UNIVERSIDADE FEDERAL DE UBERLÂNDIA
FACULDADE DE MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

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Porção variável de cadeia simples (scFv) anti-Glicosilfosfatidilinositol (GPI) de *Plasmodium falciparum* como alvo terapêutico para malária cerebral

Uberlândia
2024

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal de Uberlândia como requisito parcial para obtenção do título de mestre em Ciências Veterinárias.

Área de concentração: Saúde Animal

Orientador: Murilo Vieira da Silva

Uberlândia

2024

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

K64
2024

Klein, Sandra Gabriela, 1997-
Porção variável de cadeia simples (scFv) anti-glicosilfosfatidilinositol (GPI) de Plasmodium falciparum como alvo terapêutico para malária cerebral [recurso eletrônico] / Sandra Gabriela Klein. - 2024.

Orientador: Murilo Vieira da Silva.
Dissertação (Mestrado) - Universidade Federal de Uberlândia, Pós-graduação em Ciências Veterinárias.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.di.2024.46
Inclui bibliografia.

1. Veterinária. I. Silva, Murilo Vieira da,1988-, (Orient.). II. Universidade Federal de Uberlândia. Pós-graduação em Ciências Veterinárias. III. Título.

CDU: 619

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

Gizele Cristine Nunes do Couto - CRB6/2091
Nelson Marcos Ferreira - CRB6/3074

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	CIÊNCIAS VETERINÁRIAS				
Defesa de:	DISSERTAÇÃO DE MESTRADO ACADÊMICO PPGCVET Nº 01/2024				
Data:	06 DE FEVEREIRO DE 2024	Hora de início:	14:00	Hora de encerramento:	16:30
Matrícula do Discente:	12312MEV026				
Nome do Discente:	SANDRA GABRIELA KLEIN				
Título do Trabalho:	Porção variável de cadeia simples (scFv) anti glicosilfosfatidilinositol (GPI) de *Plasmodium falciparum* como alvo terapêutico para malária cerebral				
Área de concentração:	SAÚDE ANIMAL				
Linha de pesquisa:	INVESTIGAÇÃO ETIOLÓGICA				
Projeto de Pesquisa de vinculação:	PREVENÇÃO E CONTROLE DE MICROORGANISMOS IMPORTANTES PARA SAÚDE ANIMAL E HUMANA UTILIZANDO MÉTODOS BASEADOS EM TERANÓSTICA NANOBIOTECNOLOGIA E PELA SELEÇÃO E CARACTERIZAÇÃO DE ADITIVOS ALIMENTARES				

Reuniu-se na Sala de Reuniões da PROPP (bloco 8H), Campus Santa Mônica, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências Veterinárias, assim composta: Professores Doutores: Alessandra Aparecida Medeiros (FAMEV/UFU); Flávia Batista Ferreira (PPGBC/UFU/Suplente), Murilo Vieira da Silva (FAMEV/UFU) orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Murilo Vieira da Silva, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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Aprovado(a).

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Murilo Vieira da Silva**, **Médico(a) Veterinário(a)**, em 06/02/2024, às 16:36, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Alessandra Aparecida Medeiros Ronchi**, **Professor(a) do Magistério Superior**, em 06/02/2024, às 16:39, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Flávia Batista Ferreira França**, **Usuário Externo**, em 07/02/2024, às 11:35, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Referência: Processo nº 23117.084682/2023-98 SEI nº 5008392

AGRADECIMENTOS

Agradeço primeiramente aos animais de pesquisa que doaram a vida para que esses experimentos fossem possíveis.

Agradeço aos meus pais, Darci e Clair e ao meu irmão Eduardo que sempre me apoiaram e ajudaram na realização dos meus sonhos, especialmente na minha caminhada acadêmica.

Ao meu companheiro Igor, que me apoia, me ouve e me acolhe sempre que preciso de um ombro ou um desabafo. Além disso, sempre faz graça para tornar tudo mais leve.

Agradeço ao meu orientador e amigo Murilo, pelas oportunidades, ensinamentos, desafios e confiança desde o meu primeiro estágio no biotério até os dias de hoje.

Aos meus colegas e amigos do Laboratório de Biotecnologias em Modelos Experimentais (LABME), pós-doutorandas Flávia e Isabela, ao meu colega de mestrado Ray, a todos os alunos de iniciação científica, em especial ao Matheus e a Ludmilla que me ajudaram nesse trabalho e também à Alessandra que está sempre disponível para tudo que preciso, agradeço pela amizade, parceria, risadas e discussões.

Às demais envolvidas ao longo desses experimentos e que foram essenciais para que cada parte se tornasse possível, deixo meus sinceros agradecimentos à Mylla, Bruna, Kellen e Fabiana.

Ao corpo técnico e demais funcionários da Rede de Biotérios de Roedores (REBIR) pelas conversas, cafés e principalmente pelo trabalho que prestam, sempre em prol da qualidade e bem-estar dos nossos animais.

Agradeço à professora Wânia, por tudo que me ensinou sobre malária, pela paciência, dedicação e auxílios prestados durante meus experimentos e agora também por aceitar participar da minha banca.

À professora Alessandra por todos os ensinamentos durante a graduação e pós-graduação e por aceitar contribuir com este trabalho fazendo parte da minha banca.

Aos órgãos de fomento, Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Ministério Público do Trabalho (MPT) e Rede Mineira de Biotecnologia em Modelos Experimentais (RMBME) pelos recursos financeiros necessários para a realização dos experimentos e pela bolsa concedida.

À todos que de alguma forma contribuíram para que este trabalho fosse possível, meus sinceros agradecimentos!

“Quando vier uma onda muito grande,
mergulhe fundo.”
(DESCONHECIDO)

RESUMO

A malária é uma doença infecciosa causada por protozoários do gênero *Plasmodium* e é responsável pela morte de milhares de pessoas todos os anos. Sua complicação para o quadro cerebral afeta principalmente crianças e provoca a maioria dos óbitos e sequelas da enfermidade. Embora existam protocolos de tratamento que visam principalmente inibir a replicação do parasito, não há tratamentos eficazes para os quadros neurológicos, sendo necessário estudos de novas drogas. Com base nisso, visamos nesse estudo determinar o potencial efeito terapêutico de um fragmento variável de cadeia simples (scFv) anti-Glicosilfosfatidilinusitol (GPI) de *Plasmodium* em ensaio pré-clínico. Utilizamos camundongos da linhagem C57BL/6J infectados com *Plasmodium berghei*-ANKA (PbA) e tratados com scFv anti-GPI ou com solução salina fosfato (PBS) nos dias 0, 3 e 6 pós infecção, incluímos os controles não infectados tratados nos mesmos dias com scFv ou PBS. Os animais foram avaliados quanto a morbidade e mortalidade, peso corporal, parasitemia, hemograma, citocinas e histopatologia. Nossos resultados indicam que o tratamento com scFv anti-GPI em animais infectados com PbA resgatou 71,4% dos animais, promoveu a recuperação da perda de peso inicial provocada pela infecção, inibiu sinais neurológicos da malária cerebral (MC), bem como sinais sistêmicos da doença, reduziu a parasitemia, melhorou os parâmetros sanguíneos, mobilizou citocinas pró-inflamatórias e diminuiu hemorragias cerebrais dos animais, mostrando importante efeito terapêutico na patologia da malária cerebral experimental (MCE).

Palavras-chave: nanobiotecnologia; anticorpo; *Plasmodium berghei*; tratamento; modelo murino.

ABSTRACT

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium* and is responsible for the deaths of thousands of people every year. Its complication to cerebral involvement primarily affects children and leads to the majority of deaths and sequelae associated with the disease. While there are treatment protocols primarily targeting the replication of the parasite, effective treatments for neurological manifestations are lacking, necessitating the exploration of new drug studies. In this study, our aim was to determine the potential therapeutic effect of a single-chain variable fragment (scFv) anti-Glycosylphosphatidylinositol (GPI) of *Plasmodium* in a preclinical assay. We utilized C57BL/6J mice infected with *Plasmodium berghei*-ANKA (PbA) and treated them with scFv anti-GPI or phosphate-buffered saline (PBS) on days 0, 3, and 6 post-infection. Uninfected controls were treated on the same days with scFv or PBS. Animals were assessed for morbidity and mortality, body weight, parasitemia, complete blood count, cytokines and histopathology. Our results indicate that treatment with scFv anti-GPI in PbA infected animals rescued 71.4% of the animals, promoted recovery from the initial weight loss caused by the infection, inhibited neurological signs of cerebral malaria (CM), as well as systemic disease signs, reduced parasitemia, improved blood parameters, induced pro-inflammatory cytokines, and decreased cerebral hemorrhages, demonstrating a significant therapeutic effect in experimental cerebral malaria pathology (ECM).

Keywords: nanobiotechnology; antibody; *Plasmodium berghei*; treatment; murine model.

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CAPÍTULO 1

1 INTRODUÇÃO

A malária é uma doença causada por protozoários do gênero *Plasmodium* e transmitida pelo mosquito *Anopheles*. Sua distribuição ocorre principalmente em países de clima tropical e subtropical, sendo regiões da África subsaariana, da Ásia, América Central e América do Sul as mais afetadas. Embora haja cinco espécies no gênero *Plasmodium* capazes de afetar humanos, *P. falciparum* e *P. vivax* destacam-se epidemiologicamente, sendo *P. falciparum* predominante na África e Ásia, enquanto *P. vivax* é mais comum nas Américas (AMIR et al., 2018; WHO, 2023a).

Somente em 2022, foram registrados no mundo 249 milhões de casos e 608 mil mortes decorrentes da malária, onde a região africana é responsável por 94% dos casos e 95% dos óbitos. Os grupos mais vulneráveis são crianças menores de 5 anos, gestantes e pessoas imunossuprimidas, com sintomas que variam de leves a potencialmente fatais. Os sintomas leves incluem febre, dor de cabeça e calafrios, enquanto os graves englobam fadiga, lesões neurológicas e dificuldade respiratória (WHO, 2023a).

Os tratamentos atuais para a malária se fundamentam em antiparasitários, como cloroquina, artemisinina e artesunato, enquanto o tratamento de suporte é adaptado às necessidades do paciente. Contudo, esses tratamentos têm limitações, especialmente no contexto da malária grave, uma vez que seus mecanismos de ação estão associados ao parasitismo e não diretamente à condição de malária cerebral. A crescente resistência aos antiparasitários, notadamente à artemisinina e terapias combinadas, é uma preocupação, sobretudo no sudeste asiático, com o *P. falciparum* mostrando resistência em diversas regiões da África, Ásia e América do Sul, e o *P. vivax* apresentando resistência à cloroquina em lugares como Papua Nova Guiné e Indonésia (RASMUSSEN, ALONSO e RINGWALD, 2021; WWARN, 2023; WHO, 2023b).

Diante desses desafios, a busca por novas drogas e alvos terapêuticos se tornam necessários. Dessa forma, nosso grupo concentra-se na investigação do glicosilfosfatidilinositol (GPI), uma molécula reconhecida como padrão molecular associado a patógenos (PAMP), que desencadeia a produção de citocinas pró-inflamatórias cruciais na patogênese da malária cerebral (GOWDA e WU, 2018). Embora células eucarióticas em geral apresentem GPI em sua superfície, há uma diferença estrutural entre o GPI de *P. falciparum* e o GPI humano. Essa diferença possibilita o uso do GPI do parasito como um alvo promissor no desenvolvimento de

novos medicamentos (GOWDA, 2007; MBENGUE et al., 2016). Diante disso, nossa hipótese é que uma porção de anticorpo anti-GPI de *Plasmodium* possa ser propícia contra o agravamento da malária, fazendo do nosso objetivo testar essa molécula na malária cerebral murina induzida experimentalmente.

2 REVISÃO BIBLIOGRÁFICA

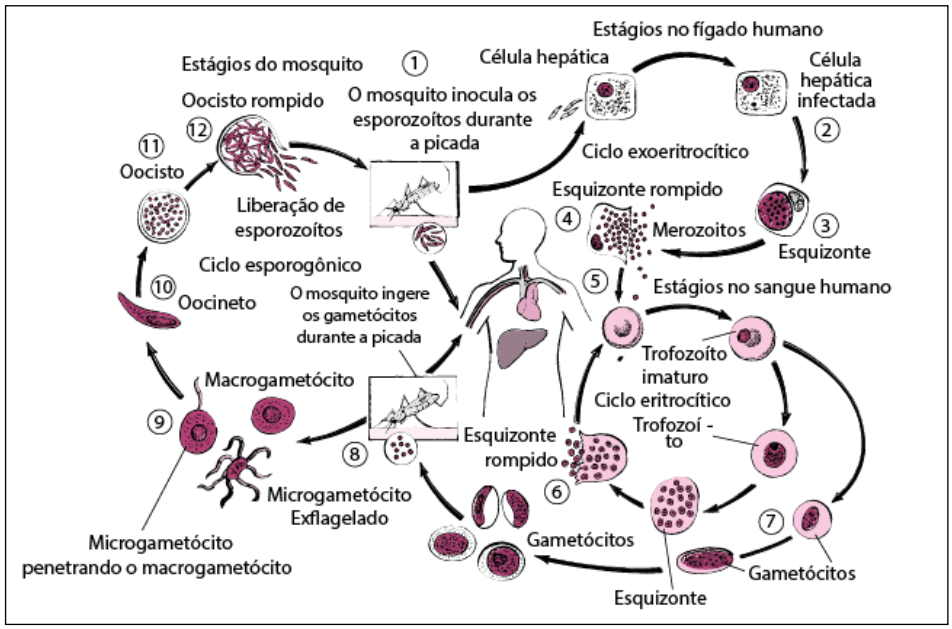
2.1. Ciclo de vida do *Plasmodium* spp.

O ciclo de vida é igual para todas as espécies de *Plasmodium* e se inicia quando a fêmea do mosquito *Anopheles* spp. se alimenta no hospedeiro intermediário, o ser humano, durante sua fase de repasto sanguíneo, transmitindo esporozoítos (Figura 1). Os esporozoítos entram na corrente circulatória e linfática, alcançando posteriormente o fígado, onde esporozoítos invadem hepatócitos transformando-se em esquizontes, se reproduzindo assexuadamente até que as células se rompam, liberando merozoítos. Essa fase inicial no fígado é conhecida como ciclo exoeritrocítico (BALAJI et al., 2020; PEARSON, 2019).

O ciclo eritrocítico tem início quando os merozoítos liberados entram na corrente sanguínea e invadem os glóbulos vermelhos. Neles, os merozoítos se desenvolvem primeiro em trofozoítos imaturos (na forma de anel) e, em seguida, em trofozoítos maduros. Os trofozoítos podem se diferenciar em esquizontes, que rompem os glóbulos vermelhos, liberando novos merozoítos, ou em gametócitos masculinos (microgametócitos) e femininos (macrogametócitos). Esses gametócitos podem ser ingeridos por um novo mosquito, dando início ao ciclo esporogônico (BALAJI et al., 2020; PEARSON, 2019).

Dentro do estômago do vetor, os gametas masculinos e femininos se fundem, formando zigotos, que se transformam em oocinetos alongados e móveis. Esses oocinetos penetram no tecido intestinal e se diferenciam em oocistos. Os oocistos se desenvolvem, liberam esporozoítos, que migram para as glândulas salivares do inseto. Durante um novo repasto sanguíneo, os esporozoítos entram no hospedeiro intermediário, iniciando assim um novo ciclo (BALAJI et al., 2020; PEARSON, 2019).

Figura 1 - Ciclo de vida do *Plasmodium* spp.



Fonte: Manual MSD, 2019.

2.2. Patogenia e síndromes associadas à malária

Na malária não complicada, os sintomas geralmente surgem após a primeira ruptura dos hepatócitos pelos esquizontes, liberando merozoítos na circulação periférica e desencadeando uma resposta imune. Esta resposta é principalmente mediada por citocinas pró-inflamatórias como Fator de Necrose Tumoral α (TNF-α), Interferon γ (IFN-γ) resultando em febre, dor de cabeça, dor muscular, vômitos e letargia e anti-inflamatória como Interleucina 10 (IL-10). Os sintomas têm duração de 6 a 12 horas e se repetem em ciclos aproximados de 48 horas, correlacionados com a liberação de novos merozoítos, variando de acordo com a espécie de *Plasmodium* envolvida na infecção (GUN et al., 2014; OAKLEY et al., 2011). Em contrapartida, a malária grave (ou complicada) é caracterizada por três síndromes sobrepostas: malária cerebral (CM), anemia grave e acidose metabólica. Essas síndromes resultam, entre outros fatores, do sequestro de hemácias parasitadas na microvasculatura, ativação endotelial, respostas inflamatórias sistêmicas e comprometimento funcional de órgãos como pulmões, rins, fígado, placenta e cérebro (WHITE et al., 2014).

2.3. Eficácia e resistência a drogas antimaláricas

Os tratamentos atuais preconizados para a malária são à base de antiparasitários. Para as formas sanguíneas utiliza-se a cloroquina em associação com a primaquina nos casos de *P. vivax ou P. ovale* e somente cloroquina para *P. malariae*. Quando se trata de *P. falciparum* a recomendação é associar artemisinina + artesunato + mefloquina + primaquina, a fim de atingir todas as formas do parasito (CDC, 2023). Nos casos de malária grave, a recomendação é que o tratamento seja feito com artesunato, o qual elimina os parasitas mais rapidamente que os demais antimaláricos e tem efeito contra as fases sexuada e assexuada do parasito (MMV, 2023). O tratamento de suporte, em relação à anemia, desidratação ou outras complicações deve ser feito de acordo com as necessidades do paciente.

As drogas antimaláricas, como cloroquina e primaquina, apresentam mecanismos de ação distintos. A cloroquina, seja na forma de fosfato ou hidroxicloroquina, atua inibindo o crescimento do *Plasmodium spp*. ao aumentar o pH nas vesículas ácidas, impedindo assim sua replicação (PINHEIRO et al., 2018). Por sua vez, a primaquina é eficaz na eliminação de hipnozoítos latentes no fígado do paciente, reduzindo o risco de recaídas (KARUNAJEEWA e JAMES, 1972). Já a mefloquina, estruturalmente relacionada ao quinino, exerce seu efeito como esquizonticida sanguíneo, embora seu mecanismo de ação ainda seja pouco compreendido (AHMAD, 2021). Enquanto isso, a artemisinina e seus derivados, como o artesunato, agem decompondo a ponte de endoperóxido mediada por heme, produzindo radicais livres que danificam alvos intracelulares específicos (MESHNICK, 2002).

No entanto, a eficácia dessas drogas é comprometida pela emergência de resistência. A artemisinina, que representa o maior avanço na terapêutica antimalárica nos últimos 20 anos, tem mostrado significativa resistência no sudeste asiático, assim como as terapias combinadas de artemisinina (ACTs). O *P. falciparum* possui resistência a todos os antimaláricos atualmente utilizados em regiões da África, Ásia e América do Sul (WWARN; VAN DER PLUIJM, 2020). Enquanto o *P. vivax* demonstrou elevada resistência à cloroquina na Papua Nova Guiné e Indonésia (PRICE et al., 2014).

A resistência à cloroquina surge devido a mutações no transportador de resistência à cloroquina de *P. falciparum* (PfCRT), localizado na membrana do vacúolo digestivo. Essas mutações impedem o acúmulo da droga, evitando sua ligação ao heme e, consequentemente, a inibição do processo de desintoxicação (WICHT et al., 2021; REED et al., 2000). Já a resistência à primaquina carece de uma definição clara, mas fatores como doses inadequadas e reinfecção contribuem para falhas no tratamento (THOMAS et al., 2016). A mefloquina

enfrenta resistência relacionada ao aumento do número de cópias do gene multirresistente 1 (pfmdr1) (PREECHAPORNKUL et al., 2009), enquanto para a artemisinina e seus derivados, as mutações no gene K13 são associadas à resistência, alterando padrões de ubiquitinação e permitindo aos parasitas resistirem ao acúmulo de proteínas poliubiquinadas (TALMAN et al., 2019).

2.4. Vacinas

Quanto à vacinação a RTS,S/AS01 (RTS,S) é a primeira vacina comprovadamente eficaz na redução significativa da malária causada por *P. falciparum*. Em 2019 foi iniciado um programa piloto coordenado pela OMS onde a vacina foi introduzida em Gana, Quénia e Malawi, alcançando mais de 1,3 milhões de crianças até março de 2023. Em outubro de 2021 a OMS recomendou sua utilização para crianças em regiões afetadas pela malária. Em outubro de 2023 a OMS atualizou a recomendação de vacinas, acrescentando a R21/Matrix-M (R21).

A vacina RTS,S consiste na formulação de uma proteína recombinante pré-eritrocítica monovalente. Ela consiste em fragmentos específicos da proteína circunsporozoíta (CSP) do *P. falciparum*, reconhecidos por sua capacidade de induzir respostas imunes humorais (região R) e celulares (região T). Estes fragmentos estão covalentemente ligados à superfície do vírus da hepatite B, atuando como antígeno (S) (LAURENS, 2020; SYED, 2022). A vacina R21 é semelhante à RTS,S, porém sem excesso de HBsAg (Antígeno de superfície da hepatite B) e, além disso, fornece maior densidade de epítopos da proteína circunsporozoíta na superfície da partícula (DATOO, 2022).

Ambas as vacinas ainda não passaram por um ensaio direto, mas até então sabe-se da eficácia de aproximadamente 75% na prevenção de episódios de malária em regiões endêmicas tanto para a RTS quanto para a R21 (WHO, 2024). Portanto, são consideradas bastantes promissoras no combate à malária, representando um marco importante no combate à doença. No entanto o surgimento dessas vacinas não dispensa a necessidade de tratamentos nos casos de pacientes incapazes de receber a imunização ou em que a vacina confira menor proteção (BEESON et al., 2022).

2.5. Estratégia técnica global para malária 2016-2030

O programa global de combate à malária até 2030, liderado pela Organização Mundial da Saúde (OMS) e parceiros internacionais, tem como objetivo principal reduzir a incidência e mortalidade causadas pela malária. A estratégia envolve a implementação de intervenções eficazes, como vacinação, diagnósticos precoces, tratamento adequado e controle vetorial. Além disso, busca fortalecer os sistemas de saúde, melhorar o acesso a serviços de qualidade e impulsionar a pesquisa e inovação para desenvolver novas ferramentas. Colaborando com governos, organizações não governamentais e a comunidade internacional, o programa visa reduzir significativamente a malária até 2030, aliviando o impacto principalmente nas regiões tropicais e subtropicais, que são as mais afetadas. O programa tem uma abordagem abrangente, buscando não apenas controlar a transmissão do parasita, mas também criar condições para a erradicação, contribuindo para melhorar a saúde global e alcançar os Objetivos de Desenvolvimento Sustentável relacionados à saúde (WHO, 2021; MMV, 2023).

2.6. Modelos animais no estudo da malária

Apesar dos avanços com diferentes tratamentos e o desenvolvimento de novas vacinas, a luta contra a malária demanda esforços contínuos. A introdução de novos medicamentos, embora promissora, enfrenta o desafio da resistência aos fármacos convencionais. Além disso, embora a vacinação seja uma estratégia positiva, sua eficácia pode ser limitada, e a distribuição abrangente pode ser restrita em estágios iniciais (WWARN; VAN DER PLUIJM, 2020). Diante desses desafios, torna-se imperativo realizar estudos aprofundados para a criação de novos medicamentos e a obtenção de conhecimentos mais abrangentes sobre os mecanismos envolvidos na patogênese da malária.

Visto isso, os modelos animais têm sido grandes aliados nos estudos da malária. Um modelo bem estabelecido para a malária cerebral é a utilização de camundongos C57BL/6 infectados com *Plasmodium berghei* – ANKA, o qual compartilha diversas similaridades com a malária cerebral humana, dentre elas o sequestro de eritrócitos infectados na barreira hematoencefálica, manguito perivascular, e sintomas neurológicos. As semelhanças entre a patogenicidade do *P. berghei* em camundongos e o *P. falciparum* em humanos permitiu a utilização desse parasita no estudo de diversos aspectos do seu desenvolvimento, interações parasita-hospedeiro, avaliação da eficácia de medicamentos e pesquisas sobre vacinas, aspectos

estes que, de outra forma, seriam inacessíveis com *P. falciparum* in vitro. Adicionalmente, os modelos murinos proporcionam inúmeras vantagens, incluindo a facilidade de manuseio, a tratabilidade experimental de todos os estágios do ciclo de vida em condições laboratoriais e a disponibilidade de uma ampla gama de sistemas de manipulação genética (CRAIG et al., 2012; DE NIZ e HEUSSLER, 2018; SIMWELA e WATERS, 2022).

Além disso, tais modelos animais proporcionam investigações que seriam dificilmente controláveis em seres humanos, como é o caso das coinfecções. Alguns estudos têm demonstrado importantes dados acerca da coinfecção entre diferentes cepas de *Plasmodium* com patógenos como *Tripanossoma*, helmintos, *Babesia*, entre outros que representam importantes marcos no estudo da doença (ADEMOLA E ODENIRAM, 2016; KNOWLES, 2011, ZAFAR et al., 2023).

2.7. Glicosilfosfatidilinositol (GPI)

O glicosilfosfatidilinositol (GPI) é um complexo glicolipídico que ancora diversas proteínas e glicoproteínas à membrana celular por meio de sua porção C terminal (KINOSHITA et al., 2008). As âncoras GPI são encontradas em todos os eucariotos, estando presentes em gêneros como *Plasmodium*, *Toxoplasma*, *Trypanosoma* e *Leishmania*, além de leveduras e mamíferos (KINOSHITA, 2016; MÜLLER e MÜLLER, 2023; DELORENZI et al., 2002; ORLEAN e MENON, 2007). Apesar de sua presença onipresente, as GPIs são encontradas em concentrações extremamente baixas nas membranas plasmáticas da maioria dos organismos, exceto nos parasitas protozoários, onde são abundantemente expressas (KINOSHITA et al., 2008).

Parasitos como o *P. falciparum*, embora compartilhem semelhanças nas GPIs, apresentam diferenças sutis nos grupamentos acil da porção lipídica (SAUER et al., 2023). Estudos indicam que as GPIs são tão pro inflamatórias quanto lipopolissacarídeos bacterianos e lipopeptídeos de *Mycoplasma*, destacando sua importância como moléculas pró-inflamatórias microbianas (ALMEIDA et al., 2000). Apesar de não serem exclusivas aos protozoários, essas âncoras são mais frequentemente utilizadas por esses organismos, apresentando diferenças funcionais significativas em relação aos eucariontes superiores (KINOSHITA et al., 2008). Em parasitos intracelulares, as GPIs desempenham papéis cruciais na invasão celular e evasão da resposta imune do hospedeiro, contribuindo assim para a sobrevivência e infectividade do parasito (KINOSHITA, 2016).

Os GPIs derivados de *P. falciparum* desempenham um papel crucial como fatores de patogenicidade na malária, evidenciado pela capacidade dessas moléculas de induzir a produção de citocinas em macrófagos e células endoteliais, assim como causar sintomas de infecção aguda em animais experimentais (NAIK et al., 2000). Estudos demonstraram que as GPIs são efetivamente fatores de patogenicidade específicos em *Plasmodium falciparum*, sendo observada uma resposta IgG específica para essas moléculas em indivíduos que vivem em áreas endêmicas de malária. Embora adultos e crianças mais velhas apresentem níveis elevados de anticorpos anti-GPI, crianças suscetíveis exibem ausência ou níveis muito baixos desses anticorpos. Além disso, a ausência de anticorpos anti-GPI em pacientes com febre e anemia sugere que esses anticorpos desempenham um papel protetor contra as formas clínicas da malária (NAIK et al., 2000, SAUER et al., 2023). Portanto, a GPI emerge como um candidato significativo para o desenvolvimento de moléculas destinadas a alternativas terapêuticas contra a malária.

2.8.Single-chain variable fragment (scFv) como proposta terapêutica

A produção de moléculas recombinantes de anticorpos artificiais, frequentemente na forma de fragmentos polipeptídicos, que mimetizam as propriedades dos anticorpos naturais, tem emergido como uma abordagem terapêutica inovadora contra diversas doenças, como câncer, doenças virais e parasitárias, e têm mostrado vantagens sobre anticorpos monoclonais e convencionais (ZHOU et al., 2017; CHEN et al., 2017; WANG et al., 2018). Um exemplo notável dessas moléculas é o scFv, que consiste em domínios variáveis de cadeias leves e pesadas conectados por um peptídeo conector (linker), apresentando um peso molecular aproximado de 30 kDa (AHMAD et al., 2012).

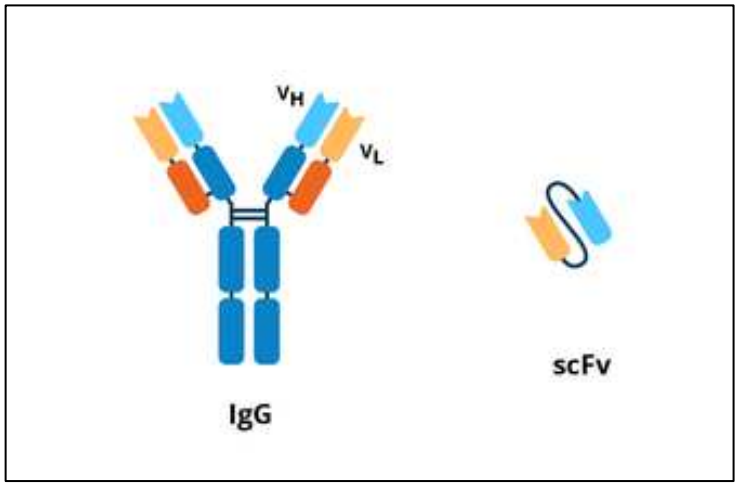
Esse tipo de engenharia foi viabilizada pela subdivisão dos anticorpos em subunidades funcionais, onde as subunidades podem ser artificialmente combinadas para originar novas moléculas, graças à estrutura em domínio dos anticorpos. Tornando esse avanço fundamental no tratamento de diversas doenças, especialmente no desenvolvimento de novas imunoglobulinas recombinantes. (AHMAD et al., 2012).

A capacidade de combinar artificialmente subunidades funcionais dos anticorpos, com base em sua estrutura de domínio, possibilita a criação de moléculas altamente específicas, direcionadas a alvos previamente determinados. Além disso, esses fragmentos de anticorpo oferecem diversas vantagens em relação ao uso de moléculas de anticorpo inteiras: (1) sua

menor dimensão permite uma distribuição mais rápida e uniforme nos tecidos, comparada a moléculas maiores; (2) apresentam baixa retenção em tecidos não alvo; (3) possuem custo comercial reduzido quando produzidos em larga escala; (4) podem ser conjugados a fármacos e radionucleotídeos, visando minimizar a exposição aos tecidos saudáveis (AHMAD et al., 2012; WANG, et al., 2018).

Assim, as âncoras GPI estão envolvidas em muitos aspectos da interação parasito-hospedeiro, além de serem capazes de induzir reposta inflamatória. Neste contexto, GPI se torna importante candidata a alvo para a seleção de clones de scFv, os quais poderão ser utilizados como alternativas terapêuticas contra doenças.

Figura 2 – Comparação entre um anticorpo e o scFv



Fonte: Addgene, 2021.

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A single-chain variable fragment (scFv) as a treatment proposal for cerebral malaria in a murine model

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Abstract: Malaria is an infectious disease caused by protozoa of the genus *Plasmodium* and is responsible for the deaths of thousands of people every year. Cerebral malaria is a serious form of the disease, mainly affecting children, pregnant women and immunosuppressed people and causing the majority of deaths and sequelae associated with the disease. While there are treatment protocols primarily targeting the replication of the parasite, effective treatments for neurological manifestations are lacking, necessitating the exploration of new drug studies. In this study, our aim was to determine the potential therapeutic effect of a single-chain variable fragment (scFv) anti-Glycosylphosphatidylinositol (GPI) of *Plasmodium* in a preclinical assay. We utilized C57BL/6J mice infected with *Plasmodium berghei*-ANKA (PbA) and treated them with scFv anti-GPI or phosphate-buffered saline (PBS) on days 0, 3, and 6 post-infection. Uninfected controls were treated on the same days with scFv or PBS. Animals were assessed for morbidity and mortality, body weight, parasitemia, complete blood count, cytokines and histopathology. Our results indicate that treatment with scFv anti-GPI in PbA infected animals rescued 71.4% of the animals, promoted recovery from the initial weight loss caused by the infection, inhibited neurological signs of cerebral malaria (CM), as well as systemic disease signs, reduced parasitemia, improved blood parameters, induced pro-inflammatory cytokines, and decreased cerebral hemorrhages, demonstrating a significant therapeutic effect in experimental cerebral malaria pathology (ECM).

Keywords: nanobiotechnology; antibody; *Plasmodium berghei*; treatment.

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

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1. Introduction

Malaria is a globally distributed and potentially fatal parasitic disease. According to estimates from the World Health Organization in 2022, there were 249 million cases and 608,000 deaths due to malaria, with the African region accounting for 94% of cases and 95% of deaths. The most vulnerable groups include children under 5 years old, pregnant women, and patients with HIV (Human Immunodeficiency Virus) or other immunosuppressive conditions [1].

Caused by protozoa of the *Plasmodium* genus and transmitted by the *Anopheles* mosquito, malaria has five species capable of causing diseases in humans. However, *P. falciparum*, *P. vivax*, and *P. malariae* are epidemiologically more significant, with *P. falciparum* predominant in Africa and Asia, and *P. vivax* in the Americas [1,2].

When infected, individuals may be asymptomatic or present symptoms ranging from mild to severe. Malaria is classified as uncomplicated, where patients exhibit mild to moderate symptoms such as headaches, muscle aches, fever, and lethargy, and

complicated, with severe symptoms including severe anemia, metabolic acidosis, and neurological symptoms [3,4].

Current malaria treatments rely on antiparasitics such as chloroquine, artemisinin, and artesunate, supplemented by supportive care based on patient needs. However, existing treatments have limitations, especially for severe malaria, as their mechanisms of action are related to parasitism rather than the condition of cerebral malaria. Resistance to antiparasitics, particularly artemisinin and combination therapies, is a growing concern, especially in Southeast Asia. *P. falciparum* has shown resistance to all antimalarials used in Africa, Asia, and South America, while *P. vivax* exhibits chloroquine resistance in Papua New Guinea and Indonesia [5, 6, 7].

In this context, new drugs and therapeutic targets are essential. Our group has explored glycosylphosphatidylinositol (GPI), a molecule recognized as a pathogen-associated molecular pattern (PAMP), triggering the production of fundamental pro-inflammatory cytokines in cerebral malaria pathogenesis. Despite eukaryotic cells generally displaying GPI on their surface, there is a structural difference between *P. falciparum* and human GPI, making the parasite's GPI a promising target for new drug development [8, 9]. Therefore, our hypothesis is that a *Plasmodium* anti-GPI antibody fragment may be promising against the worsening of malaria cases, making it our objective to test a selected anti-single chain variable fragment (scFv) against *P. falciparum* GPI in cerebral malaria. murine.

2. Materials and Methods

2.1 Ethics Statement

All studies involving mice were previously approved by the Ethics Committee on Animal Use at the Federal University of Uberlândia (CEUA/UFU), under protocol number 153/16. All animal handling and welfare procedures were conducted in accordance with the recommendations of the National Council for Animal Experimentation Control (CONCEA) through its normative resolutions and technical guidelines [10]. The animals were housed in the Central Animal Facility of the Rodent Bioterium of the Federal University of Uberlândia (REBIR-UFU), which is registered with CONCEA (CIAEP: 01.0105.2014) and the National Biosafety Technical Commission – CTNBio (CQB: 163/02).

2.2 Expression and Purification of scFv

The selection of GPI-binding scFvs was carried out using the Phage display technique, where an analysis of the specificity of the selected targets and their subsequent sequencing were carried out. With the isolated clones, scFv expression was increased and the supernatant containing scFv was subsequently purified using surface layer chromatography with the HisTrapTM HP column (GE Healthcare Life Sciences, Wau-kesha, WI, USA), following the manufacturer's instructions, on an HPLC system (AKTATM purifier, GE Healthcare Life Sciences).

After nickel immobilization on the column and the addition of binding buffer (50mM Imidazole, 0.5M NaCl, 20mM Na2HPO4, pH 7.4), the supernatant from the bacterial culture, previously obtained, was added. The scFv molecules, bound to nickel by the hexahistidine (His6) tail, were eluted through competition with elution buffer (500mM Imidazole, 0.5M NaCl, 20mM Na2HPO4, pH 7.4). The purified scFv was concentrated using a 3 kDa Amicon tube (Millipore, Billerica, MA, USA), and the protein concentration was determined by gel band quantification through electrophoresis and analysis using the ImageJ program. The treatment dose was determined to be 100 μg per animal.

2.3 Animals

For this study, we used female C57BL/6 wild-type (WT) mice aged between 6 and 8 weeks, of specific pathogen-free (SPF) status. The animals were housed in Alesco Ventilife

microisolators (Alesco, Monte Mor, SP, Brazil), with autoclaved pine shavings bedding changed weekly, filtered and autoclaved water provided ad libitum, and irradiated Nuvilab CR-1 feed (Quimtia, Colombo, PR, Brazil) also provided ad libitum. The environmental conditions were maintained at a room temperature of 22±2 °C, air humidity between 40-60%, and a 12h/12h light/dark photoperiod cycle.

2.4 Parasite Maintenance and Experimental Infection

For the maintenance of the P. berghei - ANKA parasite, BALB/c mice were used due to their greater resistance and subsequent onset of clinical signs, which are less severe compared to C57BL/6 mice [11]. An aliquot of blood infected with P. berghei - ANKA, kindly provided by Dr. Ricardo T. Gazzinelli, was thawed and immediately inoculated into BALB/c mice. After 10 days, monitoring the clinical condition and parasitemia of the animal, a blood sample was collected, and the process was repeated two more times, completing three passages before starting the experimental infection proper. These passages are allowed to stabilize the strain and maintain uniform characteristics of the parasite in all experiments [12].

On the day of experimental infection, approximately 200 μL of blood was collected from the maintenance animal, which had been previously anesthetized with 3% to 4% isoflurane and euthanized after collection. A blood smear was performed to determine the percentage of parasitized red blood cells. Subsequently, the blood was diluted in phosphate-buffered saline (PBS), and the number of red blood cells per ml was determined using a Neubauer Chamber. This allowed for the calculation of the dilution factor necessary to achieve $1x10^6$ parasitized red blood cells in each 200 μL of the solution (blood + PBS) to be inoculated into each animal.

2.5 Experimental In Vivo Design

The animals were distributed into experimental groups as follows:

- PbA + PBS: Animals infected with *Plasmodium berghei*-ANKA strain and treated with Phosphate-Buffered Saline (6 animals).
- PbA + scFv: Animals infected with *Plasmodium berghei*-ANKA strain and treated with the Single-Chain Variable Fragment against Glycosylphosphatidylinositol of *Plasmodium falciparum* (6 animals).
- PBS: Non-infected animals treated with Phosphate-Buffered Saline (3 animals).
- scFv: Non-infected animals treated with the Single-Chain Variable Fragment against Glycosylphosphatidylinositol of *Plasmodium falciparum* (3 animals).

On day 0 of the experiment, the animals received the first dose of treatment intraperitoneally, and after 4 hours, they were challenged with PbA by the same route. The second and third doses of treatment were administered on the 3rd and 6th days post-infection (dpi), respectively. Mice were evaluated for body weight daily, parasitemia (4th to 6th dpi), and morbidity score (4th to 6th dpi). Treatments, weighing, scores, and parasitemia assessments were always performed in the morning. On the 6th dpi, in the afternoon, the animals were anesthetized with 3.5 to 4% isoflurane for blood collection, subsequent euthanasia, and organ harvesting. Blood samples were used for hematological analyses. Collected organs included the brain, liver, and lungs, from which cytokine analyses (IL-10, IL-12, and IFN-γ) and histopathology analysis.

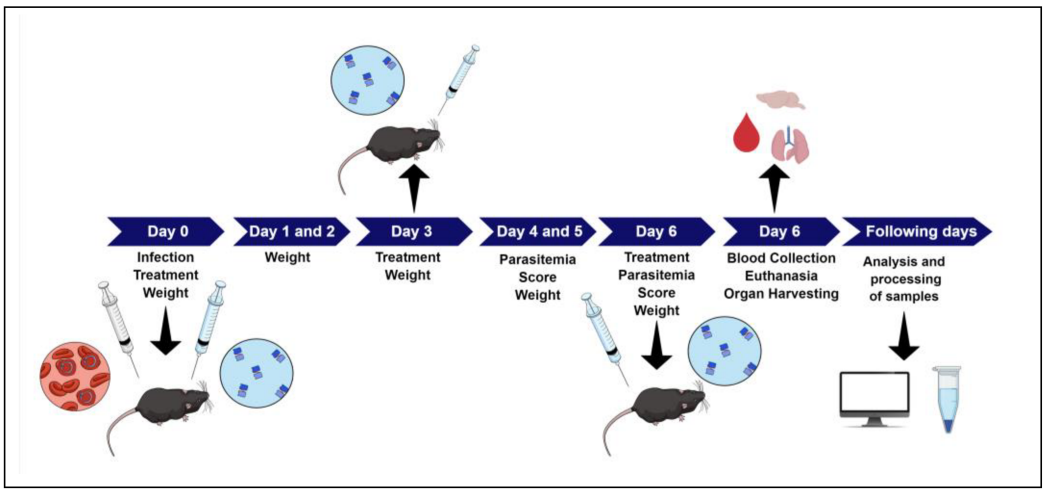


Figure 1. Representative illustration of the experimental design. On day 0 of the experiment, animals were challenged with *P. berghei*-ANKA and received the first dose of treatment; the second and third doses were administered on the 3rd and 6th days post-infection (dpi), respectively. Mice were assessed for body weight daily, parasitemia (4th to 6th dpi), and morbidity score (4th to 6th dpi). On the 6th dpi, animals were anesthetized for blood collection, subsequent euthanasia, and organ harvesting. Original image created with Mind the Graph.

2.6 *Survival Analysis*

A separate experiment was conducted to generate the survival curve, with the animal model, scFv processing, and parasite conditions following the same procedures described above. The groups included *P. berghei* ANKA + PBS (n:6) and *P. berghei* ANKA + scFv (n:7). Animals were monitored daily for 30 days to record any occurrences of mortality.

2.7 *Body Weight*

To assess whether infection and treatment could impact the animals' body weight, they were weighed from day 0 to day 6 post-infection (dpi), always in the morning.

2.8 *Morbidity*

Morbidity was assessed using the Rapid and Quantitative Scale of Murine Coma and Behavior (RMCBS), adapted from Carroll et al. (2010) [13].

This scale evaluates the clinical picture of cerebral malaria, consisting of 10 parameters, and was developed to assess manifestations of cerebral malaria in C57BL/6 mice infected with *P. berghei* - ANKA. The RMCBS allows us to monitor the progression of the clinical syndrome, validated here by correlations with intracerebral hemorrhages and edema that can cause different neurological alterations, depending on the affected brain region. It provides a tool by which animals can be identified as symptomatic or asymptomatic, as well as evaluated for the progression and/or regression of the condition during experimental treatment.

Each animal was evaluated for an average of 3 minutes. This assessment was conducted once a day on the 4th and 5th dpi and twice on the 6th dpi, once in the morning and once in the afternoon. The total score consists of 10 parameters, each of which is assigned a score ranging from 0 to 2, with 0 related to more severe symptoms, 1 to intermediate, and 2 to low or absent signs of morbidity. Thus, animals with higher scores demonstrate better clinical condition.

These parameters were compiled as a sum of scores, as well as evaluated separately.

2.9 Parasitemia

Each mouse had a drop of blood drawn through puncture of the lateral tail vein with a disposable 26G needle (13 x 0.45) and diluted in 200 uL of PBS; subsequently, the samples were analyzed using flow cytometry. The instrument used was the BD ACCURI C6, with laser excitation at 488nm. Initially, we identified erythrocytes by their size (FSC) and granularity (SSC) specificities and acquired a total of 100,000 events from each sample. Since the parasite used is genetically modified and contains green fluorescent protein (GFP) in its structure, we discriminated infected erythrocytes based on the percentage of GFP+ cells within the total erythrocyte gate.

2.10 Hematology

Blood for hematological analyses was collected via retro-orbital route using a glass capillary and a microtube with a capacity of 500 μL containing EDTA K2 anticoagulant (ethylenediaminetetraacetic acid dipotassium). The collected volume was approximately 100 μL per animal. Hematological analyses were performed using the Mindray BC-2800 vet equipment, where the following parameters were analyzed: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), in addition to the total white blood cell (WBC) analysis. With the total white blood cell data obtained from the equipment, a white blood cell differential count was performed through blood smears under optical microscopy.

2.11 Cytokines

The determination of cytokines was performed through Enzyme-Linked Immunosorbent Assay (ELISA). The concentrations of IFN-γ, IL-10, and IL-12p40 were measured in homogenates of brain, lung, spleen, and liver (100 mg of tissue) from each mouse. The quantification of cytokines was carried out using commercial ELISA kits, following the manufacturer's recommended protocols (BD Biosciences, San Diego, USA). High-affinity 96-well plates (Corning Laboratories Inc, New York, USA) were coated with specific anti-mouse capture antibodies for IFN-γ, IL-10, and IL-12p40 and incubated overnight at 4ºC. Subsequently, to block nonspecific sites, 10% fetal bovine serum in 0.01M PBS (pH 7.2) was added to the plate for 1 hour. After blocking, standard curves of the respective cytokines in serial dilutions and test samples were added to the plates and incubated for 2 hours. For quantifying the presence of cytokines in the samples, biotin-conjugated anti-mouse cytokine detection antibodies, supplemented with streptavidin-peroxidase, were incubated for 1 hour. Between each step, plates were washed with PBS-T 0.05% (PBS-T). The reaction was developed with tetramethylbenzidine (TMB), followed by the addition of 2M sulfuric acid to stop the reaction. Optical density (OD) was determined at 450 nm using a microplate reader (SpectraMax M2e, Molecular Devices, USA). Cytokine concentrations were determined from the standard curve with known cytokine concentrations, and the results were expressed in pg/mL, according to the detection limits for each assay: IFN-γ (4.1 pg/mL), IL-10 (4.5 pg/mL), and IL-12p40 (15.6 pg/mL).

2.12 Histology

Brain, lung, liver, and kidney samples were collected on the 6th day post-infection (dpi) immediately after euthanasia. The organs were fixed in 10% buffered formalin at room temperature for 24 hours, and then formalin was replaced with 70% alcohol until the paraffin embedding process. After embedding, the paraffinized organs were sectioned (5μm thick) and placed on microscopic slides, followed by deparaffinization and hydration for Hematoxylin and Eosin (H&E) staining. The slides were digitized using the Scanscope AT scanner (Leica Microsystems GmbH, Wetzlar, Germany).

2.13 Alveolar Septum Measurement

From the digitized histological slides, lung images were acquired at a 20x magnification and divided into 10 microscopic fields using the QuPath software. These fields were then analyzed for the thickness of the pulmonary septal area.

The septal thickness analysis was performed using ImageJ software. The quadrants separated in the previous process had their images converted to 8-bit quality. A threshold was applied, and subsequently, the total percentage of the "dark area," indicating the septal region, was calculated in comparison to the overall region.

2.14 Hemozoin Quantification

Quantitative analysis of hepatic hemozoin (Hz) was conducted by reading H&E-stained histological sections observed under polarized light microscopy. Image captures were performed using a 20x objective on the Nikon Eclipse Ti-5 microscope. Ten microscopic fields from each tissue section of each animal were analyzed using a polarized filter to identify the disorientation caused by Hz. Images were captured using the adapted polarized light filter and images using conventional light microscopy. Using ImageJ software, version 1.50i, each image was converted to 8 bits, transforming colored images into grayscale. The threshold was adjusted to highlight pixels covered by hemozoin in red. These areas were measured and expressed as a percentage of the total pixel area.

2.15 Statistical Analysis

Statistical methods were employed to analyze various parameters in this study. For hemogram data, a one-way ANOVA followed by Tukey's multiple comparison test was utilized to determine significant differences between groups. Body weight differences were assessed using the Student's t-test. Parasitemia levels were analyzed using two-way ANOVA, followed by Sidak's and Bonferroni's multiple comparison tests to identify variations between experimental conditions. Morbidity scores were evaluated using two-way ANOVA, followed by Tukey's multiple comparison test.Survival analysis was conducted using the Kaplan-Meier method, with the Log-rank test (Mantel-Cox) employed to compare survival curves between groups. Cytokine levels were subjected to two-way ANOVA, followed by Holm-Sidak's and Tukey's multiple comparison tests to identify significant differences For hemozoin quantification, the Kolmogorov-Smirnov test was initially applied to assess data distribution, followed by the unpaired t-test with Welch's correction to compare means between groups. Microhemorrhages in the brain were analyzed using one-way ANOVA, followed by the unpaired t-test. Furthermore, the area of septal tissue in the lungs was evaluated using the non-parametric Kruskal-Wallis test, followed by Dunns' multiple comparison tests to determine significant differences between experimental groups. The analyses were performed using GraphPad Prism Software, version 9.0, and data were considered statistically significant when $P<0.05$.

3. Results

3.1. The scFv increases survival and restores body weight in mice infected with P. berghei-ANKA

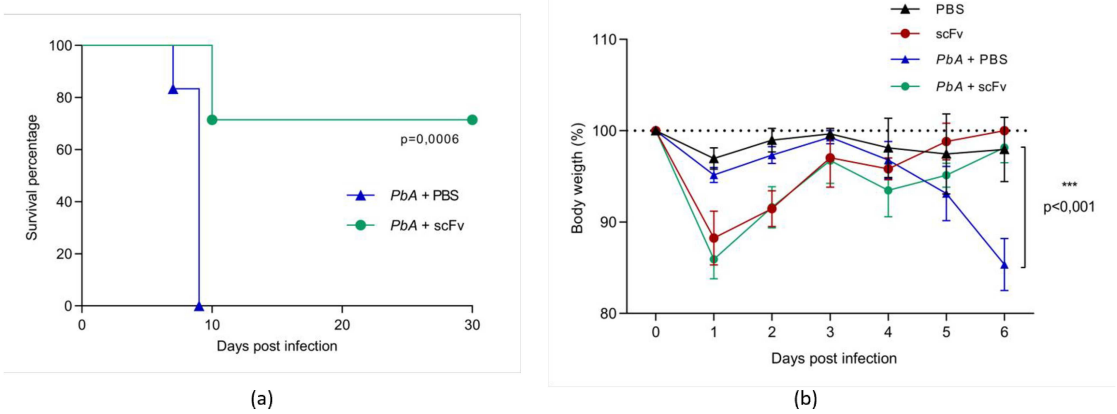


Figure 2. (a) Survival curve, demonstrating that scFv increases the survival of mice infected with Plasmodium berghei-ANKA (PbA). PbA + PBS group (n: 6), PbA + scFv group (n:7). (b) Percentage of body weight gain/loss in C57BL/6 mice infected/non-infected with PbA, treated/untreated with scFv. Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). (*** $p < 0.001$). For the survival curve, the Kaplan-Meier test followed by the Log-rank test (Mantel-Cox) was utilized, while for body weight, the Student's t-test was conducted.

During the survival monitoring (Figure 2a), it was observed that all infected animals subjected to treatment with PBS succumbed between the 7th and 9th day post-infection (dpi), displaying clinical signs of cerebral malaria. In contrast, unlike the previous group, infected animals treated with scFv remained alive and free of clinical manifestations of cerebral malaria until the 30th dpi, except for two animals that died on the 10th dpi.

Figure 2 (b) illustrates the body weight monitoring, where all animals showed an initial weight reduction. From the 3rd dpi, there was a progressive weight reduction in the PbA + PBS group until the 6th dpi. In contrast, infected animals subjected to scFv treatment exhibited body weight gain on days 2, 3, 5, and 6 post-infection.

3.2. The scFv progressively reduces the parasitic burden in mice infected with P. berghei-ANKA

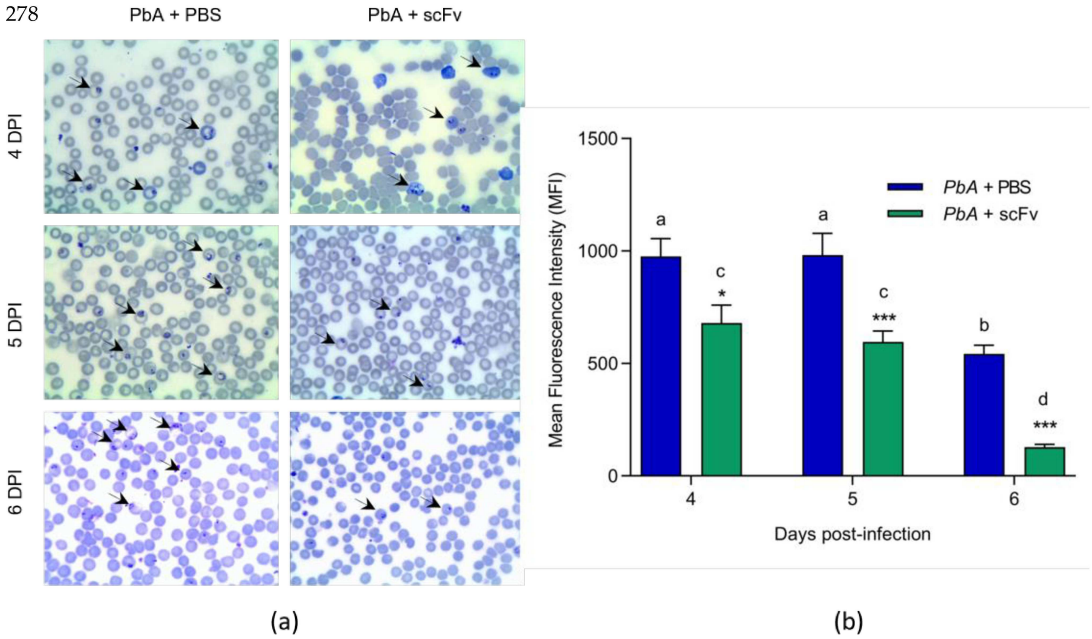
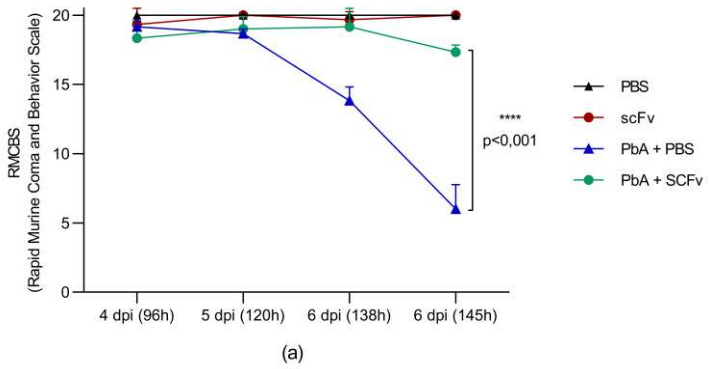


Figure 3. Parasitemia in scFv-treated and untreated anti-GPI mice infected with *Plasmodium falciparum*. In (a) illustrative image of optical microscopy showing the progression of parasitemia between the 4th and 6th dpi. In (b) parasitemia by flow cytometry, evaluated by the mean fluorescence intensity on days 4, 5, and 6 post-infection. In (b), letters indicate comparison between different days of the same group, equal letters indicate no statistically significant difference and different letters indicate statistically significant difference. Control groups (non-infected) were used to define the gate and exclude fluorescence emitted by erythrocytes. The parasite in question is GFP+, and the infecting dose was 10^6 infected erythrocytes/mouse. indicate parasitized erythrocytes. The graph presents mean and standard deviation. Groups with n of 6. (* $p < 0.05$; *** $p<0.001$). For parasitemia, a two-way ANOVA was conducted followed by Sidak and Bonferroni's multiple comparison tests.

Through flow cytometry, the mean fluorescence intensity was analyzed on the 4th, 5th, and 6th dpi (figure 3b). The PbA+PBS group maintained parasitemia on the 4th and 5th dpi and showed a reduction on the 6th dpi, as seen in Figure 3b. Mice in the PbA+scFv group exhibited significantly lower parasitemia compared to the control group on all three days of analysis, with the most pronounced difference on the 5th and 6th dpi. These results indicate that scFv plays a crucial role in reducing the parasitemia caused by PbA.

3.3. The treatment with scFv reduces clinical signs of cerebral malaria and improves the overall clinical condition of mice infected with P. berghei-ANKA



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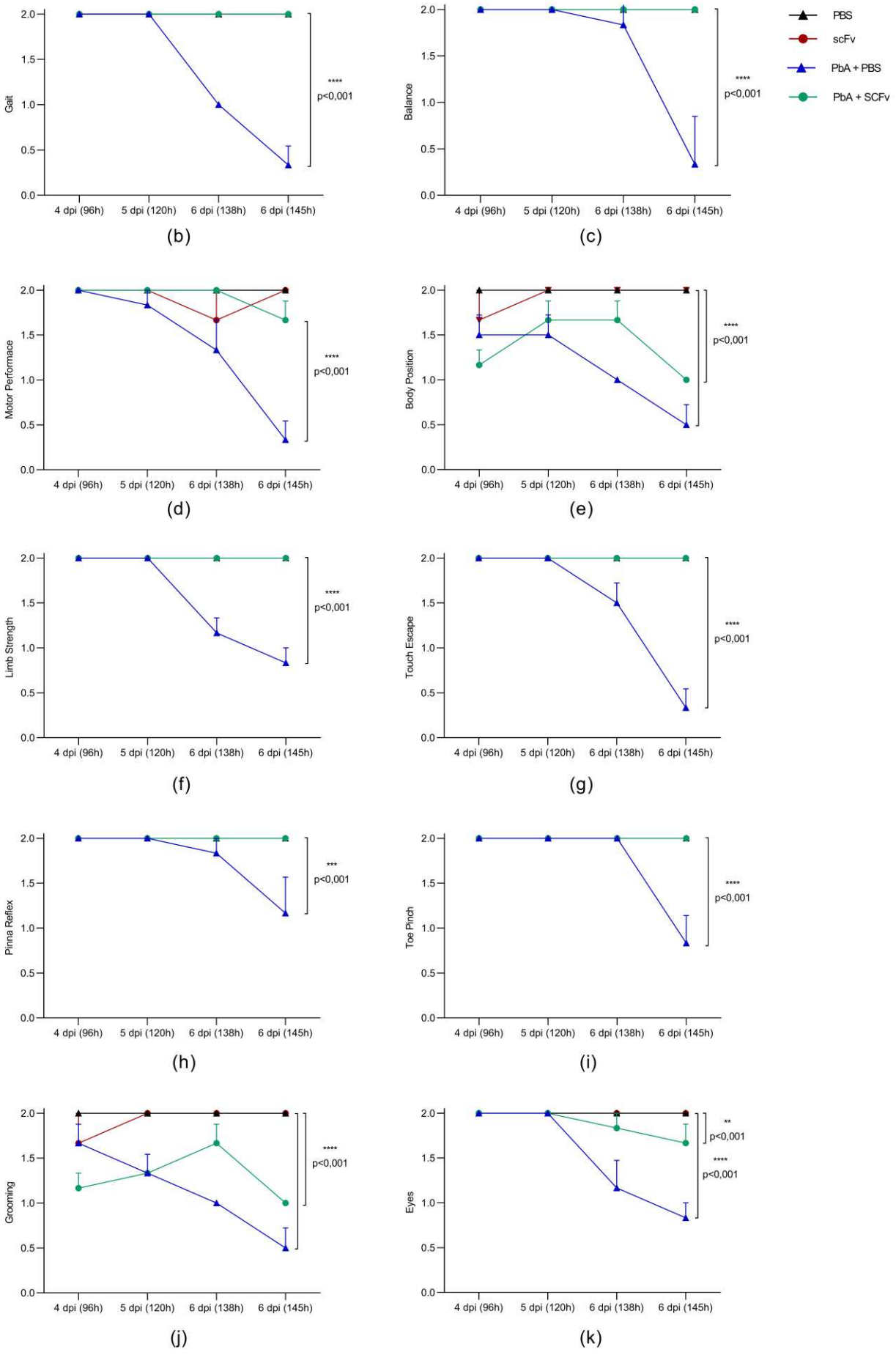
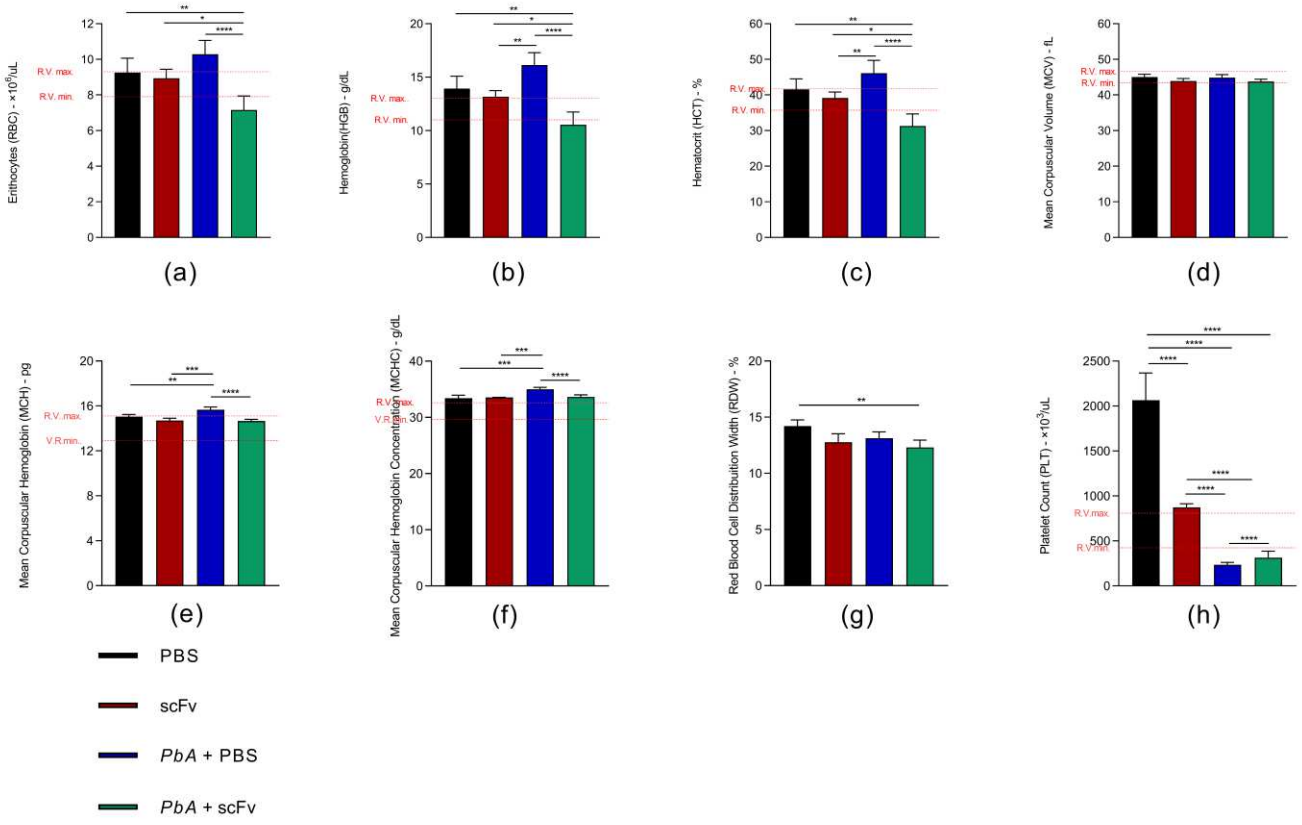

PBS
scFv
PbA + PBS
PbA + SCFv
Gait
Balance
Motor Performace
Body Position
Limb Strength
Touch Escape
Pinna Reflex
Toe Pinch
Grooming
Eyes
4 dpi (96h)
5 dpi (120h)
6 dpi (138h)
6 dpi (145h)
****
p<0,001
***
**
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)

Figure 4. Morbidity score (Rapid Murine Coma and Behavior Scale). The graph (a) presents the sum of scores, and the other graphs represent each of the analyzed parameters individually. Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). The graphs show mean and standard deviation (* $p < 0.05$; ** $p< 0.01$; *** $p<0.001$; **** $p<0.0001$). The statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test.

In the sum of all scores, the animals in the PbA + scFv group obtained scores equal to or higher than 17 points in all evaluations. On the other hand, animals in the PbA + PBS group had scores on the 4th and 5th dpi of 19.33 and 18.67, respectively. However, there was a reduction to 13.83 on the 6th dpi and to 6 points 5 minutes before euthanasia, also on the 6th dpi (figure 4a). In the evaluations of each score, the PbA + scFv group performed better than the PbA + PBS group, except for Grooming, where there was no statistical difference between the groups.

3.4. The scFv treatment improves thrombocytopenia and monocytopenia in mice infected with P. berghei-ANKA



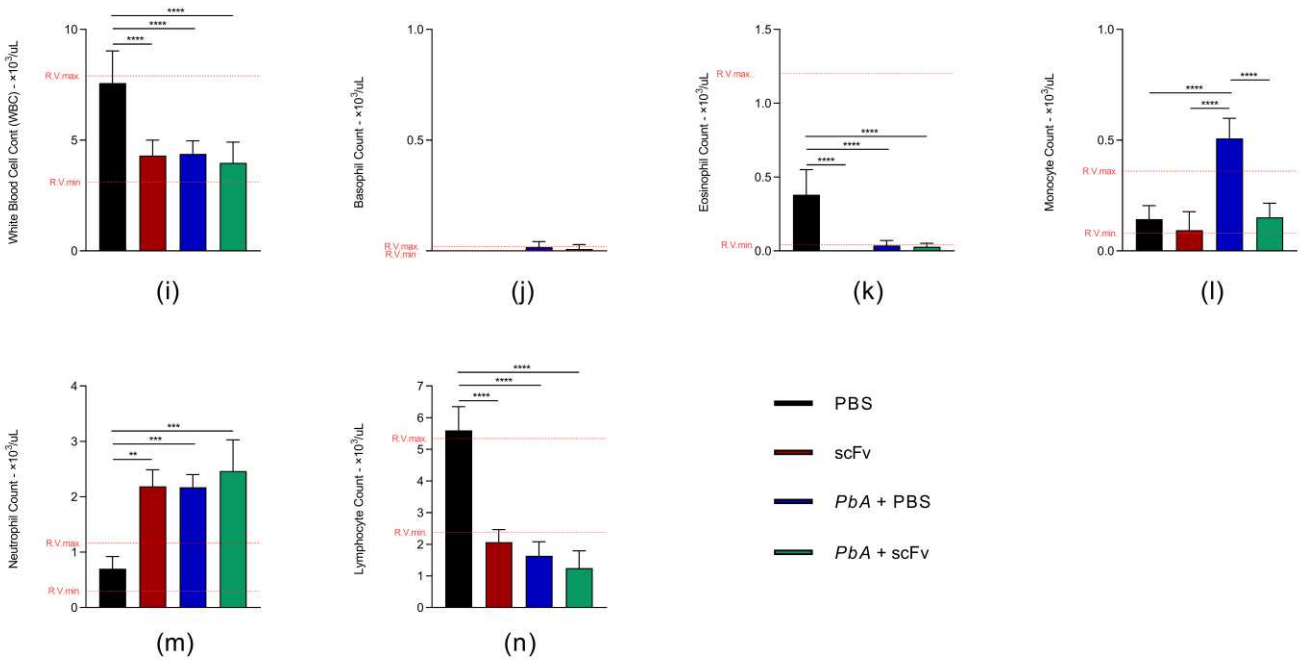


Figura 5. The graphs depict hematological parameters of C57BL/6J mice infected and uninfected with P. berghei-ANKA (PbA), treated with scFv or PBS. From (a) to (g), parameters of the red blood cell series are presented, (f) platelet count, and from (i) to (n), parameters of the white blood cell series are presented. Reference values were based on the same animal model and age from The Jackson Laboratory (JAX®). Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). Mean and standard deviation are represented (* $p < 0.05$; ** $p< 0.01$; *** $p<0.001$; **** $p<0.0001$). The statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test.

The animals in the PbA + PBS group showed polycythemia, evidenced by an increase in hematocrit (figure 5c). Although anemia is common in cases of malaria, no obvious anemia was observed due to severe dehydration on the day of collection, resulting in a reduction in circulating fluid volume and, consequently, an increase in red blood cell concentration. Additionally, an increase in MCHC was noted (figure 5f), indicative of possible hemolysis, although confirmation requires the examination of indirect bilirubin. Other findings included thrombocytopenia (figure 5h), leukopenia (figure 5i) due to lymphocytopenia (figure 5n) and eosinopenia (figure 5k), neutrophilia (figure 5m) with a right shift, and monocytosis (figure 5l).

In the PbA+scFv group, normocytic normochromic anemia (figure 5c,d,f), thrombocytopenia (figure 5h), leukopenia (figure 5i) due to lymphocytopenia (figure 5n) and eosinopenia (figure 5k), and neutrophilia (figure 5m) with a right shift were observed. These results are typical of an acute response, considering the consumption of leukocytes associated with an increase in neutrophils without signs of regeneration.

3.5. The treatment with scFv stimulates the production of pro-inflammatory cytokines in the liver and lung of animals infected with P. berghei-ANKA

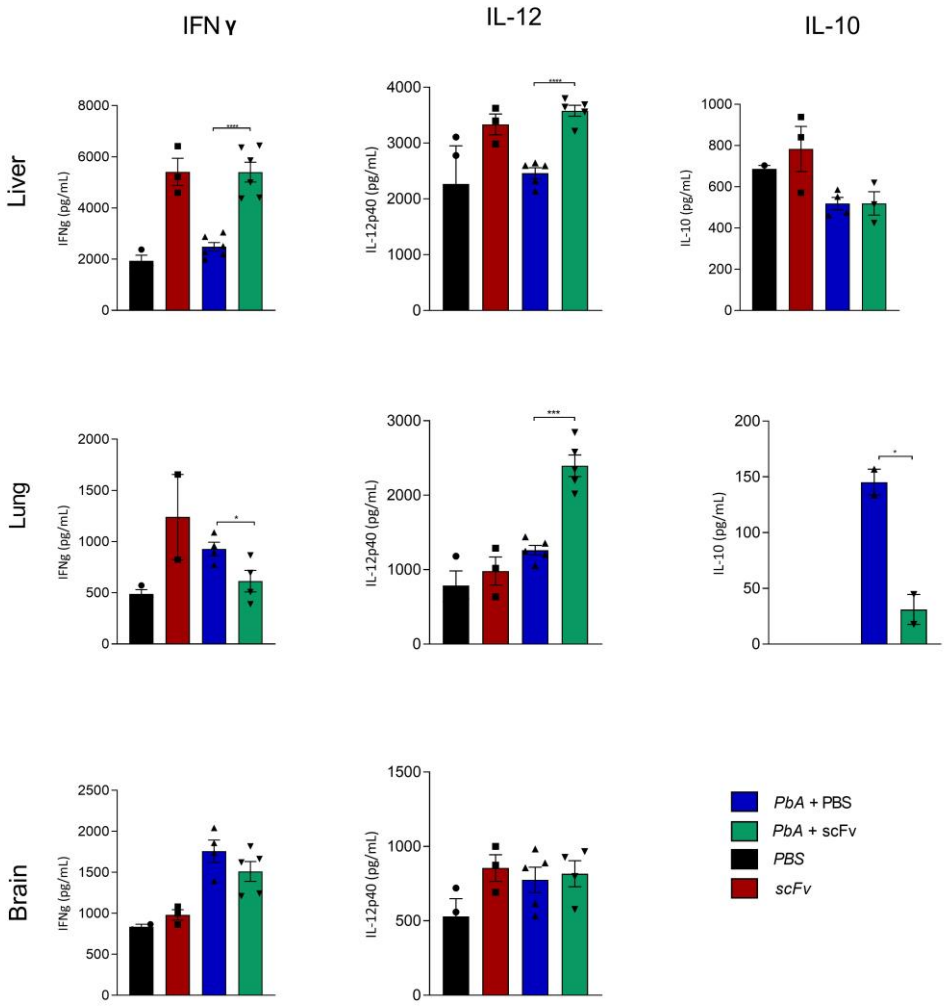


Figure 6. Cytokine panel of C57BL/6J mice infected or not with P. berghei-ANKA and treated or not with scFv anti-GPI from Plasmodium. Cytokines were measured by ELISA in liver, lung, and brain homogenates. Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). The graphs represent mean and standard error of the mean (* $p < 0.05$; ** $p < 0.01$; *** $p<0.001$; **** $p<0.0001$). The statistical analysis was conducted using two-way ANOVA followed by multiple comparisons tests of Holm-Sidak and Tukey.

The scFv treatment stimulated the production of pro-inflammatory cytokines IL-12 and IFNγ in the liver. Additionally, it decreased the production of IL-10 (anti-inflammatory) and increased IL-12 (pro-inflammatory) in the lungs. In the brain, we quantified IL-12 and IFNγ, which maintained levels close to those of the infected control group. The quantification of IL-10 in the brain was not possible to detect due to sample loss.

3.6. The scFv treatment reduces microhemorrhages in the brains of mice infected with P. berghei ANKA

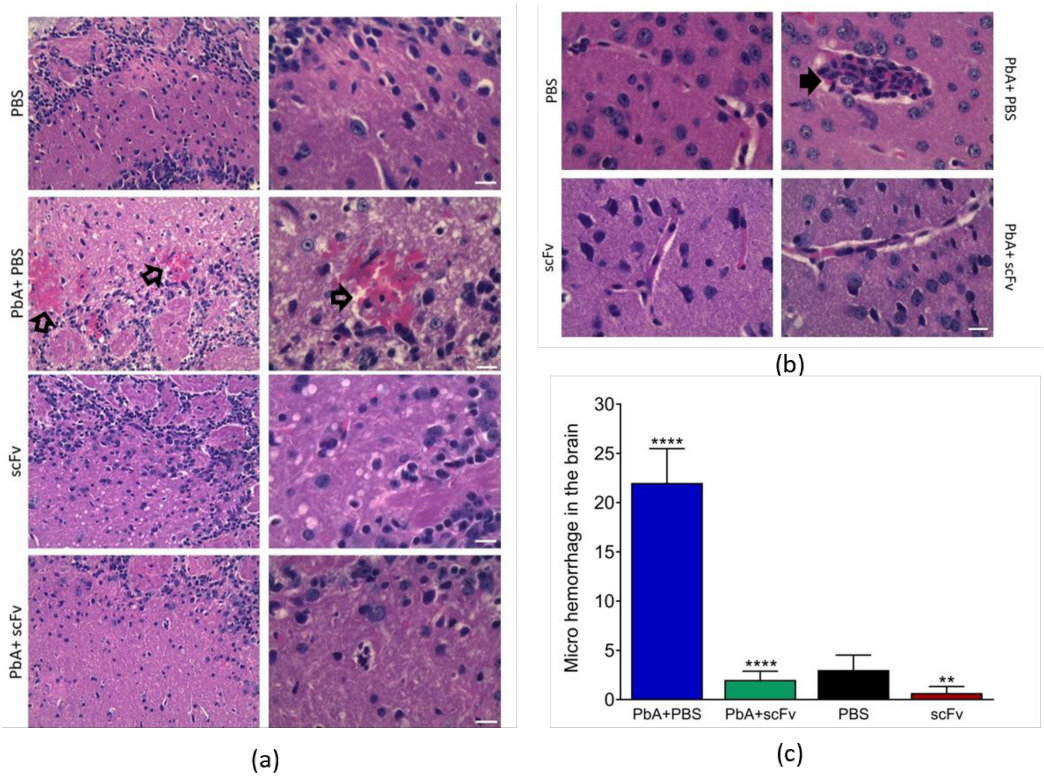


Figure 7. Photomicrographs of brains from mice infected or not with *Plasmodium berghei* ANKA (PbA) and treated or not with anti-GPI scFv from Plasmodium. (a) Microhemorrhages indicated by unfilled arrows. (b) Leukocyte migration in the brain, evidenced by the filled arrow. (c) Quantitative graph of brain microhemorrhages in the mentioned groups. Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). The graphs represent mean and standard error of the mean (** $p < 0.01$; **** $p < 0.0001$). Representative images are shown at 20x and 40x (a) and 40x (b) magnification, H&E staining. The statistical analysis was performed using one-way ANOVA followed by unpaired t-test.

Analysis of brain microhemorrhages revealed a significant statistical difference ($p < 0.0001$) between groups treated and untreated with scFv, demonstrating that scFv provides brain protection to PbA-infected animals. Microhemorrhages are illustrated in Figure 7a, indicated by unfilled arrows.

3.7. The scFv treatment does not induce alterations in alveolar septum thickening and hepatic hemozoin production in P. berghei ANKA-infected mice

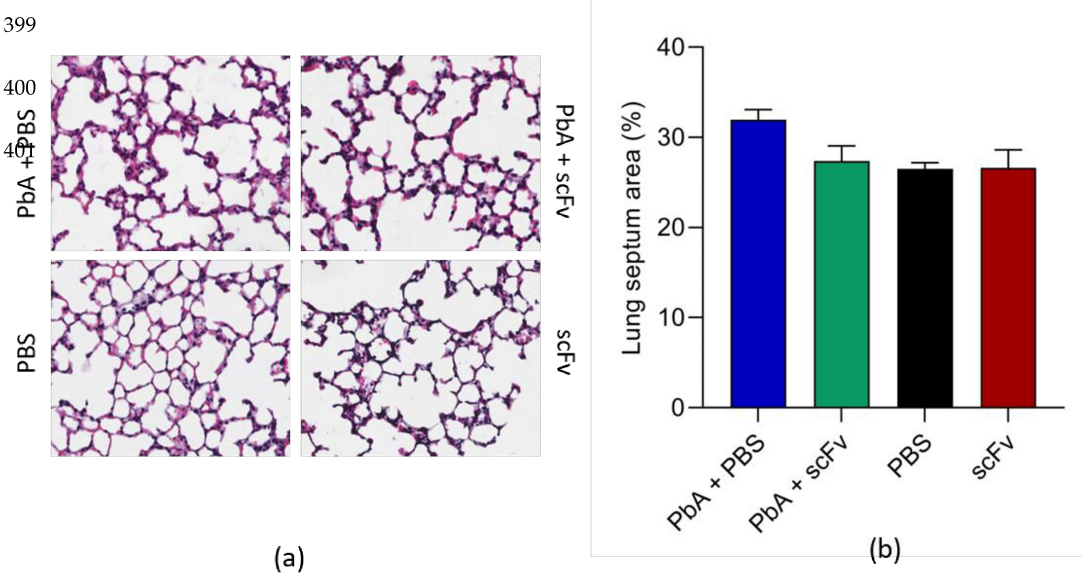


Figure 8. (a) Photomicrographs of the lung demonstrating the presence of inflammatory infiltrate and thickening of the interalveolar septum. (b) Area occupied by the alveolar septum, analyzed using the ImageJ program. Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). The graphs represent the mean and standard error of the mean. The representative images are shown at a magnification of 10x, H&E staining. The statistical analysis was conducted using the non-parametric Kruskal-Wallis test followed by Dunns' multiple comparison tests.

The lung tissue was evaluated for the area of the alveolar septum (Figure 8b), where no significant differences were found between the groups. Regarding morphological differences, no alterations were observed in terms of septal thickness and the presence of inflammatory infiltrate (Figure 8a).

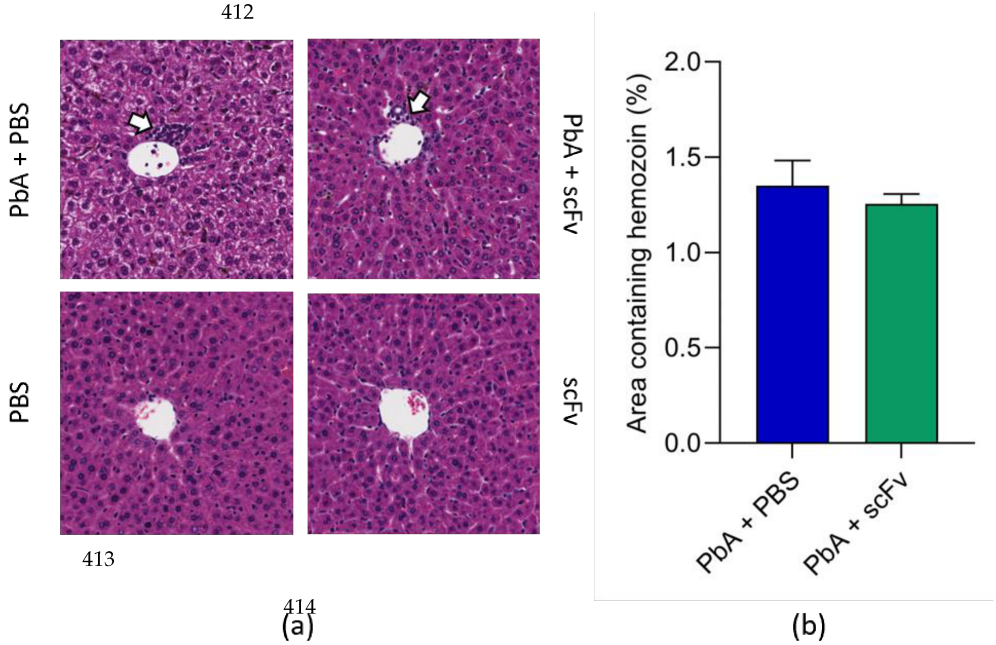


Figure 9. (a) Photomicrographs of the liver showing the presence of hemozoin. (b) Graph representing the percentage of area occupied by hemozoin in the groups infected with *Plasmodium berghei*-ANKA. In (a) the arrows indicate leukocyte infiltrate Groups: PBS (n=3), scFv (n=3), PbA+PBS (n=6), PbA+scFv (n=6). The graphs show mean and standard error of the mean. Representative images are

displayed at 20x magnification, H&E staining. The statistical analysis was performed using the Kolmogorov-Smirnov test followed by unpaired t-test with Welch's correction.

Following liver analysis, the presence of hemozoin was observed in both treated and untreated infected animals. Hemozoin was subsequently quantified via polarized light microscopy, revealing no significant difference between the groups. However, as indicated by the arrows in image 9 (a), perivascular cuffs were found in the infected groups, with a more pronounced presence in the PbA + PBS group. Additionally, in the same group, enlargement of sinusoidal capillaries within the hepatic parenchyma was also observed.

4. Discussion

Cerebral malaria is a severe neuropathological complication caused by *Plasmodium* spp. Its clinical manifestations are characterized by severe anemia, coma, and seizures, with the potential to progress to death [14]. In addition to the incomplete elucidation of its pathogenesis, Plasmodium has been displaying resistance to current treatments, representing a significant global concern [6, 15, 16].

Through the utilization of animal models, we can investigate new drugs and the mechanisms involved in the disease [17]. C57BL/6 mice infected with Plasmodium berghei ANKA constitute a well-established model that shares many similarities with human cerebral malaria, including the sequestration of infected red blood cells in the brain, neurological signs such as paralysis, ataxia, convulsions, and respiratory distress [18, 19].

Single-chain variable fragments (scFv) are structures with functions similar to antibodies but with certain advantages: they possess greater tissue penetration capacity and reach tissues faster and more uniformly due to their smaller size [20, 21]. Several scFv have been selected for specific therapies, such as cancer treatment and antiviral applications, but none have been specific to Plasmodium falciparum [22, 23].

In this study, we sought to understand the effect of a Phage display-selected *Plasmodium* anti-GPI scFv on murine cerebral malaria. Initially, we conducted a survival curve, rescuing 71.4% of animals, which is highly promising, considering that mice infected with PbA commonly succumb between the 6th and 10th days post-infection, exhibiting neurological signs in the last days or hours [23].

In addition to that, in monitoring body weight, all groups experienced initial weight loss, likely due to factors related to stress and acute inflammation caused by vehicle and parasite administration [24, 25]. The progressive weight decrease in the PbA+PBS group from the 3rd day post-infection (dpi) can be attributed to both parasitemia and a reduction in water and food intake [26]. On the other hand, animals infected and treated with scFv were active and showed an increase in body weight on days 2, 3, 5, and 6 post-infection, indicating an overall improvement in the disease condition. Since mice rapidly lose weight in the presence of infectious diseases, this evaluation can be used to predict the possibility of animal death, aligning with our survival curve and body weight results [27]. Thus, we observe that the weight recovery induced by scFv treatment indicates an improvement in the overall disease condition and higher chances of survival.

These findings align with the indications of morbidity demonstrated in the RMCBS scale (Figure 5), where statistically higher scores were observed in animals treated with scFv. It is noteworthy that higher scores correspond to fewer clinical signs, indicating that scFv protected animals from severe cerebral malaria symptoms. According to Carroll et al. [13], RMCBS scores of 10 or less characterize signs of cerebral malaria, and scores of 5 or less result in animal death between 1 and 4 hours, thus requiring euthanasia.

The score assessments can be divided into two major groups. The first includes body position, grooming, ocular assessment, and motor performance. These parameters characterize general disease signs in mice, as healthy animals dedicate the majority of their time to activities such as grooming (approximately 40%), followed by exploration, locomotion, and feeding [28, 29]. Thus, we understand that symptoms such as hunched

posture, piloerection, reduced self-cleaning, eye closure and secretion, as well as decreased exploratory activity, observed in both infected groups and more pronounced in the PbA + PBS group, are expected and represent an organism's mechanism to conserve energy in disease situations [26]. Despite the manifestation of these signs, we observed higher scores in animals from the PbA + scFv group, indicating that the treatment resulted in an improvement in the overall clinical condition.

The second major group is directed towards the evaluation of the central and peripheral nervous systems, including gait, balance, strength of thoracic limbs, response to touch, paw reflex, and pinna reflex. Animals infected and treated with scFv showed no alterations in these parameters, unlike those infected and treated with PBS, which exhibited signs ranging from moderate to severe, especially on the 6th dpi. This suggests the preservation of the integrity of the blood-brain barrier and the central nervous system concerning inflammation, edema formation, hemorrhages, and hypoxia in the scFv-treated group. These events are known to contribute to increased intracranial pressure and microlesions in brain tissue, resulting in neurological signs associated with cerebral malaria [3, 30].

Acute cerebral malaria is associated with the adherence of infected erythrocytes to the vascular endothelium, and when associated with high parasitemia, exacerbates the formation of microthrombi, microhemorrhages, and edema in various organs, especially the brain [19]. Thus, the blood parasitic load holds significant importance in the pathogenesis of cerebral malaria, as the rupture of the blood-brain barrier leads to the sudden formation of cerebral edema, causing severe neurological signs and potential fatality. With our treatment, we observed a reduction in parasitemia, particularly on days 5 and 6 post-infection, along with a significant decrease in cerebral hemorrhages. This suggests a direct correlation with improvements in morbidity and mortality indicators [31, 32, 33].

In hematological analyses, comparing the PbA + scFv group with the PbA + PBS group, a significant reduction in monocytosis, an increase in platelet count, and a decrease in MCHC were observed. The results of the infected group treated with PBS were similar to the findings of Kotepui et al. [34], who analyzed hematological parameters in 4985 human patients, including 703 with malaria. Both the mice in this study and the human patients in Kotepui et al.'s study showed low platelet, leukocyte, and lymphocyte counts, along with increased monocyte and neutrophil counts. Additional studies in humans have also reported similar alterations, such as thrombocytopenia, leukopenia, lymphocytopenia, and monocytosis [21, 34, 35, 36]. This indicates that cerebral malaria is associated with an exacerbated systemic inflammatory response, involving pro-inflammatory cytokines, activation of endothelial cells, sequestration of parasitized erythrocytes, platelets, and leukocytes, resulting in increased vascular permeability, edema, and hemorrhages [19].

Cerebral malaria exhibits a significant Th1 response, characterized by an increase in the production of cytokines IL-12p40 and IFNγ. The elevated levels of these cytokines in animals infected and treated with scFv, compared to those treated with PBS, may have reflected an early and effective immune response regulated by pro-inflammatory Th1 cytokines. Particularly, IFN-γ may have played a role in limiting the progression from uncomplicated to complicated malaria, as indicated by the morbidity scores described above [37, 38, 39].

Th1 cell responses and IFNγ production have also been associated with host resistance during the Plasmodium infection hepatic stage. Experimental evidence suggests that liver-resident T cells play a crucial role in eliminating Plasmodium-infected hepatocytes through phagocytosis by macrophages, thus preventing the emergence of merozoites and the subsequent manifestation of clinical malaria [40]. The phagocytosis of Plasmodium-infected cells, in turn, leads to hemozoin deposition in the liver, which can further induce the pro-inflammatory response [41, 42, 43]. In our treatment, we did not observe changes in hepatic hemozoin deposition, despite lower blood parasitemia, increased hepatic IFNγ and IL-12p40 levels, and decreased monocytes in circulation. This suggests

increased macrophage activity in the liver and, consequently, a better defense response against the parasite.

In the lungs, there was an increase in IL-12p40 and a decrease in IL-10 in the PbA + scFv group and a decrease in IL-12p40 and an increase in IL-10 in the PbA + PBS group (figure 6), which demonstrates a suppression in the anti- inflammatory response and increased inflammatory response caused by scFv. Despite there being no difference in the thickening of the alveolar septum (figure 8), these data are characteristic of an increase in inflammatory cells, in line with the findings of Aitken and collaborators [44], who observed a marked leukocyte infiltrate without fibrosis, since cerebral malaria presents as an acute condition.

In the brain, there was no difference in the profile of pro-inflammatory cytokines, but a significant reduction in the presence of microhemorrhages in the histopathology of the PbA + scFv group compared to the PbA + PBS group was noted. This reduction may be associated with a pathway unrelated to IFNγ and IL-12p40. Unlike viruses and bacteria, malaria protozoa do not infiltrate or infect the brain parenchyma but rather disrupt the blood-brain barrier, leading to hemorrhages and subsequent neurological alterations [19, 45]. Thus, it is inferred that scFv provided protection against blood-brain barrier disruption, as evidenced by reduced hemorrhages and clinical signs in mice.

Therefore, scFv treatment yielded numerous positive findings in malaria pathogenesis, preventing the disease from progressing to cerebral complications. Further studies are necessary for a comprehensive understanding of the involved mechanisms, but we believe that an initial step has been taken toward a potential new treatment.

5. Conclusions

With our findings, we were able to assess the effect of a *Plasmodium falciparum* anti-GPI scFv on cerebral malaria in a murine model. Therefore, we can conclude that the Plasmodium anti-GPI scFv is a promising molecule in the treatment of complicated malaria but requires further studies to understand its mechanism of action, pharmacokinetics, effects on the host, among other factors.

6. Patents

The patent registration process has started.

Author Contributions: Conceptualization, S.G.K., K.C.S.M. and M.V.S.; investigation, S.G.K., K.C.S.M., B.C.B., M.S.V., W.R.L., M.M.N., L.S.M., G.G. and F.A.A.S.; writing—original draft preparation, S.G.K.; writing—review and editing, F.B.F. and I.L.L.; supervision, M.V.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) – Rede Mineira de Biotecnologia em Modelos Experimentais (RMBME – FAPEMIG), pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e pelo Ministério Público do Trabalho de Uberlândia (MPT) and Universidade Federal de Uberlândia (UFU/PROPP).

Institutional Review Board Statement: The research was carried out in accordance with the rules of the National Council for the Control of Animal Experimentation (CONCEA) of Brazil, and international recommendations such as The ARRIVE guidelines 2.0.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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ANEXO A – Normas para publicação na revista Microorganisms

1. Manuscript Submission Overview

2. Types of Publications

Microorganisms has no restrictions on the maximum length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Microorganisms* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

Manuscripts submitted to *Microorganisms* should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

- *Article:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although short *Communications* of preliminary, but significant, results will be considered. The quality and impact of the study will be considered during peer review. i) Manuscripts consisting solely of bioinformatics analyses of public databases or genomic/transcriptomic sequencing without being accompanied by validation (biological validation in vitro or in vivo) are out of scope of the present topic; ii) Substances without clear ingredients, such as complex prescriptions, crude extracts, and herbal mixtures, will not be considered; iii) Papers that only contain clinical trials/data are not acceptable for Microorganisms.

- *Review:* The research reported within a review should be drawn from peer-reviewed publications. No new, unpublished data should be presented. These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA **guidelines.**

- *Case report:* Case reports present detailed information on the symptoms, signs, diagnosis, treatment (including all types of interventions), and outcomes of an individual patient. Case reports usually describe new or uncommon conditions that serve to enhance medical care or highlight diagnostic approaches.

3. Submission Process

Manuscripts for *Microorganisms* should be submitted online at **susy.mdpi.com**. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the **criteria to qualify for authorship**) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the **submission website**. Once you have registered, **click here to go to the submission form for *Microorganisms***. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

4. Accepted File Formats

Authors are encouraged to use the **Microsoft Word template** or **LaTeX template** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office **microorganisms@mdpi.com**. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, we encourage you to use the ***Microorganisms* Microsoft Word template file**. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.

- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (including all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, we encourage you to use the ***Microorganisms* LaTeX template files**. You can now also use the online application **writeLaTeX** to submit articles directly to *Microorganisms*. The MDPI LaTeX template file should be selected from the **writeLaTeX template gallery**.

- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see **below** for further details).

Disclaimer: Usage of these templates is exclusively intended for submission to the journal for peer-review, and strictly limited to this purpose and it cannot be used for posting online on preprint servers or other websites.

5. Free Format Submission

Microorganisms now accepts free format submission:

- We do not have strict formatting requirements, but all manuscripts must contain the required sections: Author Information, Abstract, Keywords, Introduction, Materials & Methods, Results, Conclusions, Figures and Tables with Captions, Funding Information, Author Contributions, Conflict of Interest and other Ethics Statements. Check the Journal ***Instructions for Authors*** for more details.
- Your references may be in any style, provided that you use the consistent formatting throughout. It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package *EndNote*, ***Zotero***, *Mendeley*, *Reference Manager* are recommended.
- When your manuscript reaches the revision stage, you will be requested to format the manuscript according to the journal guidelines.

6. Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

All cover letters are required to include the statements:

- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
- All authors have approved the manuscript and agree with its submission to (journal name).

7. Author Identification

Authors are encouraged to add a biography (300–1500 characters) to the submission and upload it to **SciProfiles**. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;
2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;
4. Current and previous research interests;
5. Memberships of professional societies and awards received.

If a manuscript is accepted for publication, we will add an icon linking to your online **ORCID** profile in the final version of the published paper.

8. Author Affiliation

All authors should list their current affiliation and the affiliation where most research was carried out for the preparation of their manuscript. We recommend adding as primary the affiliation where most of the research was conducted or supported, but please check with your institution for any contractual agreement requirements.

It is very important that author names and affiliations are correct. Incorrect information can mean a lack of proper attribution or incorrect citation and can even lead to problems with promotion or funding. After the publication of an article, updates or corrections to the author's address or affiliation may not be permitted.

9. Independent Researcher

If one or all the authors are not currently affiliated with a university, institution or company, or have not been during the development of the manuscript, they should list themselves as an “Independent Researcher”.

10. Manuscript Preparation

11. General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.
    - **Research manuscript sections**: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the **PRISMA** guidelines.
- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical Abstract:**

  A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

  The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, or TIFF. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

  The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.
- **Acronyms/Abbreviations/Initialisms** should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and Expression Data**.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
- **Research Data and supplementary materials:** Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about **Supplementary Materials** and Data Deposit for additional guidelines.
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- **Guidelines and standards:** MDPI follows standards and guidelines for certain types of research. See **https://www.mdpi.com/editorial_process** for further information.

12. Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used. Please do not include abbreviated or short forms of the title, such as a running title or head. These will be removed by our Editorial Office.

- **Author List and Affiliations:** One author, or at most two in limited cases, should be designated as the corresponding author. A maximum of two joint first authors can be indicated by the addition of a superscript symbol. The symbol must be defined below the affiliations by the addition of the following statement: “These authors contributed equally to this work”. The roles of the equal authors should also be adequately disclosed in the author contributions statement. Please read the criteria to qualify for authorship for further information. Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as the corresponding author. The email addresses of all authors will be displayed on published papers, and hidden by Captcha on the website as standard. It is the responsibility of the corresponding author to ensure that consent for the display of email addresses is obtained from all authors. If an author (other than the corresponding author) does not wish to have their email addresses displayed in this way, the corresponding author must indicate as such during proofreading. After acceptance, updates to author names or affiliations may not be permitted. Equal Contributions: authors who have contributed equally should be marked with a superscript symbol (†). The symbol must be included below the affiliations, and the following statement added: “These authors contributed equally to this work”. The equal roles of authors should also be adequately disclosed in the author contributions statement. Please read the criteria to qualify for authorship.

- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

13. Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.

- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.

- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.

- **Conclusions:** This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.

- **Patents:** This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

14. Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.

- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the **CRediT taxonomy** for the term explanation. For more background on CRediT, see **here**. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".

- **Funding:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published. Please add: "This research received no external funding" or "This research was funded by [name of funder] grant number [xxx]" and "The APC was funded by [XXX]" in this section. Check carefully that the details given are accurate and use the standard spelling of funding agency names at https://search.crossref.org/funding, any errors may affect your future funding.

- **Data Availability Statement:** In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "**MDPI Research Data Policies**". You might choose to exclude this statement if the study did not report any data.

- **Acknowledgments:** In this section you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Microorganisms* does not publish studies funded partially or fully by the tobacco industry. Any projects funded by industry must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study". For more details please see **Conflict of Interest**.

- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as **EndNote**, **ReferenceManager** or **Zotero** to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.

- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for **Endnote** and **Zotero** are available.

References should be described as follows, depending on the type of work:

 Journal Articles:
1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.
 Books and Book Chapters:
2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.
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 Unpublished materials not intended for publication:
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7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).
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9. Title of Site. Available online: URL (accessed on Day Month Year).
Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as **WebCite**. Archived websites should be cited using the link provided as follows:
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See the **Reference List and Citations Guide** for more detailed information.

15. Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Microorganisms* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

16. Original Images for Blots and Gels Requirements

For the main text, please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel.
- Image processing methods, such as adjusting the brightness or contrast, do not alter or distort the information in the figure and are applied to every pixel. High-contrast blots/gels are discouraged.
- Cropped blots/gels present in the main text retain all important information and bands.
- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

In order to ensure the integrity and scientific validity of blots (including, but not limited to, Western blots) and the reporting of gel data, original, uncropped and unadjusted images should be uploaded as Supporting Information files at the time of initial submission.

A single PDF file or a zip folder including all the original images reported in the main figure and supplemental figures should be prepared. Authors should annotate each original image, corresponding to the figure in the main article or supplementary materials, and label each lane or loading order. All experimental samples and controls used for one comparative analysis should be run on the same blot/gel image. For quantitative analyses, please provide the blots/gels for each independent biological replicate used in the analysis.

17. Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data. Individual journal guidelines can be found at the journal 'Instructions for Authors' page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

MDPI data policies are informed by **TOP Guidelines** and **FAIR Principles**.

Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

Data Availability Statements provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Below are suggested Data Availability Statements:

- Data available in a publicly accessible repository
  The data presented in this study are openly available in [repository name e.g., FigShare] at [**doi**], reference number [reference number].

- Data available in a publicly accessible repository that does not issue DOIs
  Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]

- Data available on request due to restrictions eg privacy or ethical
  The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]

- 3rd Party Data
  Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].

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- Data is contained within the article or supplementary material
  The data presented in this study are available in [insert article or supplementary material here]

Data citation:

- [dataset] Authors. Year. Dataset title; Data repository or archive; Version (if any); Persistent identifier (e.g., DOI).

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For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository such as **GitHub** or uploading as supplementary information to the publication. The name, version, corporation and location information for all software used should be clearly indicated. Please include all the parameters used to run software/programs analyses.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to **https://www.mdpi.com/authors/layout#_bookmark83**.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

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New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

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- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.
- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool **SPIN**). Annotated protein structure and its reference sequence must be submitted to **RCSB of Protein Data Bank**.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

18. Research and Publication Ethics

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/**), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics

committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

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If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

19. Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an

ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at **https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf**. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

20. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
21. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**
22. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
23. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

24. Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

25. Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

26. Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

27. Dual Use Research of Concern

MDPI follows the practical framework defined in **Guidance for Editors: Research, Audit and Service Evaluations** and introduced by the Committee on Publication Ethics (COPE). Research that could pose a significant threat, with broad potential consequences to public health or national security, should be clearly indicated in the manuscript, and potential dual-use research of concern should be explained in the cover letter upon submission. Potential areas of concern include but are not limited to biosecurity, nuclear and chemical threats, and research with a military purpose or application, etc. For these manuscripts to be considered for peer review, the benefits to the general public or public health must outweigh the risks. The authors have a responsibility to comply with relevant national and international laws.

28. Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

29. Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

30. Publication Ethics Statement

Microorganisms is a member of the Committee on Publication Ethics (**COPE**). We fully adhere to its **Code of Conduct** and to its **Best Practice Guidelines**.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Microorganisms* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Microorganisms* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on translations**.
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding Updating Published Papers**.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the **Rights and Permissions** page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Microorganisms* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, an investigation will take place and action taken in accordance with our policies.
    - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

      Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

      If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

  Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

31. Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on **citation manipulation** with recommendations for best practice.

32. Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper. For detailed information regarding the qualifications and responsibilities of the reviewers, please visit **https://www.mdpi.com/reviewers**.

33. Extensive English Editing

It is the authors' responsibility to submit their work in correct English. The APC includes only minor English editing, conducted by native English speakers. The APC does not include extensive English editing. If extensive editing is required, your paper could be returned to you at the English editing stage of the publication process. This could delay the publication of your work. You may have your work reviewed by an experienced English-speaking colleague or use a paid language-editing service before submitting your paper for publication. We offer rapid English editing, completed in 1 day, here: **Author Services**.

34. Preprints and Conference Papers

Microorganisms accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Microorganisms* does not publish pilot studies or studies with inadequate statistical power.

Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the **Proceedings Series journals**.

35. Authorship

MDPI follows the International Committee of Medical Journal Editors (**ICMJE**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the **International Committee of Medical Journal Editors (ICMJE)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should

keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check **MDPI ethics website**.

36. Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

37. Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

38. Conflicts of Interest

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

39. Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal’s Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript. For more details about potential conflicts of interest, please check here, **https://www.mdpi.com/reviewers#_bookmark9**.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.

- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.

- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.

- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be

asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

Please read detailed Editorial Process **here**.

40. Promoting Equity, Diversity and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

41. Resource Identification Initiative

To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

We encourage authors to include unique identifiers - RRIDs- provided by the **Resource Identification Portal** in the dedicated section of the manuscript.

To help authors quickly find the correct identifiers for their materials, there is a single **website** where all resource types can be found and a ‘cite this’ button next to each resource, that contains a proper citation text that should be included in the methods section of the manuscript.

ANEXO B – Análise final da Comissão de Ética no Uso de Animais

Universidade Federal de Uberlândia
Pró-Reitoria de Pesquisa e Pós-Graduação
Comissão de Ética na Utilização de Animais (CEUA)
Rua Ceará, S/N - Bloco 2T, sala 113 – CEP 38405-315
Campus Umuarama – Uberlândia/MG – Ramal (VoIP) 3423;
e-mail:ceua@propp.ufu.br; www.comissoes.propp.ufu.br

ANÁLISE FINAL Nº 028/17 DA COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS PARA O PROTOCOLO REGISTRO CEUA/UFU 153/16

Projeto Pesquisa: "Busca por alvos imunes para o desenvolvimento de medidas profiláticas e terapêuticas contra malária em modelo murino".

Pesquisador Responsável: Murilo Vieira Silva

O protocolo não apresenta problemas de ética nas condutas de pesquisa com animais nos limites da redação e da metodologia apresentadas. Ao final da pesquisa deverá encaminhar para a CEUA um relatório final.

Situação: PROTOCOLO DE PESQUISA APROVADO.

OBS: O CEUA/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEUA PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

Uberlândia, 17 de março de 2017.

Prof. Dr. Lúcio Vilela Carneiro Girão
Coordenador da CEUA/UFU

ANEXO C – Modelo da ficha de avaliação de morbidade (RMCBS)

Escala rápida de coma e comportamento murino (RMCBS)																					
Data: Dia de infecção: Responsável:	Score (0-2)																				
	1/1	1/2	1/3	1/4	1/5	1/6	2/1	2/2	2/3	2/4	2/5	2/6	3/1	3/2	3/3	4/1	4/2	4/3			
Marcha (nenhuma - atáxico - normal)																					
Equilíbrio (nenhuma extensão - estende só MT - eleva o corpo inteiro)																					
Desempenho motor (não explora a caixa - explora 2 a 3 cantos em 90 s - explora todos os 4 cantos em 15 s)																					
Posição corporal (de lado - curvado - extensão total)																					
Força do membro (hipotônico, sem força - recuo fraco, somente com as mãos - recuo forte, afastamento ativo)																					
Escape ao toque (nenhum - unilateral - instantâneo e bilateral) *3 tentativas																					
Reflexo de pinna (nenhum - unilateral - instantâneo e bilateral) *3 tentativas																					
Reflexo podal (nenhum - unilateral - instantâneo e bilateral) *3 tentativas																					
Grooming (irritado, com pelos bagunçados - empoeirado/piloereção - pelos limpos e com brilho)																					
Olhos (muita secreção e fechados ou quase fechados - pouca secreção e entreabertos - sem secreção e bem abertos)																					