratory, Porto Velho, RO, Brazil, **6** Laboratory of Simulids and Onchocerciasis "Malaria and Onchocerciasis Research", Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, **7** Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Emory University, Atlanta, GA, United States of America

* josue@ioc.fiocruz.br (JCLJ); lila@ioc.fiocruz.br (JO-F)

CrossMark
click for updates

OPEN ACCESS

Citation: Rodrigues-da-Silva RN, Martins da Silva JH, Singh B, Jiang J, Meyer EVS, Santos F, et al. (2016) *In silico* Identification and Validation of a Linear and Naturally Immunogenic B-Cell Epitope of the *Plasmodium vivax* Malaria Vaccine Candidate Merozoite Surface Protein-9. PLoS ONE 11(1): e0146951. doi:10.1371/journal.pone.0146951

Editor: Érika Martins Braga, Universidade Federal de Minas Gerais, BRAZIL

Received: August 13, 2015

Accepted: December 22, 2015

Published: January 20, 2016

Copyright: © 2016 Rodrigues-da-Silva et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by Brazilian National Research Council – CNPq/PAPES, (Conselho Nacional de Desenvolvimento Científico e Tecnológico/Programa de Apoio Pesquisa Estratégica em Saúde) Fiocruz, the National Institute of Health (NIH Grant #RO1 1R01AI24710), and the Yerkes National Primate Research Center Base Grant (ORIP/OD P51OD011132) awarded by the National Center for Research Resources of the

Abstract

Synthetic peptide vaccines provide the advantages of safety, stability and low cost. The success of this approach is highly dependent on efficient epitope identification and synthetic strategies for efficacious delivery. In malaria, the Merozoite Surface Protein-9 of *Plasmodium vivax* (PvMSP9) has been considered a vaccine candidate based on the evidence that specific antibodies were able to inhibit merozoite invasion and recombinant proteins were highly immunogenic in mice and humans. However the identities of linear B-cell epitopes within PvMSP9 as targets of functional antibodies remain undefined. We used several publicly-available algorithms for *in silico* analyses and prediction of relevant B cell epitopes within PMSP9. We show that the tandem repeat sequence EAAPENAEPVHENA ($PvMSP9_{E795\text{-}A808}$) present at the C-terminal region is a promising target for antibodies, given its high combined score to be a linear epitope and located in a putative intrinsically unstructured region of the native protein. To confirm the predictive value of the computational approach, plasma samples from 545 naturally exposed individuals were screened for IgG reactivity against the recombinant $PvMSP9\text{-}RIRII_{729\text{-}972}$ and a synthetic peptide representing the predicted B cell epitope $PvMSP9_{E795\text{-}A808}$. 316 individuals (58%) were responders to the full repetitive region PvMSP9-RIRII, of which 177 (56%) also presented total IgG reactivity against the synthetic peptide, confirming it validity as a B cell epitope. The reactivity indexes of anti-PvMSP9-RIRII and anti-$PvMSP9_{E795\text{-}A808}$ antibodies were correlated. Interestingly, a potential role in the acquisition of protective immunity was associated with

National Institutes of Health. JCLJ is recipient of a FAPERJ-APQ1 (E-26/111.248/2014) and CPNq-Universal research grants (445150/2014-9), JOF is recipient of CNPq Productivity Fellowship.

Competing Interests: The authors have declared that no competing interests exist.

the linear epitope, since the IgG1 subclass against PvMSP9$_{E795-A808}$ was the prevalent subclass and this directly correlated with time elapsed since the last malaria episode; however this was not observed in the antibody responses against the full PvMSP9-RIRII. In conclusion, our findings identified and experimentally confirmed the potential of PvMSP9$_{E795-A808}$ as an immunogenic linear B cell epitope within the *P. vivax* malaria vaccine candidate PvMSP9 and support its inclusion in future subunit vaccines.

Introduction

Despite global investments in the control and elimination of malaria, the disease remains a major public health burden worldwide. According to the World Health Organization (WHO), more than 3 billion people are still at risk of infection, with an estimated 197 million of cases and 584 thousand deaths [1]. Among the species that infect humans *Plasmodium falciparum* and *P. vivax* are considered the two most important malaria parasites. Although *P. falciparum* is responsible for the major number of cases and deaths, especially in children, *P. vivax* is the most prevalent species outside the African continent [1]. Aside from the enormous socioeconomic impact caused by *P. vivax* prevalence [2], an increased number of publications reporting severe disease [3–8] and the emergence of strains resistant to chloroquine [9–11] and primaquine [12–14], make the development of a safe and affordable vaccine an important component in *P. vivax* control strategies. Although the epidemiological importance of *P. vivax* malaria worldwide is evident, the research on potential *P. vivax* vaccine candidates lags behind that on *P. falciparum*. Currently, there are only four *P. vivax* vaccine candidates or components in advanced preclinical studies and only one in clinical development, while 34 *P. falciparum* candidates are as listed in the WHO's Malaria Vaccine Rainbow Tables [15]. These data show the continued global commitment to control and eliminate malaria with strategies that include vaccination, and highlight the specific need for identifying and testing additional vaccine candidates against *P. vivax*.

Recent advances in adjuvant composition, delivery systems and the design of subunit vaccine constructs, support the use of synthetic peptides containing B and T-cell epitopes as a vaccine platform against malaria. Moreover, synthetic peptide vaccines have several advantages for clinical development, such as their stability in the absence of proteases, the lack of contamination with biological agents, the fast production with good inter-batch reproducibility, and the facility to be produced using solid phase peptide synthesis technologies that do not require skilled operators [16]. In *P. vivax* vaccine studies, long synthetic peptide (LSP) vaccines have been shown to be immunogenic in New World monkeys of the genus *Aotus* [16] and they were reported to be safe and immunogenic in phase I clinical trials [17]. The LSP approach allows the combination of different epitopes of different vaccine targets, a strategy that has had success in murine malaria models [18]. The identification of antigens that induce protective responses and confirmation of their immunogenic potential are critical for effective vaccine development using synthetic platforms.

Invasion of erythrocytes is a critical step in the *Plasmodium* life cycle that is associated with clinical manifestations and complications. Vaccines targeting this stage are intended to reduce morbidity and mortality [19]. Erythrocytic vaccine strategies aim to disrupt the interaction between *Plasmodium* merozoite proteins and erythrocyte surface ligands by eliciting neutralizing antibodies [20, 21], an approach strongly supported by studies with asexual blood-stage antigens in animal models [22] and immune recognition of these antigens by exposed

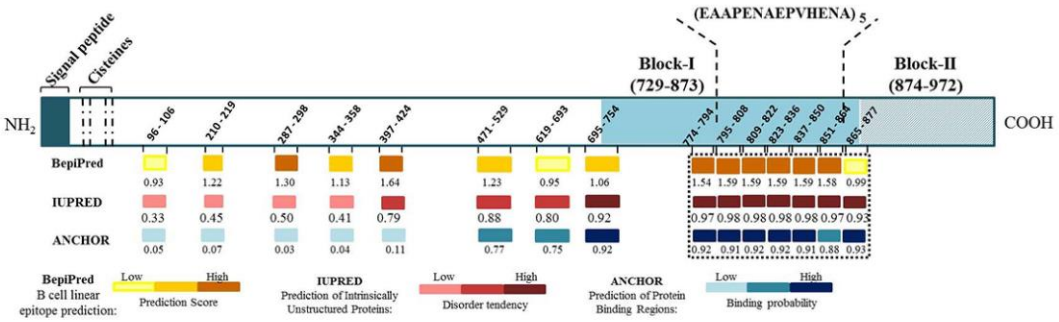


Fig 1. Schematic diagram of PvMSP9 and the predictions scores for linear B cell epitopes, intrinsically unstructured/disordered regions and protein-protein interaction regions. The region corresponding to the amino acid residues 795–808 of PvMSP9 was selected for the synthesis of a soluble peptide based on the best combination of prediction scores using BepiPred, IUPRED and ANCHOR algorithms. Yellow heat bars represent B-cell epitopes, red heat bars represent predicted unordered regions and blue heat bars represent prediction of binding regions. The prediction scores represents the average of scores for all amino acids within the region with prediction values above the cut-offs chosen for significance. The bar color intensities are proportional to the prediction scores.

doi:10.1371/journal.pone.0146951.g001

individuals in malaria-endemic areas [23–27]. In this scenario, Merozoite Surface Proteins (MSP) are a promising set of proteins, since they are expressed during schizogony and become associated with the surface of merozoites in the course of schizont development [28]. Moreover, based on their repeated exposure to the host immune system, several MSPs were described and their immunological properties were investigated [29–31]. Among these proteins, PvMSP9 has gained attention as a potential vaccine candidate. The MSP9 was initially identify in *Plasmodium falciparum* as a 101 kDa Acidic-Basic Repetitive Antigen (ABRA/PfMSP9), and then orthologous genes were identified in other *Plasmodium* species [28, 32, 33]. The phylogeny of MSP9 shows that *P. vivax* and species of *Plasmodium* that infect non-human primates are closely related [34]. Structurally, *P.vivax* MSP9 was described as a hydrophilic protein with a putative 20 amino acid signal peptide, a cluster of four cysteines, a long non-repetitive conserved N-terminal domain and a C-terminal region containing blocks of species-specific tandem repeats [28, 35] (Fig 1). Previous studies have demonstrated that the N-terminal region was immunogenic in mice [36], and naturally acquired immune responses have been described in adults [26] and children [31]. The immunogenic N-terminal region contains five promiscuous T-cell epitopes (pE, pJ, pK, pH and pL), which interact with a broad range of HLA class II molecules [36, 37]. Concerning the C-terminal region, naturally acquired immune responses of adults living in malaria endemic areas, confirmed the presence of highly antigenic blocks of tandem repeats (RI and RII). Anti-PvMSP9-RIRII antibodies are directly correlated to malaria exposure [26, 35]. These observations, allied to the ability of a PvMSP9 monoclonal antibody to inhibit *P. vivax* merozoite invasion into erythrocytes [28], suggest that PvMSP9 contains potential B-cell epitopes that could be used in the design of a multi-target vaccine candidate against *P. vivax*.

Pertinent to this context, most protein epitopes are thought to be discontinuous, composed of different parts of the polypeptide chain that are brought into spatial proximity by the folding of the protein. However, for approximately 10% of the epitopes, the corresponding antibodies are cross-reactive with a linear peptide fragment of the epitope [38], those linear or continuous epitopes are comprised of a single stretch of the polypeptide chain. In the post-genomic era, reverse vaccinology approaches have gained attention for the rational selection of antigens and

identification of key immunological epitopes [39]. Consequently, the efficient prediction and confirmation of immunogenic linear epitopes also represents a promising strategy to develop safe, viable and cost-effective vaccines. The aim of the present study was to identify an antigenic B-cell linear epitope within PvMSP9 and confirm its immunogenicity by using a synthetic peptide representing the predicted epitope for seroepidemiological studies. Our data add further support for studies of vaccines based on linear synthetic-peptides and epitope mapping strategies to characterize *Plasmodium vivax* antigens.

Material and Methods

Sequence Data

To predict possible antigenic properties and the 3-Dimensional (3D) structure of PvMSP9 (PlasmoDB ID: PVX_124060) using bioinformatic tools, the entire sequence of PvMSP9 (Belem strain, Accession Number AAL78897.1) was downloaded from the NCBI website (www.ncbi.nlm.nih.gov/protein) and used for analyses.

B Cell Epitope Prediction

The prediction of linear B-cell epitopes was carried out using the program BepiPred [38]. This software takes a single sequence in FASTA format input and each amino acid receives a prediction score based on Hidden Markov Model profiles of known antigens and incorporates propensity scale methods based on hydrophilicity and secondary structure prediction. For each input sequence the server outputs a prediction score. The positions of the linear B-cell epitopes are predicted to be located at the residues with the highest scores. In order to consider a given region as a valid linear B cell epitope for PvMSP9, the cut-off value of 0.9 was used to warrant a high specificity (0.91) and low predicted sensitivity (0.25). Therefore, the epitope score represents the average of the scores of least nine consecutive amino acids above the cut-off, and the sequences with higher mean values were chosen as potential linear epitopes.

Prediction of Intrinsically Unstructured/Disordered Regions (IURs) and Potential Binding Regions in PvMSP9

The prediction of intrinsically unstructured/disordered regions (IURs) was carried out using the IUPred algorithm [40]. IUPred takes a single sequence in FASTA format as input and predicts the potential IURs. The final output is an individual score for each amino acid that ranges from 0 (completely ordered) to 1 (completely unordered). IURs were then predicted as a region spanning at least 9 contiguous amino acids with individual IUPred prediction score for each amino acid >0.5. Additionally, the ANCHOR tool was used to predict possible regions involved in protein-protein interactions in the complete PvMSP9 sequence. This approach relies on the pairwise energy estimation approach and seeks to identify segments that reside in disordered regions, cannot form enough favorable intra-chain interactions to fold on their own and are likely to gain stabilizing energy by interacting with a globular protein partner [41]. The basic output of this prediction method is a probability score, indicating the likelihood of the residue to be part of a disordered binding region along each position in the sequence. Regions that have a score >0.5 and pass the filtering criteria are predicted as disordered binding regions.

3D Model and Electrostatic Analysis

The 3D structure of MSP9 was predicted using the Robetta algorithm [42]. The amino acid sequence was retrieved from NCBI under accession code AAL78897.1. The Robetta is an

automated algorithm for predictions of the 3D structure of proteins through *ab initio* and comparative modeling. The first step is the searching for structural homologs using BLAST [43] or PSI-BLAST [44]. In the protein sequence, the target primary structure is broken down into separated domains, or independently folding units of proteins, by comparing the sequence to structural families in the Pfam database [45]. Domains with homolog structures follow a template-based modeling protocol. The final five structures are selected by taking the lowest energy models as determined by the Rosetta energy function. The electrostatic surface was calculated with the Adaptive Poisson-Boltzmann Solver (APBS) software [46] integrated with Pymol [47]. The APBS software solves the Poisson-Boltzmann equation in order to describe electrostatic interactions between solute in aqueous solution. Continuous electrostatic plays a very important role in determining ligand-protein and protein-protein binding kinetics.

Molecular Dynamics Simulations

The GROMACS 4.6.5 package [48] was used to perform the minimization and dynamics of the PvMSP9 protein under explicit solvent. Dynamics simulations were run with the GROMOS96 53a6 force field [49] and the SPC water model [50]. The MSP9 model was energy minimized then the system was gradually heated from 0 to 300 K over 3ns using the NVT ensemble with the Berendsen thermostat. A total of 10ns was performed.

Peptide Synthesis

The consensual analysis of the *in silico* prediction tools, indicate the peptide sequence corresponding to residues E_{795}–A_{808} as a relevant epitope within PvMSP9. Therefore, the sequence EAAPENAEPVHENA was synthesized by fluorenylmethoxycarbonyl (F-moc) solid-phase chemistry [51] (GenOne Biotechnologies, Brazil). Analytical chromatography of the peptide demonstrated a purity of >95% and mass spectrometric analysis also indicated an estimated mass of 1477.50 Da, corresponding to the mass of the peptide.

Protein Expression

PvMSP9 recombinant proteins representing the C-terminal region containing the second block of repeats (PvMSP9-RII; aa874-972) [35] and containg Blocks I and II of tandem repeats (PvMSP9-RIRII; aa 729–972) [26] were initially amplified from *P. vivax* (Belem strain), expressed as GST fusion proteins and purified as previously described. The SDS-PAGE of PvMSP9-RIRII recombinant protein used in ELISA assays is shown in S1 Fig.

Samples and Survey

Plasma samples were examined from a cross-sectional cohort study involving 545 individuals from communities in the malaria endemic region of Rondônia state, Brazil, where over the last seven years, *P. vivax* malaria accounts for more than 80% of all malaria cases [52]. The individuals in the study population have been described elsewhere [26]. Briefly, they consist of rain forest natives as well as migrants from several non-endemic areas of Brazil who have resided in the region for 5 years or more. Additionally, samples from 24 individuals from non-endemic regions of Brazil, who never resided in malaria endemic areas and with no history of malaria, comprised a control group. A study survey included questions related to demographics, time of residence in the endemic area, personal histories of malaria and personal knowledge of malaria. The enrollment exclusion criteria were as follows: age <10 years old, pregnancy, breast-feeding, anti-malarial drug use, mental disorders and status as member of an indigenous population. Written informed consent was obtained from all adult donors or from parents of donors in the

case of minors. The study was reviewed and approved by the Oswaldo Cruz Foundation Ethical Committee and the National Ethical Committee of Brazil.

Detection of Specific Antibodies against the Recombinant PvMSP9-RIRII and the Predicted B Cell Epitope EAAPENAEPVHENA ($PvMSP9_{E795-A808}$)

Plasma samples from study participants were screened for the presence of naturally acquired antibodies against the PvMSP9-RIRII recombinant protein and $PvMSP9_{E795-A808}$ synthetic peptide by enzyme-linked immunosorbent assay (ELISA). Briefly, MaxiSorp 96-well plates (Nunc, Rochester, NY) were coated with 5 μg/mL of peptide or 2 μg/mL of recombinant protein. After overnight incubation at 4°C, plates were washed with PBS and blocked with PBS-Tween containing 5% non-fat dry milk (PBS-Tween- M) for 2h at 37°C. Individual plasma samples diluted 1:100 on PBS-Tween-M were added in duplicate wells and the plates incubated at 37°C for 2h. After three washes with PBS-Tween, bound antibodies were detected with peroxidase-conjugated goat anti-human IgG (Sigma St. Louis, MO) followed by o-phenylediamine and hydrogen peroxide. The absorbance was read at 492nm using an ELISA reader (Spectramax 250, Molecular Devices, Sunnyvale, CA) and specific reactivity was obtained by subtraction of the averaged OD value due to GST alone from the averaged OD value of the same plasma for the recombinant protein. The results for total IgG were expressed as reactivity indexes (RI) that were calculated by dividing the mean optical density (OD) of tested samples by the mean ODs plus 3 standard deviations (SD) of 24 non-exposed controls. Subjects were considered IgG responders to a particular antigen if the RI was higher than 1. The total IgG responders were also tested for IgG subclasses using the following peroxidase-conjugated monoclonal mouse anti-human antibodies: clones HP-6001 for IgG1, HP-6002 for IgG2, HP-6050 for IgG3 and HP-6023 for IgG4 (Sigma), as described before. As the cut-off for positivity, subclass-specific prevalence for each antigen was determined using OD values above 3 SD mean OD of 24 non-exposed controls.

Statistical Analysis

All statistics analyzes were carried out using Prism 5.0 for Windows (GraphPad Software, Inc.). The one-sample Kolmogorov-Smirnoff test was used to determine whether a variable was normally distributed. The Wilcoxon matched pairs test was used to compare reactivity indexes of synthetic peptides and recombinant protein (PvMSP9-RIRII) and the optical density (OD) against PvMSP9-RIRII on absorption ELISA. Differences in proportions of the RI of IgG subclasses were evaluated by chi-square test ($\chi 2$) and associations between antibody responses and epidemiological data were determined by the Fisher's exact test or the Spearman rank test when appropriated. A two-sided P value < 0.05 was considered significant.

Results

In silico Analysis of PvMSP9 and Identification of $PvMSP9_{E795-A808}$ as a Potential B Cell Epitope

To detect potential linear B-cell epitopes with intrinsically unstructured/disordered regions and possible components of binding regions in the protein, the full sequence of PvMSP9 was analyzed using the BepiPred, IUPRED and ANCHOR algorithms, respectively. As shown in Fig 1, nine high scored potential linear epitopes with at least nine amino acids were identified on the entire protein sequence. The prediction scores ranged from 0.93 to 1.64. However, a long fragment of 104 amino acids (E_{774} –H_{877}; prediction score mean of 1.5) was identified as a

main epitope within the known naturally immunogenic C terminal region. E_{774}-H_{877} was further characterized and seven linear epitopes were predicted: the first sequence 774–794 with a BepiPred score of 1.54, five uninterrupted tandem repeats of the sequence EAAPENAEPVHENA (E_{795}-A_{808}; E_{809}-A_{822}; E_{823}-$A_{836;}$ E_{837}-A_{850}; E_{851}-A_{864}) and the last predicted sequence 865–877 with a BepiPred score of 0.88. The five tandem repeats represented 29% of all PvMSP9-RIRII amino acid residues and also presented the highest epitope prediction score (mean = 1.59) within the repetitive C-terminal region of PvMSP9 and the second highest prediction score of the full sequence. In relation to the probability of being a binding site and having the presence of intrinsically unstructured regions, the N terminal region presented lower scores (0.06 and 0.49, respectively) and the central region had intermediate scores (0.61 and 0.86). Interestingly, the epitopes located in the C-terminal region (E_{774}-H_{877}) presented the highest disorder tendency score of the protein sequence (0.97) and presented a high probability of being a binding region (0.92). The consensual analysis of prediction scores indicated that the tandem sequence of repeats contained an important epitope within the PvMSP9 region predicted to be involved in protein-protein interactions. Therefore, the putative predicted sequence EAAPENAEPVHENA was designated $PvMSP9_{E795\text{-}A808}$ and selected for further characterization of its potential as a B cell epitope using a synthetic peptide and naturally acquired antibodies.

Molecular Modeling of the PvMSP9 RIRII Domain

The predicted PvMSP9 RIRII structure is composed of 53% alpha helices, less than 1% beta sheets and 45% turns and coils, as measured by Stride [53]. The disordered regions are located in the RIRII domain, represented by green loops (Fig 2A). The PvMSP9 surface is highly charged, with a high number of Asp and Glu residues (Fig 2C and 2D). The region encompassing the PvMSP9-RIRII predicted region seems more flexible than the rest of the structure, shown by the calculated B-factors (Fig 2B). The B-factors of protein crystal structures reflect the overall fluctuation of atoms about their average positions and are capable of providing important information about protein dynamics. This result confirms the prediction of the disordered region defined by BepiPred. Also, it is possible to notice the relative exposition to the solvent and consequently, other receptors or ligands.

Epidemiological Profile of the Studied Individuals

The epidemiological characteristics of the studied population are summarized on Table 1. The participants of the study were mostly composed of men (X^2: 14.98, p = 0.0001), with ages ranging from 10 to 85 years old. All enrolled individuals were residing in areas where malaria transmission occurs and were considered naturally exposed to *P. vivax* infections. As a consequence, 85.7% of participants reported previous malaria episodes and 73.4% reported previous infections with *P. vivax*. At the moment of diagnosis, 11.4% of the population was infected and received the appropriate treatment according to the Brazilian guidelines (Table 1).

Characterization of $PvMSP9_{E795\text{-}A808}$ as a Naturally Immunogenic B-Cell Linear Epitope within the Immunodominant Region of PvMSP9

To test if the PvMSP9-RIRII protein region is a target for naturally acquired antibodies, we assessed the IgG reactivity profile against the recombinant protein representing the two blocks of repeats from plasma samples collected from 545 individuals living in endemic areas of a western amazon region of Brazil. We observed that 58% of the studied population represented antibody responders against the recombinant PvMSP9-RIRII protein. Among the responders, the reactivity index ranged from 1.1 to 9.0 (mean = 2.4 ± 1.7), which reflected a wide spectrum

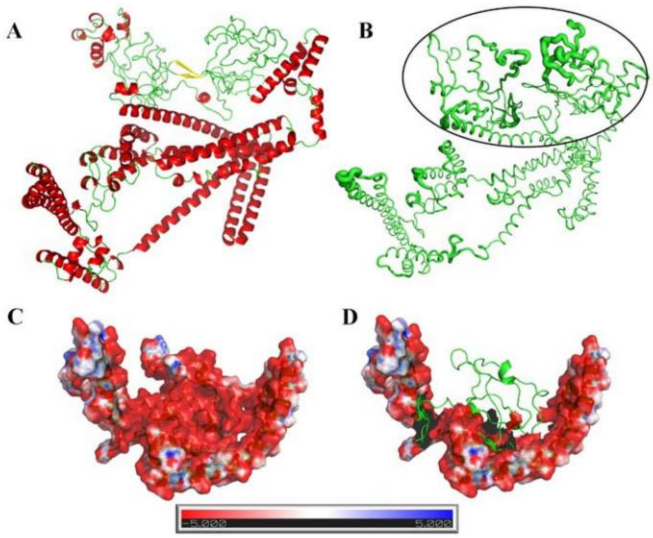


Fig 2. Three-dimensional structure prediction of the PvMSP9 RIRII domain. (A) 3D model of the PvMSP9 domain constructed using the Robetta algorithm. Red structures depict alpha helices, while the disordered region is represented in green. A small beta sheet was found between residues 850 and 858. (B) B-factor as calculated by GROMACS after 10ns simulation. The thicker segments represent the most flexible regions, while the thinnest represent the most rigid. (C) Electrostatic surface of the disordered region, showing a predominantly negative segment in red. (D) Same region show in B, without the electrostatic surface, showing the region 793–866 highlighted in green. The color scale was set from 5 kT/e (red) to 5 kT/e (blue), as calculated by APBS.

doi:10.1371/journal.pone.0146951.g002

in the magnitude of the naturally acquired IgG response and also confirmed the two blocks of repeats as an immunogenic region of PvMSP9. To test if the predicted $PvMSP9_{E795\text{-}A808}$ sequence contains a valid B-cell epitope, we characterized the antigenicity of a synthetic peptide representing this sequence. The overall frequency of responders to the peptide was 32.5%, however among the 316 antibody responder individuals to PvMSP9-RIRII, 56% presented specific IgG antibody response against the $PvMSP9_{E795\text{-}A808}$ synthetic peptide (Fig 3A). The magnitude of the anti-$PvMSP9_{E795\text{-}A808}$ specific IgG response varied with RI values ranged from 1.1 to 3.4 (mean = 1.4 ± 0.4) in responders to $PvMSP9_{E795\text{-}A808}$. Additionally, the IgG subclass profile against the synthetic $PvMSP9_{E795\text{-}A808}$ was characterized with a significantly higher frequency of IgG1 responders (68.6%) over IgG2 (42.2%; $\chi2 = 13.41$ $p<0.0003$); IgG3 (52%, $\chi2 = 5.24$ $p<0.0221$) and IgG4 (28.2%, $\chi2 = 25.86$ $p<0.00001$). A similar profile of IgG subclasses against PvMSP9-RIRII was observed with no significant differences between frequencies of IgG subclasses against the synthetic and recombinant PvMSP9 derived antigens (Fig 3B). After validating that the tandem repeat region within PvMSP9-RIRII is a linear B-cell epitope, we further evaluated the importance of $PvMSP9_{E795\text{-}A808}$ by comparing the fine specificity of the naturally acquired antibody responses. Individuals with antibody responses to $PvMSP9_{E795\text{-}A808}$ had higher IgG levels against PvMSP9-RIRII in comparison to non-responder individuals ($p<0.0001$; Fig 4A). Moreover, as shown in Fig 4A, we also observed that the significantly higher reactivity indexes against PvMSP9-RIRII when compared to

Table 1. Summary of the epidemiological data of naturally exposed individuals enrolled in the study.

	Median (IQ)	Frequency (N)
Gender		
Male	-	55.9% (305)
Female	-	44.1% (240)
Malaria exposure		
Age	33 (21–49)	-
Years of residence on endemic area	23 (16–37)	-
Number of past malaria infections	5 (2–10)	-
Months since the last malaria infection	1 (0–12)	-
Previous malaria species contracted		
Plasmodium vivax	-	15.8% (86)
Plasmodium falciparum	-	10.8% (59)
Both species	-	57.6% (314)
Never infected / Not reported	-	15.8% (86)
Diagnosis		
Plasmodium vivax	-	6.8% (37)
Plasmodium falciparum	-	4.6% (25)
P. falciparum + P. vivax	-	0% (0)
Not infected	-	89% (483)

doi:10.1371/journal.pone.0146951.t001

PvMSP9-RII recombinant protein is present only among the responders to PvMSP9$_{E795\text{-}A808}$, while this significant difference was not observed when the RIs against the repetitive regions was compared among non-responders to peptide. Lastly, as shown in Fig 4B, a weak direct correlation between the RI of IgG against PvMSP9$_{E795\text{-}A808}$ and PvMSP9-RIRII ($p = 0.0045$; $r = 0.1593$) was also observed.

Association between Epidemiological Variables and Antibody Responses to PvMSP9$_{E795\text{-}A808}$

To assess whether epidemiological factors influence the naturally acquired immune response against PvMSP9$_{E795\text{-}A808}$, different variables of the studied population were studied for correlation with the reactivity indexes of total IgG. Our data indicate that the reactivity index IgG specific against PvMSP9$_{E795\text{-}A808}$ was not correlated with number of previous malaria infections (PMI; $p = 0.733$), time of residence in endemic area (TREA; $p = 0.171$) or time since the last malaria episode (TLM; $p = 0.109$). However, among responders to PvMSP9-RIRII, both responders and non-responders to PvMSP9$_{E795\text{-}A808}$ presented significantly higher medians of time of residence in endemic areas ($p<0.0001$ and $p = 0.0001$ respectively) and time since the last malaria episode ($p = 0.0225$ and $p = 0.0278$ respectively) than non-responders to PvMSP9-RIRII (Table 2). In addition, a direct correlation between RIs against PvMS9-RIRII and time of residence in endemic areas was observed in both groups, responders ($r = 0.3619$; $p<0.0001$) and non-responders ($r = 0.2560$; $p<0.002$) to the peptide. Interestingly, the responders to PvMSP9$_{E795\text{-}A808,}$ presented a RI of IgG against PvMSP9-RIRII that directly correlated with the time since the last malaria episode ($p = 0.022$, $r = 0.177$) and inversely correlated with the number of infections in the last six months ($p = 0.0174$, $r = -0.178$), while the non- responders presented no correlation with these parameters. Lastly, in relation to the IgG subclasses against PvMSP9$_{E795\text{-}A808}$, PMI and TREA did not correlate with the RI for all tested subclasses. However, an IgG1 biased response directed to the epitope was associated with a

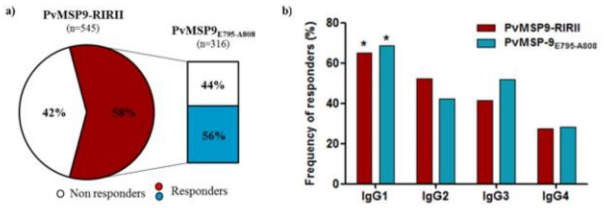


Fig 3. Frequency of total IgG and IgG subclasses responders to PvMSP9-RIRII and to $PvMSP9_{E795-A808}$. (A) Frequency of total IgG responders to PvMSP9-RIRII (red pie slice) and $PvMSP9_{E795-A808}$ (blue bar). (B) Frequency of IgG subclasses responders to PvMSP9-RIRII and $PvMSP9_{E795-A808}$ presented no statistically significant difference. (*) Indicates that the difference was significant ($p < 0.05$) for a comparison between a particular IgG subclass over the others IgG subclasses for the same antigen by chi-square test.

doi:10.1371/journal.pone.0146951.g003

protection parameter, since TLM was correlated with IgG1 reactivity indexes against $PvMSP9_{E795-A808}$ ($r = 0.2644$, $p<0.0362$) but not against PvMSP9-RIRII ($r = -0.0870$, $p- = 0.241$).

Discussion

The development of a safe, efficacious and inexpensive vaccine against *P. vivax* remains a challenge for the scientific community. Despite a considerable number of antigens that have been described as vaccine candidates, the conventional vaccinology strategies applied are especially difficult when dealing with non-cultivable microorganisms, as *P. vivax*. With the concomitant advent of whole-genome sequencing and advances in bioinformatics, the vaccinology field has been radically changed in the last few decades, providing the opportunity for description of

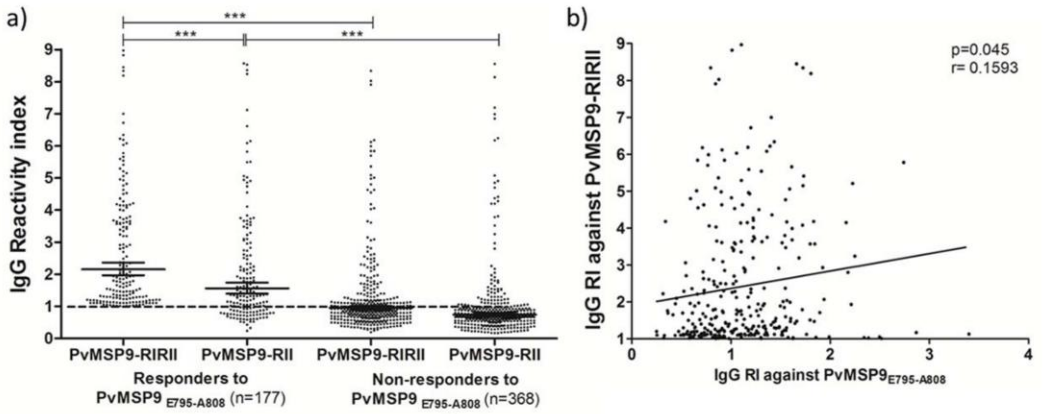


Fig 4. IgG reactivity index to PvMSP9-RIRII and $PvMSP9_{E795-A808}$. (A) Comparison of IgG reactivity index against PvMSP9-RIRII and PvMSP9-RII among responders and non-responders to $PvMSP9_{E795-A808}$. The lines indicate geometric means with 95% of confidence interval. The Mann Whitney test was used to compare medians of IgG reactivity indexes against recombinant proteins on responders and non-responders to synthetic peptide. Significant differences were indicated by *. (*) $p<0.05$; (**) $p<0.001$; *** $p<0.0001$ (B) Correlation between IgG reactivity indexes against $PvMSP9_{E795-A808}$ and against PvMSP9-RIRII. The correlation was assessed by Spearman's rank test.

doi:10.1371/journal.pone.0146951.g004

Table 2. Epidemiological parameters grouped in different IgG reactivity profiles.

Epidemiological parameters	$PvMSP9_{E795-A808}$ (+) PvMSP9-RIRII (+) (n = 177)	$PvMSP9_{E795-A808}$ (-) PvMSP9-RIRII (+) (n = 139)	$PvMSP9_{E795-A808}$ (-) PvMSP9-RIRII (-) (n = 230)
Years of residence in endemic area (Median-IQ)	29 (20–45) ###	26.5 (17–37) ***	19 (15–28) ***; ###
Months elapsed since the last malaria episode (Median-IQ)	24 (2–72) #	16 (2–72) *	12 (2–48) *; #
Number of previous malaria infections (Median-IQ)	5 (2–10)	5 (2–10)	5 (2–10)
Infections in the current year (Mean +SD)	0.44 ± 0.98	0.64 ± 1.46	0.68 ± 1.34
Infected by *P. vivax* at the moment of collection (N—%)	16 (9%)	6 (4%)	25 (11%)

Differences in proportions of number of individuals infected by *P. vivax* at the moment of collection were evaluated by the chi-square test ($\chi 2$) and comparisons of epidemiological parameters were made using the Mann Whitney test.

Significant differences between responders and non-responders to both antigens were indicated in the table by

#: p<0.05

##: p<0.001

###: p<0.0001.

Significant differences between responders to PvMSP9-RIRII only and non-responders were indicated in the table by

*: p<0.05

**: p<0.001

***: p<0.0001.

Statistical differences on epidemiological parameters were not observed between responders and non-responders to $PvMSP9_{E795-A808}$.

doi:10.1371/journal.pone.0146951.t002

novel antigens and improvement of the already known candidates. The vaccine constructs based on synthetic peptides represent one of these well succeeded reemerging strategies [54, 55], but it is strongly dependent of an efficient epitope selection. In this study we describe the identification of a B-cell linear epitope ($PvMSP9_{E795-A808}$) within the *P.vivax* MSP9 using bio-informatics tools applied to reverse vaccinology. Using conventional vaccinology approaches, we validated $PvMSP9_{E795-A808}$ as a target of antibodies by conducting a seroepidemiological assessment using a cohort of individuals naturally exposed to *P. vivax* in malaria endemic regions of western Brazil. Our results support further development of this epitope as a possible subunit in a multi-target synthetic vaccine against *P. vivax*.

Firstly, we screened the full sequence of PvMSP9 using the BepiPred algorithm. The selection of this epitope prediction algorithm was based on the fact that it is the heavily cited and widely used tool for *In silico* analyses of linear B-cell epitopes [38]. In *P. vivax* vaccine research this approach was recently used to map potential epitopes in well-known vaccine candidate PvMSP-1 [56] and also to map and validate a highly immunogenic linear epitope in PvAMA-1 [57]. The *in silico* mapping of PvMSP9 B cell epitopes by BepiPred indicated nine potential regions in the full protein sequence. In comparison to other *P. vivax* vaccine candidates, PvMSP9 presented a comparable number of epitopes predicted and higher predicted mean scores. Interestingly, the repetitive region of PvMSP9 predicted here is located within a long fragment in the C-terminal region previously identified as target of naturally acquired immune responses [26, 58], suggesting that the long sequence of 104 amino acids could be a main target of antibodies directed to $PvMSP9_{E795-A808}$. Based on the evidence that the fragment predicted as a B-cell epitope contains five uninterrupted tandem repeats of the sequence: EAAPE-NAEPVHENA ($_{E795-A808;\ E809-A822;\ E823-A836;\ E837-A850;\ E851-A864}$), each tandem sequence was analyzed as an individual epitope. The epitope based on the predicted tandem sequence of

PvMSP9 is located in a species-specific region [28] with limited polymorphism [34, 35]. These findings supported the selection of this region as a main target for a linear B-cell epitope selection. In addition, allied to the high prediction score as a linear epitope, the sequence EAAPENAEPVHENA had the highest probability to be present in an intrinsically unstructured region of the PvMSP9 sequence. Interestingly, several vaccine candidates that have been extensively studied in *P. falciparum*, were later reported to have unstructured regions, some of which serve as targets of protective immunity. For example, MSP-2 was shown to be largely unstructured [59, 60], MSP-3 and Glutamate-rich protein (GLURP) presented long unstructured regions [61] and even Apical Membrane Antigen -1 (AMA-1), though generally known as a well-structured molecule [62], contains disordered N-and C-terminal regions [63]. In *P. vivax*, a recently described linear epitope within domain II of AMA-1, which was targeted for naturally acquired antibodies, is also located in an IUR. In this context, since many disordered proteins are organized via binding to a structured partner to gain stabilizing energy and undergo a disorder-to-order transition, we also used the ANCHOR algorithm to identify potential binding sites within the disordered regions. As expected, the tandem sequence also presented the highest score within the full protein sequence. In accordance with these findings, our 3D molecular modeling and dynamic simulations of the PvMSP9 structure also indicated the tandem repetitive region as the most disordered, charged and predictably exposed at the surface of merozoites, supporting the idea of this region as critical for protein-protein interactions. The combination of prediction algorithms used for the *In silico* analyses of PvMSP9 were especially interesting given that the mechanism used by MSP9 to be located at the parasite membrane, which is not through a GPI anchor, and its role in merozoite invasion remain unknown [64]. Based on the evidence that specific antibodies against PvMSP9 are able to inhibit the parasite invasion [28], we could hypothesize that antibodies against the repeat regions could have functional activity by inhibiting the formation of MSPs at the surface of merozoites or modify the kinetics of merozoite invasion. In summary, based on the combination of an elevated predicted score in linear B-cell epitope prediction and the highest probability of being inserted in an IUR and located in a binding region of PvMSP9, the sequence EAAPENAEPVHENA designated as $PvMSP9_{E795\text{-}A808}$ was selected for validation as a linear B-cell epitope.

In a cross-sectional study carried out using plasma samples from naturally exposed individuals we firstly confirmed the previously described role of two blocks of tandem repeats at the C-terminal region of PvMSP9 (PvMSP9-RIRII) as target of immune response [26, 31]. The high frequency of responders to PvMSP9-RIRII and the RIs were also consistent with previous studies, which describe the two blocks of repeats as the most immunogenic region of PvMSP9 in adults from the Brazilian Amazon [26]. Among the responders against the recombinant protein PvMSP9-RIRII, the majority of individuals were also reactive to the synthetic peptide representing the predicted epitope $PvMSP9_{E795\text{-}A808}$, confirming that is naturally immunogenic and supporting the *in silico* prediction workflow used. Interestingly, a significant proportion of responders to PvMSP9-RIRII presented no reactivity against the synthetic peptide. Taking into account that the linear epitope is located in the first block of repeats and the RIs of IgG against PvMSP9-RIRII were higher in individuals who were also responders to $PvMSP9_{E795\text{-}A808}$, we believe that non-responders could have had their antibody responses biased towards the second block of repeats, which was also reported as highly immunogenic in our earlier studies [26]. Indeed, the lack of a significant linear epitope predicted in the second block of repeats could suggest that humoral immune responses detected in our previous studies could be directed to conformational epitopes presented in the recombinant PvMSP9-RII. In fact, the lack of peptide-based methods well-stablished for screening these conformational epitopes that we hypothesize limited our findings. On the other hand, the observation of higher antibody levels against PvMSP9-RIRII than PvMSP9-RII only in responders to the peptide and, even with a

lower coefficient, the positive correlation between reactivity indexes of IgG antibodies specific to $PvMSP9_{E795\text{-}A808}$ and PvMSP9-RIRII, suggested that $PvMSP9_{E795\text{-}A808}$ is a linear and naturally immunogenic epitope with significant effect on the humoral immune response directed against the first block of tandem repeats of PvMSP9.

The importance of a linear B-cell epitope within a vaccine candidate against *P. vivax* was also described using a similar approach to study PvAMA-1 [57]. However, even though the higher RI found in comparison with our PvMSP9 derived peptide, there was no association between the high response observed and exposure and/or protection, as well as the subclass profiling of responders. In our work, we found a prevalence of cytophilic IgG1 antibodies that were both reactive and non-reactive to $PvMSP9_{E795\text{-}A808}$. The high prevalence of cytophilic antibodies to $PvMSP9_{E795\text{-}A808}$ was an encouraging finding based on the reported function of such antibodies in the protective immune response to merozoite antigens [65, 66]. In the context of blood-stage malaria immunity against *P. falciparum*, the interaction of cytophilic antibodies (IgG1 and IgG3) with monocytes has been extensively reported as important to mediate the effective antibody-dependent cellular inhibition (ADCI) [67–70]. Conversely, the non-cytophilic responses could interfere with the opsonizing effects of IgG1 and IgG3 [68, 71]. Although the effector mechanism of opsonizing antibodies remains controversial in *P. vivax* malaria, IgG1 and IgG3 subclasses seem to be important. For examples, IgG1 and IgG3 specific to PvMSP1 were the most prevalent IgG subclasses in asymptomatic individuals from Papua New Guinea [72] and from Brazil, respectively [72–74], suggesting a protective role of these antibodies. Curiously, no correlations were observed between exposure or indicative of protection data and IgG subclass reactivity against PvMSP9-RIRII [26]. In this scenario, the prevalence of IgG1 against $PvMSP9_{E795\text{-}A808}$ and the positive ratio between cytophilic and non-cytophilic antibodies (data not show) against the selected peptide suggest the potential for this epitope in immunity acquisition.

Lastly, taking into account that the immune response and susceptibility to malaria are intrinsically linked and vary considerably under different epidemiological scenarios [75], we accessed the relationship between the specific immune response against the linear peptide. We used the years of residence in endemic areas and the self-reported number of malaria lifetime episodes as exposure parameters. Moreover, a crude approximation of protection status estimated by the length of the period (in months) since their last malaria episode and the number of infections within the last 6 months prior to the blood collection. Our first results suggested that antibodies against PvMSP9-RIRII increase with exposure and could be involved in protection, since we observed that responders to PvMSP9-RIRII presented longer time elapsed since the last malaria episode. Additionally, the positive correlation between RIs of IgG specific to recombinant protein and time of residence in the endemic area confirm the cumulative response against the block of tandem repeats in naturally exposed individuals. These findings were corroborated in comparison with previous studies in which a low frequency of antibody responses against PvMSP9-RIRII was reported in children [31] and high frequency in adults [26]. The role of anti-$PvMSP9_{E795\text{-}A808}$ IgG antibodies in this process remain unknown, since there are no significant differences in these parameters between responders and non-responders against the peptide, as well as specific correlations between reactivity indexes and exposure and/or protection parameters used. However, among responders to $PvMSP9_{E795\text{-}A808}$, the reactivity of IgG antibodies specific to PvMSP9-RIRII presented a direct correlation with time since the last malaria episode and an inverse correlation with the number of malaria episodes in the last six months. Therefore, although our results did not show a clear association between IgG against $PvMSP9_{E795\text{-}A808}$ and epidemiological parameters, the direct correlation between IgG1 and the time elapsed since the last malaria episode suggest that the response against

$PvMSP9_{E795-A808}$ could be involved in immunity acquisition mediated by naturally acquired antibodies against PvMSP9.

In conclusion, we identified and confirmed that the PvMSP9 peptide sequence EAAPE-NAEPVHENA ($PvMSP9_{E795-A808}$) contains a linear B-cell epitope. The epitope is present in the protein sequence within a tandem block of repeated amino acids and is targeted by naturally acquired IgG antibodies from individuals living in malaria endemic areas. Antibodies against the linear B-cell epitope were responsible for a significant proportion of immune responses against the entire repetitive region (PvMSP9-RIRII) expressed as a recombinant protein. Lastly, immune responses observed were mainly biased to cytophilic antibodies and the levels of specific IgG1 against the epitope were correlated with epidemiological parameters of protection. Hence, our data describes the potential of $PvMSP9_{E795-A808}$ as an immunogenic linear epitope and support its inclusion in future multi-target vaccine development assays that use synthetic peptides.

Supporting Information

S1 Fig. SDS-PAGE of PvMSP9-RIRII recombinant protein expressed, purified and used as antigen ELISA assays. Lane 1 corresponds to Pre-stained molecular mass markers from BioRad (Precision Plus protein standards, Cat# 161–0373). Lanes 2–3 correspond to rPvMSP9-RIRII in 2.5 μg and 5.0 μg. The recombinant protein migration ≈ 60 Kda confirmed the successfully expression and purity of our antigen, which has a expected molecular mass of 52.1 Kda (25.1 Kda of protein sequence and 27 Kda of GST Tag).
(EPS)

Acknowledgments

We are grateful to all individuals who participated in this study, for their cooperation and generous donation of blood, which made this study possible. We thank the Secretary of Health of Rondonia State and the Laboratorio Central–LACEN of Rondonia for supporting fieldwork. This work was supported by Brazilian National Research Council–CNPq/PAPES, (Conselho Nacional de Desenvolvimento Científico e Tecnológico/Programa de Apoio Pesquisa Estratégica em Saúde) Fiocruz, the National Institute of Health (NIH Grant #RO1 1R01AI24710), and the Yerkes National Primate Research Center Base Grant (ORIP/OD P51OD011132) awarded by the National Center for Research Resources of the National Institutes of Health. JCLJ is recipient of a FAPERJ-APQ1 (E-26/111.248/2014) and CPNq-Universal research grants (445150/2014-9), JOF is recipient of CNPq Productivity Fellowship.

Author Contributions

Conceived and designed the experiments: JCLJ RNRS JOF MRG. Performed the experiments: RNRS JCLJ JOF. Analyzed the data: RNRS AM JHMS MRG JOF JCLJ. Contributed reagents/materials/analysis tools: BS EVSM FS DMB JHMS JJ. Wrote the paper: RNRS JCLJ JOF MRG AM.

References

1. W.H.O. WORLD MALARIA REPORT 2014. 2014.
2. Richie TL, Saul A. Progress and challenges for malaria vaccines. Nature. 2002; 415(6872):694–701. doi: 10.1038/415694a PMID: 11832958.
3. Tan LK, Yacoub S, Scott S, Bhagani S, Jacobs M. Acute lung injury and other serious complications of Plasmodium vivax malaria. The Lancet Infectious diseases. 2008; 8(7):449–54. doi: 10.1016/S1473-3099(08)70153-1 PMID: 18582837.

4. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM. Vivax malaria: neglected and not benign. The American journal of tropical medicine and hygiene. 2007; 77(6 Suppl):79–87. PMID: 18165478; PubMed Central PMCID: PMC2653940.
5. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. Malaria journal. 2014; 13:481. doi: 10.1186/1475-2875-13-481 PMID: 25486908; PubMed Central PMCID: PMC4364574.
6. O'Brien AT, Ramirez JF, Martinez SP. A descriptive study of 16 severe Plasmodium vivax cases from three municipalities of Colombia between 2009 and 2013. Malaria journal. 2014; 13:404. doi: 10.1186/1475-2875-13-404 PMID: 25318617; PubMed Central PMCID: PMC4203896.
7. Gougoutsi A, Karageorgopoulos DE, Dimitriadou A, Melas N, Kranidiotis G, Voutsinas D, et al. Severe Plasmodium vivax malaria complicated with acute respiratory distress syndrome: a case associated with focal autochthonous transmission in Greece. Vector borne and zoonotic diseases. 2014; 14 (5):378–81. doi: 10.1089/vbz.2012.1192 PMID: 24745658.
8. Zubairi AB, Nizami S, Raza A, Mehraj V, Rasheed AF, Ghanchi NK, et al. Severe Plasmodium vivax malaria in Pakistan. Emerging infectious diseases. 2013; 19(11):1851–4. doi: 10.3201/eid1911.130495 PMID: 24188313; PubMed Central PMCID: PMC3837647.
9. Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ. Global extent of chloroquine-resistant Plasmodium vivax: a systematic review and meta-analysis. The Lancet Infectious diseases. 2014; 14(10):982–91. doi: 10.1016/S1473-3099(14)70855-2 PMID: 25213732; PubMed Central PMCID: PMC4178238.
10. de Santana Filho FS, Arcanjo AR, Chehuan YM, Costa MR, Martinez-Espinosa FE, Vieira JL, et al. Chloroquine-resistant Plasmodium vivax, Brazilian Amazon. Emerging infectious diseases. 2007; 13 (7):1125–6. doi: 10.3201/eid1307.061386 PMID: 18214203; PubMed Central PMCID: PMC2878224.
11. Ruebush TK 2nd, Zegarra J, Cairo J, Andersen EM, Green M, Pillai DR, et al. Chloroquine-resistant Plasmodium vivax malaria in Peru. The American journal of tropical medicine and hygiene. 2003; 69 (5):548–52. PMID: 14695094.
12. Nayar JK, Baker RH, Knight JW, Sullivan JS, Morris CL, Richardson BB, et al. Studies on a primaquine-tolerant strain of Plasmodium vivax from Brazil in Aotus and Saimiri monkeys. The Journal of parasitology. 1997; 83(4):739–45. PMID: 9267419.
13. Kristensen KL, Dragsted UB. Recurrent Plasmodium vivax malaria due to dose-dependent primaquine resistance: a case report. Scandinavian journal of infectious diseases. 2014; 46(1):63–5. doi: 10.3109/00365548.2013.822093 PMID: 23957539.
14. Arias AE, Corredor A. Low response of Colombian strains of Plasmodium vivax to classical antimalarial therapy. Tropical medicine and parasitology: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit. 1989; 40(1):21–3. PMID: 2662351.
15. Organization WH. Tables of malaria vaccine projects globally ("Rainbow Tables"). Available: http://www.who.int/immunization/research/development/Rainbow_tables/en/2015. Accessed 20 July 2015.
16. Herrera S, Bonelo A, Perlaza BL, Valencia AZ, Cifuentes C, Hurtado S, et al. Use of long synthetic peptides to study the antigenicity and immunogenicity of the Plasmodium vivax circumsporozoite protein. International journal for parasitology. 2004; 34(13–14):1535–46. doi: 10.1016/j.ijpara.2004.10.009 PMID: 15582530.
17. Herrera S, Bonelo A, Perlaza BL, Fernandez OL, Victoria L, Lenis AM, et al. Safety and elicitation of humoral and cellular responses in colombian malaria-naive volunteers by a Plasmodium vivax circumsporozoite protein-derived synthetic vaccine. The American journal of tropical medicine and hygiene. 2005; 73(5 Suppl):3–9. PMID: 16291760.
18. Caro-Aguilar I, Lapp S, Pohl J, Galinski MR, Moreno A. Chimeric epitopes delivered by polymeric synthetic linear peptides induce protective immunity to malaria. Microbes and infection / Institut Pasteur. 2005; 7(13):1324–37. doi: 10.1016/j.micinf.2005.04.020 PMID: 16253535.
19. Schwartz L, Brown GV, Genton B, Moorthy VS. A review of malaria vaccine clinical projects based on the WHO rainbow table. Malaria journal. 2012; 11:11. doi: 10.1186/1475-2875-11-11 PMID: 22230255; PubMed Central PMCID: PMC3286401.
20. Polley SD, McRobert L, Sutherland CJ. Vaccination for vivax malaria: targeting the invaders. Trends in parasitology. 2004; 20(3):99–102. PMID: 16676415.
21. Wipasa J, Elliott S, Xu H, Good MF. Immunity to asexual blood stage malaria and vaccine approaches. Immunology and cell biology. 2002; 80(5):401–14. doi: 10.1046/j.1440-1711.2002.01107.x PMID: 12225376.
22. Amante FH, Engwerda CR, Good MF. Experimental asexual blood stage malaria immunity. Current protocols in immunology / edited by Coligan John E [et al]. 2011;Chapter 19:Unit 19 4. doi: 10.1002/0471142735.im1904s93 PMID: 21462169.

23. Herrera S, Corradin G, Arevalo-Herrera M. An update on the search for a Plasmodium vivax vaccine. Trends in parasitology. 2007; 23(3):122–8. doi: 10.1016/j.pt.2007.01.008 PMID: 17258937.

24. Mahanty S, Saul A, Miller LH. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. The Journal of experimental biology. 2003; 206(Pt 21):3781–8. PMID: 14506213.

25. Lima-Junior JC, Jiang J, Rodrigues-da-Silva RN, Banic DM, Tran TM, Ribeiro RY, et al. B cell epitope mapping and characterization of naturally acquired antibodies to the Plasmodium vivax merozoite surface protein-3alpha (PvMSP-3alpha) in malaria exposed individuals from Brazilian Amazon. Vaccine. 2011; 29(9):1801–11. doi: 10.1016/j.vaccine.2010.12.099 PMID: 21215342; PubMed Central PMCID: PMC3065243.

26. Lima-Junior JC, Tran TM, Meyer EV, Singh B, De-Simone SG, Santos F, et al. Naturally acquired humoral and cellular immune responses to Plasmodium vivax merozoite surface protein 9 in Northwestern Amazon individuals. Vaccine. 2008; 26(51):6645–54. doi: 10.1016/j.vaccine.2008.09.029 PMID: 18832003; PubMed Central PMCID: PMC4431613.

27. Storti-Melo LM, Souza-Neiras WC, Cassiano GC, Taveira LC, Cordeiro AJ, Couto VS, et al. Evaluation of the naturally acquired antibody immune response to the Pv200L N-terminal fragment of Plasmodium vivax merozoite surface protein-1 in four areas of the Amazon Region of Brazil. The American journal of tropical medicine and hygiene. 2011; 84(2 Suppl):58–63. doi: 10.4269/ajtmh.2011.10–0044 PMID: 21292879; PubMed Central PMCID: PMC3032491.

28. Barnwell JW, Galinski MR, DeSimone SG, Perler F, Ingravallo P. Plasmodium vivax, P. cynomolgi, and P. knowlesi: identification of homologue proteins associated with the surface of merozoites. Experimental parasitology. 1999; 91(3):238–49. doi: 10.1006/expr.1998.4372 PMID: 10072326.

29. Osier FH, Mackinnon MJ, Crosnier C, Fegan G, Kamuyu G, Wanaguru M, et al. New antigens for a multicomponent blood-stage malaria vaccine. Science translational medicine. 2014; 6(247):247ra102. doi: 10.1126/scitranslmed.3008705 PMID: 25080477.

30. Cheng Y, Wang B, Sattabongkot J, Lim CS, Tsuboi T, Han ET. Immunogenicity and antigenicity of Plasmodium vivax merozoite surface protein 10. Parasitology research. 2014; 113(7):2559–68. doi: 10.1007/s00436-014-3907-8 PMID: 24764159.

31. Stanisic DI, Javati S, Kiniboro B, Lin E, Jiang J, Singh B, et al. Naturally acquired immune responses to P. vivax merozoite surface protein 3alpha and merozoite surface protein 9 are associated with reduced risk of P. vivax malaria in young Papua New Guinean children. PLoS neglected tropical diseases. 2013; 7(11):e2498. doi: 10.1371/journal.pntd.0002498 PMID: 24244763; PubMed Central PMCID: PMC3828159.

32. Vargas-Serrato E, Corredor V, Galinski MR. Phylogenetic analysis of CSP and MSP-9 gene sequences demonstrates the close relationship of Plasmodium coatneyi to Plasmodium knowlesi. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2003; 3(1):67–73. PMID: 12797974.

33. Lopera-Mesa TM, Kushwaha A, Mohmmed A, Chauhan VS. Plasmodium berghei merozoite surface protein-9: immunogenicity and protective efficacy using a homologous challenge model. Vaccine. 2008; 26(10):1335–43. doi: 10.1016/j.vaccine.2007.12.042 PMID: 18272263.

34. Chenet SM, Pacheco MA, Bacon DJ, Collins WE, Barnwell JW, Escalante AA. The evolution and diversity of a low complexity vaccine candidate, merozoite surface protein 9 (MSP-9), in Plasmodium vivax and closely related species. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2013; 20:239–48. doi: 10.1016/j.meegid.2013.09.011 PMID: 24044894; PubMed Central PMCID: PMC4066979.

35. Vargas-Serrato E, Barnwell JW, Ingravallo P, Perler FB, Galinski MR. Merozoite surface protein-9 of Plasmodium vivax and related simian malaria parasites is orthologous to p101/ABRA of P. falciparum. Molecular and biochemical parasitology. 2002; 120(1):41–52. PMID: 11849704.

36. Oliveira-Ferreira J, Vargas-Serrato E, Barnwell JW, Moreno A, Galinski MR. Immunogenicity of Plasmodium vivax merozoite surface protein-9 recombinant proteins expressed in E. coli. Vaccine. 2004; 22(15–16):2023–30. doi: 10.1016/j.vaccine.2003.07.021 PMID: 15121316.

37. Lima-Junior JC, Rodrigues-da-Silva RN, Banic DM, Jiang J, Singh B, Fabricio-Silva GM, et al. Influence of HLA-DRB1 and HLA-DQB1 alleles on IgG antibody response to the P. vivax MSP-1, MSP-3alpha and MSP-9 in individuals from Brazilian endemic area. PloS one. 2012; 7(5):e36419. doi: 10.1371/journal.pone.0036419 PMID: 22649493; PubMed Central PMCID: PMC3359319.

38. Larsen JE, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. Immunome research. 2006; 2:2. doi: 10.1186/1745-7580-2-2 PMID: 16635264; PubMed Central PMCID: PMC1479323.

39. Sette A, Rappuoli R. Reverse vaccinology: developing vaccines in the era of genomics. Immunity. 2010; 33(4):530–41. doi: 10.1016/j.immuni.2010.09.017 PMID: 21029963; PubMed Central PMCID: PMC3320742.

40. Dosztanyi Z, Csizmok V, Tompa P, Simon I. IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. Bioinformatics. 2005; 21 (16):3433–4. doi: 10.1093/bioinformatics/bti541 PMID: 15955779.

41. Dosztanyi Z, Meszaros B, Simon I. ANCHOR: web server for predicting protein binding regions in disordered proteins. Bioinformatics. 2009; 25(20):2745–6. doi: 10.1093/bioinformatics/btp518 PMID: 19717576; PubMed Central PMCID: PMC2759549.

42. Raman S, Vernon R, Thompson J, Tyka M, Sadreyev R, Pei J, et al. Structure prediction for CASP8 with all-atom refinement using Rosetta. Proteins. 2009; 77 Suppl 9:89–99. doi: 10.1002/prot.22540 PMID: 19701941; PubMed Central PMCID: PMC3688471.

43. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990; 215(3):403–10. Epub 1990/10/05. doi: 10.1016/s0022-2836(05)80360-2 PMID: 2231712.

44. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research. 1997; 25 (17):3389–402. Epub 1997/09/01. PMID: 9254694; PubMed Central PMCID: PMCPmc146917.

45. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: the protein families database. Nucleic acids research. 2014; 42(D1):D222–D30. doi: 10.1093/nar/gkt1223

46. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. Electrostatics of nanosystems: Application to microtubules and the ribosome. Proceedings of the National Academy of Sciences. 2001; 98 (18):10037–41. doi: 10.1073/pnas.181342398

47. Schrodinger, LLC. The PyMOL Molecular Graphics System, Version 1.7.4. 2010.

48. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC. GROMACS: Fast, flexible, and free. Journal of Computational Chemistry. 2005; 26(16):1701–18. doi: 10.1002/jcc.20291 PMID: 16211538

49. Oostenbrink C, Villa A, Mark AE, van Gunsteren WF. A biomolecular force field based on the free enthalpy of hydration and solvation: the GROMOS force-field parameter sets 53A5 and 53A6. J Comput Chem. 2004; 25(13):1656–76. Epub 2004/07/21. doi: 10.1002/jcc.20090 PMID: 15264259.

50. Berendsen HJC, Postma JPM, Gunsteren WF, Hermans J. Interaction Models for Water in Relation to Protein Hydration. In: Pullman B, editor. Intermolecular Forces. The Jerusalem Symposia on Quantum Chemistry and Biochemistry. 14: Springer Netherlands; 1981. p. 331–42.

51. Gausepohl H, Boulin C, Kraft M, Frank RW. Automated multiple peptide synthesis. Peptide research. 1992; 5(6):315–20. PMID: 1283542.

52. SVS-SIVEP-Malária MdS-. Sistema de Informação de Vigilância Epidemiológica—Notificação de casos. Available: http://portalweb04.saude.gov.br/sivep_malaria/default.asp2015. Accessed 30 July 2015.

53. Heinig M, Frishman D. STRIDE: a web server for secondary structure assignment from known atomic coordinates of proteins. Nucleic acids research. 2004; 32(Web Server issue):W500–2. Epub 2004/06/25. doi: 10.1093/nar/gkh429 PMID: 15215436; PubMed Central PMCID: PMCPmc441567.

54. Cespedes N, Arevalo-Herrera M, Felger I, Reed S, Kajava AV, Corradin G, et al. Antigenicity and immunogenicity of a novel chimeric peptide antigen based on the P. vivax circumsporozoite protein. Vaccine. 2013; 31(42):4923–30. doi: 10.1016/j.vaccine.2013.05.082 PMID: 23954378.

55. Silva-Flannery LM, Cabrera-Mora M, Jiang J, Moreno A. Recombinant peptide replicates immunogenicity of synthetic linear peptide chimera for use as pre-erythrocytic stage malaria vaccine. Microbes and infection / Institut Pasteur. 2009; 11(1):83–91. doi: 10.1016/j.micinf.2008.10.009 PMID: 19015042; PubMed Central PMCID: PMC2673111.

56. Soares LA, Evangelista J, Orlandi PP, Almeida ME, de Sousa LP, Chaves Y, et al. Genetic diversity of MSP1 Block 2 of Plasmodium vivax isolates from Manaus (central Brazilian Amazon). Journal of immunology research. 2014; 2014:671050. doi: 10.1155/2014/671050 PMID: 24741614; PubMed Central PMCID: PMC3987980.

57. Bueno LL, Lobo FP, Morais CG, Mourao LC, de Avila RA, Soares IS, et al. Identification of a highly antigenic linear B cell epitope within Plasmodium vivax apical membrane antigen 1 (AMA-1). PloS one. 2011; 6(6):e21289. doi: 10.1371/journal.pone.0021289 PMID: 21713006; PubMed Central PMCID: PMC3119695.

58. Lima JC, Banic DM, Tran TM, Meyer VSE, De-Simone SG, Santos F, et al. Promiscuous T-cell epitopes of Plasmodium merozoite surface protein 9 (PvMSP9) induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon. Vaccine. 2010; 28(18):3185–91. doi: 10.1016/j.vaccine.2010.02.046 PMID: WOS:000277064500016.

59. Zhang X, Perugini MA, Yao S, Adda CG, Murphy VJ, Low A, et al. Solution conformation, backbone dynamics and lipid interactions of the intrinsically unstructured malaria surface protein MSP2. Journal

of molecular biology. 2008; 379(1):105–21. doi: 10.1016/j.jmb.2008.03.039 PMID: 18440022; PubMed Central PMCID: PMC4432223.

60. Adda CG, Murphy VJ, Sunde M, Waddington LJ, Schloegel J, Talbo GH, et al. Plasmodium falciparum merozoite surface protein 2 is unstructured and forms amyloid-like fibrils. Molecular and biochemical parasitology. 2009; 166(2):159–71. doi: 10.1016/j.molbiopara.2009.03.012 PMID: 19450733; PubMed Central PMCID: PMC2713819.

61. Feng ZP, Zhang X, Han P, Arora N, Anders RF, Norton RS. Abundance of intrinsically unstructured proteins in P. falciparum and other apicomplexan parasite proteomes. Molecular and biochemical parasitology. 2006; 150(2):256–67. doi: 10.1016/j.molbiopara.2006.08.011 PMID: 17010454.

62. Pizarro JC, Vulliez-Le Normand B, Chesne-Seck ML, Collins CR, Withers-Martinez C, Hackett F, et al. Crystal structure of the malaria vaccine candidate apical membrane antigen 1. Science. 2005; 308 (5720):408–11. doi: 10.1126/science.1107449 PMID: 15731407.

63. Nair M, Hinds MG, Coley AM, Hodder AN, Foley M, Anders RF, et al. Structure of domain III of the blood-stage malaria vaccine candidate, *Plasmodium falciparum* apical membrane antigen 1 (AMA1). J Mol Biol. 2002; 322(4):741–53. PMID: 12270711.

64. Li X, Chen H, Oo TH, Daly TM, Bergman LW, Liu SC, et al. A co-ligand complex anchors Plasmodium falciparum merozoites to the erythrocyte invasion receptor band 3. The Journal of biological chemistry. 2004; 279(7):5765–71. doi: 10.1074/jbc.M308716200 PMID: 14630931.

65. Singh S, Soe S, Roussilhon C, Corradin G, Druilhe P. Plasmodium falciparum merozoite surface protein 6 displays multiple targets for naturally occurring antibodies that mediate monocyte-dependent parasite killing. Infection and immunity. 2005; 73(2):1235–8. doi: 10.1128/IAI.73.2.1235–1238.2005 PMID: 15664972; PubMed Central PMCID: PMC547023.

66. Singh S, Soe S, Mejia JP, Roussilhon C, Theisen M, Corradin G, et al. Identification of a conserved region of Plasmodium falciparum MSP3 targeted by biologically active antibodies to improve vaccine design. The Journal of infectious diseases. 2004; 190(5):1010–8. doi: 10.1086/423208 PMID: 15295710.

67. Shi YP, Udhayakumar V, Oloo AJ, Nahlen BL, Lal AA. Differential effect and interaction of monocytes, hyperimmune sera, and immunoglobulin G on the growth of asexual stage Plasmodium falciparum parasites. The American journal of tropical medicine and hygiene. 1999; 60(1):135–41. PMID: 9988337.

68. Bouharoun-Tayoun H, Druilhe P. Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infection and immunity. 1992; 60(4):1473–81. PMID: 1548071; PubMed Central PMCID: PMC257020.

69. Tebo AE, Kremsner PG, Luty AJ. Plasmodium falciparum: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. Experimental parasitology. 2001; 98 (1):20–8. doi: 10.1006/expr.2001.4619 PMID: 11426948.

70. Hermsen CC, Verhage DF, Telgt DS, Teelen K, Bousema JT, Roestenberg M, et al. Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial. Vaccine. 2007; 25(15):2930–40. doi: 10.1016/j.vaccine.2006.06.081 PMID: 16914240.

71. Groux H, Gysin J. Opsonization as an effector mechanism in human protection against asexual blood stages of Plasmodium falciparum: functional role of IgG subclasses. Research in immunology. 1990; 141(6):529–42. PMID: 1704637.

72. Fernandez-Becerra C, Sanz S, Brucet M, Stanisic DI, Alves FP, Camargo EP, et al. Naturally-acquired humoral immune responses against the N- and C-termini of the Plasmodium vivax MSP1 protein in endemic regions of Brazil and Papua New Guinea using a multiplex assay. Malaria journal. 2010; 9:29. doi: 10.1186/1475-2875-9-29 PMID: 20092651; PubMed Central PMCID: PMC2835717.

73. Nogueira PA, Alves FP, Fernandez-Becerra C, Pein O, Santos NR, Pereira da Silva LH, et al. A reduced risk of infection with Plasmodium vivax and clinical protection against malaria are associated with antibodies against the N terminus but not the C terminus of merozoite surface protein 1. Infection and immunity. 2006; 74(5):2726–33. doi: 10.1128/IAI.74.5.2726–2733.2006 PMID: 16622209; PubMed Central PMCID: PMC1459730.

74. Versiani FG, Almeida ME, Melo GC, Versiani FO, Orlandi PP, Mariuba LA, et al. High levels of IgG3 anti ICB2-5 in Plasmodium vivax-infected individuals who did not develop symptoms. Malaria journal. 2013; 12:294. doi: 10.1186/1475-2875-12-294 PMID: 23977965; PubMed Central PMCID: PMC3844576.

75. Cespedes N, Jimenez E, Lopez-Perez M, Rubiano K, Felger I, Alonso P, et al. Antigenicity and immunogenicity of a novel Plasmodium vivax circumsporozoite derived synthetic vaccine construct. Vaccine. 2014; 32(26):3179–86. doi: 10.1016/j.vaccine.2014.04.007 PMID: 24731811.

3.4 Artigo 4 (Aceito com major revision pela revista Vaccine) – Imunogenicidade de construções peptídicas sintéticas baseadas no $PvMSP9_{E795-A808}$, um epítopo linear de célula B da proteína-9 de superífice de merozoíta de *P. vivax*, em camundongos BALB/c.

3.4.1 Objetivos específicos:

- Sintetizar peptídeos lineares representando epítopos de célula T e B da PvMSP9;
- Avaliar a resposta imune celular e humoral induzidas pelos peptídeos sintéticos, formulados em adjuvante, em camundongos BALB/c;
- Averiguar o reconhecimento da proteína recombinante PvMSP9-RIRII pelos anticorpos induzidos pelos peptídeos sintéticos;
- Verificar o reconhecimento da proteína nativa no parasito por anticorpos gerados em camundongos imunizados com peptídeos sintéticos;
- Caracterizar fenotípicamente as subpopulações de células T e B de memória induzidas na imunização com peptídeos sintéticos.

Elsevier Editorial System(tm) for Vaccine
Manuscript Draft

Manuscript Number: JVAC-D-18-00623

Title: Immunogenicity of synthetic peptide constructs based on PvMSP9E795-A808, a linear B-cell epitope of the P. vivax Merozoite Surface Protein-9

Article Type: Original article

Keywords: Malaria
Linear B-cell Epitope
T-cell epitope
Peptide-based immunogens
Immunogenicity
Antigenicity

Corresponding Author: Mr. Josué da Costa Lima-Junior, PhD

Corresponding Author's Institution: Instituto Oswaldo Cruz

First Author: Rodrigo N Rodrigues-da-Silva, PhD

Order of Authors: Rodrigo N Rodrigues-da-Silva, PhD; Daniely Correa-Moreira, PhD; Isabela F Soares; Paula M De Luca, PhD; Paulo Renato R Totino , PhD; Fernanda N Morgado, PhD; Balwan Singh, PhD; Mary R Galinski, PhD; Alberto Moreno, PhD; Joseli Oliveira-Ferreira, PhD; Josué da Costa Lima-Junior, PhD

Abstract: Plasmodium vivax Merozoite Surface Protein-9 (PvMSP-9) is a malaria vaccine candidate naturally immunogenic in humans and able to induce high antibody titers in animals when delivered as a recombinant protein. Recently, we identified the sequence EAAPENAEPVHENA (PvMSP9E795-A808) as the main linear B-cell epitope in naturally exposed individuals. However, the potential of PvMSP9E795-A808 as an immunogen in experimental animal models remained unexplored. Here we assess the immunogenicity of PvMSP9E795-A808 using synthetic peptides. The peptides tested in BALB/c mice include two repeats of the sequence EAAPENAEPVHENA tested alone (peptide RII), or linked to an autologous (PvMSP9 peptide pL; pLRII) or heterologous (p2 tetanus toxin universal T cell epitope; TTRII) T cell epitope. Immune responses were evaluated by ELISA, FLUOROSPOT, and indirect immunofluorescence. We show that all of the peptide constructs tested were immunogenic eliciting specific IgG antibodies at different levels, with a prevalence of IgG1 and IgG2. Animals immunized with synthetic peptides containing T cell epitopes (pLRII or TTRII) had more efficient antibody responses that resulted in higher antibody titers able to recognize the native protein by immunofluorescence. Relevantly, the frequency of IFN-γ secreting SFC elicited by immunization with TTRII synthetic peptide was comparable to that reported to the PvMSP9-Nt recombinant protein. Taken together, our study indicates that PvMSP9E795-A808 is highly immunogenic in mice and further studies to evaluate its value as promising vaccine target are warranted. Moreover, our study supports the critical role of CD4 T cell epitopes to enhance humoral responses induced by subunit-based vaccines.

Suggested Reviewers: Manuel Patarroyo PhD
Fundación Instituto de Inmunología de Colombia
manuel.patarroyo@urosario.edu.co
Expert in malaria vaccines and epitope screening

Myriam Arévalo-Herrera PhD
Caucaseco Scientific Research Center, Cali, Colombia, Faculty of Health, Universidad del Valle, Cali, Colombia
marevalo@inmuno.org
Expert in peptide vaccines against P. vivax and P. falciparum

Fabio T. M. Costa PhD
Universidade Estadual de Campinas (UNICAMP)
fabiotmc72@gmail.com
High expertise in P. vivax and experimental models in malaria

Lilian Lacerda Bueno PhD
Universidade Federal de Minas Gerais
lilacerdabueno@gmail.com
Expert in epitope prediction and imune response against peptide antigens

Ivo Mueller PhD
Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
mueller@wehi.edu.au
Expert in malaria vaccines and immune response against merozoite surface proteins of Plasmodium vivax

Opposed Reviewers:

*Manuscript
Click here to view linked References

Immunogenicity of synthetic peptide constructs based on PvMSP9$_{E795-A808}$, a linear B-cell epitope of the *P. vivax* Merozoite Surface Protein-9

Rodrigo Nunes Rodrigues-da-Silva[1], Daniely Correa-Moreira[2], Isabela Ferreira Soares[1], Paula Melo de-Luca[1], Paulo Renato Rivas Totino[3], Fernanda Nazaré Morgado[4], Balwan Singh[5], Mary R. Galinski[5,6], Alberto Moreno[5,6], Joseli Oliveira Ferreira[1], Josué da Costa Lima-Junior[1*]

[1] Laboratory of Immunoparasitology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, (FIOCRUZ), Rio de Janeiro, RJ, Brazil.

[2] Laboratory of Taxonomy, Biochemistry and Fungi Bioprospecting – Oswaldo Cruz Institute - Oswaldo Cruz Foundation – Rio de Janeiro – Brazil

[3] Laboratory of Malaria Research – Oswaldo Cruz Institute - Oswaldo Cruz Foundation – Rio de Janeiro – Brazil

[4] Laboratory of Leishmaniasis Research – Oswaldo Cruz Institute - Oswaldo Cruz Foundation – Rio de Janeiro – Brazil

[5] Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA.

[6] Division of Infectious Diseases, Emory Vaccine Center, Yerkes National Primate Research Center, Emory University School of Medicine, Emory University, Atlanta, GA, USA

* e-mail: josue@ioc.fiocruz.br

Abstract

Plasmodium vivax Merozoite Surface Protein-9 (PvMSP-9) is a malaria vaccine candidate naturally immunogenic in humans and able to induce high antibody titers in animals when delivered as a recombinant protein. Recently, we identified the sequence EAAPENAEPVHENA ($PvMSP9_{E795-A808}$) as the main linear B-cell epitope in naturally exposed individuals. However, the potential of $PvMSP9_{E795-A808}$ as an immunogen in experimental animal models remained unexplored. Here we assess the immunogenicity of $PvMSP9_{E795-A808}$ using synthetic peptides. The peptides tested in BALB/c mice include two repeats of the sequence EAAPENAEPVHENA tested alone (peptide RII), or linked to an autologous (PvMSP9 peptide pL; pLRII) or heterologous (p2 tetanus toxin universal T cell epitope; TTRII) T cell epitope. Immune responses were evaluated by ELISA, FLUOROSPOT, and indirect immunofluorescence. We show that all of the peptide constructs tested were immunogenic eliciting specific IgG antibodies at different levels, with a prevalence of IgG1 and IgG2. Animals immunized with synthetic peptides containing T cell epitopes (pLRII or TTRII) had more efficient antibody responses that resulted in higher antibody titers able to recognize the native protein by immunofluorescence. Relevantly, the frequency of IFN-γ secreting SFC elicited by immunization with TTRII synthetic peptide was comparable to that reported to the PvMSP9-Nt recombinant protein. Taken together, our study indicates that $PvMSP9_{E795-A808}$ is highly immunogenic in mice and further studies to evaluate its value as promising vaccine target are warranted. Moreover, our study supports the critical role of CD4 T cell epitopes to enhance humoral responses induced by subunit based vaccines.

46

47 **Introduction**

48 After more than a century of basic research on malaria, this vector-borne disease
49 continues to be a global health threat. Although *Plasmodium falciparum* continues to
50 cause the greatest morbidity and lethality among the five species of *Plasmodium* that
51 infect humans, an increasing number of severe cases caused by *P. vivax* have been
52 reported. In 2016, *P. vivax* was responsible for about 40% of malaria cases outside of
53 Africa, representing 64% of malaria cases in the Americas, above 30% in Southeast
54 Asia and 40% in Eastern Mediterranean regions [1]. Several factors are involved in the
55 high transmissibility and spread of *P. vivax* and include: early and continuous
56 production of gametocytes during the erythrocytic cycle [2, 3], shorter development
57 cycle in the vector compared to other *Plasmodium* spp [4], and ability to relapse from
58 long-lasting dormant liver stages (hypnozoites) [5, 6]. These biological features along
59 with the enormous socioeconomic impact caused by *P. vivax* [7], the report of severe
60 and lethal *P. vivax* malaria cases [8-10] and the emergence of chloroquine [11-13] and
61 primaquine resistant strains [14-16] make the development of a safe and affordable
62 vaccine a critical component in *P. vivax* control strategies.

63 The identification and validation of potential vaccine targets against *P. vivax* have
64 been delayed, in part due to difficulties associated with the absence of a continuous,
65 long-term *in vitro* culture of this parasite, but also due to limited of investment in
66 available tools and methods [17-19]. Regardless, peptide constructs, containing B and
67 T-cell epitopes, have been considered in strategies for developing vaccines for *P. vivax*
68 and this direction has advanced for several known target antigens. This vaccine platform
69 based on the design of minimal subunits, using synthetic peptides, has the potential to

deliver precisely defined epitopes that can be produced at large-scale, high yield and relatively low cost [20, 21]. Synthetic peptides are also stable in the absence of proteases, do not have contamination with biological agents, and can be produced in a fast and reproducible manner [22]. Furthermore, peptide vaccines allow the conjugation of multiple epitopes in a single construct representing a promising approach against genetic variants of vaccine candidates that are involved in parasite escape mechanisms [20, 23] and a good strategy to develop multi-stage or multi-specific vaccines. Unfortunately, synthetic peptides have overall poor immunogenicity [24, 25]. Because this, several alternative approaches have been used to overcome this barrier, like the use of virus-like particles and the conjugation of B-cell linear epitopes to T-cell epitopes or lipid moieties [26].

T cell-independent immune responses induced in the absence of T cell help are weaker, uneven and have impaired memory responses in comparison to those elicited by T cell-dependent antigens. T-helper cells play a crucial role in linking innate and adaptive immunity and they become critical components of peptide-based vaccines [27, 28]. A challenge for testing subunit vaccines in preclinical trials is that individual epitopes could not be recognized by the experimental animal models used [29]. Recently, bioinformatics tools have been introduced for the successful *in silico* identification of potential epitopes on vaccine candidates against several pathogens [30-33]. However, the number of predicted and validated epitopes within *P. vivax* antigens, as well as the knowledge on protective efficacy is still limited.

Merozoite Surface Protein 9 (MSP9) is a conserved protein among *Plasmodium* species infecting humans, rodents and primates [34-36], which is expressed on the merozoite surface during schizont development and segmentation [37]. Antibodies

94 produced against *P. cynomolgi* and *P. knowlesi* MSP9 homologs inhibit merozoite
95 invasion of erythrocytes [36]. *P. vivax* MSP9 (PvMSP9) is also immunogenic in animal
96 models [38] and naturally exposed individuals [39]. This experimental evidence
97 supports the research and development of a *P. vivax* vaccine based on MSP9.
98 Structurally, PvMSP9 contains a long non-repetitive conserved N-terminal domain, with
99 five promiscuous CD4 T cell epitopes (pE, pJ, pK, pH and pL) [40, 41] and a C-
100 terminal domain, that contains two blocks of tandem repeats, described as the main
101 target of the humoral response in adults living in endemic areas [39]. Recently, using a
102 combination of *in silico* tools we identified the sequence EAAPENAEPVHENA
103 ($PvMSP9_{E795-A808}$) as a minimal linear B-cell epitope. The native PvMSP9 includes five
104 $PvMSP9_{E795-A808}$ tandem repeats, corresponding to 29% of the PvMSP9's two blocks of
105 repeats. Furthermore, the potential role of $PvMSP9_{E795-A808}$ in the acquisition of
106 protective immunity has been reported [42]. Based on this observation, we aimed to
107 assess the value of $PvMSP9_{E795-A808}$ for the development of a subunit-based *P. vivax*
108 vaccine by characterizing the immunogenicity of this epitope in animal models. Here
109 we characterize the immune responses elicited by immunization with peptide-based
110 immunogens that incorporate PvMSP-$9_{E795-A808}$. Peptides were synthetized representing
111 the B cell epitope alone or conjugated to well-characterized CD4 T cell epitopes. Our
112 data add further support for the development of vaccines based on linear synthetic-
113 peptides and epitope mapping strategies of *P. vivax* proteins.

114

115 **Material and Methods**

116 **Peptide Synthesis**

Three epitopes were selected to design the peptide constructs: (a) the sequence EAAPENAEPVHENAEAAPENAEPVHENA (Peptide RII), consists of two repeats of the identified B-cell linear epitope PvMSP-$9_{E795\text{-}A808}$ arrayed in tandem conformation [42]. (b) The sequence ASIDSMIDEIDFYEK (PvMSP-$9_{A443\text{-}K456}$, Peptide pL), a well-defined promiscuous and naturally immunogenic CD4 T cell epitope [41], and (c) the sequence QYIKANSKFIGITE (Peptide TT), a CD4 T cell epitope, of tetanus toxin, able to enhance the humoral response in mice [43-45]. All peptides were synthesized by fluorenylmethoxycarbonyl (F-moc) solid-phase chemistry [46] (GenOne Biotechnologies, Brazil) as single peptides (RII, pL, TT) and as hybrid peptides, containing a combination of a B-cell epitope and a T-cell epitope (pLRII and TTRII) (Table 1). Synthetic peptides containing PvMSP-$9_{E795\text{-}A808}$ were flanked by cysteine residues at N- and C-terminal regions, which allows spontaneous polymerization, a strategy that has been used to enhance immunogenicity [47-49]. Analytical chromatography of the peptide demonstrated a purity of >95% and mass spectrometric analysis also indicated an estimated mass corresponding to the mass of the peptides.

Table 1: Design of synthetic peptides used in this study. Five peptides were synthesized based on three selected epitopes. The single peptides - RII, representing two repeats of the B cell epitope PvMSP9$_{(E795\text{-}A808)2}$; Peptide pL representing the T cell epitope PvMSP9$_{(A443\text{-}K456)}$ identified within the N-terminal region of PvMSP9; Peptide TT, Tetanus toxin$_{(Q830\text{-}E843)}$ corresponding to a well-defined CD4 T cell epitope. To induce spontaneous polymerization, the peptides used for immunization were synthesized with flanked cysteine residues.

138 **Immunization of mice with synthetic peptides**

139 Female BALB/c mice of 6-8 weeks of age were obtained from the Institute of Science
140 and Technologies in Biomodels (ICTB) / FIOCRUZ. Groups of 21 mice were
141 immunized subcutaneously (s.c) three times at 3-week intervals (days 0, 21 and 42) at
142 the base of the tail with 50 μg of one of the synthetic peptides (RII, pL, TT, pLRII and
143 TTRII) emulsified in 150 μL of Montanide ISI 51 (SEPPIC, France). Controls received
144 only PBS emulsified in the same adjuvant. Mice were bled at days 0, 11, 21, 33, 42, 63,
145 84 and 132, and the sera samples were tested by enzyme-linked immunosorbent assay
146 (ELISA) for antibody responses. On day 63 three mice in each group were sacrificed,
147 and splenocytes were harvested to evaluate cellular immune responses using IFN-γ and
148 IL-5 Fluorospot assays. Twenty-one non-immunized animals were bled and sacrificed at
149 each time point to serve as additional control group.

150 All the animal studies were performed at the animal facilities of Oswaldo Cruz
151 Foundation in accordance with guidelines and protocols approved by the Ethics
152 Committee for Animal Experimentation of the Oswaldo Cruz Foundation CEUA-
153 FIOCRUZ (Protocol Nº LW-12/14).

154 **Recombinant PvMSP9-RIRII**

155 The recombinant protein PvMSP9-RIRII, containing the C-terminal blocks of tandem
156 repetitions, was expressed as a GST fusion protein, as described [39] and were used in
157 Absorption ELISA tests.

158 **Antibody assays**

The presence and levels of specific antibodies against the synthetic peptides in sera of mice were evaluated by Enzyme-linked immunosorbent assay (ELISA). Briefly, 96-microwell plates (Nunc-Maxisorb) were coated with 5µg/mL of synthetic peptides (RII, TT, and pL). After overnight incubation at 4ºC, the plates were washed with PBS and blocked with PBS-0.05% Tween 20 containing 5% non-fat dry milk (PBS-Tween-M 5%) for 1 hour at 37ºC. Individual mice serum samples at two-fold serial dilutions in PBS-Tween-M 2.5% were added to duplicate wells, and the plates were incubated at 37ºC for 2 hours. After three washes with PBS-Tween, bonded antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (Southern Biotech) followed by o-phenylenediamine and hydrogen peroxide. The absorbance was read at 492 nm using an ELISA reader (Spectramax 250, Molecular Devices, Sunnyvale, CA). The end-point titers in the mice sera were determined as the highest dilution at which immunized mice sera had optical density (OD) value three times higher than sera from control mice (the OD values in the control mice were about 0.045, 0.053 and 0.054 for peptides RII, TT, and pL, respectively). The determination of IgG subclass profile against peptide RII was also performed as described above, except that the secondary antibodies used were goat anti-mouse monoclonal antibodies specific for mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech). Moreover, to confirm the reactivity of induced antibodies against PvMSP9, an ELISA was performed following the same methodology above described, except that plates were coated with 2µg/mL of recombinant protein PvMSP9-RIRII.

Absorption ELISA

The absorption ELISAs were performed as previously described [50]. Briefly, 96-microwell plates (NUNC-Maxisorp) were coated overnight with 5 µg/mL of the

peptide RII or 2 µg/mL of PvMSP9-RIRII recombinant protein, then washed, and blocked as described. Sera were added to the plates at end-point titers and incubated for two hours at 37ºC. After incubation, sera were transferred to plates coated overnight with PvMSP9-RIRII (2 µg/mL) after appropriate washing and blocking, and the ELISAs were performed as described. After the read of absorbance, the OD values against PvMSP9-RIRII of serum before and after absorption were compared.

Indirect immunofluorescence assays

The specificity of the antibody response elicited by immunization was tested by immunofluorescence assays (IFA) using air-dried thin films of erythrocytes infected with *P. vivax* schizonts as described previously [38]. Surface expression was detected using sera from mice immunized with synthetic peptides RII, pLRII and TTRII and affinity-purified goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma, St. Louis). Pools of sera from animals of each group (RII, TTRII, pLRII) collected on days 63 and 84 were tested at 1:2 dilution. DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) (SIGMA, St. Loius) was used to confirm the presence of DNA. Serum of an individual from Brazilian Amazon, who presented high antibody titers against *P. vivax* merozoite proteins was used as positive control.

Fluorospot

The relative number of mouse antigen-specific T-cells secreting IFN-γ and IL-5 was determined by FluoroSpot (FluoroSpot kit for mouse IFN-γ /IL-5; MabTech). Briefly, Fluorospot plates were pre-wetted with 15 µL 35% ethanol for 1 min, immediately followed by washing with sterile water (200 µL/well). 100 µL of anti-mouse IFN-γ

(AN18) and anti-mouse IL-5 (TRFK5) antibodies, both diluted to 15 µg/mL in sterile PBS, were added to each well. After overnight incubation at 4ºC, plates were washed with sterile PBS (200 µL/well), and blocked with 200 µL/well with cell culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 1 mM glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 0.5 mM HEPES) for at least 30 min at room temperature. The blocking medium was removed and fresh medium with or without one of the stimulants (ConA, RII, TTRII, pLRII, pL, and TT) combined with anti-CD28 mAb at 0.2µg/mL. Splenocytes of immunized mice were added to each well (250,000 cells/well) in duplicate and incubated for 30 h at 37ºC and 5% CO_2. Cells were removed by washing the plates with PBS (200 µL/well), and 100 µL of monoclonal antibodies anti-IFN-γ (R4-6A2-BAM; 1:200) and anti-IL-5 (TRFK4-biotin; 2µg/mL) in PBS with 0.1% bovine serum albumin (PBS/BSA) were added to each well. Plates were incubated at room temperature for 2h, followed by washing as described above. Secondary detection reagents (anti-BAM-490, and SA-550) were diluted 1:200 in PBS/BSA and 100µL added to each well for 1 h at room temperature. Plates were washed as above and 50 µL fluorescence enhancer added to each well for 15 min. The enhancer was discarded thoroughly, the plate underdrain removed, and the plates left to dry protected from light. IFN-γ and IL-5 secreting cells were counted with an Immunospot reader S6UV ultra (Cellular Technology Ltd, Cleveland, OH). The number of IFN- γ, and IL-5 secreting cells per 10^6 spleen cells was expressed as the mean number of spots induced by antigen subtracted by the number of spots induced by PBS.

Statistical methods

GraphPad Prism version 5 (GraphPad Software, Inc, La Jolla, CA, USA) was used for statistical analysis. Statistical difference in categorical variables between the two

228 defined groups was determined using Fischer exact test while Mann-Whitney U test was
229 used to determine differences in continuous variables. P values of ≤ 0.05 were
230 considered statistically significant.

231

232 **Results**

233 **Synthetic peptides containing the epitope PvMSP9$_{E795\text{-}A808}$ are immunogenic in**
234 **BALB/c mice and showed enhanced activity when linked to a T helper epitope.**

235 To evaluate the immunogenicity of the linear B-cell epitope PvMSP9$_{E795\text{-}A808}$, we
236 determined antibody end-point titers elicited by immunization against peptide RII, a
237 synthetic peptide that includes two repeats of the PvMSP9$_{E795\text{-}A808}$ epitope. Plasma
238 samples from mice immunized with different synthetic peptides (RII, pLRII, TTRII, pL
239 and TT) were collected at different time points and antibody titers determined by
240 ELISA (Figure 1). Firstly, we confirmed that epitope PvMSP9$_{E795\text{-}A808}$ was
241 immunogenic in BALB/c, once that all groups immunized with synthetic peptides
242 containing the peptide RII (RII, pLRII and TTRII) presented specific IgG antibodies
243 against this peptide. Animals immunized with single peptide RII had a detectable level
244 of antibodies at day 42 after the first immunization and reached its maximum antibody
245 level (1:6400) on day 84. Moreover, the immunization with peptides linked to T cell
246 epitopes (pLRII and TTRII) elicited earlier and higher IgG antibody titers against
247 peptide RII. Mice immunized with pLRII had detectable levels of antibodies (1:800) 33
248 days after the first immunization and presented the maximum titers of antibodies
249 (1:12800) at day 63. Besides, animals immunized with TTRII presented a detectable
250 level of antibodies (1:100) 21 days after the first immunization, even before the second

251 immunization, and also reached its maximum level of antibodies (1:25600) at day 63. In
252 groups RII, pLRII and TTRII, the maximum antibodies titers against RII were
253 maintained since than reached until the kinetic last time point.

254 No cross-reactions were observed between antibodies specific to peptide RII and the T
255 cell epitopes (pL and TT), since plasma collected from mice immunized with the single
256 peptide RII, did not develop specific antibodies against peptides pL or TT
257 (Supplementary Figure 1a and 1b, respectively). Mice immunized with a single T cell
258 epitope (pL or TT) did not elicit specific antibodies against RII (Figure 1). Moreover,
259 synthetic peptides containing T cell epitopes (pL, pLRII, TT, and TTRII) elicited low
260 IgG specific responses against the T cell epitopes, 32 days after the first immunization
261 (Supplementary Figure 1). A specific response against peptide pL was observed in
262 plasma of animals immunized with the single peptide pL or the hybrid peptide pLRII
263 (Supplementary Figure 1a). In the same way, anti-peptide TT IgG antibodies were
264 identified in samples collected from mice immunized with the single peptide TT or the
265 hybrid TTRII peptides (Supplementary Figure 1b).

266

267

268 **Figure 1: Endpoint anti-RII antibody titers in mice immunized with synthetic**
269 **peptides.** The figure summarize the results of two different experiments and the values
270 represent the mean of six animals in each point of both experiments (three animals per
271 time point, in each experiment). Lines indicate the variation of antibody titer along
272 experimental kinetic in immunized groups: RII (green), pLRII (blue), TTRII (red), pL

(purple), TT (orange) and PBS (gray). All immunogens were formulated in adjuvant Montanide ISA51. Arrows indicate the immunization times.

IgG1 and IgG2 are the predominant isotypes against RII.

To evaluate the profile of anti-RII IgG subclass induced by synthetic peptides, we determined the final IgG1, IgG2a, IgG2b and IgG3 antibody titers in plasma collected at day 63. In all studied groups, a similar subclass profile was observed with no differences between induced titers of IgG1, IgG2a, and IgG2b (Figure 2). Moreover, the absence of detectable levels of IgG3 was a common finding in all groups. No significant changes in IgG isotype patterns were observed in the course of the follow-up after each immunization (Data not shown).

Figure 2: IgG subclass profile against peptide RII induced by immunization with synthetic peptides. Each point represents end-point mean values of IgG1, IgG2a, IgG2b and IgG3 antibody titers ± SEM. RII (green), pLRII (blue), TTRII (red). Samples were collected three weeks after the last immunization (day 63) from six mice per group.

Anti-RII antibodies elicited by immunization with synthetic peptides recognized the PvMSP9-RIRII recombinant protein.

To confirm the specificity of anti-RII antibodies elicited by immunization, we tested by ELISA the plasma of immunized mice against the recombinant protein PvMSP9-RIRII

295 that represents the two blocks of repeats on the C-terminal region of PvMSP9. Plasma
296 samples collected from mice immunized with the synthetic peptides constructs
297 containing the peptide RII (RII, pLRII and TTRII) were able to recognize the
298 recombinant protein at 1:100 dilution (Figure 3). The O.D. mean values were higher in
299 groups immunized with pLRII or TTRII than in group immunized with RII ($p=0.038$
300 and $p=0.031$; respectively). Moreover, mice immunized with peptide TT, pL or with
301 PBS formulated in adjuvant presented similarly low ODs than groups immunized with
302 RII, pLRII, and TTRII ($p<0.0001$).

303

304

305 **Figure 3: Evaluation of reactivity of anti-RII IgG against the recombinant protein**
306 **PvMSP9-RIRII.** Optical densities of antibodies elicited by immunization with
307 synthetic peptides TTRII (red box), pLRII (blue box), RII (green box), pL (purple box)
308 or TT (orange box) against rPvMSP9-RIRII. Synthetic peptides that include the RII
309 sequence (RII, pLRI, and TTRII) induced antibodies that recognize the recombinant
310 protein. The antibody responses are significantly higher in comparison to control mice
311 immunized with adjuvant alone ($p<0.0001$). Mice immunized with peptides containing
312 linked T and B cell epitopes (pLRII and TTRII) presented higher optical densities than
313 those immunized with the single peptide RII. Animals immunized with synthetic
314 peptides representing T cell epitopes (TT and pL) or PBS formulated in adjuvant were
315 not able to recognize the recombinant protein. Data is presented as Box and Whiskers
316 plots with lines representing 10-90 percentile and p values included. Each column
317 represents the optical densities of animals from each group of samples collected 42, 63,
318 84 and 132 days after the first immunization.

The anti-RII antibodies were able to recognize specifically the MSP9 native protein.

After having demonstrated that anti-RII antibodies recognized the recombinant protein representing the two blocks of repeats within PvMSP9, we investigated whether these antibodies recognize the native protein expressed during the blood stage of the parasite life cycle. To accomplish this, pool of sera collected from mice immunized with different synthetic peptides were tested for reactivity using IFA. Plasma from mice immunized with peptides containing the sequence $PvMSP9_{E795\text{-}A808}$ (RII, pLRII and TTRII) recognized the native MSP9 with a fluorescent pattern consistent with surface staining. No reactivity was observed in sera from naive mice, mice immunized with peptides pL or TT or mice immunized with PBS formulated in adjuvant. Representative results are shown in Figure 4.

Figure 4: RII-specific antibodies induced by synthetic peptides recognize the native PvMSP9. Binding of RII-specific IgG using a pool of sera from BALB/c mice immunized with the synthetic peptide (TTRII) and erythrocytes infected with *P. vivax* schizonts. Images were taken at 100-fold magnification. Left panels show the cell DNA stained with DAPI (4′,6-diamidino-2-phenylindole), middle panels show the fluorescein isothiocyanate (FITC) fluorescence indicating the reactivity of anti-RII IgG against the parasite, and the right panels show the merged images. Scale bar, 5 μm length. The sera

of an individual from Brazil endemic area, who presented high response to *P. vivax* MSP-9 recombinant antigen was as used as positive control.

The promiscuous T-cell epitope TT was critical to induce IFN-γ secreting T cells.

Once observed that T cell epitopes enhanced the antibody responses, we explored the effect of these epitopes on cellular immune responses. To determine the number of IFN-γ and IL-5-Spots Forming Cells (SFC) induced by immunization with synthetic peptides, we used the Fluorospot. Splenocytes derived from mice immunized with the single peptide RII and hybrid peptides (pLRII and TTRII) were collected 3 weeks after the third immunization (day 63) and were stimulated ex vivo using each peptide used for immunization (pL, TT, RII, pLRII and TTRII). Mice immunized with TTRII showed an increased number of IFN-γ-SFC, when stimulated with peptides TT or TTRII, compared to the number of SFC induced by the same peptides using splenocytes collected from mice immunized with other synthetic peptides ($p=0.024$). Moreover, no significant number of IL-5 secreting cells were observed (Figure 5). All cells stimulated with ConA have high numbers of IFN-γ and IL-5-secreting cells (Data not showed).

Figure 5: Detection of IFN-γ and IL-5 secreting cells from mice immunized with synthetic peptides collected three weeks after the last immunization (day 63). Results are express as the mean values of duplicate assays using three different animals from each group individually analyzed. Cells of each animal were individually stimulated with each peptide. (a) Number of IFN-γ SFC in 10^6 spleen cells after

stimulation with peptide pL (gray bars) or pLRII (striped bar). (b) Number of IFN-γ SFC in 10^6 spleen cells when stimulated with peptide TT (gray bars) or TTRII (striped bar). (c) Number of IL-5 SFC in 10^6 spleen cells when stimulated with peptide pL (gray bars) or pLRII (striped bar). (d) Number of IL-5 SFC in 10^6 spleen cells when stimulated with peptide TT (gray bars) or TTRII (striped bar). The corresponding immunized groups (RII, pLRII and TTRII) are presented in the X-axis. The bars represent the mean number of SFC stimulated by synthetic peptides and lines indicate the respective Standard Error Mean. The SFC values were subtracted from the SFC values obtained with the control group.

Discussion

Synthetic peptides represent a promising approach for the development of subunit vaccines [51, 52], providing a safe and inexpensive alternative to the conventional vaccine platforms. This approach can be even more effective by targeting both B and T cell epitopes known to be involved in protective efficacy aiming to induce a balanced immune response [53-56]. However, constructs containing linear B-cell epitopes from *Plasmodium* antigens have not always met with their expected success [57-59]. Both antibody-dependent and -independent T-cell-mediated protective immune mechanisms are operative at different stages of the parasite life cycle [60-64], so the ideal vaccine should combine epitopes identified as strong inducers of both antibody and cell-mediated immunity. In this study, we used synthetic peptides to immunize BALB/c mice and to verify the immunogenicity of two known epitopes described within PvMSP9, a potential vaccine candidate.

Firstly, we observed that the B cell epitope PvMSP9$_{E795-A808}$ was immunogenic and confirmed that hybrid peptides synthesized by linking the sequence to T cell epitopes exhibit enhanced antibody responses. All the peptides tested that include such a sequence (RII, pLRII, and TTRII) induced specific antibodies against RII and the antibody levels were maintained up to three months after the last immunization. Relevantly, both peptides containing B and T cell epitopes, pLRII and TTRII, elicited earlier and higher antibody titers (1:12800 and 1:25600, respectively) than the RII synthetic peptide, that includes only the B cell epitope (1:6400). The enhanced level of antibodies induced by synthetic peptides containing a T helper epitope, pLRII, and TTRII, are in agreement to previous studies [48, 49], which demonstrated the enhancement of a specific response induced by the insertion of a T helper epitope and generated a specific level of antibodies similar to our study. Moreover, although this enhanced humoral response was expected to peptide TT (Tetanus Toxin$_{(Q830-E843)}$) [43-45], we present here the first evidence that the T cell epitope PvMSP9$_{A443-K456}$ has potential as a T helper epitope. The kinetics of the antibody responses elicited by immunization with the synthetic peptides that included the PvMSP9$_{E795-A808}$ epitope reported here are similar to those described for immunization with the recombinant PvMSP9 proteins. However, consistent with the lower immunogenicity of linear peptides, higher levels of antibodies were induced by the recombinant proteins [38].

Despite differences in total IgG levels between immunized groups, similar IgG isotype patterns were elicited with similar titers of IgG1, IgG2a, and IgG2b. Interestingly, the same subclass profiles were observed when BALB/c mice were immunized with PvMSP9 recombinant proteins, formulated in Montanide ISA51 [38]. This effect could be attributed to the adjuvant effect given the fact that adjuvants

411 enhance and modulate the magnitude of adaptive immune responses to co-administered
412 antigens, impacting longevity, antigen avidity, and modulation of isotype and IgG
413 subclass switches [65, 66]. On the other hand, based on the scarce knowledge about the
414 role of each IgG subclass on protection against murine malaria, we cannot determine
415 how effective the induced profile could be in a protective response. To date, amongst
416 mouse IgG subclasses, IgG2a and IgG2b are considered to be the most potent activators
417 of complement and most used in passive transfer experiments in murine infections
418 (including malaria) [67, 68]. Besides, IgG1 is believed not to be a potent complement
419 activator [69]; to be poor at killing tumors [70]; but plays an important role in
420 controlling gastrointestinal parasites [71].

421 Despite early studies already demonstrating that antibodies against recombinant
422 MSP-9 were able to block merozoite invasion *in vitro* [36] and naturally acquired
423 antibodies correlate with exposure/protection in Brazilian Amazon [39] and Southeast
424 Asia [72], the functionality of these induced antibodies against our synthetic constructs
425 and their role on parasite recognition remained unknown. Therefore, we first observed
426 that the induced IgG anti-RII were able to recognize the recombinant protein
427 representing the PvMSP9-RIRII. This result was consistent with our previous study, in
428 which we observed that specific antibodies against RII of naturally exposed individuals
429 corresponded to 30% of antibodies against the PvMSP9-RIRII (Data not showed) [42].
430 Moreover, specific antibodies against peptide RII were also able to recognize the native
431 protein on the surface of merozoites and schizonts in immunofluorescence assays.
432 Unfortunately, we could not carry out an inhibition assay or challenge of immunized
433 mice to verify the protective potential of the anti-RII antibodies. However, considering
434 that monoclonal antibodies against PvMSP9 were able to inhibit the invasion of

Rio de Janeiro, April 16th 2018.

Dear Editor,

P. vivax malaria remains as one of the principal public health problems in tropical regions and the development of an effective vaccine certainly will benefit the control. Despite a considerable number of antigens that were described as candidates, the conventional vaccinology strategies applied are especially difficult when dealing with non-cultivable microorganisms, as *P. vivax*. However, with the concomitant advent of whole-genome sequencing and advances in bioinformatics, the vaccinology field radically change in the last few decades, providing the opportunity for a faster description of novel antigens and improvement of the already known candidates. During the past years, our previous works demonstrated that PvMSP-9 is naturally immunogenic in humans, present no HLA restriction associated to humoral response and have promiscuous T-cell epitopes. In our last published work, we screened the full sequence of PvMSP-9 vaccine candidate using reverse vaccinology bioinformatics tools aiming the identification of important B-cell epitopes. We identified and confirmed the sequence EAAPENAEPVHENA ($PvMSP9_{E795-A808}$) as a valid B-cell epitope within the vaccine candidate PvMSP-9. The epitope is present in the protein sequence as a tandem block of 5 uninterrupted repeats and were targeted by naturally acquired IgG antibodies from individuals living in endemic areas of malaria. In this scenario and aiming a multi-target vaccine constructs in the future, in this work we assess the immunogenicity of $PvMSP9_{E795-A808}$ using synthetic peptides. The peptides tested in BALB/c mice include two repeats of the sequence EAAPENAEPVHENA tested alone (peptide RII), or linked to an autologous (PvMSP9 peptide pL; pLRII) or heterologous (p2 tetanus toxin universal T cell epitope; TTRII) T cell epitope. We show that all of the peptide constructs tested were immunogenic eliciting specific IgG antibodies at different levels, with a prevalence of IgG1 and IgG2. Animals immunized with synthetic peptides containing T cell epitopes (pLRII or TTRII) had more efficient antibody responses that resulted in higher antibody titers able to recognize the recombinant protein and, more importantly, the native protein by immunofluorescence. Taken together, our study results indicates that $PvMSP9_{E795-A808}$ is highly immunogenic in mice and further studies to evaluate its value as promising vaccine target are warranted.

Best Regards,

Josué da Costa Lima Junior, PhD
Laboratory of Immunoparasitology

erythrocytes by *P. vivax* merozoites [36], that two blocks of repeats are the most immunogenic regions of the protein [39], the ability of anti-RII antibodies to recognize the recombinant protein PvMSP9-RIRII and the native protein reinforce the potential of $PvMSP9_{E795\text{-}A808}$ as a vaccine target for novel synthetic constructions.

Interestingly, even with the enhancement in humoral responses elicited by immunization with the synthetic peptide representing the B cell epitope linked to T cell epitopes, only the peptide TTRII was able to induce IFN-γ secreting cells. In our point of view, this finding could be associated with differences on presentation of T cell epitopes by MHC of different models, once for efficient induction of either B-cell or cytotoxic T cell responses, the induction of a robust T helper cell responses is crucial [73, 74]. The use of promiscuous or universal T helper epitopes, which bind several or most MHC class II molecules, respectively, offer a good alternative to design subunit vaccines able to induce a robust immune response regardless of the MHC makeup [75, 76]. Unfortunately, the evident bias on MHC presentation of synthetic peptides in humans and animal models limit several applications. For example, whereas the universal T cell epitope PADRE binds many human HLA-DR molecules with high affinity, they only show strong binding to H2I-Ab in mice [77]. Here, although the epitope $PvMSP9_{A443\text{-}K456}$ (peptide pL) was described as a promiscuous T helper epitope in humans [41], this was the first work using this as a T helper epitope in mice. Moreover, the evaluation of their prediction data suggested no binding of this peptide by mice MHC (H-2-Ib, H-2-Id, and H-2-Ed) (data not showed), whereas peptide TT ($Tetanus\ Toxin_{(Q830\text{-}E843)}$) was described as a universal epitope in human and mice [78]. On the other hand, the induction of IFN-γ secreting cells by TTRII was comparable to the number of secreting cells induced by recombinant protein PvMSP9 [38], supporting

459 that the adequate choices of T helper epitopes could potentiate the immunogenicity of
460 synthetic peptides.

461 In conclusion, this was the first work to evaluate the immunogenicity of the B-cell
462 epitope PvMSP9$_{E795\text{-}A808}$ and the T helper epitope PvMSP9$_{A443\text{-}K456}$, using synthetic
463 peptides as a vaccine platform. The B cell epitope PvMSP9$_{E795\text{-}A808}$ was immunogenic
464 in BALB/c mice, and specific antibodies to this epitope were able to recognize the
465 native parasite protein. Moreover, we confirmed that a Tetanus Toxin derived T-cell
466 epitope enhanced the humoral immune response when conjugated to B cell epitope RII,
467 once TTRII elicited an earlier and higher humoral response than a single peptide RII.
468 Besides, our data suggest that epitope PvMSP9$_{A443\text{-}K456}$ was not a potential T helper in
469 mice, disagreeing with the described promiscuity in binding to several MHC alleles in
470 humans. Our data reinforces the importance of PvMSP9$_{E795\text{-}A808}$ as a potential epitope to
471 be included in a subunit malaria vaccine against *P. vivax*.

472

473 **Acknowledgments**

474 This work was supported by Brazilian National Research Council – CNPq/PAPES
475 (Conselho Nacional de Desenvolvimento Científico e Tecnológico/Programa de Apoio
476 Pesquisa Estratégica em Saúde), Fiocruz, the National Institute of Health (NIH Grant
477 #RO1 1R01AI24710), and the Yerkes National Primate Research Center Base Grant
478 (ORIP/OD P51OD011132) awarded by the National Center for Research Resources of
479 the National Institutes of Health. JCLJ is recipient of FAPERJ APQ1 (E-26/
480 210.653/2015), Jovem Cientista do Nosso Estado (E26/203.255/2016) and CPNq-

Universal research grants (445150/2014-9), JOF and JCLJ are recipients of CNPq Productivity Fellowship.

Supplementary material:

Supplementary Figure 1: Endpoint antibody titers (a) anti-pL and (b) anti-TT. Mean end-point antibody titers derived from six mice are presented. Each line represents one group immunized with a different formulation: pLRII (blue line), TTRII (red line), pL (purple line), TT (orange line), RII (Green line) and PBS (gray line). Arrows indicate the immunization times. Samples from mice immunized with peptide RII and from group immunized with PBS formulated in adjuvant do not presented reactivity against peptides pL or TT. Besides, mice immunized with peptide TT or TTRII did not present antibodies against peptide pL, whilst those immunized with peptides pL or pLRII did not presented antibodies against peptide TT.

References:

[1] W.H.O. World Malaria Report 2017. World Malaria Report2017.

[2] Bousema T, Drakeley C. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clin Microbiol Rev. 2011;24:377-410.

[3] McKenzie FE, Jeffery GM, Collins WE. Gametocytemia and fever in human malaria infections. J Parasitol. 2007;93:627-33.

505 [4] Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, et al. Key
506 gaps in the knowledge of Plasmodium vivax, a neglected human malaria parasite.
507 Lancet Infect Dis. 2009;9:555-66.
508 [5] Dembele L, Franetich JF, Lorthiois A, Gego A, Zeeman AM, Kocken CH, et al.
509 Persistence and activation of malaria hypnozoites in long-term primary hepatocyte
510 cultures. Nat Med. 2014;20:307-12.
511 [6] White MT, Karl S, Battle KE, Hay SI, Mueller I, Ghani AC. Modelling the
512 contribution of the hypnozoite reservoir to Plasmodium vivax transmission. Elife.
513 2014;3.
514 [7] Richie TL, Saul A. Progress and challenges for malaria vaccines. Nature.
515 2002;415:694-701.
516 [8] Geleta G, Ketema T. Severe Malaria Associated with Plasmodium falciparum and P.
517 vivax among Children in Pawe Hospital, Northwest Ethiopia. Malar Res Treat.
518 2016;2016:1240962.
519 [9] Gupta BK, Gupta A, Nehra HR, Balotia HR, Meena SL, Kumar S. Clinical Profile
520 and Prognostic Indicators in Adults Hospitalized with Severe Malaria Caused by
521 Different Plasmodium Species. Infect Dis (Auckl). 2015;8:45-50.
522 [10] Mitra S, Abhilash K, Arora S, Miraclin A. A prospective study from south India to
523 compare the severity of malaria caused by Plasmodium vivax, P. falciparum and dual
524 infection. J Vector Borne Dis. 2015;52:281-6.
525 [11] de Santana Filho FS, Arcanjo AR, Chehuan YM, Costa MR, Martinez-Espinosa
526 FE, Vieira JL, et al. Chloroquine-resistant Plasmodium vivax, Brazilian Amazon.
527 Emerging infectious diseases. 2007;13:1125-6.

528 [12] Karunajeewa HA, Mueller I, Senn M, Lin E, Law I, Gomorrai PS, et al. A trial of
529 combination antimalarial therapies in children from Papua New Guinea. The New
530 England journal of medicine. 2008;359:2545-57.
531 [13] Teka H, Petros B, Yamuah L, Tesfaye G, Elhassan I, Muchohi S, et al.
532 Chloroquine-resistant Plasmodium vivax malaria in Debre Zeit, Ethiopia. Malaria
533 journal. 2008;7:220.
534 [14] Nayar JK, Baker RH, Knight JW, Sullivan JS, Morris CL, Richardson BB, et al.
535 Studies on a primaquine-tolerant strain of Plasmodium vivax from Brazil in Aotus and
536 Saimiri monkeys. J Parasitol. 1997;83:739-45.
537 [15] Kristensen KL, Dragsted UB. Recurrent Plasmodium vivax malaria due to dose-
538 dependent primaquine resistance: a case report. Scandinavian journal of infectious
539 diseases. 2014;46:63-5.
540 [16] Arias AE, Corredor A. Low response of Colombian strains of Plasmodium vivax to
541 classical antimalarial therapy. Tropical medicine and parasitology : official organ of
542 Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur
543 Technische Zusammenarbeit. 1989;40:21-3.
544 [17] Thomson-Luque R, Shaw Saliba K, Kocken CHM, Pasini EM. A Continuous,
545 Long-Term Plasmodium vivax In Vitro Blood-Stage Culture: What Are We Missing?
546 Trends Parasitol. 2017.
547 [18] Patarroyo MA, Calderon D, Moreno-Perez DA. Vaccines against Plasmodium
548 vivax: a research challenge. Expert Rev Vaccines. 2012;11:1249-60.
549 [19] Galinski MR, Tirouvanziam RM, Moreno A. *Plasmodium vivax* vaccine surrogate
550 markers of protection: dawning of a new era. Malaria Vaccine Development: Over 40
551 Years of Trials and Tribulations2014.

552 [20] Patarroyo ME, Patarroyo MA. Emerging rules for subunit-based, multiantigenic,
553 multistage chemically synthesized vaccines. Acc Chem Res. 2008;41:377-86.
554 [21] Satterthwait AC, Chiang LC, Arrhenius T, Cabezas E, Zavala F, Dyson HJ, et al.
555 The conformational restriction of synthetic vaccines for malaria. Bull World Health
556 Organ. 1990;68 Suppl:17-25.
557 [22] Herrera S, Bonelo A, Perlaza BL, Valencia AZ, Cifuentes C, Hurtado S, et al. Use
558 of long synthetic peptides to study the antigenicity and immunogenicity of the
559 Plasmodium vivax circumsporozoite protein. Int J Parasitol. 2004;34:1535-46.
560 [23] Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, et al. Association of
561 malaria parasite population structure, HLA, and immunological antagonism. Science.
562 1998;279:1173-7.
563 [24] Pishraft Sabet L, Taheri T, Memarnejadian A, Mokhtari Azad T, Asgari F,
564 Rahimnia R, et al. Immunogenicity of Multi-Epitope DNA and Peptide Vaccine
565 Candidates Based on Core, E2, NS3 and NS5B HCV Epitopes in BALB/c Mice. Hepat
566 Mon. 2014;14:e22215.
567 [25] Slingluff CL, Jr. The present and future of peptide vaccines for cancer: single or
568 multiple, long or short, alone or in combination? Cancer J. 2011;17:343-50.
569 [26] Jackson DC, Purcell AW, Fitzmaurice CJ, Zeng W, Hart DN. The central role
570 played by peptides in the immune response and the design of peptide-based vaccines
571 against infectious diseases and cancer. Curr Drug Targets. 2002;3:175-96.
572 [27] Lairmore MD, DiGeorge AM, Conrad SF, Trevino AV, Lal RB, Kaumaya PT.
573 Human T-lymphotropic virus type 1 peptides in chimeric and multivalent constructs
574 with promiscuous T-cell epitopes enhance immunogenicity and overcome genetic
575 restriction. Journal of virology. 1995;69:6077-89.

[28] Overholser J, Ambegaokar KH, Eze SM, Sanabria-Figueroa E, Nahta R, Bekaii-Saab T, et al. Anti-Tumor Effects of Peptide Therapeutic and Peptide Vaccine Antibody Co-targeting HER-1 and HER-2 in Esophageal Cancer (EC) and HER-1 and IGF-1R in Triple-Negative Breast Cancer (TNBC). Vaccines (Basel). 2015;3:519-43.

[29] Skwarczynski M, Toth I. Peptide-based synthetic vaccines. Chemical science. 2016;7:842-54.

[30] Xie Q, He X, Yang F, Liu X, Li Y, Liu Y, et al. Analysis of the genome sequence and prediction of B-cell epitopes of the envelope protein of Middle East respiratory syndrome-coronavirus. IEEE/ACM transactions on computational biology and bioinformatics. 2017.

[31] Kalaiselvan S, Sankar S, Ramamurthy M, Ghosh AR, Nandagopal B, Sridharan G. Prediction of Pan-Specific B-Cell Epitopes From Nucleocapsid Protein of Hantaviruses Causing Hantavirus Cardiopulmonary Syndrome. Journal of cellular biochemistry. 2017;118:2320-4.

[32] Kalaiselvan S, Sankar S, Ramamurthy M, Ghosh AR, Nandagopal B, Sridharan G. Prediction of B Cell Epitopes Among Hantavirus Strains Causing Hemorragic Fever With Renal Syndrome. Journal of cellular biochemistry. 2017;118:1182-8.

[33] Pourseif MM, Moghaddam G, Naghili B, Saeedi N, Parvizpour S, Nematollahi A, et al. A novel in silico minigene vaccine based on CD4(+) T-helper and B-cell epitopes of EG95 isolates for vaccination against cystic echinococcosis. Computational biology and chemistry. 2017.

[34] Lopez C, Yepes-Perez Y, Hincapie-Escobar N, Diaz-Arevalo D, Patarroyo MA. What Is Known about the Immune Response Induced by Plasmodium vivax Malaria Vaccine Candidates? Front Immunol. 2017;8:126.

600 [35] Vargas-Serrato E, Corredor V, Galinski MR. Phylogenetic analysis of CSP and
601 MSP-9 gene sequences demonstrates the close relationship of Plasmodium coatneyi to
602 Plasmodium knowlesi. Infect Genet Evol. 2003;3:67-73.
603 [36] Barnwell JW, Galinski MR, DeSimone SG, Perler F, Ingravallo P. Plasmodium
604 vivax, P. cynomolgi, and P. knowlesi: identification of homologue proteins associated
605 with the surface of merozoites. Exp Parasitol. 1999;91:238-49.
606 [37] Vargas-Serrato E, Barnwell JW, Ingravallo P, Perler FB, Galinski MR. Merozoite
607 surface protein-9 of Plasmodium vivax and related simian malaria parasites is
608 orthologous to p101/ABRA of P. falciparum. Mol Biochem Parasitol. 2002;120:41-52.
609 [38] Oliveira-Ferreira J, Vargas-Serrato E, Barnwell JW, Moreno A, Galinski MR.
610 Immunogenicity of Plasmodium vivax merozoite surface protein-9 recombinant
611 proteins expressed in E. coli. Vaccine. 2004;22:2023-30.
612 [39] Lima-Junior JC, Tran TM, Meyer EV, Singh B, De-Simone SG, Santos F, et al.
613 Naturally acquired humoral and cellular immune responses to Plasmodium vivax
614 merozoite surface protein 9 in Northwestern Amazon individuals. Vaccine.
615 2008;26:6645-54.
616 [40] Lima-Junior JC, Rodrigues-da-Silva RN, Banic DM, Jiang J, Singh B, Fabricio-
617 Silva GM, et al. Influence of HLA-DRB1 and HLA-DQB1 alleles on IgG antibody
618 response to the P. vivax MSP-1, MSP-3alpha and MSP-9 in individuals from Brazilian
619 endemic area. PLoS One. 2012;7:e36419.
620 [41] Lima-Junior JC, Banic DM, Tran TM, Meyer VS, De-Simone SG, Santos F, et al.
621 Promiscuous T-cell epitopes of Plasmodium merozoite surface protein 9 (PvMSP9)
622 induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in
623 the Brazilian Amazon. Vaccine. 2010;28:3185-91.

[42] Rodrigues-da-Silva RN, Martins da Silva JH, Singh B, Jiang J, Meyer EV, Santos F, et al. In silico Identification and Validation of a Linear and Naturally Immunogenic B-Cell Epitope of the Plasmodium vivax Malaria Vaccine Candidate Merozoite Surface Protein-9. PLoS One. 2016;11:e0146951.

[43] Kaumaya PT, Kobs-Conrad S, Seo YH, Lee H, VanBuskirk AM, Feng N, et al. Peptide vaccines incorporating a 'promiscuous' T-cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. J Mol Recognit. 1993;6:81-94.

[44] Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. Eur J Immunol. 1989;19:2237-42.

[45] Jang JI, Kim JS, Eom JS, Kim HG, Kim BH, Lim S, et al. Expression and delivery of tetanus toxin fragment C fused to the N-terminal domain of SipB enhances specific immune responses in mice. Microbiol Immunol. 2012;56:595-604.

[46] Frank R. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. J Immunol Methods. 2002;267:13-26.

[47] Silva-Flannery LM, Cabrera-Mora M, Dickherber M, Moreno A. Polymeric linear Peptide chimeric vaccine-induced antimalaria immunity is associated with enhanced in vitro antigen loading. Infect Immun. 2009;77:1798-806.

[48] Caro-Aguilar I, Lapp S, Pohl J, Galinski MR, Moreno A. Chimeric epitopes delivered by polymeric synthetic linear peptides induce protective immunity to malaria. Microbes Infect. 2005;7:1324-37.

646 [49] Caro-Aguilar I, Rodriguez A, Calvo-Calle JM, Guzman F, De la Vega P, Patarroyo
647 ME, et al. Plasmodium vivax promiscuous T-helper epitopes defined and evaluated as
648 linear peptide chimera immunogens. Infect Immun. 2002;70:3479-92.
649 [50] Santiago HC, Bennuru S, Boyd A, Eberhard M, Nutman TB. Structural and
650 immunologic cross-reactivity among filarial and mite tropomyosin: implications for the
651 hygiene hypothesis. J Allergy Clin Immunol. 2011;127:479-86.
652 [51] Cespedes N, Arevalo-Herrera M, Felger I, Reed S, Kajava AV, Corradin G, et al.
653 Antigenicity and immunogenicity of a novel chimeric peptide antigen based on the P.
654 vivax circumsporozoite protein. Vaccine. 2013;31:4923-30.
655 [52] Silva-Flannery LM, Cabrera-Mora M, Jiang J, Moreno A. Recombinant peptide
656 replicates immunogenicity of synthetic linear peptide chimera for use as pre-
657 erythrocytic stage malaria vaccine. Microbes Infect. 2009;11:83-91.
658 [53] Herrera MA, de Plata C, Gonzalez JM, Corradin G, Herrera S. Immunogenicity of
659 multiple antigen peptides containing Plasmodium vivax CS epitopes in BALB/c mice.
660 Mem Inst Oswaldo Cruz. 1994;89 Suppl 2:71-6.
661 [54] Marussig M, Renia L, Motard A, Miltgen F, Petour P, Chauhan V, et al. Linear and
662 multiple antigen peptides containing defined T and B epitopes of the Plasmodium yoelii
663 circumsporozoite protein: antibody-mediated protection and boosting by sporozoite
664 infection. Int Immunol. 1997;9:1817-24.
665 [55] Nardelli B, Tam JP. The MAP system. A flexible and unambiguous vaccine design
666 of branched peptides. Pharm Biotechnol. 1995;6:803-19.
667 [56] Nardin EH, Oliveira GA, Calvo-Calle JM, Nussenzweig RS. The use of multiple
668 antigen peptides in the analysis and induction of protective immune responses against
669 infectious diseases. Adv Immunol. 1995;60:105-49.

[57] Dolan SA, Miller LH, Wellems TE. Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. J Clin Invest. 1990;86:618-24.

[58] Ballou WR, Hoffman SL, Sherwood JA, Hollingdale MR, Neva FA, Hockmeyer WT, et al. Safety and efficacy of a recombinant DNA Plasmodium falciparum sporozoite vaccine. Lancet. 1987;1:1277-81.

[59] Herrington DA, Clyde DF, Losonsky G, Cortesia M, Murphy JR, Davis J, et al. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites. Nature. 1987;328:257-9.

[60] Doolan DL, Hoffman SL. The complexity of protective immunity against liver-stage malaria. J Immunol. 2000;165:1453-62.

[61] Tetteh KK, Polley SD. Progress and challenges towards the development of malaria vaccines. BioDrugs. 2007;21:357-73.

[62] Malkin E, Dubovsky F, Moree M. Progress towards the development of malaria vaccines. Trends Parasitol. 2006;22:292-5.

[63] Mahanty S, Saul A, Miller LH. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. J Exp Biol. 2003;206:3781-8.

[64] Brown GV. Progress in the development of malaria vaccines: context and constraints. Parassitologia. 1999;41:429-32.

[65] Di Pasquale A, Preiss S, Tavares Da Silva F, Garcon N. Vaccine Adjuvants: from 1920 to 2015 and Beyond. Vaccines (Basel). 2015;3:320-43.

[66] Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity. 2010;33:492-503.

[67] Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science. 2005;310:1510-2.

694 [68] Spencer Valero LM, Ogun SA, Fleck SL, Ling IT, Scott-Finnigan TJ, Blackman
695 MJ, et al. Passive immunization with antibodies against three distinct epitopes on
696 Plasmodium yoelii merozoite surface protein 1 suppresses parasitemia. Infect Immun.
697 1998;66:3925-30.
698 [69] Woof JM. Immunology. Tipping the scales toward more effective antibodies.
699 Science. 2005;310:1442-3.
700 [70] Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, Kusser K, et al.
701 Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus.
702 Immunity. 2009;30:421-33.
703 [71] McCoy KD, Stoel M, Stettler R, Merky P, Fink K, Senn BM, et al. Polyclonal and
704 specific antibodies mediate protective immunity against enteric helminth infection. Cell
705 Host Microbe. 2008;4:362-73.
706 [72] Stanisic DI, Javati S, Kiniboro B, Lin E, Jiang J, Singh B, et al. Naturally acquired
707 immune responses to P. vivax merozoite surface protein 3alpha and merozoite surface
708 protein 9 are associated with reduced risk of P. vivax malaria in young Papua New
709 Guinean children. PLoS Negl Trop Dis. 2013;7:e2498.
710 [73] Testa JS, Philip R. Role of T-cell epitope-based vaccine in prophylactic and
711 therapeutic applications. Future Virol. 2012;7:1077-88.
712 [74] Lanier JG, Newman MJ, Lee EM, Sette A, Ahmed R. Peptide vaccination using
713 nonionic block copolymers induces protective anti-viral CTL responses. Vaccine.
714 1999;18:549-57.
715 [75] Moyle PM, Toth I. Modern subunit vaccines: development, components, and
716 research opportunities. ChemMedChem. 2013;8:360-76.

717 [76] Alexander J, del Guercio MF, Maewal A, Qiao L, Fikes J, Chesnut RW, et al.
718 Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce
719 specific high titer IgG antibody responses. J Immunol. 2000;164:1625-33.
720 [77] Alexander J, Sidney J, Southwood S, Ruppert J, Oseroff C, Maewal A, et al.
721 Development of high potency universal DR-restricted helper epitopes by modification
722 of high affinity DR-blocking peptides. Immunity. 1994;1:751-61.
723 [78] Valmori D, Pessi A, Bianchi E, Corradin G. Use of human universally antigenic
724 tetanus toxin T cell epitopes as carriers for human vaccination. J Immunol.
725 1992;149:717-21.
726

Tables

Nomenclature	Topology	Amino acid sequence	MW (Da)
RII	cys-PvMSP9$_{(E795-A808)2}$-cys	CEAAPENAEPVHENAEAAPENAEPVHENAC	3143.25
pL	PvMSP9$_{(A443-K456)}$	ASIDSMIDEIDFYEK	1775.93
TT	Tetanus toxin$_{(Q830-E843)}$	QYIKANSKFIGITE	1611.84
pLRII	cys-PvMSP9$_{(A443-K456)}$PvMSP9$_{(E795-A808)2}$-cys	CASIDSMIDEIDFYEKEAAPENAEPVHENAEAAPENAEPVHENAC	4901.17
TTRII	cys-Tetanus toxin$_{(Q830-E843)}$PvMSP9$_{(E795-A808)2}$-cys	CQYIKANSKFIGITEEAAPENAEPVHENAEAAPENAEPVHENAC	4737.07

Figure 1
Click here to download high resolution image

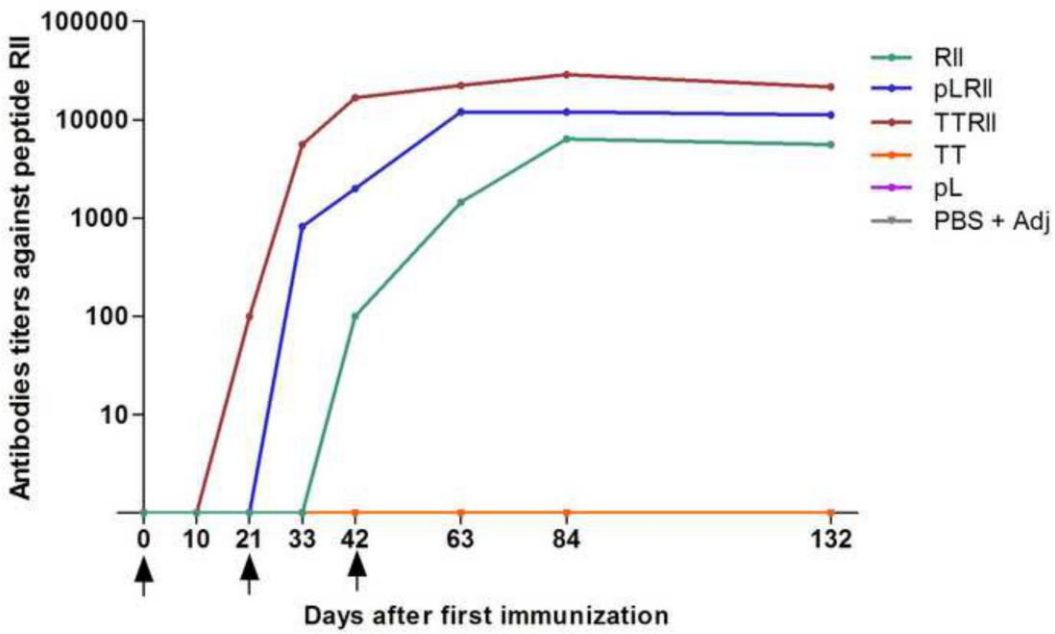


Figure 2
Click here to download high resolution image

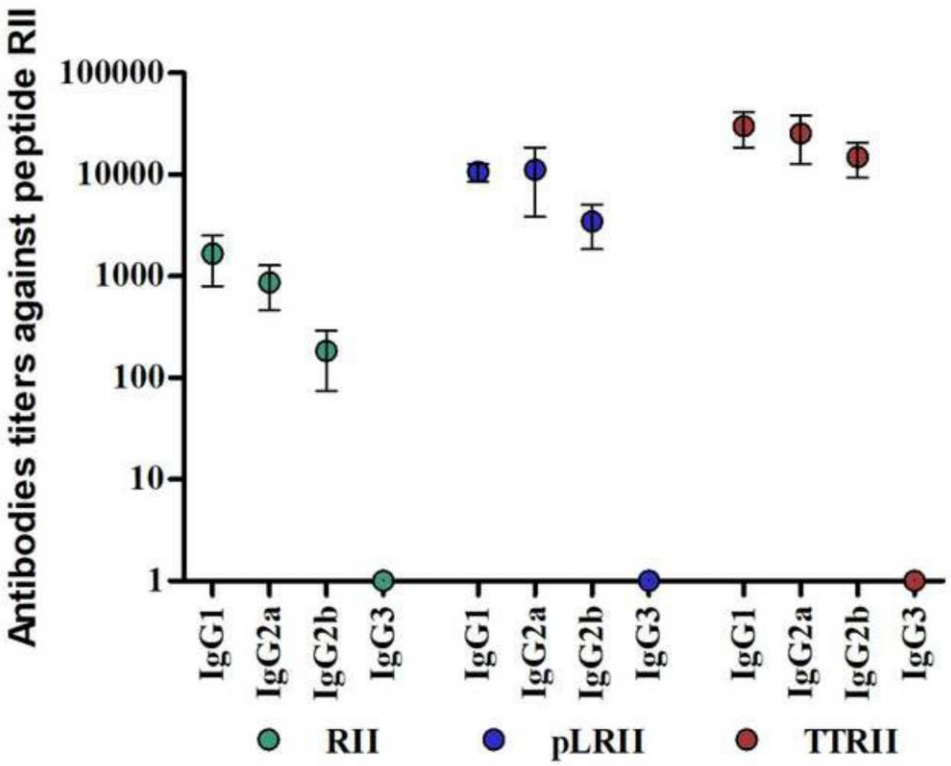


Figure 3
Click here to download high resolution image

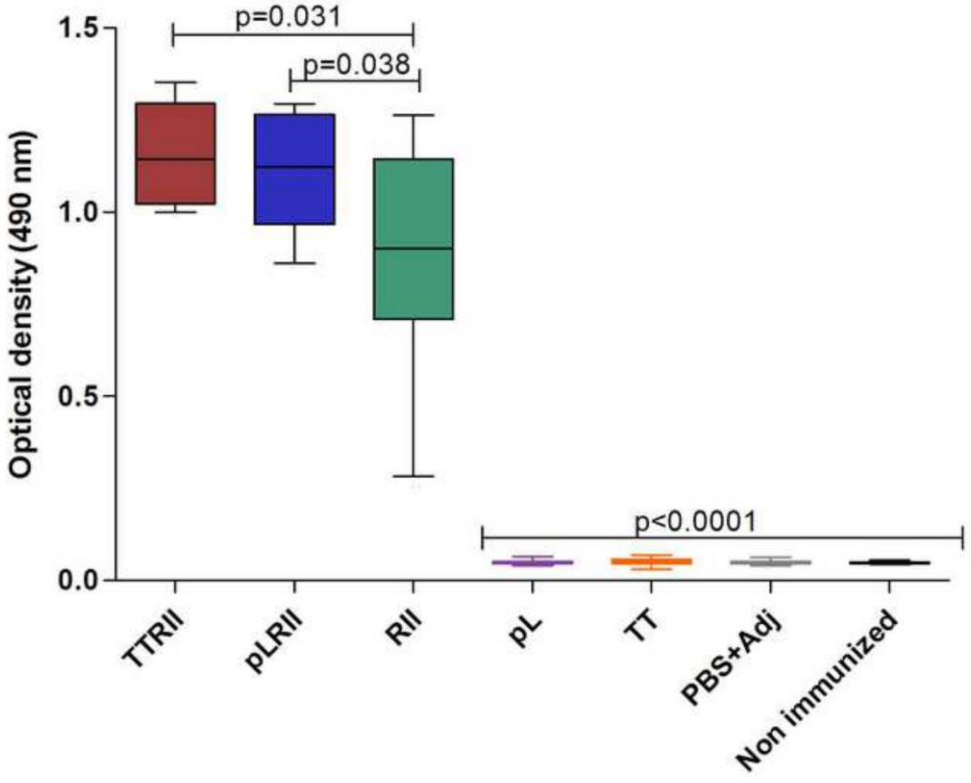


Figure 4
Click here to download high resolution image

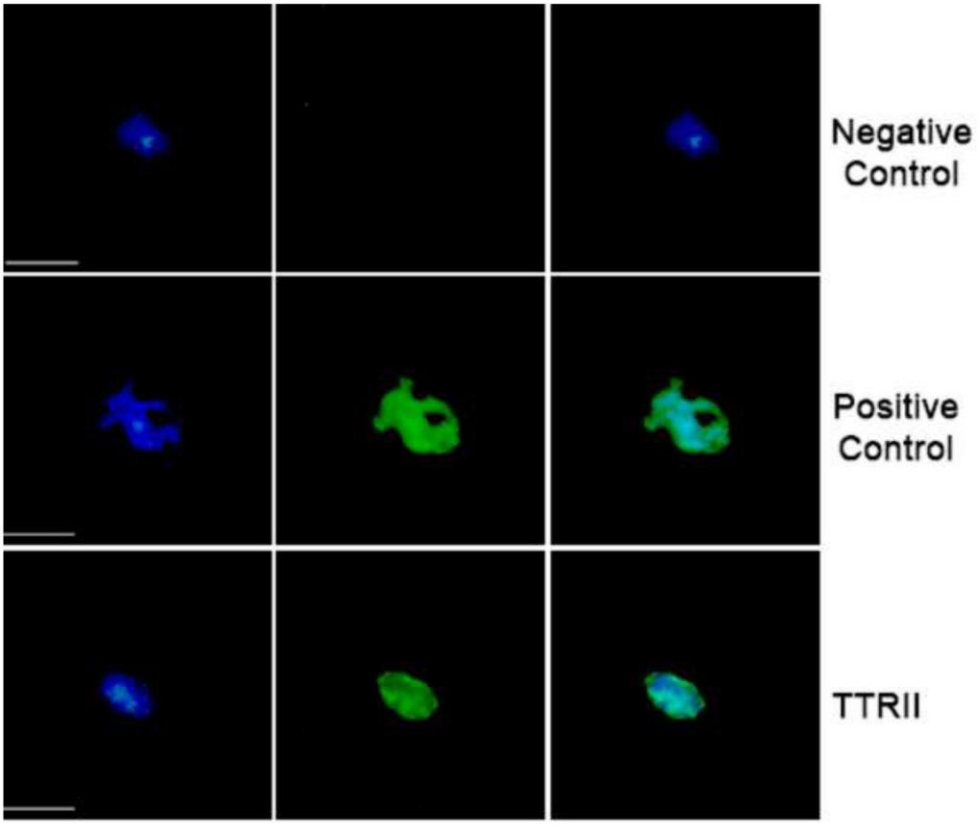
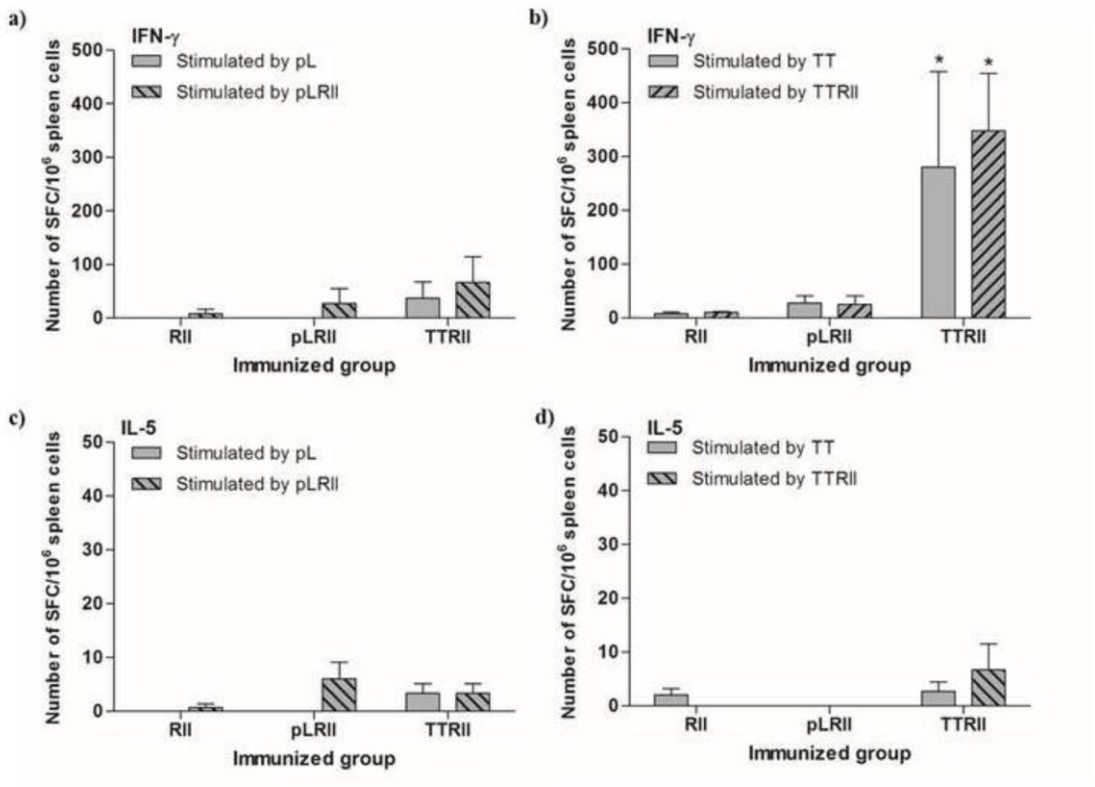
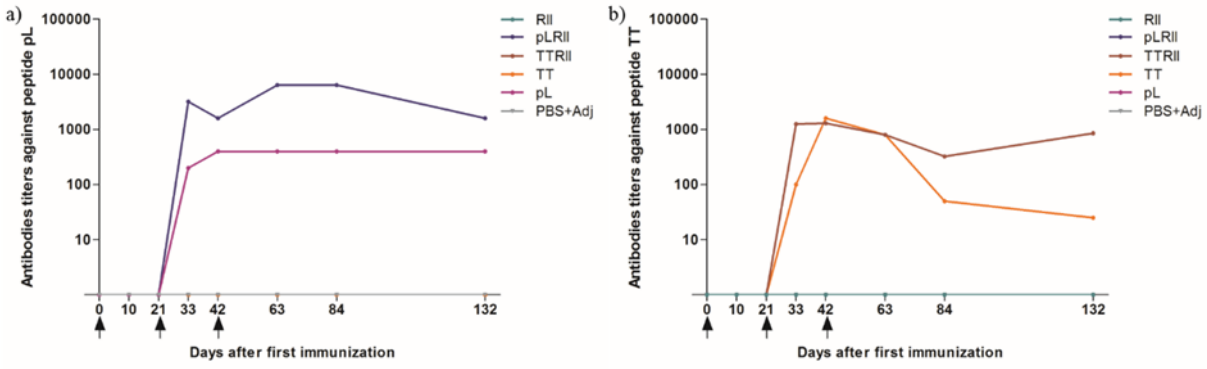


Figure 5
Click here to download high resolution image



Supplementary Figure 1



Highlights:

PvMSp9$_{E795-A808}$ synthetic constructs were immunogenic in mice;

IgG1, IgG2a and IgG2b were prevalent in humoral response induced by synthetic peptides

T-cell epitopes enhanced the humoral response

Anti-PvMSP9$_{E795-A808}$ antibodies were able to recognize the native PvMSP9 by Immunofluorescence assay.

DISCUSSÃO

O desenvolvimento de uma vacina segura, eficaz e de baixo custo contra o *P. vivax* persiste um desafio para a comunidade científica. Apesar do considerado número de antígenos descritos nos últimos anos, as estratégias de vacinologia convencional são particularmente dificultadas quando lidam com parasitos não-cultiváveis, como *P. vivax*. Contudo, com o advento do sequenciamento completo do genoma e avanços na área de bioinformática, o campo da vacinologia foi radicalmente modificado, permitindo a descrição de novos antígenos e o aprimoramento de candidatos vacinais já conhecidos através da vacinologia reversa. Nesse contexto, construções vacinais baseadas em peptídeos sintéticos representam uma estratégia reemergente bem-sucedida (Cespedes et al. 2013; Silva-Flannery et al. 2009b), mas extremamente dependente da adequada seleção de epítopos.

Baseados neste racional e considerando estudos que apontam a proteína de "Travessia Celular de Oocinetos e Esporozoítas" (CelTOS) de *P. falciparum* como uma potencial candidata vacinal (Bergmann-Leitner et al. 2010; Kariu et al. 2006; Kusi et al. 2014), a primeira parte de nosso estudo buscou testar duas hipóteses centrais: A proteína CelTOS de *P. vivax* (PvCelTOS) pode ser considerada uma candidata vacinal promissora contra o *P. vivax*. E, é possível, utilizando ferramentas de predição *in silico*, identificar com sucesso epítopos de célula B em proteínas candidatas vacinais de *P. vivax*.

Deste modo, a fim de confirmar a primeira hipótese, avaliamos a resposta imune naturalmente adquirida contra a PvCelTOS em uma população formada por 528 indivíduos da Amazônia brasileira, naturalmente expostos a malária. Primeiramente, verificamos que cerca de 18% da população estudada apresentou anticorpos específicos contra a PvCelTOS, confirmando, pela primeira vez, a imunogenicidade naturalmente induzida por esta proteína em indivíduos da Amazônia brasileira. Interessantemente, a frequência de respondedores observada em nosso estudo foi similar a observada na Tailândia por Longley e seus colaboradores (Longley et al. 2016), sugerindo um padrão de resposta similar em populações geneticamente distintas. Cabe ressaltar que a baixa reatividade é um achado comum em estudos que avaliam resposta naturalmente adquirida contra antígenos de fase pré-eritrocítica e pode ser justificada pelo curto período de contato do esporozoíta com o sistema imune (Oliveira-Ferreira et al. 2004a; Storti-Melo et al.

2012; Yildiz Zeyrek et al. 2011). Em contraponto, apesar da baixa frequência de respondedores, observamos a prevalência de anticorpos do isotipo IgG1, um achado relevante, visto que anticorpos citofílicos (IgG1 e IgG3) são frequentemente prevalentes em amostras de indivíduos imunes de áreas de alta-transmissão (Bouharoun-Tayoun& Druilhe 1992; Chelimo et al. 2005; John et al. 2005; Stanisic et al. 2009) e costumeiramente associados a imunidade protetora (Aribot et al. 1996; Metzger et al. 2003; Nebie et al. 2008).

Ainda avaliando o potencial vacinal desta proteína, buscamos por associações entre parâmetros epidemiológicos de proteção, susceptibilidade ou exposição da população e a resposta humoral contra PvCelTOS. Neste ponto do trabalho, constatamos que os níveis de IgG se mostraram diretamente relacionados ao número de infecções anteriores de malária, sugerindo um efeito cumulativo de anticorpos com novas infecções, e inversamente correlacionados com o tempo desde o último episódio de malária, indicando um efeito protetor dos anticorpos anti-PvCelTOS. Em contraponto, ao analisarmos os índices de reatividade das subclasses, notamos que os níveis de IgG3 se mostraram diretamente associados ao número de episódios recentes de malária, e inversamente associado com o tempo decorrido desde a última malária, sugerindo que infecções recentes possam induzir maiores níveis de IgG3.

Além disso, considerando que trabalhos anteriores demonstraram associações entre altos níveis de anticorpos contra antígenos pré-eritrocíticos e risco reduzido de malária clínica em crianças (John et al. 2008) e também com a diminuição do risco de infecção em adultos (John et al. 2005), resolvemos agrupar os indivíduos respondedores para PvCelTOS, em alto-respondedores (índice de reatividade > 2) e baixo-respondedores (1> índice de reatividade ≤2), a fim de evidenciar diferenças nos perfis epidemiológicos que pudessem justificar a ampla magnitude de resposta observada e indicar um eventual papel protetor do alto nível de anticorpos contra PvCelTOS. Nesse sentido, nossa primeira constatação foi que indivíduos baixo-respondedores e não respondedores apresentavam perfis epidemiológico extremamente similares, fato que nos levou a cogitar uma eventual restrição genética na resposta para PvCelTOS. Sobre este tema, trabalhos anteriores demonstraram associação entre diferentes alelos de HLA-II e ausência de resposta humoral contra alvos de estágio pré-eritrocítico de *P. vivax* (Oliveira-Ferreira et al. 2004a; Storti-Melo et al. 2012). Contudo, nosso desenho experimental não nos

permite determinar se há alguma influência de diferentes alelos de HLA na resposta ou ausência de resposta à PvCelTOS.

Independente dos fatores determinantes na habilidade de desenvolver resposta humoral contra PvCelTOS, quando comparados a não respondedores ou a baixo respondedores, os indivíduos com altos níveis de anticorpos anti-PvCelTOS apresentaram maior número de episódios anteriores de malária e maior frequência de episódios recentes da doença. Esses dados são contrários a hipótese de que a proteína PvCelTOS seja uma boa candidata vacinal, uma vez que, embora naturalmente imunogênica, não observamos nenhuma associação marcante entre esta resposta e proteção. Em contraponto, um trabalho recente de nosso grupo evidenciou que a PvCelTOS é altamente conservada entre cepas de diferentes regiões geográficas (Bitencourt Chaves et al. 2017), indicando um baixo polimorfismo da proteína, que pode ser considerado um fator positivo para indicá-la como um candidato vacinal, contudo, a baixa reatividade observada em diferentes populações também pode indicar o efeito de uma baixa pressão seletiva sobre a mesma. Infelizmente, esse primeiro conjunto de dados não foi suficiente para definir a PvCelTOS como uma boa candidata vacinal, nem a descartar deste papel. Acreditamos que somente a identificação de suas regiões imunogênicas, combinada a estratégias de imunização de modelos animais e o uso de parasitos quiméricos em ensaios de desafio poderão determinar de fato o papel dos anticorpos anti-PvCelTOS na imunidade.

Desse modo, demos sequência ao estudo buscando avaliar nossa segunda hipótese: “É possível identificar *in silico* epítopos de célula B de proteínas candidatas vacinais de *P. vivax*”. Para tal, utilizando 2 algoritmos de bioinformática (BepiPred e EMINI surface accessibility), identificamos *in* silico 4 sequências de aminoácidos como epítopos lineares de célula B, hipoteticamente expressos na superfície da PvCelTOS ($PvCelTOS_{K6\text{-}N13,}$ $PvCelTOS_{G38\text{-}R57}$, $PvCelTOS_{I136\text{-}E143}$ e $PvCelTOS_{K166\text{-}S191}$). Paralelamente, por peptide array, testamos as 94 amostras respondedoras para PvCelTOS contra 32 peptídeos lineares sintéticos, formados por 15 aminoácidos, com sobreposição de 9 aminoácidos, que representavam toda sequência da proteína. Deste modo, realizamos o mapeamento de epítopos da PvCelTOS, identificando ao todo 10 peptídeos (N13-L27; S19-V33; E73-I87; L79-K93; S97-A111; P127-V141; I133-G147; P139-V153; L181-L195; E182-D196) que foram reconhecidos por mais de 20% dos respondedores para proteína

recombinante. Com base nesses resultados, os peptídeos reconhecidos que apresentavam sobreposição de aminoácidos foram agrupados em 5 regiões imunogênicas ($PvCelTOS_{N13-V33}$; $PvCelTOS_{E73-K93}$; $PvCelTOS_{S97-A111}$; $PvCelTOS_{P127-V153}$ and $PvCelTOS_{L181-D196}$) nas quais epítopos de célula B estariam inseridos. Assim, pela combinação da análise *in silico* e o mapeamento de epítopos de célula B da PvCelTOS, foi possível verificar que 50% das sequências preditas como epítopos lineares de célula B, se encontravam em regiões imunogênicas, validando a metodologia de predição. Adicionalmente, as duas regiões imunogênicas que apresentaram maiores frequências de reconhecimento possuíam epítopos preditos em seu interior, sendo o peptídeo I133-G147, identificado como imunodominante na PvCelTOS e reconhecido por mais de 90% dos respondedores para proteína recombinante, o único peptídeo a conter inteiramente a sequência de um epítopo predito ($PvCelTOS_{I136-E143}$). Nesse contexto, utilizando o peptide-array foi possível confirmar nossa hipótese secundária e validar o uso de ferramentas de predição para identificação ou caracterização de epítopos alvo dentro de proteínas candidatas de *P. vivax*. Como perspectiva para este trabalho, sendo a PvCelTOS uma proteína de fase pré-eritrocítica, presente em esporozoítas e essencial ao seu processo de travessia entre os hepatócitos, pretendemos identificar epítopos de célula TCD4 e TCD8 nesta proteína e verificar a resposta celular induzida por eles em PBMC de indivíduos naturalmente expostos a malária.

Com a conclusão do trabalho anterior, iniciamos uma nova etapa em nosso estudo, que envolveu a aplicação de abordagens de bioinformática para identificar epítopos de célula B na PvMSP9, uma reconhecida candidata vacinal de estágio eritrocítico de *P. vivax*. Nesta etapa, duas novas hipóteses foram levantadas: (I) É possível identificar *in silico* um epítopo imunodominante na PvMSP9 e (ii) validá-lo como epítopo de célula B naturalmente imunogênico na proteína. Para testar nossa primeira hipótese, utilizando a combinação de 3 diferentes algoritmos de predição (BepiPred, IUPRED e Anchor), foi possível identificar 9 sequências de diferentes tamanhos como epítopos lineares de célula B na PvMSP9. Interessantemente, apenas a maior das sequências, formada por 104 aminoácidos ($PvMSP9_{E774-H877}$) se encontrava inteiramente inserida na região descrita como mais imunogênica da PvMSP9, seus dois blocos de repetição na região C-terminal (Lima-Junior et al. 2008; Lima et al. 2010). Guiados por esta informação, resolvemos explorar este longo fragmento e identificamos que o mesmo era formado por 3 diferentes

sequências, sendo a segunda destas formada por 14 aminoácidos que se repetiam em tandem (E795-A808; E809-A822; E823-A836; E837-A850; E851-A864). Assim, considerando que a sequência EAAPENAEPVHENA se repete cinco vezes ininterruptas em tandem, corresponde a 29% da região mais imunogênica da PvMSP9 e apresentou o segundo maior score de predição como epítopo linear de célula B (BepiPred), associado a altas probabilidades de estar inserida em uma região desordenada (IUPRED) da proteína e servir como sítio de interação entre proteínas (Anchor), nós assumimos que esta sequência, denominada $PvMSP9_{E795-A808}$, poderia ser considerada um potencial epítopo de célula B.

Recentemente, Bueno e seus colaboradores adotaram uma estratégia similar para mapear e validar um epítopo linear de célula B altamente imunogênico na candidata vacinal PvAMA-1 (Bueno et al. 2011). Embasados neste estudo, nós demos sequência ao teste de nossa hipótese, buscando validar o epítopo predito $PvMSP9_{E795-A808}$ como um epítopo imunodominante na PvMSP9. Para tal, utilizamos amostras de 316 indivíduos residentes na Amazônia brasileira, que, sabidamente, apresentavam anticorpos específicos para proteína recombinante PvMSP9-RIRII, que representa os dois blocos de repetição da PvMSP9. Assim, inicialmente confirmamos que o epítopo $PvMSP9_{E795-A808}$ é naturalmente imunogênico em populações expostas a malária, visto que 56% da população analisada apresentava anticorpos específicos para o peptídeo sintético representando a sequência. Mais do que isso, baseados no estudo de Bueno e colaboradores, através do ensaio de ELISA de absorção, confirmamos que os anticorpos anti-$PvMSP9_{E795-A808}$ correspondiam a cerca de 30% dos anticorpos anti-PvMSP9-RIRII, sugerindo então, que este seja de fato um epítopo imunodominante situado no primeiro bloco de repetições da proteína. Interessantemente, a resposta específica contra o epítopo era formada prevalentemente por anticorpos do subtipo IgG1, uma subclasse citofílica, estando seus níveis diretamente associados ao tempo decorrido desde o último episódio de malária. Deste modo, confirmamos nossas hipóteses, não apenas validando o epítopo $PvMSP9_{E795-A808}$ como um linear de célula B na PvMSP9, mas dando suporte ao potencial desta sequência em construções vacinais.

Por fim, com a conclusão desta etapa, demos início a etapa final de nosso estudo, buscando confirmar a hipótese de que “o epítopo $PvMSP9_{E795-A808}$ é imunogênico em modelos animais em construções peptídicas sintéticas”. Para validar esta hipótese e considerando que o epítopo validado se repete cinco vezes

em tandem na PvMSP9, peptídeos lineares foram sintetizados tendo como base 3 epítopos: duas repetições da sequência EAAPENAEPVHENA [peptídeo RII: $PvMSP9_{(E795\text{-}A808)2}$]; a sequência ASIDSMIDEIDFYEK (Peptídeo pL), um epítopo T-helper promíscuo presente na região N-terminal da PvMSP9, naturalmente imunogênico em populações expostas a malária (Lima-Junior et al. 2010) e a sequência QYIKANSKFIGITE (peptídeo TT), um epítopo T-helper da toxina tetânica, já bem caracterizado por sua capacidade de potencializar a resposta humoral em camundongos (Jang et al. 2012; Kaumaya et al. 1993; Panina-Bordignon et al. 1989). Todos os peptídeos foram sintetizados em sua forma simples (RII, pL e TT) e na forma conjugada, com um epítopo T-helper fusionado ao epítopo $PvMSP9_{(E795\text{-}A808)2}$ (pLRII e TTRII), formulados em adjuvante Montanide ISA-51 e utilizados na imunização de camundongos BALB/c, a fim de avaliar a resposta imune humoral e celular induzida pelas diferentes construções, bem como o perfil fenotípico de subpopulações de memória nos esplenócitos ao final das imunizações.

Desse modo, nosso primeiro resultado nesta etapa já validava nossa hipótese, visto que todas as construções vacinais contendo o epítopo $PvMSP9_{(E795\text{-}A808)2}$ se mostraram capazes de induzir anticorpos específicos contra o peptídeo RII. Mais do que isso, conforme observado em estudos anteriores de Caro-Aguilar, a inserção de epítopos T-helper nas construções foi capaz de potencializar a resposta humoral, induzindo mais rapidamente, maiores níveis de anticorpos que o epítopo de célula B isolado (Caro-Aguilar et al. 2005; Caro-Aguilar et al. 2002). No entanto, embora os níveis de anticorpos obtidos pelas construções conjugadas sejam comparáveis a estudos similares, estes se mostraram aquém daqueles obtidos pela imunização com proteínas recombinantes (Oliveira-Ferreira et al. 2004b), reforçando a ideia de peptídeos sintéticos sejam uma plataforma vacinal pouco imunogênica, e evidenciando a necessidade de se buscarem novas ferramentas a fim de ampliar esta imunogenicidade. Apesar desta discrepância no nível de anticorpos induzidos por peptídeos sintéticos e proteínas recombinantes, cabe ressaltar que, considerando uma cinética de imunizações similar, com o mesmo adjuvante, ambas as plataformas vacinais induziram tanto perfis similares de imunogenicidade, com soroconversão após o primeiro booster vacinal e manutenção dos níveis de anticorpos até o final da cinética experimental, quanto um mesmo padrão de subclasses, caracterizado pela ausência de IgG3 a similar prevalência de IgG1, IgG2a e IgG2b (Oliveira-Ferreira et al. 2004b). As semelhanças observadas entre os

dois estudos sugerem uma importante participação do adjuvante na determinação do perfil de subclasses e indução de anticorpos.

Acerca do papel protetor dos anticorpos induzidos, nosso desenho experimental foi limitado pela falta de um desafio vacinal. Apesar disso, os anticorpos anti-PvMSP9$_{(E795-A808)2}$ se mostraram capazes de reconhecer especificamente a proteína recombinante PvMSP9-RIRII e, mais do que isso, capazes de reconhecer a proteína nativa do parasito em ensaios de imunofluorescência. Deste modo, considerando que o epítopo PvMSP9$_{(E795-A808)}$ é um epítopo imunodominante na região mais imunogênica da proteína e que anticorpos anti-PvMSP9 já se mostraram capazes de inibir a invasão de hemácias pelos merozoítas *in vitro* (Barnwell et al. 1999), acreditamos que a sequência PvMSP9$_{(E795-A808)2}$ possa ser um bom candidato vacinal. Contudo, consideramos que o uso de parasitos quiméricos, expressando a PvMSP9, seja a melhor maneira de comprovar inquestionavelmente o papel protetor destes anticorpos.

Por outro lado, ao avaliarmos a resposta celular induzida pelas diferentes construções peptídicas, constatamos que apenas o peptídeo TTRII foi capaz de induzir um número significativo de células secretoras de IFN-γ, comparável ao induzido por proteínas recombinantes (Oliveira-Ferreira et al. 2004b). Cabe ressaltar que este foi o primeiro trabalho avaliando a imunogenicidade do epítopo pL em modelos animais. Curiosamente, embora este epítopo seja caracterizado como um epítopo T-helper promíscuo em seres humanos e que quando fusionado ao epítopo PvMSP9$_{(E795-A808)2}$ tenha induzido uma potencialização da resposta humoral contra este epítopo, ao que parece o epítopo pL não se mostrou capaz de estimular um aumento específico no número de células produtoras de IFN-γ. Este dado não chega a ser uma surpresa, visto que a análise *in silico* de sua sequência sugeria que esta não seria facilmente reconhecida por MHC-II de camundongos BALB/c (H2-IA^d e H2-IE^d).

Por fim, ainda buscando caracterizar a resposta imune induzida pelo epítopo PvMSP9$_{(E795-A808)2}$ em peptídeos sintéticos, analisamos o perfil de subpopulações de memória no baço dos animais imunizados, 21 dias após a última imunização. Contudo, não foi possível observar nenhuma alteração marcante na frequência de células encontradas, fato que pode ser atribuído ao longo período decorrido entre a imunização e a análise. Reinhardt e seus colaboradores observaram que a frequência de células de memória induzidas por peptídeos de ovalbumina no baço tem seu pico cerca de 3 dias após a injeção do antígeno, mas retorna a níveis

basais em torno de 20 dias (Reinhardt et al. 2001). Deste modo, acreditamos que as células de memória induzidas pelos peptídeos sintéticos já deveriam ter migrado quando observamos, estando alojadas na medula, linfonodos ou mesmo circulantes na corrente sanguínea. Embasados por isso, em estudos futuros pretendemos realizar uma alteração no desenho experimental, observando perfis fenotípicos no baço 3 dias após a última imunização.

Concluindo, nosso estudo reforça o uso da vacinologia reversa como uma alternativa viável para identificar e explorar candidatos vacinais contra o *P. vivax*, caracterizando seus epítopos e permitindo a elaboração de novas e melhores construções vacinais, baseadas em multi-epítopos. Além disso, destacamos a importância do desenvolvimento de parasitos transgênicos como modelos para melhor avaliar o potencial de novas construções vacinais, acreditando que em conjunto estas abordagens possam representar um marco no desenvolvimento no de vacinas contra a espécie plasmodial mais dispersa no mundo e hoje vista como a mais difícil de se eliminar.

PERSPECTIVAS

- Identificar *in silico* e validar experimentalmente epítopos de célula T-CD4 e T-CD8 na PvCelTOS;
- Sintetizar construções vacinais contendo epítopos de célula T e B validados na PvCelTOS e avaliar seu potencial antigênico e imunogênico em modelos animais;
- Desenvolver esporozoítas quiméricos, expressando a PvCelTOS, a fim de avaliar o papel protetor de vacinas baseadas em epítopos da PvCelTOS;
- Desenvolver merozoítas quiméricos expressando a PvMSP9, para avaliar o papel protetor dos anticorpos anti-PvMSP9$_{(E795\text{-}A808)2}$;
- Desenvolver construções vacinais multi-epítopos e multi-estágio, contendo epítopos de fase pré-eritrocítica, eritrocítica e sexuada, para ensaios de imunização em modelos animais.

CONCLUSÕES

- A combinação de diferentes algoritmos de predição se mostrou uma estratégia para identificação de epítopos imunogênicos;
- A PvCelTOS é naturalmente imunogênica em habitantes da Amazônia brasileira, com prevalência de anticorpos IgG1;
- 5 regiões imunogênicas foram identificadas, em diferentes níveis, na PvCelTOS;
- Todas as regiões imunogênicas se mostraram expostas e acessíveis em solução;
- O peptídeo $PvCelTOS_{A133-F147}$ foi o epítopo imunodominante;
- A sequência predita EAAPENAEPVHENA ($PvMSP9_{E795-A808}$) pode ser considerada uma potencial candidata vacinal, uma vez que foi validada como naturalmente imunogênica em indivíduos expostos à malária e se mostrou imunogênica em modelos animais;
- Anticorpos específicos contra $PvMSP9_{(E795-A808)2}$ foram capazes de reconhecer a proteína recombinante PvMSP9-RIRII e a proteína nativa no parasito;
- Epítopos T-helper conjugados em peptídeos sintéticos se mostraram capazes de potencializar a resposta humoral contra epítopos de célula B.

REFERÊNCIAS BIBLIOGRÁFICAS

Acharya P, Pallavi R, Chandran S, Chakravarti H, Middha S, Acharya J, Kochar S, Kochar D, Subudhi A, Boopathi AP, Garg S, Das A, Tatu U 2009. A glimpse into the clinical proteome of human malaria parasites Plasmodium falciparum and Plasmodium vivax. *Proteomics Clin Appl*, 3, 1314-1325.

Adams JH, Mueller I 2017. The Biology of Plasmodium vivax. *Cold Spring Harb Perspect Med*, 7.

Ahmed MA, Cox-Singh J 2015. Plasmodium knowlesi - an emerging pathogen. *ISBT Sci Ser*, 10, 134-140.

Alexander J, del Guercio MF, Maewal A, Qiao L, Fikes J, Chesnut RW, Paulson J, Bundle DR, DeFrees S, Sette A 2000. Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. *J Immunol*, 164, 1625-1633.

Alexander J, Sidney J, Southwood S, Ruppert J, Oseroff C, Maewal A, Snoke K, Serra HM, Kubo RT, Sette A, et al. 1994. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity*, 1, 751-761.

Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, Menard R 2006a. Quantitative imaging of Plasmodium transmission from mosquito to mammal. *Nat Med*, 12, 220-224.

Amino R, Thiberge S, Shorte S, Frischknecht F, Menard R 2006b. Quantitative imaging of Plasmodium sporozoites in the mammalian host. *C R Biol*, 329, 858-862.

Anders RF 1986. Multiple cross-reactivities amongst antigens of Plasmodium falciparum impair the development of protective immunity against malaria. *Parasite Immunol*, 8, 529-539.

Angrisano F, Tan YH, Sturm A, McFadden GI, Baum J 2012. Malaria parasite colonisation of the mosquito midgut--placing the Plasmodium ookinete centre stage. *Int J Parasitol*, 42, 519-527.

Arama C, Troye-Blomberg M 2014. The path of malaria vaccine development: challenges and perspectives. *J Intern Med*, 275, 456-466.

Arevalo-Herrera M, Herrera S 2001. Plasmodium vivax malaria vaccine development. *Mol Immunol*, 38, 443-455.

Arias AE, Corredor A 1989. Low response of Colombian strains of Plasmodium vivax to classical antimalarial therapy. *Tropical medicine and parasitology : official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit*, 40, 21-23.

Aribot G, Rogier C, Sarthou JL, Trape JF, Balde AT, Druilhe P, Roussilhon C 1996. Pattern of immunoglobulin isotype response to Plasmodium falciparum blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). *Am J Trop Med Hyg*, 54, 449-457.

Awono-Ambene HP, Robert V 1998. Estimation of Plasmodium falciparum oocyst survival in Anopheles arabiensis. *Ann Trop Med Parasitol*, 92, 889-890.

Baird JK 1998. Age-dependent characteristics of protection v. susceptibility to Plasmodium falciparum. *Ann Trop Med Parasitol*, 92, 367-390.

Ballou WR, Hoffman SL, Sherwood JA, Hollingdale MR, Neva FA, Hockmeyer WT, Gordon DM, Schneider I, Wirtz RA, Young JF, et al. 1987. Safety and efficacy of a recombinant DNA Plasmodium falciparum sporozoite vaccine. *Lancet*, 1, 1277-1281.

Banerjee R, Liu J, Beatty W, Pelosof L, Klemba M, Goldberg DE 2002. Four plasmepsins are active in the Plasmodium falciparum food vacuole, including a protease with an active-site histidine. *Proc Natl Acad Sci U S A*, 99, 990-995.

Barnwell JW, Galinski MR, DeSimone SG, Perler F, Ingravallo P 1999. Plasmodium vivax, P. cynomolgi, and P. knowlesi: identification of homologue proteins associated with the surface of merozoites. *Exp Parasitol*, 91, 238-249.

Bassat Q, Velarde M, Mueller I, Lin J, Leslie T, Wongsrichanalai C, Baird JK 2016. Key Knowledge Gaps for Plasmodium vivax Control and Elimination. *Am J Trop Med Hyg*, 95, 62-71.

Baton LA, Ranford-Cartwright LC 2005a. Do malaria ookinete surface proteins P25 and P28 mediate parasite entry into mosquito midgut epithelial cells? *Malar J*, 4, 15.

—— 2005b. How do malaria ookinetes cross the mosquito midgut wall? *Trends Parasitol*, 21, 22-28.

Beeson JG, Drew DR, Boyle MJ, Feng G, Fowkes FJ, Richards JS 2016. Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS Microbiol Rev*, 40, 343-372.

Beeson JG, Osier FH, Engwerda CR 2008. Recent insights into humoral and cellular immune responses against malaria. *Trends Parasitol*, 24, 578-584.

Belnoue E, Kayibanda M, Vigario AM, Deschemin JC, van Rooijen N, Viguier M, Snounou G, Renia L 2002. On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. *J Immunol*, 169, 6369-6375.

Bennink S, Kiesow MJ, Pradel G 2016. The development of malaria parasites in the mosquito midgut. *Cell Microbiol*, 18, 905-918.

Bergmann-Leitner ES, Mease RM, De La Vega P, Savranskaya T, Polhemus M, Ockenhouse C, Angov E 2010. Immunization with pre-erythrocytic antigen CelTOS from Plasmodium falciparum elicits cross-species protection against heterologous challenge with Plasmodium berghei. *PLoS One*, 5, e12294.

Bianucci R, Mattutino G, Lallo R, Charlier P, Jouin-Spriet H, Peluso A, Higham T, Torre C, Rabino-Massa E 2008. Immunological evidence of Plasmodium falciparum infection in an Egyptian child mummy from the Early Dynastic Period. *Journal of Archaeological Science*, 35, 6.

Bitencourt Chaves L, Perce-da-Silva DS, Rodrigues-da-Silva RN, Martins da Silva JH, Cassiano GC, Machado RL, Pratt-Riccio LR, Banic DM, Lima-Junior JD 2017. Plasmodium vivax Cell Traversal Protein for Ookinetes and Sporozoites (PvCelTOS) gene sequence and potential epitopes are highly conserved among isolates from different regions of Brazilian Amazon. *PLoS Negl Trop Dis*, 11, e0005344.

Blackman MJ 2000. Proteases involved in erythrocyte invasion by the malaria parasite: function and potential as chemotherapeutic targets. *Curr Drug Targets*, 1, 59-83.

Blagborough AM, Musiychuk K, Bi H, Jones RM, Chichester JA, Streatfield S, Sala KA, Zakutansky SE, Upton LM, Sinden RE, Brian I, Biswas S, Sattabonkot J, Yusibov V 2016. Transmission blocking potency and immunogenicity of a plant-produced Pvs25-based subunit vaccine against Plasmodium vivax. *Vaccine*, 34, 3252-3259.

Blagborough AM, Sinden RE 2009. Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. *Vaccine*, 27, 5187-5194.

Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA 2004. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. *Cell*, 116, 661-670.

Blume M, Hliscs M, Rodriguez-Contreras D, Sanchez M, Landfear S, Lucius R, Matuschewski K, Gupta N 2011. A constitutive pan-hexose permease for the Plasmodium life cycle and transgenic models for screening of antimalarial sugar analogs. *FASEB J*, 25, 1218-1229.

Bouharoun-Tayoun H, Druilhe P 1992. Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect Immun*, 60, 1473-1481.

Bousema T, Drakeley C 2011. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. *Clin Microbiol Rev*, 24, 377-410.

Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, Russell B, Ginsburg H, Nosten F, Day NP, White NJ, Carlton JM, Preiser PR 2008. The transcriptome of Plasmodium vivax reveals divergence and diversity of transcriptional regulation in malaria parasites. *Proc Natl Acad Sci U S A*, 105, 16290-16295.

Brasil P, Zalis MG, de Pina-Costa A, Siqueira AM, Junior CB, Silva S, Areas ALL, Pelajo-Machado M, de Alvarenga DAM, da Silva Santelli ACF, Albuquerque HG, Cravo P, Santos de Abreu FV, Peterka CL, Zanini GM, Suarez Mutis MC, Pissinatti A, Lourenco-de-Oliveira R, de Brito CFA, de Fatima Ferreira-da-Cruz M, Culleton R, Daniel-Ribeiro CT 2017. Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. *Lancet Glob Health*, 5, e1038-e1046.

Brown GV 1999. Progress in the development of malaria vaccines: context and constraints. *Parassitologia*, 41, 429-432.

Bueno LL, Lobo FP, Morais CG, Mourao LC, de Avila RA, Soares IS, Fontes CJ, Lacerda MV, Chavez Olortegui C, Bartholomeu DC, Fujiwara RT, Braga EM 2011. Identification of a highly antigenic linear B cell epitope within Plasmodium vivax apical membrane antigen 1 (AMA-1). *PLoS One*, 6, e21289.

Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K 1998. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat Med*, 4, 358-360.

Campanella GS, Tager AM, El Khoury JK, Thomas SY, Abrazinski TA, Manice LA, Colvin RA, Luster AD 2008. Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. *Proc Natl Acad Sci U S A*, 105, 4814-4819.

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.

Caro-Aguilar I, Lapp S, Pohl J, Galinski MR, Moreno A 2005. Chimeric epitopes delivered by polymeric synthetic linear peptides induce protective immunity to malaria. *Microbes Infect*, 7, 1324-1337.

Caro-Aguilar I, Rodriguez A, Calvo-Calle JM, Guzman F, De la Vega P, Patarroyo ME, Galinski MR, Moreno A 2002. Plasmodium vivax promiscuous T-helper epitopes defined and evaluated as linear peptide chimera immunogens. *Infect Immun*, 70, 3479-3492.

Carter R, Chen DH 1976. Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature*, 263, 57-60.

Carter R, Mendis KN, Miller LH, Molineaux L, Saul A 2000. Malaria transmission-blocking vaccines--how can their development be supported? *Nat Med*, 6, 241-244.

Carvalho LH, Sano G, Hafalla JC, Morrot A, Curotto de Lafaille MA, Zavala F 2002a. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat Med*, 8, 166-170.

Carvalho LJ, Daniel-Ribeiro CT, Goto H 2002b. Malaria vaccine: candidate antigens, mechanisms, constraints and prospects. *Scand J Immunol*, 56, 327-343.

Cespedes N, Arevalo-Herrera M, Felger I, Reed S, Kajava AV, Corradin G, Herrera S 2013. Antigenicity and immunogenicity of a novel chimeric peptide antigen based on the P. vivax circumsporozoite protein. *Vaccine*, 31, 4923-4930.

Chelimo K, Ofulla AV, Narum DL, Kazura JW, Lanar DE, John CC 2005. Antibodies to Plasmodium falciparum antigens vary by age and antigen in children in a malaria-holoendemic area of Kenya. *Pediatr Infect Dis J*, 24, 680-684.

Chen DH, Tigelaar RE, Weinbaum FI 1977. Immunity to sporozoite-induced malaria infeciton in mice. I. The effect of immunization of T and B cell-deficient mice. *J Immunol*, 118, 1322-1327.

Cheng Y, Wang B, Sattabongkot J, Lim CS, Tsuboi T, Han ET 2014. Immunogenicity and antigenicity of Plasmodium vivax merozoite surface protein 10. *Parasitology research*, 113, 2559-2568.

Chin W, Coatney GR 1971. Relapse activity in sporozoite-induced infections with a West African strain of Plasmodium ovale. *Am J Trop Med Hyg*, 20, 825-827.

Cockburn I 2013. Chimeric parasites as tools to study Plasmodium immunology and assess malaria vaccines. *Methods Mol Biol*, 923, 465-479.

Coffman RL, Sher A, Seder RA 2010. Vaccine adjuvants: putting innate immunity to work. *Immunity*, 33, 492-503.

Coggeshall LT, Kumm HW 1937. Demonstration of Passive Immunity in Experimental Monkey Malaria. *J Exp Med*, 66, 177-190.

Cohen S, Mc GI, Carrington S 1961. Gamma-globulin and acquired immunity to human malaria. *Nature*, 192, 733-737.

Collins WE 1974. Primate malarias. *Adv Vet Sci Comp Med*, 18, 1-23.

Collins WE, Jeffery GM 2007. Plasmodium malariae: parasite and disease. *Clin Microbiol Rev*, 20, 579-592.

Collins WE, Skinner JC, Broderson JR, Pappaioanou M, Filipski V, Sutton BB, Stanfill PS, Huong AY, Roberts J, Wilson C 1989. The Uganda I/CDC strain of Plasmodium malariae in Aotus lemurinus griseimembra monkeys. *J Parasitol*, 75, 61-65.

Couper KN, Blount DG, Wilson MS, Hafalla JC, Belkaid Y, Kamanaka M, Flavell RA, de Souza JB, Riley EM 2008. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog*, 4, e1000004.

Cox FE 2010. History of the discovery of the malaria parasites and their vectors. *Parasit Vectors*, 3, 5.

Crispe IN 2003. Hepatic T cells and liver tolerance. *Nat Rev Immunol*, 3, 51-62.

Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, Miller LH, Barillas-Mury C, Pierce SK 2014. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu Rev Immunol*, 32, 157-187.

Cunningham D, Lawton J, Jarra W, Preiser P, Langhorne J 2010. The pir multigene family of Plasmodium: antigenic variation and beyond. *Mol Biochem Parasitol*, 170, 65-73.

de Pina-Costa A, Brasil P, Di Santi SM, de Araujo MP, Suarez-Mutis MC, Santelli AC, Oliveira-Ferreira J, Lourenco-de-Oliveira R, Daniel-Ribeiro CT 2014. Malaria in Brazil: what happens outside the Amazonian endemic region. *Mem Inst Oswaldo Cruz*, 109, 618-633.

de Santana Filho FS, Arcanjo AR, Chehuan YM, Costa MR, Martinez-Espinosa FE, Vieira JL, Barbosa M, Alecrim WD, Alecrim M 2007. Chloroquine-resistant Plasmodium vivax, Brazilian Amazon. *Emerg Infect Dis*, 13, 1125-1126.

Dembele L, Franetich JF, Lorthiois A, Gego A, Zeeman AM, Kocken CH, Le Grand R, Dereuddre-Bosquet N, van Gemert GJ, Sauerwein R, Vaillant JC, Hannoun L, Fuchter MJ, Diagana TT, Malmquist NA, Scherf A, Snounou G, Mazier D 2014. Persistence and activation of malaria hypnozoites in long-term primary hepatocyte cultures. *Nat Med*, 20, 307-312.

Dharia NV, Bright AT, Westenberger SJ, Barnes SW, Batalov S, Kuhen K, Borboa R, Federe GC, McClean CM, Vinetz JM, Neyra V, Llanos-Cuentas A, Barnwell JW, Walker JR, Winzeler EA 2010. Whole-genome sequencing and microarray analysis of ex vivo Plasmodium vivax reveal selective pressure on putative drug resistance genes. *Proc Natl Acad Sci U S A*, 107, 20045-20050.

Di Pasquale A, Preiss S, Tavares Da Silva F, Garcon N 2015. Vaccine Adjuvants: from 1920 to 2015 and Beyond. *Vaccines (Basel)*, 3, 320-343.

Dolan SA, Miller LH, Wellems TE 1990. Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. *J Clin Invest*, 86, 618-624.

Doolan DL, Hoffman SL 2000. The complexity of protective immunity against liver-stage malaria. *J Immunol*, 165, 1453-1462.

Douglas RG, Amino R, Sinnis P, Frischknecht F 2015. Active migration and passive transport of malaria parasites. *Trends Parasitol*, 31, 357-362.

Douradinha B, Doolan DL 2011. Harnessing immune responses against Plasmodium for rational vaccine design. *Trends Parasitol*, 27, 274-283.

Draheim M, Wlodarczyk MF, Crozat K, Saliou JM, Alayi TD, Tomavo S, Hassan A, Salvioni A, Demarta-Gatsi C, Sidney J, Sette A, Dalod M, Berry A, Silvie O, Blanchard N 2017. Profiling MHC II immunopeptidome of blood-stage malaria reveals that cDC1 control the functionality of parasite-specific CD4 T cells. *EMBO Mol Med*.

Druilhe P, Pradier O, Marc JP, Miltgen F, Mazier D, Parent G 1986. Levels of antibodies to Plasmodium falciparum sporozoite surface antigens reflect malaria transmission rates and are persistent in the absence of reinfection. *Infect Immun*, 53, 393-397.

Duffy PE, Pimenta P, Kaslow DC 1993. Pgs28 belongs to a family of epidermal growth factor-like antigens that are targets of malaria transmission-blocking antibodies. *J Exp Med*, 177, 505-510.

Duncan CJ, Sheehy SH, Ewer KJ, Douglas AD, Collins KA, Halstead FD, Elias SC, Lillie PJ, Rausch K, Aebig J, Miura K, Edwards NJ, Poulton ID, Hunt-Cooke A, Porter DW, Thompson FM, Rowland R, Draper SJ, Gilbert SC, Fay MP, Long CA, Zhu D, Wu Y, Martin LB, Anderson CF, Lawrie AM, Hill AV, Ellis RD 2011. Impact on malaria parasite multiplication rates in infected volunteers of the protein-in-adjuvant vaccine AMA1-C1/Alhydrogel+CPG 7909. *PLoS One*, 6, e22271.

Ersmark K, Feierberg I, Bjelic S, Hamelink E, Hackett F, Blackman MJ, Hulten J, Samuelsson B, Aqvist J, Hallberg A 2004. Potent inhibitors of the Plasmodium falciparum enzymes plasmepsin I and II devoid of cathepsin D inhibitory activity. *J Med Chem*, 47, 110-122.

Ewer KJ, O'Hara GA, Duncan CJ, Collins KA, Sheehy SH, Reyes-Sandoval A, Goodman AL, Edwards NJ, Elias SC, Halstead FD, Longley RJ, Rowland R, Poulton ID, Draper SJ, Blagborough AM, Berrie E, Moyle S, Williams N, Siani L, Folgori A, Colloca S, Sinden RE, Lawrie AM, Cortese R, Gilbert SC, Nicosia A, Hill AV 2013. Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. *Nat Commun*, 4, 2836.

Farrance CE, Rhee A, Jones RM, Musiychuk K, Shamloul M, Sharma S, Mett V, Chichester JA, Streatfield SJ, Roeffen W, van de Vegte-Bolmer M, Sauerwein RW,

Tsuboi T, Muratova OV, Wu Y, Yusibov V 2011. A plant-produced Pfs230 vaccine candidate blocks transmission of Plasmodium falciparum. *Clin Vaccine Immunol*, 18, 1351-1357.

Fauconnier M, Palomo J, Bourigault ML, Meme S, Szeremeta F, Beloeil JC, Danneels A, Charron S, Rihet P, Ryffel B, Quesniaux VF 2012. IL-12Rbeta2 is essential for the development of experimental cerebral malaria. *J Immunol*, 188, 1905-1914.

Fornaciari G, Giuffra V, Ferroglio E, Bianucci R 2010a. Malaria was "the killer" of Francesco I de' Medici (1531-1587). *Am J Med*, 123, 568-569.

Fornaciari G, Giuffra V, Ferroglio E, Gino S, Bianucci R 2010b. Plasmodium falciparum immunodetection in bone remains of members of the Renaissance Medici family (Florence, Italy, sixteenth century). *Trans R Soc Trop Med Hyg*, 104, 583-587.

Frank R 2002. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. *J Immunol Methods*, 267, 13-26.

Freitas do Rosario AP, Lamb T, Spence P, Stephens R, Lang A, Roers A, Muller W, O'Garra A, Langhorne J 2012. IL-27 promotes IL-10 production by effector Th1 CD4+ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. *J Immunol*, 188, 1178-1190.

Freitas do Rosario AP, Langhorne J 2012. T cell-derived IL-10 and its impact on the regulation of host responses during malaria. *Int J Parasitol*, 42, 549-555.

Frias L, Leles D, Araujo A 2013. Studies on protozoa in ancient remains--a review. *Mem Inst Oswaldo Cruz*, 108, 1-12.

Galinski MR, Corredor-Medina C, Povoa M, Crosby J, Ingravallo P, Barnwell JW 1999. Plasmodium vivax merozoite surface protein-3 contains coiled-coil motifs in an alanine-rich central domain. *Mol Biochem Parasitol*, 101, 131-147.

Garcia-Basteiro AL, Bassat Q, Alonso PL 2012. Approaching the target: the path towards an effective malaria vaccine. *Mediterr J Hematol Infect Dis*, 4, e2012015.

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.

Garver LS, de Almeida Oliveira G, Barillas-Mury C 2013. The JNK pathway is a key mediator of Anopheles gambiae antiplasmodial immunity. *PLoS Pathog*, 9, e1003622.

Geleta G, Ketema T 2016. Severe Malaria Associated with Plasmodium falciparum and P. vivax among Children in Pawe Hospital, Northwest Ethiopia. *Malar Res Treat*, 2016, 1240962.

Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, Kwiatkowski D, Greenwood BM, Whittle HC, Hill AV 1998. Association of malaria parasite population structure, HLA, and immunological antagonism. *Science*, 279, 1173-1177.

Gimenez AM, Lima LC, Francoso KS, Denapoli PMA, Panatieri R, Bargieri DY, Thiberge JM, Andolina C, Nosten F, Renia L, Nussenzweig RS, Nussenzweig V, Amino R, Rodrigues MM, Soares IS 2017. Vaccine Containing the Three Allelic Variants of the Plasmodium vivax Circumsporozoite Antigen Induces Protection in Mice after Challenge with a Transgenic Rodent Malaria Parasite. *Front Immunol*, 8, 1275.

Good MF, Doolan DL 1999. Immune effector mechanisms in malaria. *Curr Opin Immunol*, 11, 412-419.

Goodman AL, Blagborough AM, Biswas S, Wu Y, Hill AV, Sinden RE, Draper SJ 2011. A viral vectored prime-boost immunization regime targeting the malaria Pfs25 antigen induces transmission-blocking activity. *PLoS One*, 6, e29428.

Goulielmaki E, Siden-Kiamos I, Loukeris TG 2014. Functional characterization of Anopheles matrix metalloprotease 1 reveals its agonistic role during sporogonic development of malaria parasites. *Infect Immun*, 82, 4865-4877.

Gruner AC, Mauduit M, Tewari R, Romero JF, Depinay N, Kayibanda M, Lallemand E, Chavatte JM, Crisanti A, Sinnis P, Mazier D, Corradin G, Snounou G, Renia L 2007. Sterile protection against malaria is independent of immune responses to the circumsporozoite protein. *PLoS One*, 2, e1371.

Gueirard P, Tavares J, Thiberge S, Bernex F, Ishino T, Milon G, Franke-Fayard B, Janse CJ, Menard R, Amino R 2010. Development of the malaria parasite in the skin of the mammalian host. *Proc Natl Acad Sci U S A*, 107, 18640-18645.

Guermonprez P, Helft J, Claser C, Deroubaix S, Karanje H, Gazumyan A, Darasse-Jeze G, Telerman SB, Breton G, Schreiber HA, Frias-Staheli N, Billerbeck E, Dorner M, Rice CM, Ploss A, Klein F, Swiecki M, Colonna M, Kamphorst AO, Meredith M, Niec R, Takacs C, Mikhail F, Hari A, Bosque D, Eisenreich T, Merad M, Shi Y, Ginhoux F, Renia L, Urban BC, Nussenzweig MC 2013. Inflammatory Flt3l is essential to mobilize dendritic cells and for T cell responses during Plasmodium infection. *Nat Med*, 19, 730-738.

Gupta BK, Gupta A, Nehra HR, Balotia HR, Meena SL, Kumar S 2015. Clinical Profile and Prognostic Indicators in Adults Hospitalized with Severe Malaria Caused by Different Plasmodium Species. *Infect Dis (Auckl)*, 8, 45-50.

Gupta L, Molina-Cruz A, Kumar S, Rodrigues J, Dixit R, Zamora RE, Barillas-Mury C 2009. The STAT pathway mediates late-phase immunity against Plasmodium in the mosquito Anopheles gambiae. *Cell Host Microbe*, 5, 498-507.

Gwadz RW 1976. Successful immunization against the sexual stages of Plasmodium gallinaceum. *Science*, 193, 1150-1151.

Gwadz RW, Green I 1978. Malaria immunization in Rhesus monkeys. A vaccine effective against both the sexual and asexual stages of Plasmodium knowlesi. *J Exp Med*, 148, 1311-1323.

Haeberlein S, Chevalley-Maurel S, Ozir-Fazalalikhan A, Koppejan H, Winkel BMF, Ramesar J, Khan SM, Sauerwein RW, Roestenberg M, Janse CJ, Smits HH, Franke-Fayard B 2017. Protective immunity differs between routes of administration of attenuated malaria parasites independent of parasite liver load. *Sci Rep*, 7, 10372.

Han YS, Thompson J, Kafatos FC, Barillas-Mury C 2000. Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J*, 19, 6030-6040.

Handayani S, Chiu DT, Tjitra E, Kuo JS, Lampah D, Kenangalem E, Renia L, Snounou G, Price RN, Anstey NM, Russell B 2009. High deformability of Plasmodium vivax-infected red blood cells under microfluidic conditions. *J Infect Dis*, 199, 445-450.

Hansen DS, D'Ombrain MC, Schofield L 2007. The role of leukocytes bearing Natural Killer Complex receptors and Killer Immunoglobulin-like Receptors in the immunology of malaria. *Curr Opin Immunol*, 19, 416-423.

Hansen R, deSilva S, Strickland GT 1979. Antisporozoite antibodies in mice immunized with irradiation-attenuated Plasmodium berghei sporozoites. *Trans R Soc Trop Med Hyg*, 73, 574-578.

Hermsen CC, Verhage DF, Telgt DS, Teelen K, Bousema JT, Roestenberg M, Bolad A, Berzins K, Corradin G, Leroy O, Theisen M, Sauerwein RW 2007. Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial. *Vaccine*, 25, 2930-2940.

Herrera MA, de Plata C, Gonzalez JM, Corradin G, Herrera S 1994. Immunogenicity of multiple antigen peptides containing Plasmodium vivax CS epitopes in BALB/c mice. *Mem Inst Oswaldo Cruz*, 89 Suppl 2, 71-76.

Herrera S, Bonelo A, Perlaza BL, Valencia AZ, Cifuentes C, Hurtado S, Quintero G, Lopez JA, Corradin G, Arevalo-Herrera M 2004. Use of long synthetic peptides to study the antigenicity and immunogenicity of the Plasmodium vivax circumsporozoite protein. *Int J Parasitol*, 34, 1535-1546.

Herrington DA, Clyde DF, Losonsky G, Cortesia M, Murphy JR, Davis J, Baqar S, Felix AM, Heimer EP, Gillessen D, et al. 1987. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites. *Nature*, 328, 257-259.

Hippocrates 1985. *The Genuine Work of Hippocrates.* Birmingham, Birmingham, AL.

Hodder AN, Crewther PE, Anders RF 2001. Specificity of the protective antibody response to apical membrane antigen 1. *Infect Immun*, 69, 3286-3294.

Hoffman SL, Doolan DL 2000. Malaria vaccines-targeting infected hepatocytes. *Nat Med*, 6, 1218-1219.

Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci J, de la Vega P, Dowler M, Paul C, Gordon DM, Stoute JA, Church LW, Sedegah M, Heppner DG, Ballou WR, Richie TL 2002. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis*, 185, 1155-1164.

Hoffman SL, Wistar R, Jr., Ballou WR, Hollingdale MR, Wirtz RA, Schneider I, Marwoto HA, Hockmeyer WT 1986. Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of Plasmodium falciparum. *N Engl J Med*, 315, 601-606.

Hollingdale MR, Hogh B, Petersen E, Wirtz RA, Bjorkmann A 1989. Age-dependent occurrence of protective anti-Plasmodium falciparum sporozoite antibodies in a holoendemic area of Liberia. *Trans R Soc Trop Med Hyg*, 83, 322-324.

Hollingdale MR, Nardin EH, Tharavanij S, Schwartz AL, Nussenzweig RS 1984. Inhibition of entry of Plasmodium falciparum and P. vivax sporozoites into cultured cells; an in vitro assay of protective antibodies. *J Immunol*, 132, 909-913.

Holz LE, Fernandez-Ruiz D, Heath WR 2016. Protective immunity to liver-stage malaria. *Clin Transl Immunology*, 5, e105.

Horowitz A, Newman KC, Evans JH, Korbel DS, Davis DM, Riley EM 2010. Cross-talk between T cells and NK cells generates rapid effector responses to Plasmodium falciparum-infected erythrocytes. *J Immunol*, 184, 6043-6052.

Howland SW, Poh CM, Renia L 2015. Activated Brain Endothelial Cells Cross-Present Malaria Antigen. *PLoS Pathog*, 11, e1004963.

Huff CG, Marchbank DF, Shiroishi T 1958. Changes in infectiousness of malarial gametocytes. II. Analysis of the possible causative factors. *Exp Parasitol*, 7, 399-417.

Imai T, Ishida H, Suzue K, Hirai M, Taniguchi T, Okada H, Suzuki T, Shimokawa C, Hisaeda H 2013. CD8(+) T cell activation by murine erythroblasts infected with malaria parasites. *Sci Rep*, 3, 1572.

Jackson DC, Purcell AW, Fitzmaurice CJ, Zeng W, Hart DN 2002. The central role played by peptides in the immune response and the design of peptide-based vaccines against infectious diseases and cancer. *Curr Drug Targets*, 3, 175-196.

Jang JI, Kim JS, Eom JS, Kim HG, Kim BH, Lim S, Bang IS, Park YK 2012. Expression and delivery of tetanus toxin fragment C fused to the N-terminal domain of SipB enhances specific immune responses in mice. *Microbiol Immunol*, 56, 595-604.

Janssen CS, Phillips RS, Turner CM, Barrett MP 2004. Plasmodium interspersed repeats: the major multigene superfamily of malaria parasites. *Nucleic Acids Res*, 32, 5712-5720.

Jimah JR, Salinas ND, Sala-Rabanal M, Jones NG, Sibley LD, Nichols CG, Schlesinger PH, Tolia NH 2016. Malaria parasite CelTOS targets the inner leaflet of cell membranes for pore-dependent disruption. *Elife*, 5.

John CC, Moormann AM, Pregibon DC, Sumba PO, McHugh MM, Narum DL, Lanar DE, Schluchter MD, Kazura JW 2005. Correlation of high levels of antibodies to multiple pre-erythrocytic Plasmodium falciparum antigens and protection from infection. *Am J Trop Med Hyg*, 73, 222-228.

John CC, Tande AJ, Moormann AM, Sumba PO, Lanar DE, Min XM, Kazura JW 2008. Antibodies to pre-erythrocytic Plasmodium falciparum antigens and risk of clinical malaria in Kenyan children. *J Infect Dis*, 197, 519-526.

Josling GA, Llinas M 2015. Sexual development in Plasmodium parasites: knowing when it's time to commit. *Nat Rev Microbiol*, 13, 573-587.

Kariu T, Ishino T, Yano K, Chinzei Y, Yuda M 2006. CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Molecular microbiology*, 59, 1369-1379.

Karunajeewa HA, Mueller I, Senn M, Lin E, Law I, Gomorrai PS, Oa O, Griffin S, Kotab K, Suano P, Tarongka N, Ura A, Lautu D, Page-Sharp M, Wong R, Salman S, Siba P, Ilett KF, Davis TM 2008. A trial of combination antimalarial therapies in children from Papua New Guinea. *N Engl J Med*, 359, 2545-2557.

Kaumaya PT, Kobs-Conrad S, Seo YH, Lee H, VanBuskirk AM, Feng N, Sheridan JF, Stevens V 1993. Peptide vaccines incorporating a 'promiscuous' T-cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. *J Mol Recognit*, 6, 81-94.

Kebaier C, Voza T, Vanderberg J 2009. Kinetics of mosquito-injected Plasmodium sporozoites in mice: fewer sporozoites are injected into sporozoite-immunized mice. *PLoS Pathog*, 5, e1000399.

Kimura D, Miyakoda M, Kimura K, Honma K, Hara H, Yoshida H, Yui K 2016. Interleukin-27-Producing CD4(+) T Cells Regulate Protective Immunity during Malaria Parasite Infection. *Immunity*, 44, 672-682.

Kristensen KL, Dragsted UB 2014. Recurrent Plasmodium vivax malaria due to dose-dependent primaquine resistance: a case report. *Scandinavian journal of infectious diseases*, 46, 63-65.

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

—— 1989. The hypnozoite and malarial relapse. *Prog Clin Parasitol*, 1, 1-19.

Kuk S 2007. [CD8+ and CD4+ T lymphocyte responses against malaria]. *Mikrobiyol Bul*, 41, 329-339.

Kumar S, Gupta L, Han YS, Barillas-Mury C 2004. Inducible peroxidases mediate nitration of anopheles midgut cells undergoing apoptosis in response to Plasmodium invasion. *J Biol Chem*, 279, 53475-53482.

Kusi KA, Bosomprah S, Dodoo D, Kyei-Baafour E, Dickson EK, Mensah D, Angov E, Dutta S, Sedegah M, Koram KA 2014. Anti-sporozoite antibodies as alternative markers for malaria transmission intensity estimation. *Malar J*, 13, 103.

Ladeia-Andrade S, Ferreira MU, de Carvalho ME, Curado I, Coura JR 2009. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. *Am J Trop Med Hyg*, 80, 452-459.

Langhorne J, Ndungu FM, Sponaas AM, Marsh K 2008. Immunity to malaria: more questions than answers. *Nat Immunol*, 9, 725-732.

Lanier JG, Newman MJ, Lee EM, Sette A, Ahmed R 1999. Peptide vaccination using nonionic block copolymers induces protective anti-viral CTL responses. *Vaccine*, 18, 549-557.

Liehl P, Zuzarte-Luis V, Chan J, Zillinger T, Baptista F, Carapau D, Konert M, Hanson KK, Carret C, Lassnig C, Muller M, Kalinke U, Saeed M, Chora AF, Golenbock DT, Strobl B, Prudencio M, Coelho LP, Kappe SH, Superti-Furga G, Pichlmair A, Vigario AM, Rice CM, Fitzgerald KA, Barchet W, Mota MM 2014. Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection. *Nat Med*, 20, 47-53.

Lima-Junior JC, Banic DM, Tran TM, Meyer VS, De-Simone SG, Santos F, Porto LC, Marques MT, Moreno A, Barnwell JW, Galinski MR, Oliveira-Ferreira J 2010. Promiscuous T-cell epitopes of Plasmodium merozoite surface protein 9 (PvMSP9) induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon. *Vaccine*, 28, 3185-3191.

Lima-Junior JC, Rodrigues-da-Silva RN, Banic DM, Jiang J, Singh B, Fabricio-Silva GM, Porto LC, Meyer EV, Moreno A, Rodrigues MM, Barnwell JW, Galinski MR, de

Oliveira-Ferreira J 2012. Influence of HLA-DRB1 and HLA-DQB1 alleles on IgG antibody response to the P. vivax MSP-1, MSP-3alpha and MSP-9 in individuals from Brazilian endemic area. *PLoS One*, 7, e36419.

Lima-Junior JC, Tran TM, Meyer EV, Singh B, De-Simone SG, Santos F, Daniel-Ribeiro CT, Moreno A, Barnwell JW, Galinski MR, Oliveira-Ferreira J 2008. Naturally acquired humoral and cellular immune responses to Plasmodium vivax merozoite surface protein 9 in Northwestern Amazon individuals. *Vaccine*, 26, 6645-6654.

Lima JC, Banic DM, Tran TM, Meyer VSE, De-Simone SG, Santos F, Porto LCS, Marques MTQ, Moreno A, Barnwell JW, Galinski MR, Oliveira-Ferreira J 2010. Promiscuous T-cell epitopes of Plasmodium merozoite surface protein 9 (PvMSP9) induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon. *Vaccine*, 28, 3185-3191.

Lin E, Kiniboro B, Gray L, Dobbie S, Robinson L, Laumaea A, Schopflin S, Stanisic D, Betuela I, Blood-Zikursh M, Siba P, Felger I, Schofield L, Zimmerman P, Mueller I 2010. Differential patterns of infection and disease with P. falciparum and P. vivax in young Papua New Guinean children. *PLoS One*, 5, e9047.

Lindner SE, Miller JL, Kappe SH 2012. Malaria parasite pre-erythrocytic infection: preparation meets opportunity. *Cell Microbiol*, 14, 316-324.

Long CA 1993. Immunity to blood stages of malaria. *Curr Opin Immunol*, 5, 548-556.

Long CA, Zavala F 2017. Immune Responses in Malaria. *Cold Spring Harb Perspect Med*, 7.

Longley RJ, Reyes-Sandoval A, Montoya-Diaz E, Dunachie S, Kumpitak C, Nguitragool W, Mueller I, Sattabongkot J 2015. Acquisition and Longevity of Antibodies to Plasmodium vivax Preerythrocytic Antigens in Western Thailand. *Clin Vaccine Immunol*, 23, 117-124.

—— 2016. Acquisition and Longevity of Antibodies to Plasmodium vivax Preerythrocytic Antigens in Western Thailand. *Clin Vaccine Immunol*, 23, 117-124.

Lopez C, Yepes-Perez Y, Hincapie-Escobar N, Diaz-Arevalo D, Patarroyo MA 2017. What Is Known about the Immune Response Induced by Plasmodium vivax Malaria Vaccine Candidates? *Front Immunol*, 8, 126.

Lusingu JP, Vestergaard LS, Alifrangis M, Mmbando BP, Theisen M, Kitua AY, Lemnge MM, Theander TG 2005. Cytophilic antibodies to Plasmodium falciparum

glutamate rich protein are associated with malaria protection in an area of holoendemic transmission. *Malar J*, 4, 48.

M. ER, Nicoletta Cerutti A, S. MD 2000. MALARIA IN ANCIENT EGYPT: PALEOIMMUNOLOGICAL INVESTIGATION ON PREDYNASTIC MUMMIFIED REMAINS. *Chungara, Revista de Antropología Chilena*, 32, 3.

Mahanty S, Saul A, Miller LH 2003. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. *J Exp Biol*, 206, 3781-3788.

Malkin E, Dubovsky F, Moree M 2006. Progress towards the development of malaria vaccines. *Trends Parasitol*, 22, 292-295.

Manwell RD, Goldstein F 1940. Passive Immunity in Avian Malaria. *J Exp Med*, 71, 409-423.

March S, Ng S, Velmurugan S, Galstian A, Shan J, Logan DJ, Carpenter AE, Thomas D, Sim BK, Mota MM, Hoffman SL, Bhatia SN 2013. A microscale human liver platform that supports the hepatic stages of Plasmodium falciparum and vivax. *Cell Host Microbe*, 14, 104-115.

Margos G, Navarette S, Butcher G, Davies A, Willers C, Sinden RE, Lachmann PJ 2001. Interaction between host complement and mosquito-midgut-stage Plasmodium berghei. *Infect Immun*, 69, 5064-5071.

Marty P, Chapdelaine B, Le Fichoux Y, Chabert JM 1987. [Anemic Plasmodium ovale malaria after 45 months' incubation]. *Presse Med*, 16, 357.

Marussig M, Renia L, Motard A, Miltgen F, Petour P, Chauhan V, Corradin G, Mazier D 1997. Linear and multiple antigen peptides containing defined T and B epitopes of the Plasmodium yoelii circumsporozoite protein: antibody-mediated protection and boosting by sporozoite infection. *Int Immunol*, 9, 1817-1824.

McCoy KD, Stoel M, Stettler R, Merky P, Fink K, Senn BM, Schaer C, Massacand J, Odermatt B, Oettgen HC, Zinkernagel RM, Bos NA, Hengartner H, Macpherson AJ, Harris NL 2008. Polyclonal and specific antibodies mediate protective immunity against enteric helminth infection. *Cell Host Microbe*, 4, 362-373.

McGregor IA 1964. Studies in the Acquisition of Immunity of Plasmodium Falciparum Infections in Africa. *Trans R Soc Trop Med Hyg*, 58, 80-92.

McKenzie FE, Jeffery GM, Collins WE 2002. Plasmodium vivax blood-stage dynamics. *J Parasitol*, 88, 521-535.

—— 2007. Gametocytemia and fever in human malaria infections. *J Parasitol*, 93, 627-633.

Mellouk S, Mazier D, Druilhe P, Berbiguier N, Danis M 1986. In vitro and in vivo results suggesting that anti-sporozoite antibodies do not totally block Plasmodium falciparum sporozoite infectivity. *N Engl J Med*, 315, 648.

Menard R, Tavares J, Cockburn I, Markus M, Zavala F, Amino R 2013. Looking under the skin: the first steps in malarial infection and immunity. *Nat Rev Microbiol*, 11, 701-712.

Mendis KN, Targett GA 1979. Immunisation against gametes and asexual erythrocytic stages of a rodent malaria parasite. *Nature*, 277, 389-391.

Metzger WG, Okenu DM, Cavanagh DR, Robinson JV, Bojang KA, Weiss HA, McBride JS, Greenwood BM, Conway DJ 2003. Serum IgG3 to the Plasmodium falciparum merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. *Parasite Immunol*, 25, 307-312.

Michon P, Cole-Tobian JL, Dabod E, Schoepflin S, Igu J, Susapu M, Tarongka N, Zimmerman PA, Reeder JC, Beeson JG, Schofield L, King CL, Mueller I 2007. The risk of malarial infections and disease in Papua New Guinean children. *Am J Trop Med Hyg*, 76, 997-1008.

Miller LH, Hoffman SL 1998. Research toward vaccines against malaria. *Nat Med*, 4, 520-524.

Miller RL, Ikram S, Armelagos GJ, Walker R, Harer WB, Shiff CJ, Baggett D, Carrigan M, Maret SM 1994. Diagnosis of Plasmodium falciparum infections in mummies using the rapid manual ParaSight-F test. *Trans R Soc Trop Med Hyg*, 88, 31-32.

Milligan P, Flach C, Theisen M 2017. Efficacy of the GMZ2 malaria vaccine in African children. *Vaccine*, 35, 202.

Mirabello L, Conn JE 2006. Molecular population genetics of the malaria vector Anopheles darlingi in Central and South America. *Heredity*, 96, 311-321.

Mitchell GH, Thomas AW, Margos G, Dluzewski AR, Bannister LH 2004. Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. *Infect Immun*, 72, 154-158.

Mitra S, Abhilash K, Arora S, Miraclin A 2015. A prospective study from south India to compare the severity of malaria caused by Plasmodium vivax, P. falciparum and dual infection. *J Vector Borne Dis*, 52, 281-286.

Mizutani M, Fukumoto S, Soubeiga AP, Soga A, Iyori M, Yoshida S 2016. Development of a Plasmodium berghei transgenic parasite expressing the full-length Plasmodium vivax circumsporozoite VK247 protein for testing vaccine efficacy in a murine model. *Malar J*, 15, 251.

Mlambo G, Kumar N 2008. Transgenic rodent Plasmodium berghei parasites as tools for assessment of functional immunogenicity and optimization of human malaria vaccines. *Eukaryot Cell*, 7, 1875-1879.

Mlambo G, Maciel J, Kumar N 2008. Murine model for assessment of Plasmodium falciparum transmission-blocking vaccine using transgenic Plasmodium berghei parasites expressing the target antigen Pfs25. *Infect Immun*, 76, 2018-2024.

Moorthy VS, Good MF, Hill AV 2004. Malaria vaccine developments. *Lancet*, 363, 150-156.

Mora M, Veggi D, Santini L, Pizza M, Rappuoli R 2003. Reverse vaccinology. *Drug Discov Today*, 8, 459-464.

Mota MM, Hafalla JC, Rodriguez A 2002. Migration through host cells activates Plasmodium sporozoites for infection. *Nat Med*, 8, 1318-1322.

Moyle PM, Toth I 2013. Modern subunit vaccines: development, components, and research opportunities. *ChemMedChem*, 8, 360-376.

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.

Nacer A, Movila A, Sohet F, Girgis NM, Gundra UM, Loke P, Daneman R, Frevert U 2014. Experimental cerebral malaria pathogenesis--hemodynamics at the blood brain barrier. *PLoS Pathog*, 10, e1004528.

Naing C, Whittaker MA, Nyunt Wai V, Mak JW 2014. Is Plasmodium vivax malaria a severe malaria?: a systematic review and meta-analysis. *PLoS Negl Trop Dis*, 8, e3071.

Nardelli B, Tam JP 1995. The MAP system. A flexible and unambiguous vaccine design of branched peptides. *Pharm Biotechnol*, 6, 803-819.

Nardin EH, Nussenzweig RS, McGregor IA, Bryan JH 1979. Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. *Science*, 206, 597-599.

Nardin EH, Oliveira GA, Calvo-Calle JM, Nussenzweig RS 1995. The use of multiple antigen peptides in the analysis and induction of protective immune responses against infectious diseases. *Adv Immunol*, 60, 105-149.

Nayar JK, Baker RH, Knight JW, Sullivan JS, Morris CL, Richardson BB, Galland GG, Collins WE 1997. Studies on a primaquine-tolerant strain of Plasmodium vivax from Brazil in Aotus and Saimiri monkeys. *J Parasitol*, 83, 739-745.

Nebie I, Diarra A, Ouedraogo A, Soulama I, Bougouma EC, Tiono AB, Konate AT, Chilengi R, Theisen M, Dodoo D, Remarque E, Bosomprah S, Milligan P, Sirima SB 2008. Humoral responses to Plasmodium falciparum blood-stage antigens and association with incidence of clinical malaria in children living in an area of seasonal malaria transmission in Burkina Faso, West Africa. *Infect Immun*, 76, 759-766.

Nerlich AG, Schraut B, Dittrich S, Jelinek T, Zink AR 2008. Plasmodium falciparum in ancient Egypt. *Emerg Infect Dis*, 14, 1317-1319.

Nimmerjahn F, Ravetch JV 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*, 310, 1510-1512.

Nussenzweig R, Vanderberg J, Most H 1969. Protective immunity produced by the injection of x-irradiated sporozoites of Plasmodium berghei. IV. Dose response, specificity and humoral immunity. *Mil Med*, 134, 1176-1182.

Nussenzweig RS, Vanderberg J, Most H, Orton C 1967. Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. *Nature*, 216, 160-162.

Nussenzweig V, Nussenzweig RS 1989. Circumsporozoite proteins of malaria parasites. *Bull Mem Acad R Med Belg*, 144, 493-504.

Oakley MS, Sahu BR, Lotspeich-Cole L, Solanki NR, Majam V, Pham PT, Banerjee R, Kozakai Y, Derrick SC, Kumar S, Morris SL 2013. The transcription factor T-bet regulates parasitemia and promotes pathogenesis during Plasmodium berghei ANKA murine malaria. *J Immunol*, 191, 4699-4708.

Okie S 2005. Betting on a malaria vaccine. *N Engl J Med*, 353, 1877-1881.

Oliveira-Ferreira J, Lacerda MV, Brasil P, Ladislau JL, Tauil PL, Daniel-Ribeiro CT 2010. Malaria in Brazil: an overview. *Malar J*, 9, 115.

Oliveira-Ferreira J, Pratt-Riccio LR, Arruda M, Santos F, Daniel Ribeiro CT, Goldberg AC, Banic DM 2004a. HLA class II and antibody responses to circumsporozoite protein repeats of P. vivax (VK210, VK247 and P. vivax-like) in individuals naturally exposed to malaria. *Acta Trop*, 92, 63-69.

Oliveira-Ferreira J, Vargas-Serrato E, Barnwell JW, Moreno A, Galinski MR 2004b. Immunogenicity of Plasmodium vivax merozoite surface protein-9 recombinant proteins expressed in E. coli. *Vaccine*, 22, 2023-2030.

Oliveira Gde A, Lieberman J, Barillas-Mury C 2012. Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. *Science*, 335, 856-859.

Olotu A, Fegan G, Wambua J, Nyangweso G, Leach A, Lievens M, Kaslow DC, Njuguna P, Marsh K, Bejon P 2016. Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. *N Engl J Med*, 374, 2519-2529.

Osier FH, Mackinnon MJ, Crosnier C, Fegan G, Kamuyu G, Wanaguru M, Ogada E, McDade B, Rayner JC, Wright GJ, Marsh K 2014. New antigens for a multicomponent blood-stage malaria vaccine. *Science translational medicine*, 6, 247ra102.

Othman AS, Marin-Mogollon C, Salman AM, Franke-Fayard BM, Janse CJ, Khan SM 2017. The use of transgenic parasites in malaria vaccine research. *Expert Rev Vaccines*, 16, 1-13.

Outchkourov NS, Roeffen W, Kaan A, Jansen J, Luty A, Schuiffel D, van Gemert GJ, van de Vegte-Bolmer M, Sauerwein RW, Stunnenberg HG 2008. Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice. *Proc Natl Acad Sci U S A*, 105, 4301-4305.

Overstreet MG, Chen YC, Cockburn IA, Tse SW, Zavala F 2011. CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites. *PLoS One*, 6, e15948.

PAHO 2016. Report on the Situation of Malaria in the Americas, 2000-2015. In, Regional Malaria Program, Pan American Health Organization.

Palacpac NM, Ntege E, Yeka A, Balikagala B, Suzuki N, Shirai H, Yagi M, Ito K, Fukushima W, Hirota Y, Nsereko C, Okada T, Kanoi BN, Tetsutani K, Arisue N, Itagaki S, Tougan T, Ishii KJ, Ueda S, Egwang TG, Horii T 2013. Phase 1b randomized trial and follow-up study in Uganda of the blood-stage malaria vaccine candidate BK-SE36. *PLoS One*, 8, e64073.

Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol*, 19, 2237-2242.

Parashar A, Aikat BK, Sehgal S, Naik S 1977. Cell mediated and humoral immunity in experimental Plasmodium berghei infection. *Trans R Soc Trop Med Hyg*, 71, 474-480.

Patarroyo MA, Calderon D, Moreno-Perez DA 2012. Vaccines against Plasmodium vivax: a research challenge. *Expert Rev Vaccines*, 11, 1249-1260.

Patarroyo ME, Patarroyo MA 2008. Emerging rules for subunit-based, multiantigenic, multistage chemically synthesized vaccines. *Acc Chem Res*, 41, 377-386.

Malaria Vaccine Initiative [homepage on the Internet]: Washington, DC 20001 | USA 2017. Available from: http://www.malariavaccine.org/malaria-and-vaccines/first-generation-vaccine/rtss.

Payne RO, Silk SE, Elias SC, Milne KH, Rawlinson TA, Llewellyn D, Shakri AR, Jin J, Labbe GM, Edwards NJ, Poulton ID, Roberts R, Farid R, Jorgensen T, Alanine DG, de Cassan SC, Higgins MK, Otto TD, McCarthy JS, de Jongh WA, Nicosia A, Moyle S, Hill AV, Berrie E, Chitnis CE, Lawrie AM, Draper SJ 2017a. Human vaccination against Plasmodium vivax Duffy-binding protein induces strain-transcending antibodies. *JCI Insight*, 2.

Payne RO, Silk SE, Elias SC, Miura K, Diouf A, Galaway F, de Graaf H, Brendish NJ, Poulton ID, Griffiths OJ, Edwards NJ, Jin J, Labbe GM, Alanine DG, Siani L, Di Marco S, Roberts R, Green N, Berrie E, Ishizuka AS, Nielsen CM, Bardelli M, Partey FD, Ofori MF, Barfod L, Wambua J, Murungi LM, Osier FH, Biswas S, McCarthy JS, Minassian AM, Ashfield R, Viebig NK, Nugent FL, Douglas AD, Vekemans J, Wright GJ, Faust SN, Hill AV, Long CA, Lawrie AM, Draper SJ 2017b. Human vaccination against RH5 induces neutralizing antimalarial antibodies that inhibit RH5 invasion complex interactions. *JCI Insight*, 2.

Perez-Mazliah D, Ng DH, Freitas do Rosario AP, McLaughlin S, Mastelic-Gavillet B, Sodenkamp J, Kushinga G, Langhorne J 2015. Disruption of IL-21 signaling affects T cell-B cell interactions and abrogates protective humoral immunity to malaria. *PLoS Pathog*, 11, e1004715.

Pishraft Sabet L, Taheri T, Memarnejadian A, Mokhtari Azad T, Asgari F, Rahimnia R, Alavian SM, Rafati S, Samimi Rad K 2014. Immunogenicity of Multi-Epitope DNA and Peptide Vaccine Candidates Based on Core, E2, NS3 and NS5B HCV Epitopes in BALB/c Mice. *Hepat Mon*, 14, e22215.

Polhemus ME, Remich SA, Ogutu BR, Waitumbi JN, Otieno L, Apollo S, Cummings JF, Kester KE, Ockenhouse CF, Stewart A, Ofori-Anyinam O, Ramboer I, Cahill CP, Lievens M, Dubois MC, Demoitie MA, Leach A, Cohen J, Ballou WR, Heppner DG, Jr. 2009. Evaluation of RTS,S/AS02A and RTS,S/AS01B in adults in a high malaria transmission area. *PLoS One*, 4, e6465.

Porcherie A, Mathieu C, Peronet R, Schneider E, Claver J, Commere PH, Kiefer-Biasizzo H, Karasuyama H, Milon G, Dy M, Kinet JP, Louis J, Blank U, Mecheri S 2011. Critical role of the neutrophil-associated high-affinity receptor for IgE in the pathogenesis of experimental cerebral malaria. *J Exp Med*, 208, 2225-2236.

Pouvelle B, Gormley JA, Taraschi TF 1994. Characterization of trafficking pathways and membrane genesis in malaria-infected erythrocytes. *Mol Biochem Parasitol*, 66, 83-96.

Pouvelle B, Spiegel R, Hsiao L, Howard RJ, Morris RL, Thomas AP, Taraschi TF 1991. Direct access to serum macromolecules by intraerythrocytic malaria parasites. *Nature*, 353, 73-75.

Prato S, Maxwell T, Pinzon-Charry A, Schmidt CW, Corradin G, Lopez JA 2005. MHC class I-restricted exogenous presentation of a synthetic 102-mer malaria vaccine polypeptide. *Eur J Immunol*, 35, 681-689.

Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W 2014. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. *Malar J*, 13, 481.

Ramharter M, Willheim M, Winkler H, Wahl K, Lagler H, Graninger W, Winkler S 2003. Cytokine profile of Plasmodium falciparum-specific T cells in non-immune malaria patients. *Parasite Immunol*, 25, 211-219.

Ramirez JL, Garver LS, Brayner FA, Alves LC, Rodrigues J, Molina-Cruz A, Barillas-Mury C 2014. The role of hemocytes in Anopheles gambiae antiplasmodial immunity. *J Innate Immun*, 6, 119-128.

Ramphul UN, Garver LS, Molina-Cruz A, Canepa GE, Barillas-Mury C 2015. Plasmodium falciparum evades mosquito immunity by disrupting JNK-mediated apoptosis of invaded midgut cells. *Proc Natl Acad Sci U S A*, 112, 1273-1280.

Ranawaka GR, Fleck SL, Blanco AR, Sinden RE 1994. Characterization of the modes of action of anti-Pbs21 malaria transmission-blocking immunity: ookinete to oocyst differentiation in vivo. *Parasitology*, 109 (Pt 4), 403-411.

Rappuoli R 2000. Reverse vaccinology. *Curr Opin Microbiol*, 3, 445-450.

—— 2001. Reverse vaccinology, a genome-based approach to vaccine development. *Vaccine*, 19, 2688-2691.

Rappuoli R, Aderem A 2011. A 2020 vision for vaccines against HIV, tuberculosis and malaria. *Nature*, 473, 463-469.

Read D, Lensen AH, Begarnie S, Haley S, Raza A, Carter R 1994. Transmission-blocking antibodies against multiple, non-variant target epitopes of the Plasmodium falciparum gamete surface antigen Pfs230 are all complement-fixing. *Parasite Immunol*, 16, 511-519.

Recht J, Siqueira AM, Monteiro WM, Herrera SM, Herrera S, Lacerda MVG 2017. Malaria in Brazil, Colombia, Peru and Venezuela: current challenges in malaria control and elimination. *Malar J*, 16, 273.

Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature*, 410, 101-105.

Renia L, Goh YS 2016. Malaria Parasites: The Great Escape. *Front Immunol*, 7, 463.

Rey L 1991. *Parasitologia Médica.*

Richie TL, Saul A 2002. Progress and challenges for malaria vaccines. *Nature*, 415, 694-701.

Richter J, Franken G, Mehlhorn H, Labisch A, Haussinger D 2010. What is the evidence for the existence of Plasmodium ovale hypnozoites? *Parasitol Res*, 107, 1285-1290.

Risco-Castillo V, Topcu S, Marinach C, Manzoni G, Bigorgne AE, Briquet S, Baudin X, Lebrun M, Dubremetz JF, Silvie O 2015. Malaria Sporozoites Traverse Host Cells within Transient Vacuoles. *Cell Host Microbe*, 18, 593-603.

Rodrigues-da-Silva RN, Martins da Silva JH, Singh B, Jiang J, Meyer EV, Santos F, Banic DM, Moreno A, Galinski MR, Oliveira-Ferreira J, Lima-Junior Jda C 2016. In silico Identification and Validation of a Linear and Naturally Immunogenic B-Cell Epitope of the Plasmodium vivax Malaria Vaccine Candidate Merozoite Surface Protein-9. *PLoS One*, 11, e0146951.

Rodrigues J, Brayner FA, Alves LC, Dixit R, Barillas-Mury C 2010. Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes. *Science*, 329, 1353-1355.

Rodrigues M, Nussenzweig RS, Zavala F 1993. The relative contribution of antibodies, CD4+ and CD8+ T cells to sporozoite-induced protection against malaria. *Immunology*, 80, 1-5.

Roland J, Soulard V, Sellier C, Drapier AM, Di Santo JP, Cazenave PA, Pied S 2006. NK cell responses to Plasmodium infection and control of intrahepatic parasite development. *J Immunol*, 177, 1229-1239.

Romero P, Eberl G, Casanova JL, Cordey AS, Widmann C, Luescher IF, Corradin G, Maryanski JL 1992. Immunization with synthetic peptides containing a defined malaria epitope induces a highly diverse cytotoxic T lymphocyte response. Evidence that two peptide residues are buried in the MHC molecule. *J Immunol*, 148, 1871-1878.

Rosenthal PJ 2015. The RTS,S/AS01 vaccine continues to show modest protection against malaria in African infants and children. *Evid Based Med*, 20, 179.

Rts SCTP 2015. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet*, 386, 31-45.

Ruppert EE, Barnes RD 1996. *Zoologia de Invertebrados.* São Paulo: Rocca, 1 pp.

Ruppert EE, Fox RS, Barnes RD 2005. *Zoologia dos invertebrados: uma abordagem funcional-evolutiva.* São Paulo: Rocca.

Safeukui I, Gomez ND, Adelani AA, Burte F, Afolabi NK, Akondy R, Velazquez P, Holder A, Tewari R, Buffet P, Brown BJ, Shokunbi WA, Olaleye D, Sodeinde O,

Kazura J, Ahmed R, Mohandas N, Fernandez-Reyes D, Haldar K 2015. Malaria induces anemia through CD8+ T cell-dependent parasite clearance and erythrocyte removal in the spleen. *MBio*, 6.

Sagara I, Dicko A, Ellis RD, Fay MP, Diawara SI, Assadou MH, Sissoko MS, Kone M, Diallo AI, Saye R, Guindo MA, Kante O, Niambele MB, Miura K, Mullen GE, Pierce M, Martin LB, Dolo A, Diallo DA, Doumbo OK, Miller LH, Saul A 2009. A randomized controlled phase 2 trial of the blood stage AMA1-C1/Alhydrogel malaria vaccine in children in Mali. *Vaccine*, 27, 3090-3098.

Salman AM, Mogollon CM, Lin JW, van Pul FJ, Janse CJ, Khan SM 2015. Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins. *Methods Mol Biol*, 1325, 257-286.

Salman AM, Montoya-Diaz E, West H, Lall A, Atcheson E, Lopez-Camacho C, Ramesar J, Bauza K, Collins KA, Brod F, Reis F, Pappas L, Gonzalez-Ceron L, Janse CJ, Hill AVS, Khan SM, Reyes-Sandoval A 2017. Rational development of a protective P. vivax vaccine evaluated with transgenic rodent parasite challenge models. *Sci Rep*, 7, 46482.

Santiago HC, Bennuru S, Boyd A, Eberhard M, Nutman TB 2011. Structural and immunologic cross-reactivity among filarial and mite tropomyosin: implications for the hygiene hypothesis. *J Allergy Clin Immunol*, 127, 479-486.

Satterthwait AC, Chiang LC, Arrhenius T, Cabezas E, Zavala F, Dyson HJ, Wright PE, Lerner RA 1990. The conformational restriction of synthetic vaccines for malaria. *Bull World Health Organ*, 68 Suppl, 17-25.

Sebina I, James KR, Soon MS, Fogg LG, Best SE, Labastida Rivera F, Montes de Oca M, Amante FH, Thomas BS, Beattie L, Souza-Fonseca-Guimaraes F, Smyth MJ, Hertzog PJ, Hill GR, Hutloff A, Engwerda CR, Haque A 2016. IFNAR1-Signalling Obstructs ICOS-mediated Humoral Immunity during Non-lethal Blood-Stage Plasmodium Infection. *PLoS Pathog*, 12, e1005999.

Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH, Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M, Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK, Ledgerwood JE, Graham

BS, Hoffman SL, Team VRCS 2013. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science*, 341, 1359-1365.

Shiao SH, Whitten MM, Zachary D, Hoffmann JA, Levashina EA 2006. Fz2 and cdc42 mediate melanization and actin polymerization but are dispensable for Plasmodium killing in the mosquito midgut. *PLoS Pathog*, 2, e133.

Sieber KP, Huber M, Kaslow D, Banks SM, Torii M, Aikawa M, Miller LH 1991. The peritrophic membrane as a barrier: its penetration by Plasmodium gallinaceum and the effect of a monoclonal antibody to ookinetes. *Exp Parasitol*, 72, 145-156.

Silva-Flannery LM, Cabrera-Mora M, Dickherber M, Moreno A 2009a. Polymeric linear Peptide chimeric vaccine-induced antimalaria immunity is associated with enhanced in vitro antigen loading. *Infect Immun*, 77, 1798-1806.

Silva-Flannery LM, Cabrera-Mora M, Jiang J, Moreno A 2009b. Recombinant peptide replicates immunogenicity of synthetic linear peptide chimera for use as pre-erythrocytic stage malaria vaccine. *Microbes Infect*, 11, 83-91.

Silvie O, Mota MM, Matuschewski K, Prudencio M 2008. Interactions of the malaria parasite and its mammalian host. *Curr Opin Microbiol*, 11, 352-359.

Sinden RE, Gilles HM 2002. The malaria parasites. In GH Warrell DA, *Essential Malariology*, 4th ed., Arnold, London, p. 8–34.

Singh B, Cabrera-Mora M, Jiang J, Moreno A 2012. A hybrid multistage protein vaccine induces protective immunity against murine malaria. *Infect Immun*, 80, 1491-1501.

Sirima SB, Durier C, Kara L, Houard S, Gansane A, Loulergue P, Bahuaud M, Benhamouda N, Nebie I, Faber B, Remarque E, Launay O, Group AM-DS 2017. Safety and immunogenicity of a recombinant Plasmodium falciparum AMA1-DiCo malaria vaccine adjuvanted with GLA-SE or Alhydrogel(R) in European and African adults: A phase 1a/1b, randomized, double-blind multi-centre trial. *Vaccine*, 35, 6218-6227.

Sirima SB, Mordmuller B, Milligan P, Ngoa UA, Kironde F, Atuguba F, Tiono AB, Issifou S, Kaddumukasa M, Bangre O, Flach C, Christiansen M, Bang P, Chilengi R, Jepsen S, Kremsner PG, Theisen M, Group GMZTS 2016. A phase 2b randomized, controlled trial of the efficacy of the GMZ2 malaria vaccine in African children. *Vaccine*, 34, 4536-4542.

SIVEP - SIG Malária: Sistema de Informações e Gerenciamento [database on the Internet]2017 [cited 25/10/2017].

Slingluff CL, Jr. 2011. The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? *Cancer J*, 17, 343-350.

Smith RC, Barillas-Mury C 2016. Plasmodium Oocysts: Overlooked Targets of Mosquito Immunity. *Trends Parasitol*, 32, 979-990.

Smith RC, Barillas-Mury C, Jacobs-Lorena M 2015. Hemocyte differentiation mediates the mosquito late-phase immune response against Plasmodium in Anopheles gambiae. *Proc Natl Acad Sci U S A*, 112, E3412-3420.

Smith RC, Vega-Rodriguez J, Jacobs-Lorena M 2014. The Plasmodium bottleneck: malaria parasite losses in the mosquito vector. *Mem Inst Oswaldo Cruz*, 109, 644-661.

Smith TG, Ayi K, Serghides L, McAllister CD, Kain KC 2002. Innate immunity to malaria caused by Plasmodium falciparum. *Clin Invest Med*, 25, 262-272.

Soe S, Theisen M, Roussilhon C, Aye KS, Druilhe P 2004. Association between protection against clinical malaria and antibodies to merozoite surface antigens in an area of hyperendemicity in Myanmar: complementarity between responses to merozoite surface protein 3 and the 220-kilodalton glutamate-rich protein. *Infect Immun*, 72, 247-252.

Soria-Guerra RE, Nieto-Gomez R, Govea-Alonso DO, Rosales-Mendoza S 2015. An overview of bioinformatics tools for epitope prediction: implications on vaccine development. *Journal of biomedical informatics*, 53, 405-414.

Spencer Valero LM, Ogun SA, Fleck SL, Ling IT, Scott-Finnigan TJ, Blackman MJ, Holder AA 1998. Passive immunization with antibodies against three distinct epitopes on Plasmodium yoelii merozoite surface protein 1 suppresses parasitemia. *Infect Immun*, 66, 3925-3930.

Stanisic DI, Javati S, Kiniboro B, Lin E, Jiang J, Singh B, Meyer EV, Siba P, Koepfli C, Felger I, Galinski MR, Mueller I 2013. Naturally acquired immune responses to P. vivax merozoite surface protein 3alpha and merozoite surface protein 9 are associated with reduced risk of P. vivax malaria in young Papua New Guinean children. *PLoS Negl Trop Dis*, 7, e2498.

Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, Gilson PR, Murphy VJ, Anders RF, Mueller I, Beeson JG 2009. Immunoglobulin G subclass-specific responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infect Immun*, 77, 1165-1174.

Stephens R, Langhorne J 2010. Effector memory Th1 CD4 T cells are maintained in a mouse model of chronic malaria. *PLoS Pathog*, 6, e1001208.

Stevenson MM, Riley EM 2004. Innate immunity to malaria. *Nat Rev Immunol*, 4, 169-180.

Storti-Melo LM, da Costa DR, Souza-Neiras WC, Cassiano GC, Couto VS, Povoa MM, Soares Ida S, de Carvalho LH, Arevalo-Herrera M, Herrera S, Rossit AR, Cordeiro JA, de Mattos LC, Machado RL 2012. Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with Plasmodium vivax. *Acta Trop*, 121, 152-155.

Struik SS, Riley EM 2004. Does malaria suffer from lack of memory? *Immunol Rev*, 201, 268-290.

Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, Krueger A, Pollok JM, Menard R, Heussler VT 2006. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*, 313, 1287-1290.

Sutherland CJ, Tanomsing N, Nolder D, Oguike M, Jennison C, Pukrittayakamee S, Dolecek C, Hien TT, do Rosario VE, Arez AP, Pinto J, Michon P, Escalante AA, Nosten F, Burke M, Lee R, Blaze M, Otto TD, Barnwell JW, Pain A, Williams J, White NJ, Day NP, Snounou G, Lockhart PJ, Chiodini PL, Imwong M, Polley SD 2010. Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. *J Infect Dis*, 201, 1544-1550.

Suwanarusk R, Cooke BM, Dondorp AM, Silamut K, Sattabongkot J, White NJ, Udomsangpetch R 2004. The deformability of red blood cells parasitized by Plasmodium falciparum and P. vivax. *J Infect Dis*, 189, 190-194.

Swanson PA, 2nd, Hart GT, Russo MV, Nayak D, Yazew T, Pena M, Khan SM, Janse CJ, Pierce SK, McGavern DB 2016. CD8+ T Cells Induce Fatal Brainstem Pathology during Cerebral Malaria via Luminal Antigen-Specific Engagement of Brain Vasculature. *PLoS Pathog*, 12, e1006022.

Takala SL, Plowe CV 2009. Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'. *Parasite Immunol*, 31, 560-573.

Tan SY, Ahana A 2009. Charles Laveran (1845-1922): Nobel laureate pioneer of malaria. *Singapore Med J*, 50, 657-658.

Tapchaisri P, Chomcharn Y, Poonthong C, Asavanich A, Limsuwan S, Maleevan O, Tharavanij S, Harinasuta T 1983. Anti-sporozoite antibodies induced by natural infection. *Am J Trop Med Hyg*, 32, 1203-1208.

Targett G 2015. Phase 3 trial with the RTS,S/AS01 malaria vaccine shows protection against clinical and severe malaria in infants and children in Africa. *Evid Based Med*, 20, 9.

Taylor-Robinson A 1999. Immunity to asexual blood stages of: is resistance to acute malaria adaptive or innate? - a response. *Parasitol Today*, 15, 208.

Teka H, Petros B, Yamuah L, Tesfaye G, Elhassan I, Muchohi S, Kokwaro G, Aseffa A, Engers H 2008. Chloroquine-resistant Plasmodium vivax malaria in Debre Zeit, Ethiopia. *Malar J*, 7, 220.

Testa JS, Philip R 2012. Role of T-cell epitope-based vaccine in prophylactic and therapeutic applications. *Future Virol*, 7, 1077-1088.

Tetteh KK, Polley SD 2007. Progress and challenges towards the development of malaria vaccines. *BioDrugs*, 21, 357-373.

Tewari K, Flynn BJ, Boscardin SB, Kastenmueller K, Salazar AM, Anderson CA, Soundarapandian V, Ahumada A, Keler T, Hoffman SL, Nussenzweig MC, Steinman RM, Seder RA 2010. Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to Plasmodium falciparum circumsporozoite protein (CSP) and alphaDEC-CSP in non human primates. *Vaccine*, 28, 7256-7266.

Tewari R, Patzewitz EM, Poulin B, Stewart L, Baker DA 2014. Development of a transgenic Plasmodium berghei line (Pb pfpkg) expressing the P. falciparum cGMP-dependent protein kinase, a novel antimalarial drug target. *PLoS One*, 9, e96923.

Thomson-Luque R, Shaw Saliba K, Kocken CHM, Pasini EM 2017. A Continuous, Long-Term Plasmodium vivax In Vitro Blood-Stage Culture: What Are We Missing? *Trends Parasitol*.

Todryk SM, Bejon P, Mwangi T, Plebanski M, Urban B, Marsh K, Hill AV, Flanagan KL 2008. Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4 CD25 high T cells with susceptibility in Kenyans. *PLoS One*, 3, e2027.

Tuteja R 2007. Malaria - an overview. *FEBS J*, 274, 4670-4679.

Valencia SH, Rodriguez DC, Acero DL, Ocampo V, Arevalo-Herrera M 2011. Platform for Plasmodium vivax vaccine discovery and development. *Mem Inst Oswaldo Cruz*, 106 Suppl 1, 179-192.

Valmori D, Pessi A, Bianchi E, Corradin G 1992. Use of human universally antigenic tetanus toxin T cell epitopes as carriers for human vaccination. *J Immunol*, 149, 717-721.

Van den Steen PE, Deroost K, Van Aelst I, Geurts N, Martens E, Struyf S, Nie CQ, Hansen DS, Matthys P, Van Damme J, Opdenakker G 2008. CXCR3 determines strain susceptibility to murine cerebral malaria by mediating T lymphocyte migration toward IFN-gamma-induced chemokines. *Eur J Immunol*, 38, 1082-1095.

Vanderberg JP 2014. Imaging mosquito transmission of Plasmodium sporozoites into the mammalian host: immunological implications. *Parasitol Int*, 63, 150-164.

Vandoolaeghe P, Schuerman L 2016. The RTS,S/AS01 malaria vaccine in children 5 to 17 months of age at first vaccination. *Expert Rev Vaccines*, 15, 1481-1493.

Vargas-Serrato E, Barnwell JW, Ingravallo P, Perler FB, Galinski MR 2002. Merozoite surface protein-9 of Plasmodium vivax and related simian malaria parasites is orthologous to p101/ABRA of P. falciparum. *Mol Biochem Parasitol*, 120, 41-52.

Vargas-Serrato E, Corredor V, Galinski MR 2003. Phylogenetic analysis of CSP and MSP-9 gene sequences demonstrates the close relationship of Plasmodium coatneyi to Plasmodium knowlesi. *Infect Genet Evol*, 3, 67-73.

Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, Stumhofer JS, Hafalla JC, Blount DG, Hunter CA, Riley EM, Couper KN 2012. IFN-gamma-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. *J Immunol*, 189, 968-979.

Villegas-Mendez A, Inkson CA, Shaw TN, Strangward P, Couper KN 2016. Long-Lived CD4+IFN-gamma+ T Cells rather than Short-Lived CD4+IFN-gamma+IL-10+ T

Cells Initiate Rapid IL-10 Production To Suppress Anamnestic T Cell Responses during Secondary Malaria Infection. *J Immunol*, 197, 3152-3164.

Vlachou D, Schlegelmilch T, Runn E, Mendes A, Kafatos FC 2006. The developmental migration of Plasmodium in mosquitoes. *Curr Opin Genet Dev*, 16, 384-391.

Volohonsky G, Steinert S, Levashina EA 2010. Focusing on complement in the antiparasitic defense of mosquitoes. *Trends Parasitol*, 26, 1-3.

Voza T, Miller JL, Kappe SH, Sinnis P 2012. Extrahepatic exoerythrocytic forms of rodent malaria parasites at the site of inoculation: clearance after immunization, susceptibility to primaquine, and contribution to blood-stage infection. *Infect Immun*, 80, 2158-2164.

W.H.O 2015. World Malaria Report 2015. In.

W.H.O. 2016. World Malaria Report. In, World Health Organization, Geneva: World Health Organization.

Westenberger SJ, McClean CM, Chattopadhyay R, Dharia NV, Carlton JM, Barnwell JW, Collins WE, Hoffman SL, Zhou Y, Vinetz JM, Winzeler EA 2010. A systems-based analysis of Plasmodium vivax lifecycle transcription from human to mosquito. *PLoS Negl Trop Dis*, 4, e653.

White MT, Karl S, Battle KE, Hay SI, Mueller I, Ghani AC 2014. Modelling the contribution of the hypnozoite reservoir to Plasmodium vivax transmission. *Elife*, 3.

White NJ 2011. Determinants of relapse periodicity in Plasmodium vivax malaria. *Malar J*, 10, 297.

Date Accessed. Malaria Vaccine Rainbow Tables. [homepage on the Internet]2017 [updated 17/07/2017]. Available from: http://www.who.int/vaccine_research/links/Rainbow/en/index.html.

Wilson KL, Xiang SD, Plebanski M 2016. A Model to Study the Impact of Polymorphism Driven Liver-Stage Immune Evasion by Malaria Parasites, to Help Design Effective Cross-Reactive Vaccines. *Front Microbiol*, 7, 303.

Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, Kusser K, Honjo T, Mohrs K, Mohrs M, Randall T, Lund FE 2009. Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus. *Immunity*, 30, 421-433.

Woof JM 2005. Immunology. Tipping the scales toward more effective antibodies. *Science*, 310, 1442-1443.

Yamauchi LM, Coppi A, Snounou G, Sinnis P 2007. Plasmodium sporozoites trickle out of the injection site. *Cell Microbiol*, 9, 1215-1222.

Yildiz Zeyrek F, Palacpac N, Yuksel F, Yagi M, Honjo K, Fujita Y, Arisue N, Takeo S, Tanabe K, Horii T, Tsuboi T, Ishii KJ, Coban C 2011. Serologic markers in relation to parasite exposure history help to estimate transmission dynamics of Plasmodium vivax. *PLoS One*, 6, e28126.

Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science*, 207, 71-73.

Zander RA, Guthmiller JJ, Graham AC, Pope RL, Burke BE, Carr DJ, Butler NS 2016. Type I Interferons Induce T Regulatory 1 Responses and Restrict Humoral Immunity during Experimental Malaria. *PLoS Pathog*, 12, e1005945.

ANEXOS: ARTIGOS EM COAUTORIA.

4.1 Artigo 1 – Correlação de APRIL com produção de citocinas inflamatórias durante a malária aguda na Amazônia brasileira.

Immunity, Inflammation and Disease

Open Access

ORIGINAL RESEARCH

Correlation of APRIL with production of inflammatory cytokines during acute malaria in the Brazilian Amazon

Raquel A. Pinna[1], Adriana C. dos Santos[2], Daiana S. Perce-da-Silva[1], Luciene A. da Silva[1], Rodrigo N. Rodrigues da Silva[3], Marcelo R. Alves[4], Fátima Santos[5], Joseli de Oliveira Ferreira[3], Josué C. Lima-Junior[3], Déa M. Villa-Verde[6], Paula M. De Luca[3], Carla E. Carvalho-Pinto[2], & Dalma M. Banic[1]

[1]Laboratory of Clinical Immunology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil 21040-360
[2]Laboratory of Experimental Pathology, Institute of Biology, Fluminense Federal University, Niterói, RJ, Brazil 24020-140
[3]Laboratory of Imunoparasitology Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil 21040-360
[4]Laboratory of Research in Pharmacogenetics, National Institute of Infectology, Oswaldo Cruz Foundation, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil 21040-360
[5]Laboratory of Entomology, LACEN/RO, Rua Anita Garibalde, 4130 – Costa e Silva, Porto Velho, RO, Brazil 76803-620
[6]Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil 21040-360

Keywords
APRIL/BAFF, malaria, TACI

Correspondence
Dalma M. Banic, Laboratory of Clinical Immunology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil 21040-360. Tel: 55 21 38658152.
Fax: +55 21 22900479.
E-mail: banic@ioc.fiocruz.br

Funding information
This work was financially supported by IOC/Fiocruz. RAP and DSPS are recipients of CAPES Doctoral Fellowships.

Received: 10 May 2017; Revised: 18 October 2017; Accepted: 20 October 2017.
Final version published online 3 January 2018.

Immunity, Inflammation and Disease **2018; 6(2): 207–220**

doi: 10.1002/iid3.208

[Correction added on 9 February 2018, after first online publication: The name of the author Rodrigo Nunes Rodrigues da Silva has been correctly detailed on this version.]

Abstract

Introduction: A proliferation-inducing ligand (APRIL) and B cell activation factor (BAFF) are known to play a significant role in the pathogenesis of several diseases, including BAFF in malaria. The aim of this study was to investigate whether APRIL and BAFF plasma concentrations could be part of inflammatory responses associated with *P. vivax* and *P. falciparum* malaria in patients from the Brazilian Amazon.
Methods: Blood samples were obtained from *P. vivax* and *P. falciparum* malaria patients ($n = 52$) resident in Porto Velho before and 15 days after the beginning of treatment and from uninfected individuals ($n = 12$). We investigated APRIL and BAFF circulating levels and their association with parasitaemia, WBC counts, and cytokine/chemokine plasma levels. The expression levels of transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) on PBMC from a subset of 5 *P. vivax*-infected patients were analyzed by flow cytometry.
Results: APRIL plasma levels were transiently increased during acute *P. vivax* and *P. falciparum* infections whereas BAFF levels were only increased during acute *P. falciparum* malaria. Although *P. vivax* and *P. falciparum* malaria patients have similar cytokine profiles during infection, in *P. vivax* acute phase malaria, APRIL but not BAFF levels correlated positively with IL-1, IL-2, IL-4, IL-6, and IL-13 levels. We did not find any association between *P. vivax* parasitaemia and APRIL levels, while an inverse correlation was found between *P. falciparum* parasitaemia and APRIL levels. The percentage of TACI positive CD4+ and CD8+ T cells were increased in the acute phase *P. vivax* malaria.
Conclusion: These findings suggest that the APRIL and BAFF inductions reflect different host strategies for controlling infection with each malaria species.

Introduction

Malaria is one of the most important human parasitic diseases. Nearly half the world's population is at risk of contracting malaria, with an estimate global annual incidence of about 212 million clinical cases and almost 429,000 deaths [1]. Among the five *Plasmodium* species that infect humans, *P. falciparum*

© 2018 The Authors. Immunity, Inflammation and Disease Published by John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

4.2 Artigo 2 – Associação do genótipo IL-10A com baixos níveis circulantes de IL-10 em indivíduos infectados com malária de área endêmica da Amazônia brasileira.

Pereira *et al. Malaria Journal* (2015) 14:30
DOI 10.1186/s12936-015-0548-z

MALARIA JOURNAL

RESEARCH Open Access

IL10A genotypic association with decreased IL-10 circulating levels in malaria infected individuals from endemic area of the Brazilian Amazon

Virginia A Pereira[1], Juan C Sánchez-Arcila[1], Antonio Teva[2], Daiana S Perce-da-Silva[3], Mariana PA Vasconcelos[4], Cleoni AM Lima[5], Cesarino JL Aprígio[6], Rodrigo N Rodrigues-da-Silva[1], Davi O Santos[7], Dalma M Banic[3], Maria G Bonecini-Almeida[7], Josué C Lima-Júnior[1] and Joseli Oliveira-Ferreira[1*]

Abstract

Background: Cytokines play an important role in human immune responses to malaria and variation in their production may influence the course of infection and determine the outcome of the disease. The differential production of cytokines has been linked to single nucleotide polymorphisms in gene promoter regions, signal sequences, and gene introns. Although some polymorphisms play significant roles in susceptibility to malaria, gene polymorphism studies in Brazil are scarce.

Methods: A population of 267 individuals from Brazilian Amazon exposed to malaria was genotyped for five single nucleotide polymorphisms (SNPs), *IFNG + 874 T/A*, *IL10A-1082G/A*, *IL10A-592A/C*, *IL10A-819 T/C* and *NOS2A-954G/C*. Specific DNA fragments were amplified by polymerase chain reaction, allowing the detection of the polymorphism genotypes. The polymorphisms *IL10A-592A/C* and *IL10A-819 T/C* were estimated by a single analysis due to the complete linkage disequilibrium between the two SNPs with D' = 0.99. Plasma was used to measure the levels of IFN-γ and IL-10 cytokines by Luminex and nitrogen radicals by Griess reaction.

Results: No differences were observed in genotype and allelic frequency of *IFNG + 874 T/A* and *NOS2A-954G/C* between positive and negative subjects for malaria infection. Interesting, the genotype NOS2A-954C/C was not identified in the study population. Significant differences were found in *IL10A-592A/C* and *IL10A-819 T/C* genotypes distribution, carriers of IL10A -592A/-819 T alleles (genotypes AA/TT + AC/TC) were more frequent among subjects with malaria than in negative subjects that presented a higher frequency of the variant C allele ($p < 0.0001$). The presence of the allele C was associated with low producer of IL-10 and low parasitaemia. In addition, the GTA haplotypes formed from combinations of investigated polymorphisms in *IL10A* were significantly associated with malaria (+) and the CCA haplotype with malaria (–) groups. The *IL10A-1082G/A* polymorphism showed high frequency of heterozygous AG genotype in the population, but it was not possible to infer any association of the polymorphism because their distribution was not in Hardy Weinberg equilibrium.

Conclusion: This study shows that the *IL10A-592A/C* and *IL10A-819 T/C* polymorphisms were associated with malaria and decreased IL-10 levels and low parasite density suggesting that this polymorphism influence IL-10 levels and may influence in the susceptibility to clinical malaria.

Keywords: Malaria disease, Cytokines, Nitric oxide synthase, Polymorphism

* Correspondence: lila@ioc.fiocruz.br
[1]Laboratório de Imunoparasitologia, Instituto Oswaldo Cruz/Fiocruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, Brazil
Full list of author information is available at the end of the article

BioMed Central

© 2015 Pereira et al.; licensee BioMed Central. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

4.3 Artigo 3 – Coinfecção com parasitas intestinais não altera o perfil de citocinas plasmáticas induzido em episódios de malária aguda em indivíduos de área endêmica do Brasil.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 857245, 12 pages
http://dx.doi.org/10.1155/2014/857245

Research Article

Intestinal Parasites Coinfection Does Not Alter Plasma Cytokines Profile Elicited in Acute Malaria in Subjects from Endemic Area of Brazil

Juan Camilo Sánchez-Arcila,[1] Daiana de Souza Perce-da-Silva,[2] Mariana Pinheiro Alves Vasconcelos,[3] Rodrigo Nunes Rodrigues-da-Silva,[1] Virginia Araujo Pereira,[1] Cesarino Junior Lima Aprígio,[4] Cleoni Alves Mendes Lima,[5] Bruna de Paula Fonseca e Fonseca,[6] Dalma Maria Banic,[2] Josué da Costa Lima-Junior,[1] and Joseli Oliveira-Ferreira[1]

[1] *Laboratório de Imunoparasitologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21040-900 Rio de Janeiro, RJ, Brazil*
[2] *Laboratório de Simulídeos e Oncocercose, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21040-900 Rio de Janeiro, RJ, Brazil*
[3] *Instituto de Infectologia Emílio Ribas, 01246-900 São Paulo, SP, Brazil*
[4] *Agencia de Vigilância em Saúde da Secretaria de Estado da Saúde (AGEVISA), 78900-000 Porto Velho, RO, Brazil*
[5] *Centro Interdepartamental de Biologia Experimental e Biotecnologia, Universidade Federal de Rondonia, 78900-000 Porto Velho, RO, Brazil*
[6] *Laboratório de Tecnologia Diagnóstica, Bio-Manguinhos, Fundação Oswaldo Cruz, 21040-900 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Joseli Oliveira-Ferreira; lila@ioc.fiocruz.br

Received 13 June 2014; Accepted 1 September 2014; Published 16 September 2014

Academic Editor: Mauricio Martins Rodrigues

Copyright © 2014 Juan Camilo Sánchez-Arcila et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In Brazil, malaria is prevalent in the Amazon region and these regions coincide with high prevalence of intestinal parasites but few studies explore the interaction between malaria and other parasites. Therefore, the present study evaluates changes in cytokine, chemokine, C-reactive protein, and nitric oxide (NO) concentrations in 264 individuals, comparing plasma from infected individuals with concurrent malaria and intestinal parasites to individuals with either malaria infection alone and uninfected. In the studied population 24% of the individuals were infected with *Plasmodium* and 18% coinfected with intestinal parasites. Protozoan parasites comprised the bulk of the intestinal parasites infections and subjects infected with intestinal parasites were more likely to have malaria. The use of principal component analysis and cluster analysis associated increased levels of IL-6, TNF-α, IL-10, and CRP and low levels of IL-17A predominantly with individuals with malaria alone and coinfected individuals. In contrast, low levels of almost all inflammatory mediators were associated predominantly with individuals uninfected while increased levels of IL-17A were associated predominantly with individuals with intestinal parasites only. In conclusion, our data suggest that, in our population, the infection with intestinal parasites (mainly protozoan) does not modify the pattern of cytokine production in individuals infected with *P. falciparum* and *P. vivax*.

1. Introduction

The geographic distribution of *Plasmodium* and intestinal parasites are overlapped over the world; therefore malaria coinfection with intestinal parasites is common in tropical regions of the planet [1]. Although it is well known that polyparasitism is a common condition in human populations, its real impact on the immunopathology of other diseases, including malaria, has not been fully explored. In Brazil, malaria is endemic in the Amazon region and this

4.4 Artigo 4 – Alterações em citocinas e parâmetros hematológicos durante as fases aguda e de convalescência de infecções por *Plasmodium falciparum* e *Plasmodium vivax*.

Alterations in cytokines and haematological parameters during the acute and convalescent phases of *Plasmodium falciparum* and *Plasmodium vivax* infections

Rodrigo Nunes Rodrigues-da-Silva[1], Josué da Costa Lima-Junior[1], Bruna de Paula Fonseca e Fonseca[2], Paulo Renato Zuquim Antas[3], Arlete Baldez[4], Fabio Luiz Storer[5], Fátima Santos[6], Dalma Maria Banic[7], Joseli de Oliveira-Ferreira[1]/+

[1]Laboratório de Imunoparasitologia [3]Laboratório de Imunologia Clínica [7]Laboratório de Simulídeos e Oncocercose, Instituto Oswaldo Cruz [2]Laboratório de Tecnologia Diagnóstica, Bio-Manguinhos-Fiocruz, Rio de Janeiro, RJ, Brasil [4]Agência de Vigilância em Saúde, Secretaria de Estado da Saúde, Porto Velho, RO, Brasil [5]Faculdade São Lucas, Porto Velho, RO, Brasil [6]Odebrecht Energia/Usina Hidrelétrica Santo Antônio, Porto Velho, RO, Brasil

Haematological and cytokine alterations in malaria are a broad and controversial subject in the literature. However, few studies have simultaneously evaluated various cytokines in a single patient group during the acute and convalescent phases of infection. The aim of this study was to sequentially characterise alterations in haematological patters and circulating plasma cytokine and chemokine levels in patients infected with Plasmodium vivax *or* Plasmodium falciparum *from a Brazilian endemic area during the acute and convalescent phases of infection. During the acute phase, thrombocytopaenia, eosinopaenia, lymphopaenia and an increased number of band cells were observed in the majority of the patients. During the convalescent phase, the haematologic parameters returned to normal. During the acute phase,* P. vivax *and* P. falciparum *patients had significantly higher interleukin (IL)-6, IL-8, IL-17, interferon-γ, tumour necrosis factor (TNF)-α, macrophage inflammatory protein-1β and granulocyte-colony stimulating factor levels than controls and maintained high levels during the convalescent phase. IL-10 was detected at high concentrations during the acute phase, but returned to normal levels during the convalescent phase. Plasma IL-10 concentration was positively correlated with parasitaemia in* P. vivax *and* P. falciparum*-infected patients. The same was true for the TNF-α concentration in* P. falciparum*-infected patients. Finally, the haematological and cytokine profiles were similar between uncomplicated* P. falciparum *and* P. vivax *infections.*

Key words: cytokines - chemokines - platelets - *P. falciparum* - *P. vivax*

Malaria remains a major health problem worldwide, with 300-500 million cases annually and nearly one million deaths (Murray et al. 2012). Although *Plasmodium falciparum* malaria represents the majority of these cases and is responsible for almost all of the associated mortality, *Plasmodium vivax* malaria has a wider geographic distribution and is responsible for high morbidity worldwide. Despite this widespread prevalence, *P. vivax* has long been overshadowed by the burden caused by *P. falciparum*. In Brazil, *P. vivax* accounts for more than 70% of all malaria cases. The infections are chronic, can produce profound anaemia, can be incapacitating for days or weeks and have the added complication of recurrent clinical episodes due to the developmental reactivation of hypnozoites, the dormant liver stage form (Mendis et al. 2001, Sina 2002).

It is well documented that disease severity depends strongly on the previous immunological experience of the host (Schofield & Mueller 2006). Therefore, in areas of high malaria transmission, infants and young children are more frequently affected, whereas in malaria-endemic areas, where transmission is unstable, adults are the most commonly affected population (Schofield & Mueller 2006). Although sterile immunity is most likely never achieved, individuals can develop essentially complete protection from severe illness and death after continuous exposure (Langhorne et al. 2008). Clinical malaria infection causes a range of symptoms from asymptomatic infection to severe disease complication. Although different theories have been proposed to explain the disease in humans, malaria pathogenesis remains controversial (Miller et al. 2002, Weatherall et al. 2002).

Although there is an extensive body of literature describing variations in haematological parameters and immune cytokine responses during malaria infection, their link to disease manifestation is still a subject of much debate. The subject of haematological changes is controversial in the malaria field, although anaemia and thrombocytopaenia are the most prominent alterations during both *P. falciparum* and *P. vivax* infections (Agarwal et al. 1983, Lacerda et al. 2011). Severe malaria has long been associated with high circulating levels of inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6. Studies have demonstrated a link between TNF-α, IL-6, IL-10 and the severity of the disease in human malaria (Akanmori et

doi: 10.1590/0074-0276140275
Financial support: DECIT, CNPq
+ Corresponding author: lila@ioc.fiocruz.br
Received 24 May 2013
Accepted 6 December 2013

online | memorias.ioc.fiocruz.br