Ministério da Saúde
Fundação Oswaldo Cruz
Centro de Pesquisas René Rachou
Programa de Pós-Graduação em Ciências da Saúde

Papel dos Receptores do tipo Toll na malária

por

Bernardo Simões Franklin

Belo Horizonte
Setembro / 2009

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Tese apresentada com vistas à obtenção do Título de Doutor em Ciências da Saúde na área de concentração de Biologia Celular e Molecular
Orientação: Dr. Ricardo Tostes Gazzinelli

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"Um pouco de ciência nos afasta de Deus. Muito, nos aproxima."
Louis Pasteur

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LISTA DE ABREVIATURAS E SÍMBOLOS

Abreviatura	*Definição*
AT9	Anti-TLR9
CBA	Cytometric Bead Array
CM	Malária Cerebral
DMEM	Meio essencial mínimo de Eagle modificado por Dulbeccos' (*Dulbeccos' Modified Eagle Medium*)
ELISA	Ensaio imunoenzimático (*Enzyme-linked immunosorbent assay*)
FIOCRUZ	Fundação Oswaldo Cruz
IgG	Imunoglobulina da classe G
iRBCs	Eritrócitos infectados (*Infected Red Blood Cells*)
IFN	Interferon
IL6	Interleucina 6
IL12	Interleucina 12
IL1	Interleucina 1
IL10	Interleucina 10
mM	Milimolar
MyD88	Molécula adaptadora dos TLRs (*myeloid differentiation primary-response protein 88*)
µg	Micrograma
µl	Microlitro
µM	Micromolar
ng	Nanograma
pmoles	Picomoles
PBS	Salina tamponada com fosfato (*Phosphate-buffered saline*)
qPCR	Reação em cadeia da polimerase em tempo real (*Real Time Polymerase Chain Reaction*)
SBF	Soro bovino fetal (Fetal bovine serum)
TNF	Fator de necrose tumoral
TLRs	Toll-like Receptors
Wt	Camundongos selvagens (*Wild-type*)
UMASSMED	University of Massachusetts Medical School

Resumo

Desde sua descoberta, os receptores do tipo Toll (TLRs) têm sido envolvidos em quase todas as doenças que afetam a saúde humana. Seu papel na proteção contra vários patógenos, incluindo protozoários está bem estabelecido. Entretanto, pouco se sabe sobre o papel dos TLRs na malária. No presente estudo, investigamos o papel dos TLRs durante a malária murina e humana. Nossos resultados mostraram que camundongos com deficiência para MyD88, um adaptador essencial para a sinalização dos TLRs, produzem níveis de citocinas pró-inflamatórias significativamente menores e apresentam sintomas mais amenos durante a infecção por *Plasmodium chabaudi*. Entretanto, estes animais retêm a capacidade de controlar a parasitemia sugerindo que os TLRs possuam um papel na patogênese e não na proteção contra a malária. Posteriormente, mostramos que ambas, a infecção natural humana por *P. falciparum* e a experimental murina por *P. chabaudi,* aumentam a expressão e a responsividade dos TLRs nas células do sistema imune inato. O estado hiper-responsivo das células durante a malária é derivado da ativação de TLR9 e a produção de IFNγ por células T, levando a uma alta susceptibilidade ao choque séptico durante a malária aguda. Finalmente, em colaboração com a EISAI Research Institute, desenvolvemos um antagonista de TLR9 e testamos seu efeito na Malária Cerebral (CM), uma das manifestações clínicas mais graves da malária. O tratamento oral com este composto inibiu os sintomas, tais como extravasamento vascular cerebral, protegendo camundongos da morte por CM. Em conjunto, nossos resultados mostram um importante papel dos TLRs, especialmente TLR9, na patogênese da malária e que a intervenção na função destes receptores é uma potencial quimioterapia anti-inflamatória contra essa doença.

Abstract

Toll-like receptors (TLRs) have been involved in almost every know disease that afflict human health so far and their role in resistance to several pathogens, including protozoan parasites, has been well established. The role of TLRs in malaria, however, still remains to be elucidated. Here we studied the role of TLRs in experimental and naturally acquired malaria infection. We showed that mice with deficiency to MyD88, an essential adaptor to TLRs signaling, produce significant lower levels of pro-inflammatory cytokines and commensurate better clinical outcome upon infection with *Plasmodium chabaudi.* Nevertheless, these mice can still control parasite loads suggesting that TLRs are involved in pathogenesis rather than protection during malaria. We further studied cellular responsiveness of innate immune responses during human and murine malaria. We showed that both natural acquired *P. falciparum* infection in humans and experimental infection of mice with *P. chabaudi* increase TLR expression in innate immune cells causing pro-inflammatory priming of TLR responses. The cellular hyper-responsiveness during Malaria is caused by TLR9 activation and IFNγ production by T cells conferring high susceptibility to septic shock during the acute disease. Finally, in collaboration effort with EISAI Research Institute, we develop and tested an antagonist of TLR9, on Cerebral Malaria (CM), one of the most severe manifestations of malaria. Oral treatment of mice with this compound inhibited CM symptoms, such as vascular leakage, and prevented death from CM. All together our results show an important role of TLRs, especially TLR9, in malaria pathogenesis and that the therapeutic targeting of TLRs is a potential anti-inflammatory chemotherapy against malaria.

1 Introdução

1 INTRODUÇÃO

A malária é ainda um grande obstáculo ao desenvolvimento econômico mundial. Considerando as taxas de mortalidade, esta doença está entre as mais letais do mundo e assim tem sido por milênios. Para cada pessoa morta pela malária, há outras centenas que são infectadas pelo parasito. Os números exatos são difíceis de definir devido ao fato do flagelo da malária ser maior em algumas das regiões mais pobres do mundo (Snow *et al.* 2005). A organização Mundial de Saúde (WHO) acredita que a malária mata de um a dois milhões de pessoas por ano, a maioria crianças abaixo de seis anos de idade. Muitas crianças que sobrevivem a casos graves sofrem algum tipo de dano cerebral, deficiência cognitiva e têm dificuldades de aprendizagem.

Além do enorme fardo da mortalidade, os mais de 213 milhões de casos de malária levam a um acumulado de mais de 800 milhões de dias de internações/doença, por pessoa/ano na África (Breman *et al.* 2004). A situação é tão dramática que economistas determinaram a malária como causa definitiva de pobreza em muitas regiões afligidas (Sachs *et al.* 2002). Tudo isso coloca a malária, junto com a AIDS e a tuberculose, entre as três doenças infecciosas mais letais do mundo. Nas regiões endêmicas para a malária, o efeito da doença é também manifestado pela sua influência na genética humana, resultando na preservação de variantes de genes humanos potencialmente deletérios (ex: talassemia, anemia falciforme) devido ao fato da heterozigose de muitos destes genes conferirem vantagens contra as complicações da doença e contra a malária fatal.

No Brasil, a transmissão da malária está tipicamente restrita à Amazônia Legal que compreende os estados do Acre, Amapá, Amazonas, Mato Grosso, Pará, Rondônia, Roraima e Tocantins e parte do estado do Maranhão (Secretaria de Vigilância à Saúde 2007) onde condições socioeconômicas e ambientais favorecem a exposição de grandes contingentes populacionais ao risco de infecção. A incidência da doença nestas áreas aumentou dramaticamente nas décadas de 70 e 80, devido, principalmente, aos projetos de colonização, expansão da fronteira agrícola, construção de estradas e hidrelétricas, projetos agropecuários, extração de madeira e mineração (Sawyer 1986; Sawyer 1993). Nestas regiões, o *Plasmodium vivax* é responsável por mais de 90% dos casos de malária. Apesar dos números de casos de infecção pelo *P. falciparum* terem diminuído nas últimas décadas, a malária ainda é uma preocupação constante nessas regiões. A

infecção pelo *P. vivax* geralmente não é letal, mas a doença causa alta morbidade e acarreta uma grande perda econômica.

Das mais de 100 espécies de *Plasmodium*, 4 infectam o homem: *P. falciparum*, *P. vivax*, *P. ovale* e *P. malariae* (Mueller *et al.* 2007). Tem sido relatados casos de infecção humana por uma quinta espécie, o *P. knowlesi*, que sabidamente infecta macacos (Singh *et al.* 2004).

A infecção humana ocorre através da picada das fêmeas de mosquitos do gênero *Anopheles* e da inoculação das formas esporozoítas do *Plasmodium* na pele. Cerca de 30 minutos após a inoculação, os esporozoítos atingem a corrente sanguínea e alcançam o fígado. Uma vez no fígado, estes protozoários infectam os hepatócitos e se multiplicam de forma assexuada produzindo milhares de merozoítos. O parasito evade o fígado de forma indetectável se recobrindo com restos da membrana celular das células hospedeiras (Sturm *et al.* 2006). O período de replicação do parasito no fígado é assintomático e pode levar de 6 – 15 dias. Após a ruptura das células hospedeiras, os merozoítos são liberados na corrente sanguínea e invadem as hemácias dando início à fase eritrocítica da doença (Bledsoe 2005). Dentro das hemácias, o parasito se multiplica de forma assexuada e periódica rompendo as células infectadas e infectando outras novas. Os ciclos de febre coincidem com o rompimento de hemácias e a liberação de um grande número de protozoários no sangue do hospedeiro. Dentre as formas parasitas das hemácias, há as formas gametócitos, que quando ingeridas por mosquitos, darão início a fase sexuada do ciclo. Os mosquitos se contaminam ao picar os portadores da doença, tornando-se o principal vetor de transmissão (Marcucci *et al.* 2004; Talman *et al.* 2004).

Em quase todo o ciclo no hospedeiro vertebrado, o parasito fica relativamente protegido do ataque do sistema imune uma vez que se multiplica dentro dos hepatócitos ou dentro das hemácias, tornando-se relativamente invisível à vigilância das células imunes. Mesmo assim, as hemácias infectadas são destruídas no baço. O *P. falciparum* expressa moléculas adesivas na superfície das hemácias causando sua adesão às paredes dos capilares sanguíneos (através de sua interação com moléculas de adesão celulares CD36, ICAM e VCAM) sequestrando estas células e impedindo dessa forma sua passagem pelo baço. Acredita-se que este fenômeno é o principal fator que gera as complicações hemorrágicas (anemia grave, coagulação intravascular disseminada, trombocitopenia, etc.) da malária. A obstrução dos capilares sanguíneos está também envolvida nas complicações observadas na gravidez e na patogênese da malária cerebral

(CM). Na CM, as hemácias sequestradas podem romper a barreira hematoencefálica levando ao extravasamento (derrame) do sangue para o tecido cerebral causando hipóxia, convulsões, coma e até a morte (Adams *et al.* 2002).

Os sintomas da malária compreendem intensas dores abdominais, dores nas articulações, mialgias e cefaléias, febre alta, calafrios, vômitos, anemia (causada pela hemólise), hemoglobinúria, danos na retina e convulsões. Os sintomas clássicos da malária são a ocorrência cíclica de calafrios seguida de febre e sudorese que podem durar até 6 horas ocorrendo a cada dois dias na infecção por *P. vivax* e *P. ovale* e a cada três dias na infecção por *P. malarie* e a cada 36 a 48 horas na infecção por *P. falciparum* (http://www.malaria.am/eng/pathogenesis.php). Por razões que ainda não são inteiramente conhecidas, mas que possivelmente se devem ao aumento da pressão intracranial, crianças com malária frequentemente desenvolvem postura anormal, indicativa de danos cerebrais graves (Idro *et al.* 2005). A segunda maior causa de morte por malária, após a CM, é a anemia grave (hemoglobina < 50 g/L e hematócrito < 15%), responsável por grande parte da morbidade e fatalidade da doença (Anstey *et al.* 2009).

É importante ressaltar que vários dos sintomas da malária, principalmente as mialgias, cegaléias, a febre alta, a rigidêz e os calafrios são muito característicos de uma resposta inflamatória onde há a liberação de citocinas altamente pirogênicas principalmente fator de necrose tumoral (TNFα), Interleucinas 1 (IL1β) e 6 (IL6). Estas citocinas são produzidas por células da imunidade inata do hospedeiro (tais como macrófagos, neutrófilos, células dendríticas e células NK). IL1β atua no sistema nervoso central (SNC) aumentando a sensibilidade à dor e TNFα e IL6 atuam no hipotálamo, o órgão responsável pela termoregulação causando a febre. O desequilíbrio, geralmente a produção exagerada, destas citocinas está implicado na patologia de várias doenças humanas, incluindo o câncer (Locksley *et al.* 2001). De fato, na malária tem sido relatada a presença de níveis elevados destas citocinas (Clark *et al.* 2006; Clark 2007; Clark *et al.* 2008) no soro de pacientes e vários estudos relatam que polimorfismos nos genes que codificam para estas citocinas têm sido associados à severidade da infecção (Aidoo *et al.* 2001; Gyan *et al.* 2002; Hananantachai *et al.* 2007; Atkinson *et al.* 2008; Clark *et al.* 2009).

Além disso, TNFα e IFNγ aumentam a expressão de moléculas de adesão celular, tais como CD36, ICAM e VCAM, que contribuem para o aumento do sequestramento de hemácias infectadas nos capilares sanguíneos e, consequentemente, para a malária cerebral. Os mecanismos da anemia grave na malária são pouco conhecidos. É muito

provável que envolva a destruição de hemácias não infectadas do sangue periférico e a supressão da hematopoiese entre outros mecanismos. A supressão da hematopoiese é sabidamente regulada por citocinas (McDevitt *et al.* 2004; Thawani *et al.* 2006; Thawani *et al.* 2009). A destruição de hemácias não infectadas pode ocorrer devido à deposição de moléculas do parasito nestas células. Durante a ruptura dos eritrócitos, os níveis plasmáticos de antígenos do parasito aumentam muito. Muitas dessas moléculas podem aderir a outras células sadias, possivelmente resultando em destruição destas hemácias pelo sistema do complemento ou por anticorpos anti-*Plasmodium* (Waitumbi *et al.* 2000; Goka *et al.* 2001; Stoute *et al.* 2003). Dessa forma, pode se concluir que as citocinas pro-inflamatórias estão envolvidas em todos os processos patológicos da malária.

Na malária murina as citocinas pró-inflamatórias também desempenham um importante papel na patogenia da infecção (Cordeiro *et al.* 1983; Grau *et al.* 1989; de Kossodo *et al.* 1993; Amani *et al.* 1998; Amani *et al.* 2000; Angulo *et al.* 2002; Li *et al.* 2003). Os modelos murinos de malária têm sido muito utilizados e fornecem uma importante ferramenta para o estudo dos mecanismos imunológicos envolvidos na proteção do hospedeiro e na patogênese da doença (Lamb *et al.* 2006). Algumas diferenças entre os modelos humano e murino, como esperado, são observadas. Em camundongos, os mecanismos de ação de IL1β, TNFα e IL6 são muito similares aos de seres humanos. Estas citocinas atuam no SNC e no centro de termoregulação no hipotálamo. Entretanto, assim como na sepsis murina, o resultado é a hipotermia ao invés de febre (Leon 2002; Utsuyama *et al.* 2002). Os mecanismos da febre na malária ainda não são bem compreendidos, mas já está estabelecido que as citocinas produzidas durante a imunidade inata desempenham um importante papel.

A imunidade inata é um das primeiras barreiras que o organismo possui para detectar a presença de patógenos invasores e rapidamente eliminá-los ou iniciar uma resposta imune adaptativa protetora. Na malária, já foi demonstrado que a imunidade inata do hospedeiro é ativada ainda nas fases iniciais da infecção e que a sobrevivência do hospedeiro, principalmente no caso da malária murina, está ligada a capacidade do animal em controlar a parasitemia nos primeiros 7 – 14 dias de infecção (Stevenson *et al.* 2004). Usando um modelo que mimetiza a infecção natural pelo *P. falciparum*, Fidel Zavala e sua equipe na Universidade John Hopkins em Baltimore (EUA) demonstraram que os linfócitos T $CD8^{+}$, que medeiam a imunidade protetora contra a malaria, são primados por células dendríticas nos linfonodos cutâneos ainda nas primeiras horas de

infecção durante a entrada dos esporozoítos (Chakravarty *et al.* 2007). Posteriormente, Kim e colaboradores observaram uma resposta de células NK com um domínio de genes induzidos por interferons (IFN) na resposta inicial (primeiras 16 horas de infecção) de camundongos infectados com *P. chabaudi* (Kim *et al.* 2008). Estas observações evidenciam um papel primordial da imunidade inata na defesa do hospedeiro contra a infecção por *Plasmodium*.

Por muito tempo, a imunidade inata foi tida como inespecífica e não tão evoluída quanto a imunidade adquirida e era estudada apenas por pesquisadores que tinham interesse em inflamação. Entretanto, uma nova era no estudo da imunidade inata teve início quando, no início dos anos 90, a pesquisadora alemã Christiane Nüsslein-Volhard recebeu o prêmio Nobel de Fisiologia e Medicina pelo seu trabalho que identificou genes que eram responsáveis pela orientação dorso-ventral durante o desenvolvimento embrionário da larva da mosca *Drosophila melanogaster (Nusslein-Volhard et al. 1980).* A estes genes foi dado o nome Toll (que em alemão significa legal, espantoso). Moscas que eram deficientes para Toll apresentavam deformações graves na sua morfologia. Em 1996, o pesquisador Jules A. Hoffman e sua equipe observaram que os genes de Toll codificavam para receptores transmembrana que possuíam domínio intracelular com alta similaridade ao receptor da citocina pro-inflamatória IL1 de mamíferos (Lemaitre *et al.* 1996). Estes mesmos autores demosntraram que os Tolls possuíam um papel essencial na imunidade da mosca contra infecções por fungos devido ao fato da ativação dos Tolls induzir a síntese de peptídeos antimicrobianos (Lemaitre *et al.* 1996). Em 1994, Nomura e sua equipe na Escola Médica de Nippon em Kanagawa (Japão) identificaram pela primeira vez em seres humanos a presença de receptores do tipo Toll (TLRs) – nome dado devido à sua semelhança com os receptores Toll da *Drosophila* (Nomura *et al.* 1994). Em 1997, Charles Janeway e Ruslan Medzhitov demonstraram que o até então desconhecido receptor de lipopolisacarídeo (LPS), componente da membrana de bactérias gram-negativas, em mamíferos era na verdade um TLR (o TLR4) o qual, quando ativado por LPS, induzia a expressão de genes necessários para uma resposta imune adquirida (Medzhitov *et al.* 1997). Desde então, muito do que se sabe hoje da imunidade inata deve-se à descoberta dos TLRs neste período (Medzhitov 2001; Takeda *et al.* 2005). Extensas análises destes receptores da imunidade inata revelaram especificidade em relação ao reconhecimento de vários ligantes, expressão em diferentes tipos celulares (Tabela 1) e um papel destes receptores em múltiplas doenças envolvendo a imunidade inata e adquirida.

Hoje é sabido que os TLRs são capazes de reconhecer moléculas características de grupos de microorganismos, tais como: i) Lipopolissacarídeo (LPS), presente na parede celular de bactérias gram-negativas; ii) ácido lipoteitóico, presente em bactérias gram-positivas,; iii) proteínas do flagelo de bactérias e protozoários; iv) seqüências de DNA ou RNA de origem bacteriana, viral ou protozoária, entre outros (Tabela 1). Atualmente, 11 membros da família TLRs foram identificados em mamíferos. O número de ligantes reconhecidos por estes receptores continua a crescer, e parece evidente que múltiplos ligantes existem para um mesmo receptor (Barton *et al.* 2002).

Tabela 1 – Os receptores do tipo Toll, seus ligantes e sua localização celular

Receptor	Ligante(s)	Localização do ligante	Moléculas adaptadoras	Localização	Tipo celular
TLR1	Múltiplos Lipopetídios Triacil	Bactéria	MyD88/MAL	Superfície celular	• Monócitos/Macrófagos • Subtipos de Células Dendríticas • Linfócitos B
TLR2	Glicolípides Lipopeptídios Lipoproteínas HSP70 Zimozan Glicofosfatidil Inositol (GPI)	Bactéria Bactéria Bactéria Célula hospedeira Fungos Protozoários	MyD88/MAL	Superfície celular	• Monócitos/Macrófagos • Células Dendríticas • Mastócitos
TLR3	RNAs de fita dupla, Poly:IC	Virus	TRIF	Endosoma celular	• Células Dendríticas • Linfócitos B
TLR4	Lipopolisacarídeo (LPS) Várias proteínas *"heat shock"* Fibrinogênio Glicofosfatidil Inositol (GIPL) Ácido hialorônico	Bactérias gram-negativas Célula hospedeira Célula hospedeira *Trypanosoma cruzi* Célula hospedeira	MyD88/MAL/TRIF/TRAM	Superfície celular	• Monócitos/Macrófagos • Células Dendríticas mielóides • Mastócitos • Epitélio intestinal
TLR5	Flagelina	Bactéria	MyD88	Superfície	• Monócitos/Macrófagos

				Celular	• Subtipos de Células Dendríticas • Epitélio intestinal
TLR6	Múltiplos lipopeptídeos Diacil	Micoplasma	MyD88/MAL	Superfície Celular	• Monócitos/Macrófagos • Mastócitos • Linfócitos B
TLR7	Imidazolquinolina loxoribina bropirimina RNAs de fita única	Compostos Sintéticos pequenos	MyD88	Endosoma celular	• Monócitos/Macrófagos • Células Dendríticas Plasmacitóides • Linfócitos B
TLR8	Compostos Sintéticos pequenos RNAs de fita única		MyD88	Endosoma celular	• Monócitos/Macrófagos • Subtipos de Células Dendríticas • Mastócitos
TLR9	CpG DNA não metilado	Bactéria	MyD88	Endosoma celular	• Monócitos/Macrófagos • Células Dendríticas Plasmacitóides • Linfócitos B
TLR10	desconhecido	desconhecido	desconhecido	Superfície celular	• Monócitos/Macrófagos • Linfócitos B
TLR11	profilina	*Toxoplasma gondii*	MyD88	Superfície celular	• Monócitos/Macrófagos • Hepatócitos • Rim • Epitélio da bexiga

A ativação dos TLRs resulta na estimulação da resposta imune inata através de vias de sinalização celulares envolvendo moléculas adaptadoras e fatores de transcrição que levam a produção de citocinas (Takeda *et al.* 2005) (figura 1). De uma maneira geral, pode-se dizer que o principal produto da ativação destes receptores são as citocinas pró-inflamatórias.

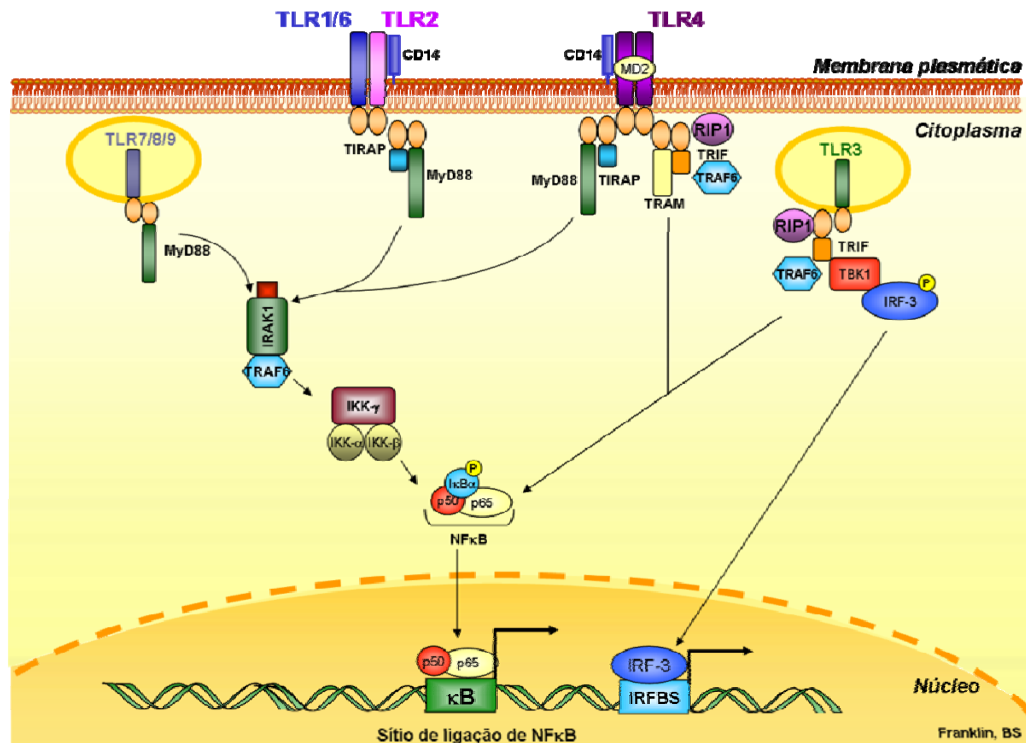

Figura 1 - Esquema da via de sinalização celular dos TLRs. Os TLRs são expressos em diferentes compartimentos celulares onde reconhecem diferentes ligantes. Uma vez ativados por seus respectivos ligantes, os TLRs recrutam a proteína adaptadora Myd88 *(myeloid differentiation primary-response protein 88)*. Esta, por sua vez, ativa IRAKs *(IL-1R-associated kinases)* e TRAF6 (*tumour-necrosis-factorreceptor-associated factor 6*) e leva à ativação do complexo IKK *(inhibitor of nuclear factor-κB (IκB)-kinase complex)*. Esta via de sinalização é utilizada por TLR1, 2, 4, 5, 6, 7, 8 e 9 e libera fator nuclear κB (NF-κB) do seu complexo inibidor permitindo sua translocação para o núcleo e a indução da expressão de citocinas pró-inflamatórias. MAL/TIRAP, uma segunda proteína adaptadora contendo um domínio TIR está envolvida na via de sinalização de TLR2 e 4. Outras duas proteínas adaptadoras que contém domínios TIR e que também estão envolvidas na sinalização independente de Myd88 são TRIF e TRAM (Takeda *et al.* 2005). A ativação dessa via leva a fosforilação do fator de transcrição IRF3 e à indução de genes de IFNs do tipo I.

Em várias doenças infecciosas, a produção de citocinas pró-inflamatórias pelo sistema imune inato em resposta à infecção protege o organismo hospedeiro controlando o crescimento do patógeno. Entretanto, como mencionado anteriormente, uma produção exagerada destas citocinas resulta em quadros patológicos tais como sepsis (El-Menyar *et al.* 2009), falha múltipla dos órgãos e doenças auto-imunes (ex: lúpus eritromatoso) (Lourenco *et al.* 2009; Nalbandian *et al.* 2009).

Isso é especialmente importante no caso da malária onde é observada uma inflamação sistêmica exagerada que pode ocasionar a morte do hospedeiro. Acredita-se também que as manifestações mais graves da malária, como a malária cerebral, podem ser resultado de uma resposta inflamatória disseminada mais do que aos danos teciduais

causados pelo sequestramento de hemácias infectadas e a consequente hipóxia. Isso é evidenciado em vários casos de autópsias de vítimas de malária cerebral nos quais não foi observada a presença de células infectadas no cérebro. De fato, a malaria cerebral geralmente ocorre quando a parasitemia ainda é baixa (< 10%) (Doolan *et al.* 2009).

Estes achados sugerem que a febre e os sintomas clínicos da malária não são apenas causados pela presença do parasito no sangue e sim, por uma resposta excessiva do sistema imune do hospedeiro. Nesse sentido, a malária se assemelha à síndrome séptica (Hemmer *et al.* 2008) e a outras doenças caracterizadas por uma reação inflamatória exagerada. De fato, os relatos de indivíduos residentes em áreas de transmissão holoendêmica que são continuamente infectados pelo parasito e, no entanto, não desenvolvem sintomas contribuem para essa hipótese. Nos últimos anos tem havido um consenso de que grande parte da patogenia envolvida na malária pode ser resultado de uma hiper-ativação de vias inflamatórias, resultando em uma produção excessiva de citocinas, tais como TNFα, linfotoxina (LT), interferon-gamma (IFNγ), IL6 e IL12 (Clark *et al.* 2000; Lou *et al.* 2001; Schofield *et al.* 2005; Clark *et al.* 2006; Walther *et al.* 2006).

Dada a importância dos TLRs para a síntese de citocinas pró-inflamatórias, e ao fato de terem sido identificados ligantes destes receptores em uma variedade de patógenos que infectam o homem (Barton *et al.* 2002; Gazzinelli *et al.* 2006), o papel dos TLRs na imunidade do hospedeiro contra infecções tem sido muito estudado nas últimas décadas. Nesse âmbito, camundongos deficientes para os TLRs ou para a proteína adaptadora Myd88 ($Myd88^{-/-}$), responsável pela sinalização celular da maioria dos TLRs (Figura 1), têm se mostrado um modelo valioso para o estudo destes receptores em infecções por vírus, protozoários, bactérias e fungos. De uma maneira geral, animais $Myd88^{-/-}$ são imunocomprometidos e sucumbem à infecção por diferentes patógenos, incluindo vírus como o *Herpes simplex* (Mansur *et al.* 2005), bactérias como a *Brucela abortus* (Weiss *et al.* 2005; Macedo *et al.* 2008), e os protozoários *Toxoplasma gondii* (Chen *et al.* 2002; Scanga *et al.* 2002), *Trypanosoma cruzi* (Campos *et al.* 2004), *Leishmania major* (de Veer *et al.* 2003; Muraille *et al.* 2003) e *Mycobacterium tuberculosis* (Fremond *et al.* 2004; Scanga *et al.* 2004). Em todos estes modelos, a susceptibilidade aumentada de animais $Myd88^{-/-}$ foi associada com a deficiência destes animais em produzir níveis ideais de citocinas pró-inflamatórias necessários para o controle da parasitemia nos primeiros estágios da infecção e para a geração de uma imunidade adquirida protetora e memória imunológica.

A partir destes estudos é possível concluir que os TLRs possuem papel essencial na proteção do hospedeiro contra infecções por protozoários. Seu papel na malária, no entanto, ainda não é conhecido.

2 Justificativa

2 JUSTIFICATIVA

Apesar não ser reconhecida por muitos pesquisadores como uma doença inflamatória, a malária é caracterizada por uma a inflamação sistêmica com a presença de níveis séricos elevados de citocinas pró-inflamatórias. Estes níveis de citocinas geralmente são deletérios para o organismo podendo levar ao choque séptico, a falha múltipla dos órgãos e à morte. Os mecanismos para essa hiper produção de citocinas encontrada em pacientes com malária ainda não são conhecidos. No entanto, é evidente que as citocinas pró-inflamatórias possuem um papel fundamental na patogenia da malária.

Em infecções por outros protozoários parasitos como o *T. cruzi*, *T. gongii* e *Leishmania,* as citocinas possuem um papel essencial no controle da replicação do parasito e na proteção do hospedeiro. Dada a importância da ativação dos TLRs para a síntese destas citocinas seu papel na proteção do hospedeiro contra a infecção por estes protozoários foi demonstrado. Da mesma forma, seu envolvimento na patogenia da malária é muito provável.

O presente trabalho se justifica devido à necessidade de se compreender o papel dos TLRs na malária. Acreditamos que o entendimento dos mecanismos patológicos envolvidos na malária pode ajudar no desenvolvimento de novas terapias para tratamento e prevenção da doença ou para a geração de novas estratégias de vacinação.

3 Objetivos

3 OBJETIVOS

3.1 Objetivo geral

Avaliar o papel dos receptores do tipo Toll (TLRs) na malária.

3.2 Objetivos específicos

Avaliar o papel dos receptores do tipo Toll (TLRs) na imunidade inata e adquirida contra a malária.

Identificar qual TLR reconhece o *Plasmodium* falciparum ou *P. chabaudi* e ativa as respostas imunes na malária humana e murina respectivamente.

Avaliar como a interferência com a função dos TLRs pode influenciar a produção de citocinas e os sintomas da malaria grave.

4 Material e Métodos

4 MATERIAL E MÉTODOS

4.1 Pacientes

Com a colaboração dos Drs. Luiz Hildebrando, Mauro Tada, Cor Jesus e Dhélio Pereira, todos do Centro de Pesquisas em Medicina Tropical de Rondônia (CEPEM). Selecionamos 57 indivíduos na fase aguda da infecção por *P. falciparum* residentes nas proximidades do município de Porto Velho (RO). Todos os indivíduos relatavam a presença de sintomas nas últimas 24 horas e tiveram a infecção por *P. falciparum* comprovada pelo exame de sangue à microscopia óptica em gota espessa. Casos de co-infecção por *P. vivax* foram excluídos após confirmação por PCR.

4.2 Comitê de Ética

Um termo de consentimento foi obtido de cada indivíduo incluído no estudo. Este estudo foi aprovado pelo Conselho de Ética e Pesquisa do Instituto René Rachou, Conselho nacional de Ética e Pesquisa (CONEP) e IRB Universidade de Massachusetts. Depois de detectada a positividade da infecção, os indivíduos foram imediatamente tratados com mefloquina (4 comprimidos por adulto). Amostras de sangue periférico destes indivíduos foram coletadas antes e 30 dias após o tratamento antimalárico.

4.3 Obtenção das células mononucleares do sangue periférico (PBMCs)

O sangue periférico (~20 ml) dos indivíduos selecionados foi coletado em tubos de coleta a vácuo e heparinizados. Células mononucleares do sangue periférico (PBMCs) foram isoladas segundo protocolo previamente estabelecido (Ockenhouse *et al.* 2006). Brevemente, aos 20 ml de sangue foram adicionados a 10 ml de PBS estéril. Essa mistura foi depositada sobre 15 ml de Ficoll Paque (GE Healthcare) e centrifugada a 1500 *x g* por 20 minutos à temperatura ambiente.

4.4 Estimulação ex vivo de PBMCs

As PBMCs (2×10^5) isoladas de pacientes em fase aguda da infecção por *P. falciparum* foram estimuladas por 12 horas com diferentes concentrações de agonistas de TLRs

sintéticos de alta pureza (Invivogen). Para a estimulação de TLR4 foi utilizado LPS, Pam2cysk4 para TLR2/1, Poly:IC para TLR3, CL075 para TLR7/8 e CpG ODN para TLR9. Os níveis de IL1β, IL12, TNFα e IL10 foram medidos no sobrenadante das culturas por ELISA (R&D Systems).

4.5 Obtenção de RNA de PBMCs de pacientes com malária por P. falciparum

O RNA total foi extraído de PBMCs de pacientes com malária aguda usando o kit RNeasy (Qiagen) segundo instruções do fabricante. A integridade e pureza das amostras de RNA foram confirmadas usando-se o sistema de eletroforese automatizado (Experion Bio-Rad, Hercules, CA) e um espectrofotômetro ND-1000 (NanoDrop Technologies Inc, Wilmington, DE). O RNA total foi extraído de cada paciente para análise durante a fase aguda da doença e 30 dias após tratamento anti-malárico específico. Assim, cada paciente serviu como seu controle para a análise do perfil de expressão gênica. Após extraído, o RNA total foi convertido em cDNA usando-se o kit SuperScript III (Invitrogen) segundo as instruções do fabricante. Este material foi marcado com fluorocromos e analisado em ensaios de *microarray* realizados na Universidade de Massachusetts. Os genes que apresentarem alterações significativas em sua expressão foram submetidos à análise por Real Time PCR para confirmação.

4.6 Experimentos de Microarray

Um total de 23 pacientes foi selecionado para o estudo de microarray por apresentarem febre no momento da coleta de material. Ao final, dois foram desqualificados do estudo; um devido à falha no tratamento e persistência da parasitemia e outro devido à co-infecção por *P. vivax* detectada após exame de PCR. Amostras de 21 pacientes foram amplificadas usando o kit SenseAMP (Genisphere) e o ensaio de *Microarray* realizado na *Microarray Facilities* do Hospital Geral de Massachusetts (Cambridge, MA) usando-se um chip Operon (desenhado pelo Dr. B

Seed e M. Freeman, da Harvard University). Este chip é composto por ~17000 sondas de cDNAs que abrangem diversas vias de sinalização celulares.

4.7 Análise dos Microarrays

As amostras submetidas ao *microarray* foram analisadas por filtragem simples, normalização e média utilizando-se o software BioArray Software Enviroment (BASE https://base.mgh.harvard.edu). Para a análise e visualização dos agrupamentos, utilizamos o software Tiger Multi Experiment Viewer (TMEV, http://www.tm4.org). Os valores de "p" foram obtidos por permutação através da correção Bon Ferroni padrão. Os genes que passaram no critério de significância foram analisados com o algoritmo KMC (K median).

A anotação e determinação da função biológica dos genes cuja expressão foi aumentada durante a doença em ambos os *arrays* foi realizada com o software Onto Express (http://vortex.cs.wayne.edu/projects.htm). A identificação de sítios de ligação para fatores de transcrição nos genes aumentados nos *arrays* foi realizada através do software online Advanced Biomedical Computer Center ABCC (http://grid.abcc.ncifcrf.gov/promoters/comparePromoters.php).

4.8 Camundongos

Em todos os experimentos os animais pertenciam à linhagem C57BL/6 produzidos por retrocruzamento por pelo menos 8 gerações. Foram utilizados animais deficientes para TLRs (TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$), para a proteína adaptadora MyD88 (MyD88$^{-/-}$) e o co-receptor CD14 (CD14$^{-/-}$), para a proteína Unc93b (3D), que participa do translocamento dos TLRs 3, 7, 8 e 9 do retículo endoplasmático para o endosoma celular, bem como animais deficientes para as citocinas IL12 (IL12$^{-/-}$) IFN-γ (IFN-γ$^{-/-}$). Os animais foram mantidos sob condições padrões no biotério do Centro de Pesquisas René Rachou/FIOCRUZ e da Universidade de Massachusetts.

4.9 Parasitos

Para os experimentos de infecção experimental, foram utilizadas as cepas AS de *P. chabaudi* e ANKA de *P. berghei*. Os camundongos foram infectados com injeção intraperitoneal com 10^5 eritrócitos infectados (iRBCs) e monitorados para patologia associada à malária ao longo da infecção. Esta avaliação incluiu porcentagem de parasitemia no sangue periférico por esfregaço sangüíneo corado com Giemsa (Merk, Darmstadt, Germany), variações de temperatura e peso, mortalidade e sintomas da malária cerebral no caso da infecção por *P. berghei* ANKA tais como: pêlo arrepiado, postura anormal, paralisia, convulções, coma e morte(Coban *et al.* 2007).

4.10 Cultura de esplenócitos

Células esplênicas dos animais foram obtidas macerando os baços por uma membrana de nylon. Os eritrócitos foram lisados com solução de lise (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA; pH 7.4). Os esplenócitos foram ressuspendidos em meio RPMI (Gibco, USA) com 5% soro bovino fetal (SBF) (Gibco, USA), 1% de antibiótico gentamicina (Schering Plough, RJ, Brazil) e 2,5 x 10^6 células foram cultivadas por 48 horas na ausência de estímulo.

4.11 Avaliação dos níveis de citocinas

Os níveis de citocinas (IFNγ, TNFα, IL1β, IL6, IL10 e IL12) foram avaliados no soro e em sobrenadantes de cultura de esplenócitos usando kits de ELISA convencionais (R&D Systems, San Diego, CA, USA) e o Kit Cytokine Bead Array (CBA) (BD Biosciences) de acordo com as especificações do fabricante.

4.12 Marcação intracelular de citocinas

Para avaliação dos níveis intracelulares de citocinas, 5 x 10^5 células esplênicas foram tratadas com Brefeldina A (1mg/ml) por 4h em placas de 96 poços, lavadas e incubadas com anticorpos marcados com FITC (fluorescein isothiocyanate) ou PE

(phycoerythrin) por 20 minutos a 4°C. Após incubação, as células foram lavadas e fixadas com PBS 1X contendo 2% de formaldeído (Sigma Chemical Co., St. Louis, MO). As células fixadas foram então permeabilizadas com saponina, marcadas utilizando-se anticorpos monoclonais anti-citocinas, re-fixadas e analisadas por citometria de fluxo (FACs). Os anticorpos utilizados para marcação foram anti-imunoglobulinas FITC e PE (utilizados como controle da especificidade da marcação), anti-TCR-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-NK-FITC, anti-CD69-FITC, anti-CD25-FITC, anti-DC-FITC, anti-11b-FITC, anti-CD4-PE, anti-CD8-PE, anti-IL-6-PE, anti-IL-12-PE, anti-IL-1a-PE, anti-TNF-a-PE, anti-IFN-g-PE, anti-CD4-PE-Cy, anti-CD8-PE-Cy (PharMingen, San Diego, CA). A análise de Linfócitos, células NK e células dendríticas foi realizada considerando-se sua distribuição, característica de células mononucleares, baseada nos perfis de tamanho e granulosidade das células, além da expressão de seus marcadores específicos. A intensidade de florescência detectada nas diferentes condições experimentais foi avaliada usando-se o software Cell Quest (Becton & Dickinson, San Jose, CA).

4.13 PCR quantitativo em tempo real (Real Time PCR)

Real Time PCR (qPCR) foi utilizado para a validação de genes identificados no microarray como diferencialmente expressos durante a malária humana e de camundongos infectados com *P. chabaudi,* após vários dias de infecção, foi realizado utilizando-se kits SYBR Green e o termociclador *ABI Prism 7000 Sequence Detection System* (Applied Biosystems). Os iniciadores (*primers* - Tabela 2) utilizados nas reações de *Real Time PCR* foram desenhados com o auxílio do software *Primer3 Express* (Applied Biosystems). Todos os *primers* foram desenhados para uma temperatura de anelamento a 60°C. As condições da reação de *Real Time PCR* foram: 95°C por 2 min, 95°C por 15 segundos e 60°C por 1 min (25 ciclos).

Os níveis de mRNA dos genes alvo foram avaliados utilizando-se a metodologia de "quantificação relativa" que faz uso da fórmula $2^{-\Delta\Delta Ct}$ onde os dados das amostras de animais infectados são normalizados contra os níveis de mRNA detectados para o gene da β-actina e expressados em "*fold change*" comparados com os das amostras de animais não-infectados.

Tabela 2 – Iniciadores *(Primers)* utilizados nas reações de Real Time PCR.

Primer	Forward (5' - 3')	Reverse (5' - 3')
TLR2	CGTTGTTCCCTGTGTTGCT	AAAGTGGTTGTCGCCTGCT
TLR3	TTGCGTTGCGAAGTGAAG	TAAAAAGAGCGAGGGGACAG
TLR4	TTCACCTCTGCCTTCACTACA	GGGACTTCTCAACCTTCTCAA
TLR7	GCTGTGTGGTTTGTCTGGTG	CCCCTTTATCTTTGCTTTCC
TLR9	GAAAGCATCAACCACACCAA	ACAAGTCCACAAAGCGAAGG
B-actin	GGATGCAGAAGGAGATTACTG	CGATCCACACAGAGTACTTG

4.14 Análises estatísticas

Cada experimento foi realizado três ou quatro vezes utilizando-se oito animais C57BL/6 e número correspondente de Knockouts infectados ou não com *P. chabaudi* ou *P. berghei* ANKA analisados individualmente. A média aritmética e erro padrão da média dos dados de parasitemia, peso, temperatura, níveis de citocinas no soro e baço, bem como número/porcentagem de células produzindo citocinas observadas nos experimentos de FACs foram calculados. A determinação da significância estatística das diferenças observadas foi realizada utilizado-se o teste T de *Student* ou Mann-Whitney quando a distribuição dos dados não atingia os critérios de uma distribuição Gaussiana. Diferenças foram consideradas significativas quando $p < 0.05$. O software Graphpad Instat 4.0 foi utilizado para as análises estatísticas.

5 Resultados: Artigos

ARTIGO 1

Myd88-dependent activation of Dendritic Cells and CD4$^+$ T lymphocytes mediates symptoms, but is not required for the immunological control of parasites during rodent malaria

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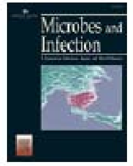
Este artigo apresenta a síntese dos resultados relativos ao objetivo específico 1:

1) Avaliar o papel dos receptores do tipo Toll (TLRs) na imunidade inata e adquirida contra a malária.

Neste trabalho demonstramos que Myd88 e os TLRs são essenciais para a produção de citocinas pró-inflamatórias que orquestram a patogenia da malária por *P. chabaudi*. Entretanto, os TLRs não são essenciais para o controle da parasitemia nem para resolução da infecção, ou mesmo para a produção de anticorpos de memória contra o parasito. Mais especificamente, mostramos que as Células Dendríticas (DCs) são cruciais para iniciar as respostas de células T e promover os sintomas da malária de maneira dependente de TLR.



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Original article

MyD88-dependent activation of dendritic cells and CD4^{+} T lymphocytes mediates symptoms, but is not required for the immunological control of parasites during rodent malaria

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Abstract

We investigated the role of different TLRs and MyD88 in host resistance to infection and malaria pathogenesis. TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$ or CD14$^{-/-}$ mice showed no change in phenotypes (parasitemia, body weight and temperature) when infected with *Plasmodium chabaudi chabaudi* (AS). MyD88$^{-/-}$ mice displayed comparable ability to wild type animals in controlling and clearing parasitemia. Importantly, MyD88$^{-/-}$ mice exhibited impaired production of TNF-α and IFN-γ as well as attenuated symptoms, as indicated by changes in body weight and temperature during parasitemia. Consistently, CD11b^{+} monocytes and CD11c^{+} dendritic cells from infected MyD88$^{-/-}$ mice were shown impaired for production of pro-inflammatory cytokines, and in initiating CD4^{+} T cell responses. Importantly, the inhibition of T cell activation with anti-CD134L, mostly inhibited IFN-γ, partially inhibited TNF-α production, and protected the animals from malaria symptoms. Our findings suggest that MyD88 and possibly its associated TLRs expressed by dendritic cells play an important role in pro-inflammatory responses, T cell activation, and pathogenesis of malaria, but are not critical for the immunological control of the erythrocytic stage of *P. chabaudi*.
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Keywords: Toll-like receptors; Innate immunity; Cytokines; Pathogenesis; Malaria and *Plasmodium chabaudi*

1. Introduction

Toll-like receptors (TLRs) are activated by diverse microbial molecular structures and share a cytoplasmic domain with high homology to the IL-1 receptor (IL-1R) known as the Toll-like receptor/IL-1 resistance (TIR) domain. The TIR domains of TLRs interact with cytoplasmic adapter molecules that also contain TIR domains. The best-studied example of a TIR domain containing adapter protein is the myeloid differentiation primary-response gene 88 (MyD88), which transduces signals for all of the known TLRs (except for TLR3) and the IL-1/IL-18 Receptors [1]. Another key element, shared by TLR2 and TLR4 receptors is CD14, which is a co-receptor with lipotransferase activities that potentiates the activation of TLR2 and TLR4 by lipid containing microbial molecules [2].

To date, TLRs have been implicated in every known category of microorganism that causes human disease, including

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protozoan parasites [3]. The early elimination of invasive microorganisms is the primary goal of the innate immune system. The TLRs are critical for all aspects of this process, including the recruitment of phagocytes to infected tissue, activation of effector mechanisms and subsequent microbial killing [1]. Further, dendritic cells (DCs) activated by TLR agonists are key elements for initiating and shaping acquired immunity and development of memory lymphocytes and long term immune responses [4].

Acute infection with *Plasmodium* results in the production of high levels of pro-inflammatory cytokines, that orchestrate host immunity to infection, but at the same cause many of the observed symptoms [5,6]. We hypothesize that TLRs are central mediators in the pathogenesis of malaria. Evidence exists that TLRs are critical for eliciting the synthesis of pro-inflammatory cytokines during infection with protozoan parasites. For example glycosylphosphatidylinositol (GPI) anchors derived from protozoan parasites [3,7], including *Plasmodium falciparum* [8] have been shown to trigger the synthesis of pro-inflammatory cytokines through TLR2 and TLR4. In addition, hemozoin carrying parasite DNA have been shown to activate human and mouse DCs via TLR9 [9,10]. Consistently, microarray analysis of RNA derived from peripheral blood mononuclear cells from individuals infected with *P. falciparum*, indicate that gene activation by TLR signaling through NF-κB is significantly upregulated [11]. Altered frequency of functional single nucleotide polymorphisms (SNPs) in *TLR2*, *TLR4*, and *TLR9* genes has been shown in individuals from endemic regions *P. falciparum* infection and associated with severe malaria in pregnancy [12,13]. Importantly, $MyD88^{-/-}$, $TLR2^{-/-}$ and $TLR9^{-/-}$ mice have attenuated symptoms when infected with *Plasmodium berghei* [14,15].

Here, we investigated the role of various TLRs into two critical immunological aspects of malaria: (i) cytokine-mediated clinical symptoms; and (ii) control of parasitemia. We observed that $TLR2^{-/-}$, $TLR4^{-/-}$, $TLR6^{-/-}$, $TLR9^{-/-}$ and $CD14^{-/-}$ mice showed similar parasitemia, production of pro-inflammatory cytokines and pathological parameters, when compared to wild type (WT) mice. The $MyD88^{-/-}$ mice also showed comparable parasitemia to WT mice, which was nevertheless associated with an impaired production of TNF-α and IFN-γ and attenuated symptoms. Importantly, DCs from $MyD88^{-/-}$ showed impaired production of pro-inflammatory cytokines, and were less able to initiate T cell responses in infected mice. Consistently, blockage of T cell activation during primary *Plasmodium chabaudi* infection, greatly inhibited IFN-γ and TNF-α production, protecting animals against the malaria symptoms. In conclusion, our findings suggest that MyD88, in coordination with a combination of TLRs expressed by dendritic cells, plays a role in pro-inflammatory responses, T cell activation and pathogenesis of malaria, but are not critical for the immunological control of *P. chabaudi* infection.

2. Material and methods

2.1. Rodent model of malaria and knockout mice

The *P. chabaudi* AS strain was used in our experimental infections [16]. Mice were infected with 10^5 infected erythrocytes, and checked daily for survival; moribund animals were scored as dead, and euthanized. Laboratory values that reflect malaria-associated pathology were determined every 2–3 days throughout the 30 day study period. This evaluation included percent parasitemia by Giemsa stained blood smears (Merk, Darmstadt, Germany), determination of temperature by rectal thermocouple, and measurement of body weight [16,17]. $MyD88^{-/-}$, $TLR2^{-/-}$, $TLR4^{-/-}$, $TLR6^{-/-}$, $CD14^{-/-}$ and IFN-$\gamma^{-/-}$ all backcrossed at least eight generations into the C57BL/6 background were used in the experiments described above.

2.2. Mouse treatment with monoclonal antibodies

Isotype matched monoclonal antibodies anti-b-galactosidase (GL113 − control), anti-CD4 (GK 1.5) and anti-CD134L (OX89) were purified from ascites, and given intraperitoneally, at a dose of 0.5 mg/mouse diluted in 200 µl of PBS, at days 7 and 1 one day prior infection and once a week thereafter.

2.3. Cytokine measurements

Supernatant of splenocyte cultures and sera were collected from each mouse, for measuring the levels of cytokines (i.e., IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70). Splenocytes were obtained by macerating spleens through a nylon mesh, erythrocytes lysed with ice-cold isotonic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA; pH 7.4). Splenocytes were then suspended in RPMI, 5% fetal calf serum (Cultilab, Campinas Brazil), 1% gentamicyn (Schering Plough, RJ, Brazil) at concentration of 5×10^6 cells/ml and cultured in 24-well plate for 48 h without stimulum. Cytokine levels were measured in sera and culture supernatants by using Cytokine bead array kit (CBA) (BD Biosciences), according to the manufacture's protocol.

2.4. Staining to determine splenocytes profile and single-cell cytoplasmic cytokine staining

Five hundred thousand cells were analyzed after 4 h cultured in 96-well plates in 200 µl medium with brefeldin-A (1 µg/ml). After that, the cells were washed and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- or phycoerythrin-cy-chrome (PE-Cy) labeled antibody solutions for 20 min at 4 °C. After washed twice, the preparations were fixed with 200 µl of 2% formaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS. The fixed cells were permeabilized with a solution of saponin, stained, using anti-cytokine monoclonal antibodies, fixed and analyzed using FACS. At least 35,000-gated events were acquired for later analysis. The antibodies used for the staining were immunoglobulin FITC and PE controls, anti-TCR-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-NK-FITC, anti-CD69-FITC, anti-CD25-FITC, anti-DC-FITC, anti-11b-FITC, anti-CD4-PE, anti-CD8-PE, anti-IL-6-PE, anti-IL-12-PE, anti-IL-1α-PE, anti-TNF-α-PE, anti-IFN-γ-PE, anti-CD4-PE-Cy, anti-CD8-PE-Cy (PharMingen, San Diego, CA).

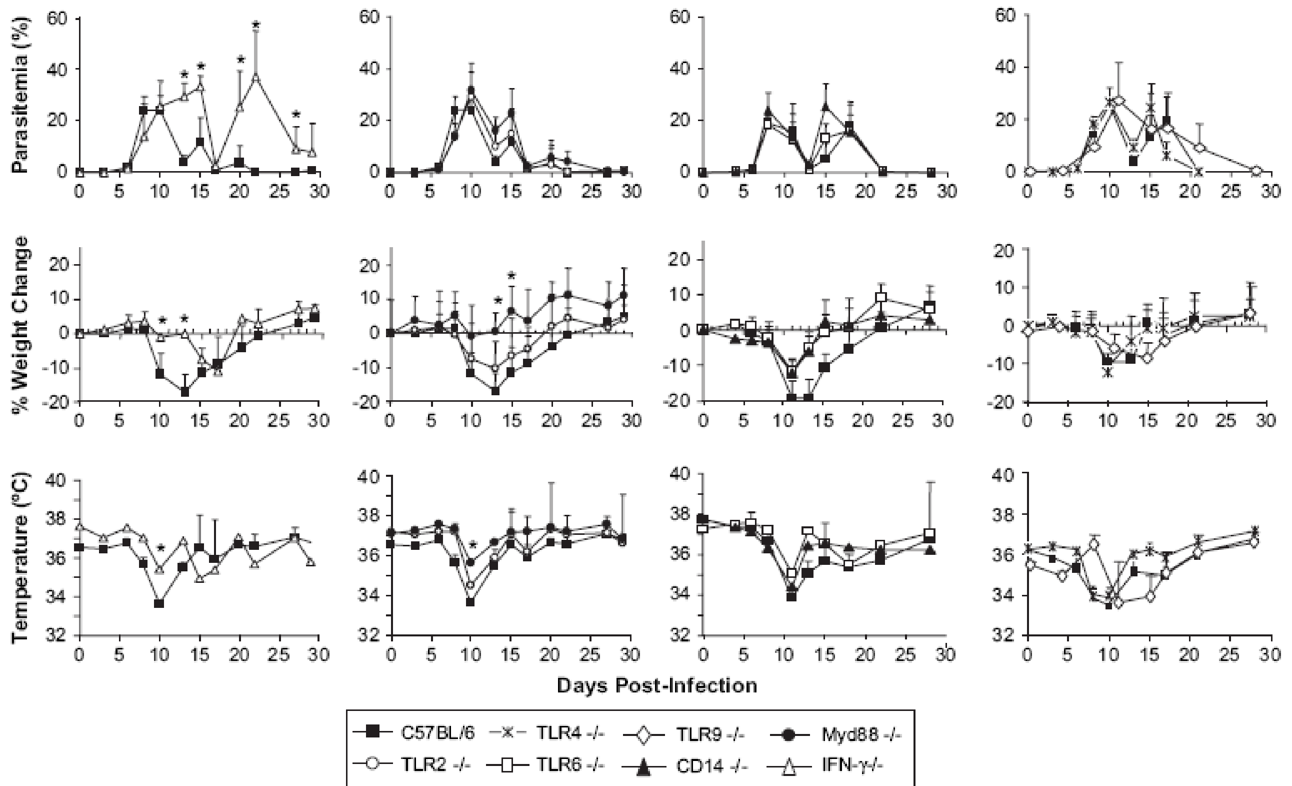

Fig. 1. C57BL/6 (WT), TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$, CD14$^{-/-}$, MyD88$^{-/-}$ and IFN-$\gamma^{-/-}$ mice were challenged with 1 × 10E5 infected erythrocytes and followed every three days for parasitemia (top panels), body weight (middle panel), and body temperature (bottom panel). The results are averages of eight animals from a representative of one out of three experiments that yielded similar results. Asterisk indicates that difference is statistically significant ($p < 0.05$) when comparing results from a specific knockout lineage (i.e. MyD88$^{-/-}$ or IFN-$\gamma^{-/-}$) with the wild type (C57BL/6) mice.

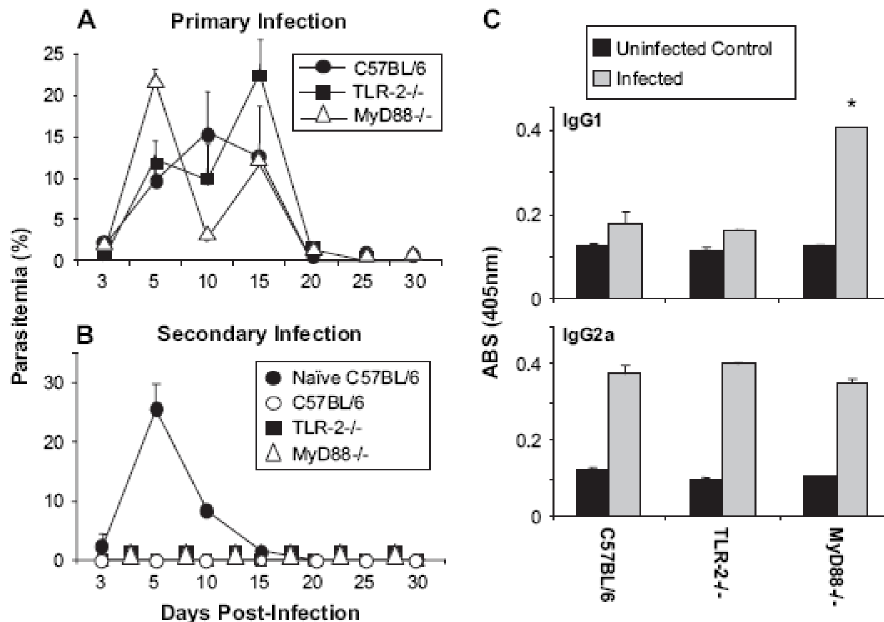

Fig. 2. (A) C57BL/6 (WT), TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were challenged with 1 × 10E5 infected erythrocytes and followed every three days for parasitemia; (B) C57BL/6 (WT), TLR2$^{-/-}$, and MyD88$^{-/-}$ mice challenged with 1 × 10E5 infected erythrocytes were re-challenged with 1 × 10E8 infected erythrocytes at 90 days after initial infection, and naïve C57BL/6 control mice receiving the same inoculums were use as controls; (C) the levels of anti-*P. chabaudi* specific IgG1 and IgG2a antibodies were measured in the sera of C57BL/6, TLR2$^{-/-}$ and MyD88$^{-/-}$ at 90 days post-infection, as well as in the sera of uninfected control mice. The results are averages of eight animals from a representative out of two experiments that yielded similar results. Asterisk indicates that difference in parasite specific IgG1 level is statistically significant ($p < 0.05$) when comparing results from a MyD88$^{-/-}$ and the wild type (C57BL/6) mice.

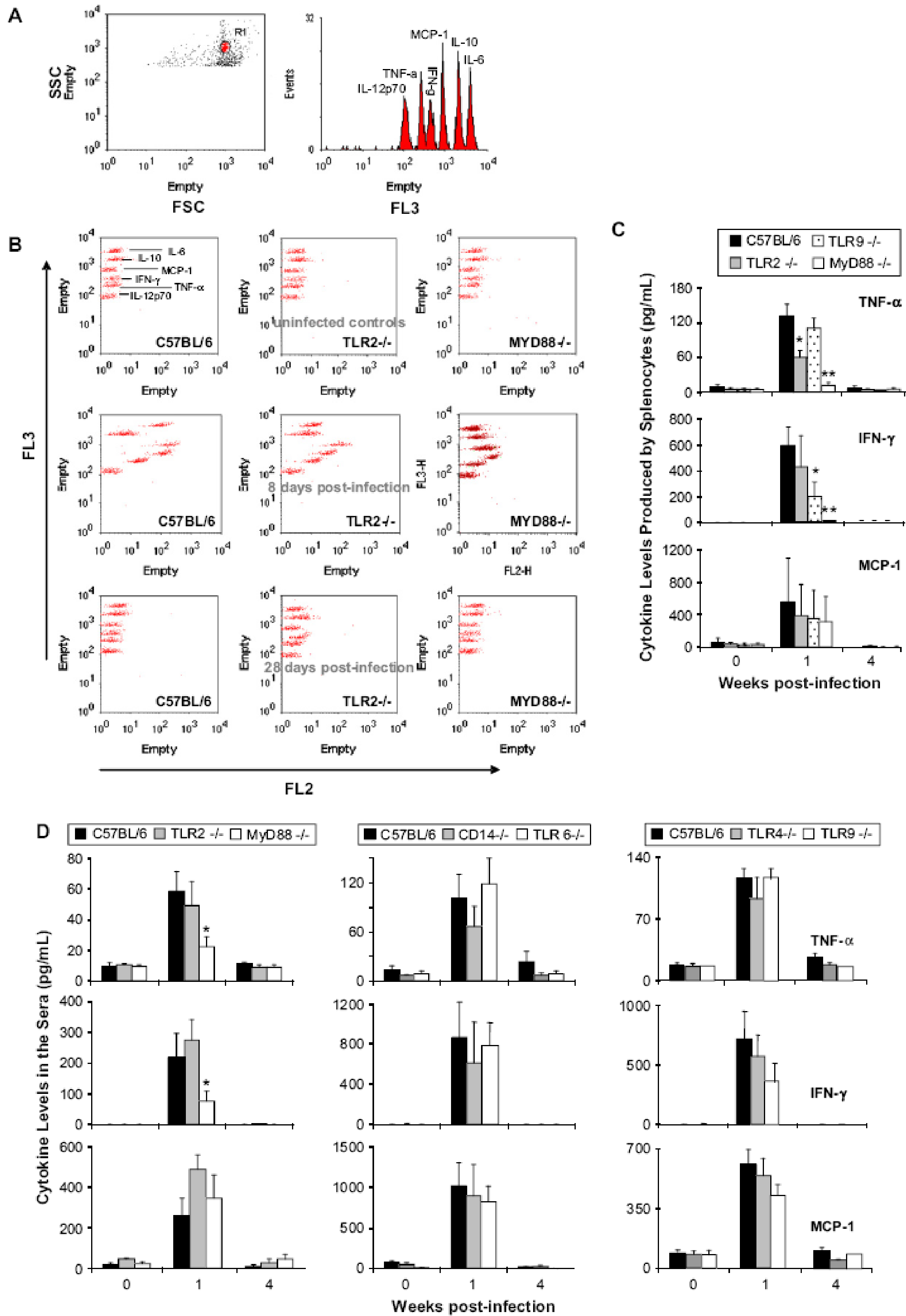

Fig. 3. Splenocytes from C57BL/6 (WT), MyD88$^{-/-}$, and TLR2$^{-/-}$ mice were harvested before (day 0), during (day 8) and after (day 28) the peak of parasitemia and cultured in absence of any exogenous stimulation. After 48 h of culture supernatants were frozen at −70 °C until use. Cytokine levels in the supernatant were

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2.5. Analysis of FACS data

Lymphocytes, NK cells, and DCs were analyzed, making use of known positioning of mononuclear cells based on size and granularity profiles, for their intracellular cytokine expression patterns and for surface markers in a number of ways using the Cell Quest (Becton & Dickinson, San José, CA). Limits for the quadrant markers were always set based on negative populations and isotype controls, and cellular staining analyzed in a FACScan flow cytometer and analyzed using Cell Quest software (Becton Dickinson, San José, CA).

2.6. Purification of $CD11c^+$ splenic DCs

Spleens from naïve or infected mice were treated for 30 min at 37 °C with 1 mg/ml collagenase IV (Sigma Aldrich). $CD11c^+$ splenic DCs were isolated using CD11c microbeads (Miltenyi Biotec) accordingly to manufactures instructions. Cells (3×10^5) were Fc blocked with 2.4G2 mAb (BD Pharmingen San Diego, CA) and labeled with FITC-conjugated anti-CD11c, PE-Cy-conjugated anti-CD40 and CD80 mAbs (BD Pharmingen San Diego, CA). A non-related IgG mAb was used as a control for staining specificity. For intracellular cells were process as described above.

2.7. Statistical analysis

Each experiment was performed using eight individual C57BL6 mice and correspondent group of knockout animals infected with *P. chabaudi*. Each experiment was repeated two or three times. Each animal was analyzed individually. The numbers of animals used for cytokine measurements and FACS analysis are indicated in the figure legends. Arithmetic means (parasitemia, temperature, body weight, cytokines, antibody levels and cell numbers/percentage obtained in FACS analysis) and standard errors of the means were calculated. T student's *t*-test was used to determine the statistical significance of the observed differences. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Role of Toll-Like Receptors (TLR2, TLR4, TLR6 or TLR9), related co-receptor (CD14) and MyD88 in host resistance and malaria pathogenesis

Infection with *P. chabaudi*, a rodent of malaria, induces high serum levels of IFN-γ, MCP-1, TNF-α, IL-6 and IL-10 that coincides with parasitemia, weight loss and drop in temperature in the wild type C57BL/6 (WT) mice (data not shown). Here, we evaluated the role of different TLRs and associated molecules in host resistance and pathogenesis of infection with *P. chabaudi*. As controls, we used the WT and IFN-$\gamma^{-/-}$ mice that are resistant and susceptible to *P. chabaudi* infection, respectively, as indicated by their ability to clear or not parasitemia after 15–20 days of infection. The results presented in Fig. 1 show that parasitemia was not different, when comparing MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$ and CD14$^{-/-}$ to WT mice. Except for the IFN-$\gamma^{-/-}$ mice, all the knockout mice, were capable of clearing parasitemia, at the same time of the WT mice. Consistently, by end of the experiment, at 28 days post-infection, we observed significantly increased lethality (>50%) only in the IFN-$\gamma^{-/-}$ mice infected with *P. chabaudi*. In the other knockout mouse strains the lethality varied from 0% to 20% and was not statistically different from WT mice (data not shown).

Similar to the WT mice, the TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$ and CD14$^{-/-}$ mice had a clear drop in the temperature and weight loss ($p < 0.05$) that corresponded to the peak of parasitemia (Fig. 1). Interestingly, the MyD88$^{-/-}$ infected with *P. chabaudi*, showed no significant change in temperature and body weight. In contrast, parasitemia was similar to the WT mice. To further confirm that MyD88 was not required for development of protective acquired immunity, we infected WT, MyD88$^{-/-}$ or TLR2$^{-/-}$ mice with 10E5 infected erythrocytes and then gave a secondary challenge using high *P. chabaudi* (i.e. 10E8 infected erythrocytes) dose at 90 days after the initial infection (Fig. 2A and B). The MyD88$^{-/-}$ infected with *P. chabaudi* showed immune response skewed towards a Th2 phenotype, as indicated by the enhancement of parasite specific IgG1 antibody isotypes (Fig. 2C) and lower IL-12 and IFN-γ (Figs. 3–5) production. Nevertheless, MyD88$^{-/-}$ mice were completely resistant to the secondary challenge, as indicated by parasitemia, weight loss and change in temperature.

3.2. Cytokinemia during infection with P. chabaudi in mice deficient in specific TLRs (TLR2, TLR4, TLR6 or TLR9), the TLR co-receptor (CD14) or the adapter molecule, MyD88

We next evaluated the cytokine responses of knockout mice at 8 and 28 days post-infection by the Cytomation Bead Arrays. Fig. 3A (left panel) shows the size (SSC) and forward (FSC) scattering and indicate in R1 the population of beads selected for analysis. Fig. 3A (right panel) shows the FL3

measured by using the pro-inflammatory cytokines – Cytometrix Bead Array (CBA). (A) Gated population of beads analyzed, and the correspondence between the bead stain intensity (FL3) and cytokine (FL2) analyzed, respectively. (B) Analysis of splenocyte supernatants from representative individual mice from each lineage analyzed at 0, 8 and 28 days post-infection. (C) Cytokine levels in the supernatant of splenocyte cultures obtained from C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$ and MyD88$^{-/-}$ mice. The results represent an average of eight mice per group pooled from two different experiments. (D) Sera from C57BL/6 (WT), TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$, CD14$^{-/-}$, and MyD88$^{-/-}$ mice were obtained before (day 0), during (day 8) and after (day 28) the peak of parasitemia and frozen at −70 °C until use. Cytokine levels in the sera were measured by using the pro-inflammatory cytokines Cytometrix Bead Array (CBA). Each panel shows the analysis of a representative individual mice from different mouse lineage analyzed at 0, 8 and 28 days post-infection. The results represent an average of eight mice per group pooled from two different experiments. One ($p < 0.05$) and two ($p < 0.01$) asterisks indicate that difference is statistically significant when comparing results from a specific knockout lineage (i.e. MyD88$^{-/-}$, TLR2$^{-/-}$ or TLR9$^{-/-}$) with the wild type (C57BL/6) mice.

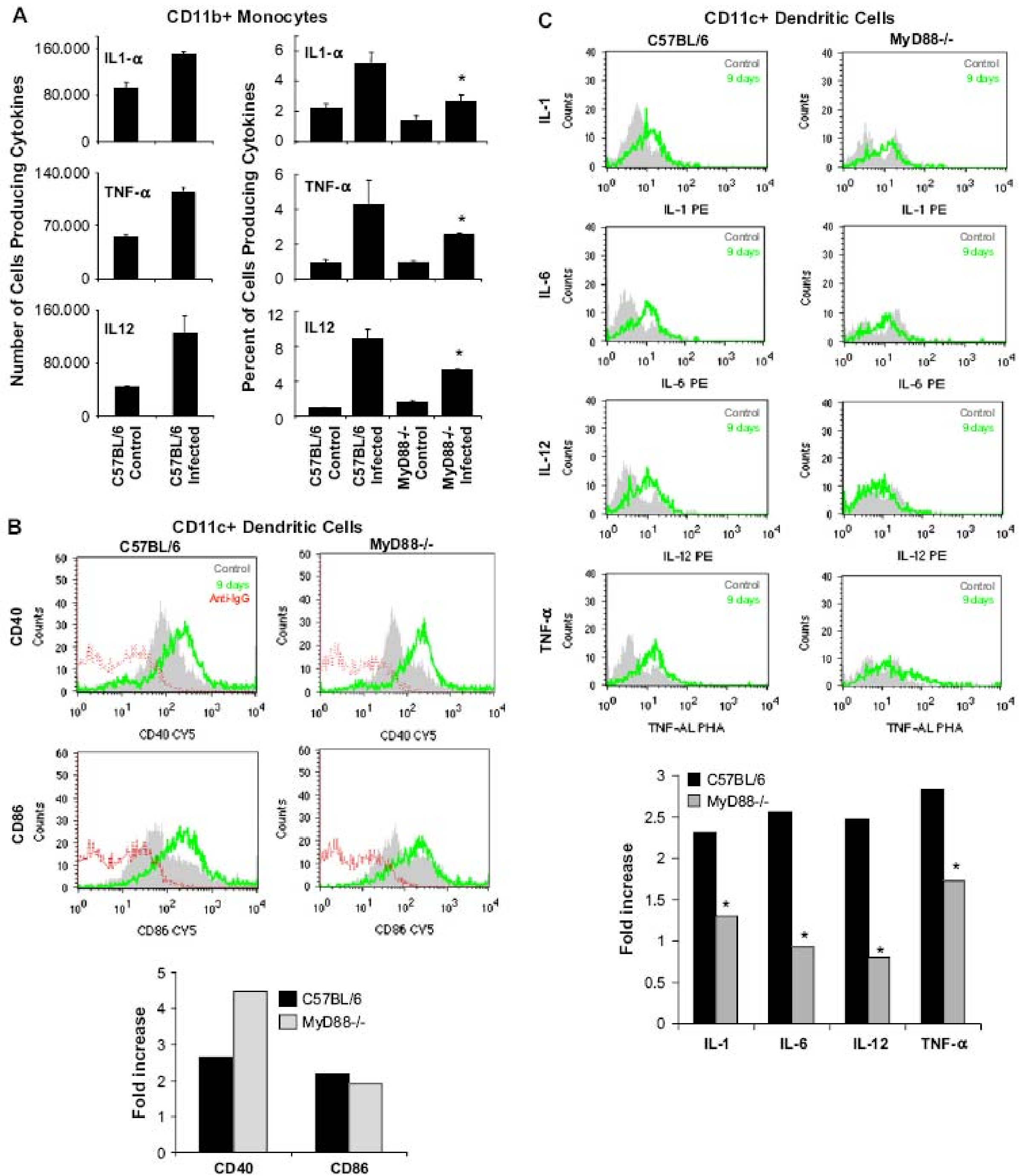

Fig. 4. (A) Splenocytes from C57BL/6 and MyD88$^{-/-}$ mice were harvested on day 0 (Control) and day 8 post-infection (peak of parasitemia and cytokinemia) and cytokine production evaluated by intracellular staining and FACS analysis employing anti-CD11b-FITC to label monocytes and anti-IL-1-α-PE, TNF-α-PE and IL-12(p70)-PE for intracellular staining of cytokine producing cells. CD11c^{+} splenic DCs (3×10^5) from naïve and *P. chabaudi*-infected (9 days post-infection) mice were purified using CD11c microbeads (Miltenyi Biotec), Fc blocked and stained for the surface expression of CD11c-FITC, CD40, CD86-Cy5 (B) or intracellular expression of IL-1, IL-6, IL-12 and TNF-α (C). (B) Histograms show the CD40 and CD80 expression on splenic CD11C^{+} DCs of naïve (shaded background) and 9 days infected (continuous line) mice. A non-related PE-conjugated IgG mAb was used as a control for staining specificity (dotted line). (C) For intracellular staining of cytokines, 3×10^5 DCs were treated with brefeldin-A, Fc blocked, stained for surface expression of CD11c and fixed. Cells from naïve mice (shaded background) and infected mice (continuous line) were then permeabilized with saponin, and stained for intracellular cytokines IL-1, IL-6, IL-12 and TNF-α using specific PE-conjugated mABs. The grafts were designed based on the reason of percentages obtained by FACs analysis from cells of infected/control mice. Data are representative of two independent experiments. Asterisk indicates that difference in parasite specific IgG1 level is statistically significant ($p < 0.05$) when comparing results from a MyD88$^{-/-}$ and the wild type (C57BL/6) mice.

indicating the peak of intensity for fluorescence of beads coated with specific cytokine antibody (lower intensity for IL-12 and higher intensity IL-6). Fig. 3B shows a representative of a single wild type, TLR2$^{-/-}$ and MyD88$^{-/-}$ prior infection (uninfected controls), at 8 and 28 days post-infection. FL3 shows the differential staining of beads coated with specific anti-cytokine antibodies, the level of FL2 fluorescence staining is determined by the levels and of cytokine present in the splenocyte culture supernatants and their interaction with each of the beads. The results show no detection of cytokinemia in days 0 and 28 post-infection, whereas cytokines, mainly IFN-γ, TNF-α and MCP-1 were augmented on day 8 post-infection. The results presented in bars represent pooled data from three different experiments and are shown in Fig. 3C (culture supernatants) and 3D (sera). We observed an impaired synthesis of TNF-α and IFN-γ, but not MCP-1, by splenocytes from MyD88$^{-/-}$ mice at day 8 post-infection ($p < 0.01$) (Fig. 3C). We also observed a partial impairment in the synthesis of TNF-α and IFN-γ by splenocytes from TLR2$^{-/-}$ and TLR9$^{-/-}$ mice, respectively ($p < 0.05$). No impairment of cytokine production, was observed in spleen cells from TLR4$^{-/-}$, TLR6$^{-/-}$ or CD14$^{-/-}$ mice at 8 days post-infection (data not shown).

Serum cytokine levels (Fig. 3D) trended along the same lines as those of the splenocytes. While statistically significant ($p < 0.01$), the impairment of TNF-α and IFN-γ levels in MyD88$^{-/-}$ mice infected with *P. chabaudi* was only partial. MCP-1 was produced in a MyD88-independent manner. The levels of TNF-α, IFN-γ and MCP-1 were not altered in the sera of TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$ or CD14$^{-/-}$ mice infected with *P. chabaudi*.

3.3. MyD88 is a critical element for cytokine production by monocytes, DCs and T lymphocytes during acute episodes of malaria in mice infected with P. chabaudi

DCs have been shown to be activated by *Plasmodium* products as well as play distinct roles during rodent malaria [18]. Exposure of BMDCs to erythrocytes infected with *P. chabaudi* elicited the production of IL-12. The levels were relatively low, as compared to LPS (TLR4 agonist) and Pam3Cys (TLR2 agonist). Importantly, the IL-12 response was ablated in DCs from MyD88$^{-/-}$ mice (data not shown). We next sought to identify, which cells are the main in vivo source of monokines, i.e. IL-1α, TNF-α and IL-12 employing the FACS assay for intracellular cytokine staining. We found that in infected mice, the frequency of monocytes (CD11b^{+} cells) producing these cytokines was significantly increased at day 8 post-infection. The production of all three cytokines was partially impaired in monocytes from MyD88$^{-/-}$ mice infected with *P. chabaudi* (Fig. 4A). The results shown in Fig. 4B indicate that based on expression of CD40 and CD86 on CD11c positive cells, the maturation of DCs is not affected in MyD88$^{-/-}$ mice infected with *P. chabaudi*. However, the production of IL-1, IL-6, IL-12 and TNF-α was impaired in DCs derived from MyD88$^{-/-}$ mice, as compared to CD11c positive cells from infected WT mice (Fig. 4C).

On the other hand, αβTCR^{+} lymphocytes and NK cells were shown to be the main source of IFN-γ. The αβTCR^{+} lymphocytes also contributed as a source of TNF-α during acute infection with *P. chabaudi* (Fig. 5A). Among T lymphocytes, CD4^{+} T cells, but not CD8^{+} T cells, were shown to be the main source of cytokines. Consistently, the expression of the CD25 and CD69 activation markers was enhanced in CD4^{+} T cells, but not in CD8^{+} T cells from mice at eight days post-infection (Fig. 5B). Importantly, the cytokine production and expression of activation markers by CD4^{+} T cells was impaired in MyD88$^{-/-}$ mice infected with *P. chabaudi* (Fig. 5A–C).

3.4. T cells are the main source of IFN-γ and mediate clinical symptoms during acute episodes of malaria in mice infected with P. chabaudi

DCs have a primary role in initiating T cell responses during infectious diseases. In order to evaluate our hypothesis that primary activation of T cells by DCs is an important step for cytokinemia and pathogenesis during acute infection with *P. chabaudi*, we treated animals, with anti-CD4 (GK 1.5) or anti-CD134 (OX89) mAbs that deplete CD4^{+} T lymphocytes or block the activation of CD4^{+} T cells, respectively. The results presented in Fig. 6 show that either treatment with mAbs resulted in decrease weight loss and drop in body temperature in infected mice, as compared to mice treated with the control mAb, GL113. The attenuation of clinical symptoms in mice depleted of CD4^{+} T cells or treated with OX89 that blocks T cell activation, was associated with decreased serum levels as well as blockade of IFN-γ and partial inhibition of TNF-α production by spleen cells from infected mice (Fig. 6). TNF-α production stayed high at day 28 post-infection, in mice depleted of CD4^{+} T cells, which did not clear parasites from blood, further suggesting the activation of innate immune cells to produce TNF-α.

4. Discussion

The strongest data suggesting the importance of the TLR signaling pathway in host resistance and pathogenesis during parasitic diseases are those obtained from infections of MyD88$^{-/-}$ mice with *Toxoplasma gondii*, *Leishmania major*, *Trypanosoma cruzi* or *Trypanosoma brucei* [19–22]. Increased susceptibility to infection with these protozoa is associated with impaired IL-12 and IFN-γ production [19–22]. Similarly, we found that MyD88 plays an important role in the production of pro-inflammatory cytokines, during infection with *P. chabaudi*. Unexpectedly, and unlike infection with the protozoan parasites mentioned above, we show that MyD88 is not essential for control and parasite clearance in rodent malaria. Importantly, despite the skewed response towards Th2 (indicated by higher parasite specific IgG1 levels), after primary infection, the MyD88$^{-/-}$ mice were completely protected, showing no parasites in the blood upon a secondary challenge with *P. chabaudi*. This came to us as a surprise since previous studies have shown MyD88 is a critical element for the production of IL-12 and the development of Th1

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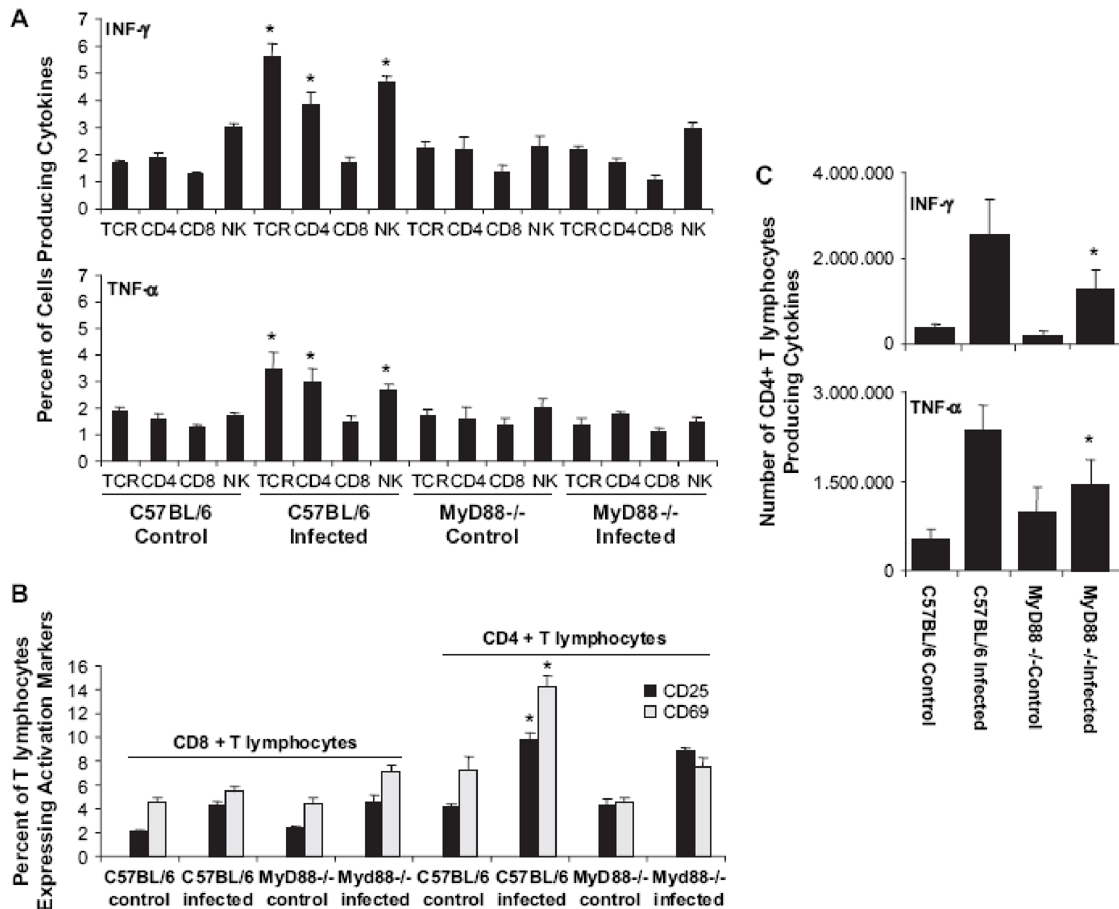

Fig. 5. Splenocytes from C57BL/6 and MyD88$^{-/-}$ mice were harvested on day 0 (uninfected controls) and day 8 post-infection and T cell activation markers evaluated by FACS analysis employing anti-TCR-FITC, anti-CD4-FITC, anti-CD8-FITC and anti-NK-FITC to label lymphocyte/NK cells and anti-IFN-γ-PE and anti-TNF-α-PE for cytokine intracellular staining (A). Alternatively, spleen cells were labeled with anti-CD4-FITC, and anti-CD8-FITC to label T cells and anti-CD25PE and anti-CD69PE to quantify activated lymphocytes (B). Splenocytes were harvested on day 0 (uninfected controls) and day 8 post-infection and labeled with anti-CD4-FITC and anti-IFN-γ-PE and TNF-α-PE for intracellular staining of cytokine and quantification of cytokine producing CD4^{+} T cells in C57BL/6 and MyD88$^{-/-}$ mice (C). The results represent an average of three mice. Similar results were obtained in three experiments. Asterisk indicates that difference in parasite specific IgG1 level is statistically significant ($p < 0.05$) when comparing results from a MyD88$^{-/-}$ and the wild type (C57BL/6) mice.

lymphocytes, known as important components for host resistance to *P. chabaudi* [23]. Thus, our findings indicate that activation of TLRs and MyD88, may contribute, but is not essential for the development of protective immunity in mice challenged with *P. chabaudi*.

Nevertheless, it was clear that the MyD88$^{-/-}$ mice had less symptoms (i.e., drop in body weight and temperature) than the WT mice infected with *P. chabaudi*. Based on the results presented here, we concluded that MyD88 mediates symptoms by being essential for systemic production of pro-inflammatory cytokines (i.e., TNF-α and IFN-γ) observed during acute episodes of malaria. We observed that CD4^{+} T lymphocytes and to a less extent NK cells were the major source of IFN-γ, whereas CD4^{+} T lymphocytes, monocytes, and DCs contributed as a source of TNF-α.

Our results also show that DCs from MyD88$^{-/-}$ mice infected with *P. chabaudi*, were impaired for production of IL-1, IL-6 and IL-12 resulting and less effective in inducing IFN-γ producing CD4^{+} T cells. These findings lead us to evaluate the ability of OX89 to block excessive production of IFN-γ/TNF-α and cytokine-mediated symptoms observed during acute episode of rodent malaria. OX89 is an anti-CD134L monoclonal antibody that blocks the interaction of the co-stimulatory molecule CD134L (OX40L) in DCs with CD134 (OX40) in T lymphocytes, which is essential for the primary activation of T cells. Treatment with OX89 has been proposed as therapy for diseases where excessive T cell response is detrimental [24,25]. We found that treatment with anti-CD134L significantly ablated the production of IFN-γ, and to a less extent affected the TNF-α synthesis in infected mice. In agreement, with the critical role of these cytokines in the clinical symptoms of animals infected with *P. chabaudi*, the anti-CD134L treated animals showed no drop in body weight or temperature.

A recent study has shown that IL-18 is an important cytokine that activates and expand T cells leading to the production of IFN-γ mediated by CD134/CD134L interaction [26]. Further, IL-18 has been shown to be involved in IFN-γ

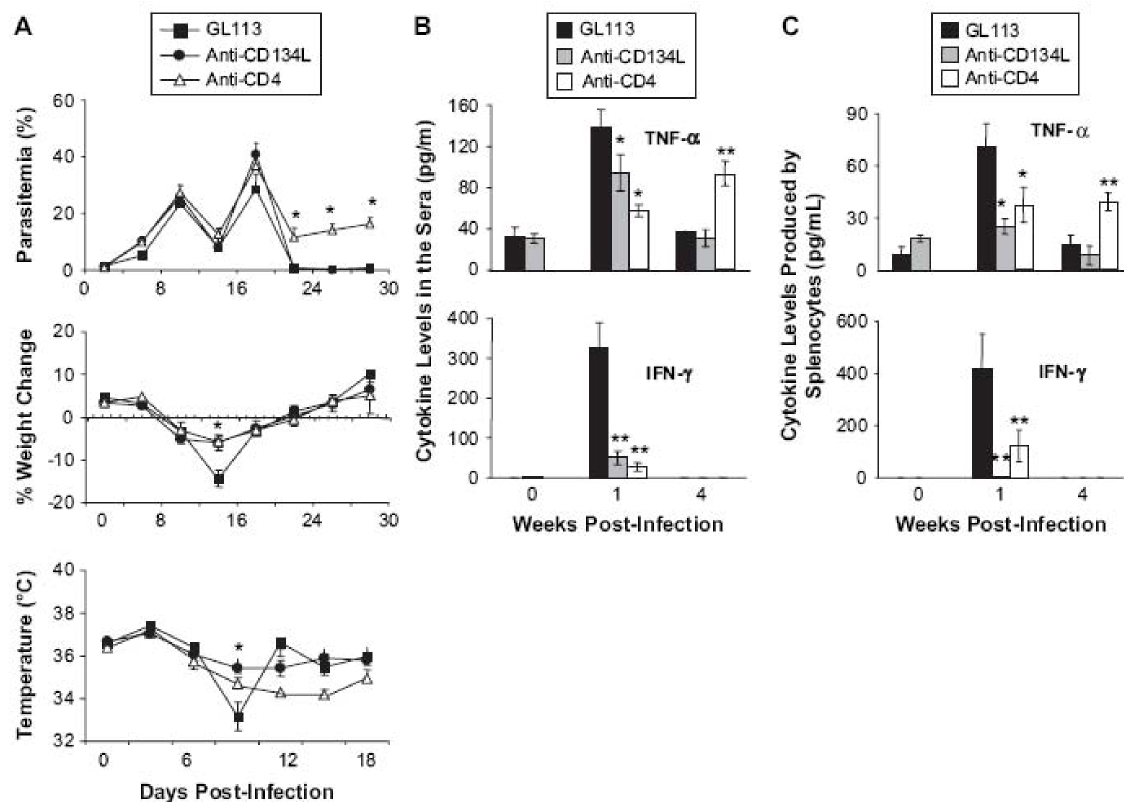

Fig. 6. C57BL/6 mice were treated with GL113 (control mAb), OX89 (anti-OX40 mAb) or GK 1.5 (anti-CD4) a week before challenge and weekly thereafter. (A) Mice were challenged with $1 \times 10E5$ infected erythrocytes and followed every four days for parasitemia (top panel), body weight (middle panel) and temperature (bottom panel). The results are averages of eight animals from a representative out of two experiments that yielded similar results. (B) Cytokine levels in supernatants of sera derived from C57BL/6 treated with either GL113, OX89 or GK 1.5 and harvested at days 0 (uninfected controls) and at 1 and 4 weeks post-infection. (C) Cytokine levels in supernatants of splenocyte cultures derived from C57BL/6 treated with either GL113, OX89 or GK 1.5 and harvested at days 0 (uninfected controls) and at 1 and 4 weeks post-infection. The cytokine measurements represent an average of four mice per group. Similar results were obtained in a second experiment. One ($p < 0.05$) and two ($p < 0.01$) asterisks indicate that difference is statistically significant when comparing results from infected wild type (C57BL/6) mice treated with control mAb (GL113) and those receiving anti-CD4 (GK 1.5) or anti-CD134L (OX89).

induction and host resistance during mouse infection with *P. berghei* or *Plasmodium yoelii* [27] and in a in vitro system for induction of IFN-γ production by NK cells exposed to *P. falciparum* [28]. However, consistent with studies performed by Adachi et al. [14], we could not reproduce the findings obtained with *P. berghei* and *P. yoelii* infections [27], and found no changes in parasitemia, cytokinemia (IFN-γ and TNF-α) and symptoms during infection of IL-$18^{-/-}$ with *P. chabaudi* (data not shown).

Together our results show that primary T cell activation by DCs is partially dependent on MyD88, and has a critical role in the pathogenesis of malaria as early as 8 days post-infection. We also show that single deficiency of TLR2, TLR6, TLR4, CD14 (co-receptor for TLR2 and TLR4), or TLR9 had no major effect on immunological control of parasite replication, cytokinemia and cytokine-mediated symptoms associated with rodent malaria. These results, contrast with recent publication from Coban et al. [15], who demonstrated a critical role for single deficiency of either TLR2 or TLR9, making mice more resistant to cerebral malaria. The assignment of single TLR responsible for the control of protozoan infections has been a consistently difficult task [3,14,19,21,22,29]. Our main hypothesis is that protozoan parasites are recognized by several TLRs. Indeed, this seems the case with *T. cruzi* experimental infection in TLR2/TLR9 double knockouts that are almost as susceptible as the MyD$88^{-/-}$ mice infected with *T. cruzi* [30].

Altogether, our results show that MyD88 plays a critical role in malaria pathogenesis. We also define a main mechanism by which MyD88 is essential in mediating cytokinemia and pathogenesis during acute malaria. The role of MyD88 appears to be critical for eliciting production of pro-inflammatory cytokines, and an exuberant primary activation of $CD4^+$ T cells, resulting in excessive production of TNF-α and IFN-γ during the peak of parasitemia. Importantly, treatment with anti-CD134L that blocks co-stimulation of T cells, had a beneficial effect blocking excessive T cell response, cytokinemia and associated symptoms during acute malaria, with no detrimental effect in terms of parasitemia and parasite clearance. Thus, primary T cell activation by DCs is a key step to interfere with malaria pathogenesis, and may be proven useful to treat patients with malaria. Further, our studies suggest that TLR antagonists maybe useful in partially blocking DC activation to prevent the excessive T cell activation and cytokine-mediated symptoms observed during acute malaria episodes.

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ARTIGO 2

Role of TLRs/MyD88 in host resistance and pathogenesis during protozoan infection: lessons from malaria

Catherine Ropert, Bernardo S. Franklin, and Ricardo T. Gazzinelli

Semin Immunopathol (2008) 30:41–51

Neste artigo de revisão, dissertamos sobre o contrastante papel de MyD88 em doenças causadas por protozoários e na malária. Alem disso, fazemos um levantamento criterioso da literatura envolvendo estudos com modelos murinos onde o papel das citocinas pró-inflamatórias e dos TLRs nas infecções por diferentes protozoários parasitos foi investigado. MyD88 é uma molécula adaptadora chave para a produção de citocinas pró-inflamatórias derivada da ativação dos TLRs e desempenha um importante papel na proteção contra várias doenças causadas por protozoários, tais como *T. cruzi*, *T. gondii* e *Leishmania sp*. No caso da malária, no entanto, a ativação dos TLRs dependentes de MyD88 e a resultante produção de citocinas pró-inlfamatórias possui um papel na gravidade dos sintomas e na patogênese da malária. Dessa forma, MyD88 e os TLRs podem ser um favorável alvo terapêutico para a prevenção das respostas inflamatórias excessivas e deletérias durante a malária.

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REVIEW

Role of TLRs/MyD88 in host resistance and pathogenesis during protozoan infection: lessons from malaria

Catherine Ropert · Bernardo S. Franklin · Ricardo T. Gazzinelli

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Abstract Toll-like receptors (TLRs) are important to initiate the innate immune response to a wide variety of pathogens. The protective role of TLRs during infection with protozoan parasites has been established. In this regard, malaria represents an exception where activation of TLRs seems to be deleterious to the host. In this article, we review the recent findings indicating the contrasting role of Myeloid Differentiation Primary-Response gene 88 (MyD88) and TLRs during malaria and infection with other protozoa. These findings suggest that MyD88 may represent an Achilles' heel during *Plasmodium* infection.

Keywords Innate immunity · Toll-like receptors · MyD88 · Protozoan parasites · Cytokines · Dendritic cells

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Introduction

Infections with protozoan parasites are a major global health problem, affecting over half a billion people world wide. Several of these diseases (such as malaria, African trypanosomiasis, and leishmaniasis) represent major causes of morbidity and mortality leading to economic losses and political instability in the developing countries (TDR Diseases website, http://www.who.int/tdr/diseases/default.htm). Furthermore, no effective prophylactic vaccines are available to prevent these diseases. Thus, a better understanding of the immunological mechanisms involved in resistance as well as in pathogenesis during infection with protozoan parasites is urgently needed. This will contribute to the development of immunological-based clinical settings to prevent or to treat these devastating diseases.

A large body of evidence shows that activation of the cellular components of the innate immune system has a central role in the outcome of infection with protozoan parasites. Proinflammatory cytokines produced by cells of the innate immune system drive the activation of effector mechanisms responsible for limiting the spread of pathogens during the early stages of infection.

For a time, innate immunity was considered to be nonspecific and not as evolved as the adaptive immunity. The dogma of the nonselective nature of innate immune response, in particular the presumed nonspecific recognition of microorganisms by phagocytic cells, has been challenged. In fact, we are no longer ignorant about the nature of innate immune receptors and how they detect pathogens before the establishment of acquired immunity. The discovery of a class of receptors, namely Toll-like receptors (TLRs), which are able to recognize pathogen-associated molecular patterns (PAMPs), has filled a void in our knowledge of immunology. TLRs represent a primary

line of defense against invading pathogens in mammals [1–5]. The members of the TLR family are pattern-recognition receptors with the ability to recognize lipid, carbohydrate, peptide, and nucleic acid structures that are broadly expressed by specific groups of microorganisms. Reports in the literature support the idea that TLRs are involved in the initial recognition of protozoan parasites by the immune system of the vertebrate host in early resistance to infection, for development of acquired immunity as well as pathology [6, 7].

In this article, we review the most recent concepts in recognition of protozoan parasites by TLRs. We discuss the consequences of TLR activation during infection with this class of pathogens. Whether MyD88 activation is a double-edged sword during infection with protozoan parasites is an important consideration developed in this review.

Balancing proinflammatory and anti-inflammatory cytokines during infection with protozoan

After infection with protozoan parasites (i.e., *Trypanosoma cruzi* and *Toxoplasma gondii*), strong proinflammatory signals are generated by the host innate immune system. In the case of *T. cruzi*, the etiologic agent of Chagas disease, as well as *T. gondii*, the importance of the host innate immune system was demonstrated in mice lacking functional genes for interleukin (IL) 12, interferon (IFN) γ, tumor necrosis factor (TNF) α receptor, or for the inducible nitric oxide synthase [8–14]. The ability to survive the infection is dependent upon IFN-γ, which is recognized as a major mediator of host resistance against *T. cruzi* [11] and *T. gondii* [15]. IFN-γ promotes the production of reactive nitrogen intermediates, which are very toxic to parasites and are considered as the main products involved in the microbicidal effect. At the same time, high levels of anti-inflammatory cytokines are released to control the inflammatory reaction. These cytokines are crucial for host protection against deleterious effects of overwhelming inflammation and important to tissue repair. There are two major anti-inflammatory cytokines, i.e., IL-10 and transforming growth factor (TGF) β, which face the challenging task of limiting the induction of the proinflammatory response. This is illustrated by studies using IL-10$^{-/-}$ mice that succumb to the normally nonlethal infection with *T. cruzi* [16] or *T. gondii* [17] because of overproduction of IL-12, TNF-α, and IFN-γ .

In the case of another member of Trypanosomatidae family, the genus *Leishmania*, these eukaryotic pathogens have evolved with the vertebrate immune system and typically produce long-lasting chronic infections [18]. The infection with *L. major* in a susceptible host is characterized by an unbalanced induction of Th1 to Th2 leading to a lack of IFN-γ and a sustained production of IL-4 in mice [19]. In contrast, the resistant host develops a strong Th1 response that is characterized by production of IFN-γ and IL-10 commonly encountered in clinical leishmaniasis [20]. This response may represent a good example of concomitant immunity leading to simultaneous secretion of IL-10 and IFN-γ, apparently sufficient for controlling parasite replication but not enough to completely eliminate the parasite.

An overall characteristic that emerges from the above examples is that generally, the Th1 response is effectively controlled during infection. However, this conclusion is not clear-cut in the case of malaria. In fact, the intense proinflammatory response observed during the initial stages of malaria appears to be largely responsible for the clinical symptoms [21]. This suggests that the initial innate response may have far-reaching consequences on disease outcome. More precisely, high levels of cytokines like TNF-α and IFN-γ appear to have a pivotal role in the pathologic process observed during acute episodes of malaria [22–26]. In a recent series of studies, essential roles have been demonstrated for both IL-10 and TGF-β in the regulation of Th1 response during rodent malaria infection [27–29]. For instance, during *Plasmodium berghei* ANKA infection, IL-10 was shown to protect against fatal Th1-driven pathogenesis [30]. The protective role of IL-10 was confirmed in experiments using IL-10$^{-/-}$ mice infected with *P. chabaudi* where an enhanced pathology mediated by excess production of proinflammatory cytokines including TNF-α is observed [31, 32].

From the above discussion, it appears that parasites carefully regulate the production of cytokines during early infection as a mean of determining their fate in the host. One can predict that parasite–host interactions differ among protozoan parasites and that TLR activation may differentially affect the outcome of these diseases.

Protozoan PAMPs and TLRs

The principal function of TLRs in innate immune cells is to detect foreign invaders and to mount an immuno-inflammatory response. To date, 11 human TLRs and 13 mouse TLRs have been identified. TLRs signal via a common pathway that leads to the expression of diverse inflammatory genes. In addition, each TLR elicits specific cellular response to pathogens owing to a differential usage of the intracellular adapter proteins MyD88, MyD88-adaptor-like (MAL, also known as TIRAP), Toll/IL-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) [2, 4]. Furthermore, the involvement of accessory molecules may help to confer ligand specificity and response. For example,

TLR4 in association with CD14 and MD-2 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria [33]. TLR2 forms association with TLR1, TLR6, and CD36 allowing the discrimination of a large variety of TLR ligands, including peptidoglycan, lipopeptides, and liproteins from Gram-positive bacteria, mycoplasma lipopeptides, and fungal zymosan [34].

TLRs can be classified into two groups based on cellular localization. The first group includes 3, 7, 8, and 9, which localize at intracellular compartments as endosomes. Intracellular TLRs sense viral and bacterial nucleic acids in particular. The second group includes TLR1, 2, 4, 5, and 6, which are all present at the plasma membrane. In contrast to intracellular TLRs, which utilize MyD88 or TRIF, plasma membrane-localized TLRs use TIRAP and/or TRAM as additional adapters suggesting a link between adapter usage and TLR localization (Fig. 1).

Recent progress has revealed that TLR responses are tightly controlled by multiple mechanisms to induce appropriate responses against diverse microbial pathogens and to prevent excessive inflammation. The modulation of TLR expression by IL-10 and TGF-β represents one of these mechanisms [35]. In addition, negative regulators (such as Myd88s, IRAKM, suppressors of cytokine signaling protein) are induced by TLR ligands themselves to avoid "overheating" the immune system (see review [36]).

TLR2 and TLR4 agonists

To predict the roles of TLRs in vivo during parasite infection, experiments in vitro have been designed to associate parasite component immune activity to TLR activation. Biochemical and immunological studies have revealed an important role of molecules present at the surface of the parasite. Among them, the glycosylphosphatidylinositols (GPIs) are dominant glycolipids that cover the surface of most protozoan parasites. These molecules may be expressed in a free form as glycoinositolphospholipid (GIPL) or linked to proteins [37–39] and are involved in the modulation of host immune responses. For instance, lipophosphoglycan (LPG), the most abundant cell surface molecule of the promastigote stage of *Leishmania*, has been involved in the subversion of the host immune system [40]. By contrast, other studies demonstrated that GPI anchors from other parasites play a role in activation of different cells from the immune system. For *T. cruzi* trypomastigotes derived from mammalian cells, the proinflammatory activity of GPI anchors covalently linked to mucin-like glycoproteins (GPI-mucin) depends on the GPI anchor's fine structure [41–44]. In a same way, GPI-anchored glycoproteins purified from *P. falciparum* [45, 46] are capable of inducing the synthesis of proinflammatory cytokines, such as TNF-α and IL-1, by macrophages. Initial data showed that the endotoxin activity of major surface antigens released during squizont rupture (i.e., merozoite surface protein [MSP] 1, MSP-2) is restricted to the GPI moiety, as protein denaturation and exhaustive digestion with pronase do not abolish or reduce the potency of these molecules [45]. Accumulated evidences indicate that GPI anchors from *P. falciparum* contribute to the pathology of infection. GPI has long been considered the toxin in malaria. Indeed, the original study showed that when administered to mice, GPI alone is sufficient to cause symptoms similar to acute malaria infection such as transient fever, hypoglycemia, and death because of TNF-α-mediated sepsis [45]. More recently, GPI anchors from another apicomplexan parasite, *T. gondii*, have been shown to induce TNF-α production in macrophages [47].

The proposed involvement of TLRs in cell signaling by the parasite glycolipids was based on the observation that the pattern of macrophage activation by *T. cruzi* mucin-derived GPI anchors was analogous to that of bacterial LPS [48]. Cell signaling in response to GPI-mucin from *T. cruzi* has been shown to be through recognition by TLR2, resulting in activation of the MyD88-dependent mitogen-activated protein kinase and nuclear factor κB (NF-κB) pathways and downstream expression of cytokines and nitric oxide [49, 50]. More recently, we have shown that TLR6 associates with TLR2 and CD14 to form a receptor complex for the recognition of GPI (unpublished observation). In addition, a *T. cruzi*-derived free GPI anchor containing a ceramide as a lipid tail (GIPL-ceramide) was shown to stimulate the innate immune response through TLR4 [51]. In parallel, LPG from *L. major* was identified as a TLR2 agonist [52].

Considering the GPI from apicomplexan parasites, currently available literature shows that they share similar specificity: recognition of GPI from *P. falciparum* or *T. gondii* parasites seems to be mediated through TLR2 and TLR4. More precisely, GPI from *P. falciparum* has been shown to induce proinflammatory cytokine production in macrophages through the interaction of the three fatty acyl chains of the GPI anchor with the TLR2/TLR1 complex, which also involves a minor contribution of TLR4 [53, 54]. GPI anchors purified from *T. gondii*, as well as synthetic fragments of the proposed structure of these GPI anchors, were shown to stimulate TNF-α synthesis by macrophages through TLR2 and TLR4 [55]. It is noteworthy that all GPI anchors from these different parasites use TLRs located at the cell surface.

TLR9 agonists

While most of the small body of literature on innate immune activators from protozoan parasites has focused

upon GPI anchors, another molecule may also play an important role in the proinflammatory response. Similar to unmethylated bacterial CpG deoxyribonucleic acid (DNA) motifs, DNA derived from various parasites, such as *T. cruzi*, *T. brucei*, and *Babesia bovis*, are able to stimulate macrophages and dendritic cells (DCs) to produce proinflammatory cytokines [56–59]. Induction of inflammatory mediators by protozoan DNA was dependent on the presence of unmethylated CpG dinucleotides. Shoda et al. [56] have correlated the frequency of CG dinucleotides in the genome and the amount of IL-12, TNF-α, and NO induced by protozoan DNA.

According to these findings, it is also becoming clear that TLR9, well known as a receptor for unmethylated bacterial CpG DNA motifs [60], has an important role for induction of proinflammatory cytokines during infection with protozoan parasites. Indeed, the proinflammatory activity of *T. cruzi* and *T. brucei* genomic DNA has been shown to depend on TLR9 [59, 61]. The presence of such a system that detects the accumulation of internalized pathogen may represent an efficient means to trigger the generation of activating signals that result in optimal intracellular killing by innate phagocytic cells.

Major highlights in malaria research include the role of hemozoin, a product of *Plasmodium* hemoglobin digestion during infection. The role of this compound as an immunomodulator has been extensively studied. Hemozoin has been reported to induce [62, 63] or inhibit [64–67] DC maturation and to induce the production of proinflammatory cytokines such as TNF-α and IL-12, chemokines [68, 69], as well as IL-10 [70, 71]. Another function has been attributed to hemozoin and concerns the impairment of phagocytosis and reduction in major histocompatibility complex II, CD11c and intercellular adhesion molecule 1 expression in monocytes and macrophages [72, 73]. The divergences observed may be due to the differences in hemozoin preparations because these preparations are heterogeneous, containing uncharacterized bioactive phospholipids, glycolipids, or nucleic acid derived from *P. falciparum* [74]. Indeed, even synthetic hemozoin may vary in its activity depending on the source of the heme employed to prepare the β-hematin [63, 75]. This compound was tested as a TLR agonist, and in this regard, a TLR9/MyD88-dependent pathway was shown to mediate murine DC activation by hemozoin during malaria—the first identification of a non-DNA ligand for TLR9 [63]. These results were quite surprising because, as mentioned before, TLR9 had been described convincingly as a receptor for DNA, mainly of unmethylated, CpG-containing DNA. More recently, it was shown that hemozoin plays a specific role in presenting the DNA to the intracellular TLR9, but is unable to stimulate the innate immune system by itself [74].

TLR11 agonists

Finally, an agonist for the latest discovered TLR has been identified in *T. gondii*, the profilin-like protein (PFTG). Purified PFTG has been found to induce IL-12 in mouse DCs in a TLR11-dependent manner [76]. In addition, mice lacking TLR11 show increased susceptibility to infection with *T. gondii*. The human TLR11 gene has a premature stop codon and therefore encodes a nonfunctional form of this receptor. As expected, PFTG does not activate human DCs. Thus, the exact function of PFTG in human toxoplasmosis is still unknown.

Different PAMPs for a same parasite: implications in vivo

As presented above, different pathogens contain several agonists that trigger TLRs. This indicates that TLRs present in several different compartments may act in concert to properly detect and respond to invasive microbial pathogens. The concept that multiple TLR–ligand interactions are required for the induction of effective host resistance to pathogens is probably the rule in "real life." In fact, the assignment of a single TLR responsible for signaling during infection has been consistently a difficult task. For example, lack of TLR2, which recognizes GPI anchors, does not affect susceptibility to infection with *T. cruzi* and unexpectedly the absence of this receptor contributes to the increased Th1 response during infection [77]. In contrast, although as not susceptible as $MyD88^{-/-}$, $TLR9^{-/-}$ mice show a significant decrease in Th1 response, which correlates with increase in IL-10 levels in vivo [59]. In this report, it was proposed that TLR2 and TLR9 cooperate to optimize host response during infection with *T. cruzi* [59]. No major phenotype is observed in terms of parasitism or immune responses when either $TLR2^{-/-}$ or $TLR4^{-/-}$ mice are infected with *T. brucei*, *T. gondii*, *P. berghei*, or *L. major*. These findings reinforce the idea that host defense against protozoan pathogens depends on engagement of multiple TLRs [78].

MyD88/TLRs: deleterious role during malaria?

Evidences for the role of MyD88/TLRs during malaria

The role of MyD88 and TLRs during malaria is still under debate, and only a small amount of information is available whether TLRs or their signaling pathways are involved in human malaria infection [79]. Indirect evidence for the role of TLRs during human malaria comes from studies where the genome of patients was analyzed and related to disease susceptibility. The TLR4 frequent single-nucleotide polymorphism (SNP), Asp299Gly, is associated with severe

malaria [80] and risk of maternal malaria, whereas the TLR9 SNP, T-1486C, increased the risk of maternal malaria but was not associated with severe malaria [81]. More recently, it was reported that heterozygosity for a variant of MAL/TIRAP, which is a critical adaptor molecule for TLR2 and TLR4 is associated with protection against of *P. falciparum* infection [82]. However, these data must be analyzed with caution, and the mechanisms involved remain to be defined.

Another evidence for the participation of TLRs in malaria concerns the regulation of TLR expression. Upregulation of TLR expression in patients infected with *P. falciparum* has been reported [83]. Consistent with these findings, study with experimental human malaria has shown that TLR responses are primed during the acute phase toward a more proinflammatory response [84, 85]. Reinforcing this observation, upregulation of messenger ribonucleic acid transcripts coding for various components of inflammatory pathways (including a number of TLRs, MyD88, NF-κB, and IFN-γ) was noted in PBMC from patients during presymptomatic infection [85]. It was hypothesized that the rupture of blood-stage schizonts releasing *Plasmodium*-derived TLR ligands, i.e., either GPI moieties or hemozoin-bound nucleic acids, induces

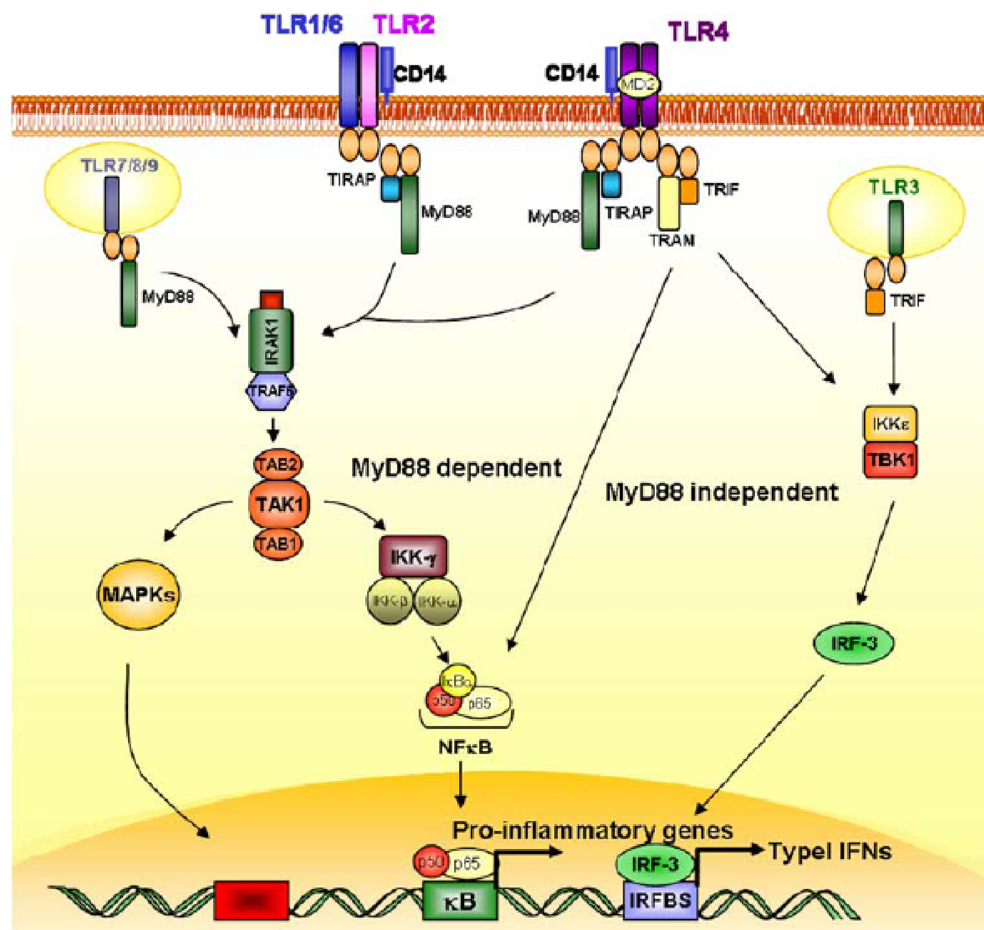

Fig. 1 Toll-like receptor signaling pathway. Toll-like receptors (*TLRs*; with the exception of TLR3) induce nuclear factor-κB (*NF-κB*)-dependent cytokine production through a pathway involving the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88). However, TLR3 (and TLR4) transduce signals through a MyD88-independent signaling pathway that involves the adaptor molecule Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (*TRIF*; also known as TICAM1). Further complexity in the TLR signaling pathways results from the obligatory use of the adaptor molecule TIR-domain-containing adaptor protein (*TIRAP*; also known as MAL) in association with MyD88 by TLR2 and TLR4 and the adaptor TRIF-related adaptor molecule (*TRAM*; also known as TICAM2) in association with TRIF by TLR4. Activation of TLR3 or TLR4 induces type I interferon (*IFN*) production through the adaptor TRIF, which associates with the kinase TANK-binding kinase 1 (*TBK1*) and induces the phosphorylation and nuclear translocation of IFN-regulatory factor 3 (*IRF3*). In TLR2 and TLR4 signaling, CD14, MD-2, and TIRAP/Mal are required for recruiting MyD88 to the receptor. After IRAK-1 associates with MyD88, it is phosphorylated by the activated IRAK-4 and subsequently associates with TNFR-associated factor 6 (*TRAF6*). A complex composed of TGF-β-activated kinase 1 (*TAK1*) and the TAK1-binding proteins, TAB1, TAB2, and TAB3, is also recruited to TRAF6. TAK1, phosphorylates both MAPK and the IKK complex (inhibitor of nuclear factor-κB [*IκB*] kinase complex), which consists of IKK-α, IKK-β, and IKK-γThe IKK complex then phosphorylates IκB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes

differences in TLR expression. A correlation between TLR expression and severity of malaria has been evoked. It is interesting to note that a priming effect leading to an increase in cytokine release in response to various TLR ligands is observed during the acute phase of *P. chabaudi* infection in a MyD88-dependent manner (unpublished data from our laboratory).

Biphasic responses are common to homeostasis in general, and malaria is not an exception to the rule. Indeed, after the early production of proinflammatory cytokines, a decline in the face of continuing parasitemia is observed in mice [86]. The mechanism by which this occurs remains poorly understood. Recently, it was proposed that the initial priming effect leading to a hyper-responsiveness to TLR agonist is followed by a hyporesponsiveness similar to a tolerance phenomenon. This hypothesis is supported by a study exploring a murine model in which DC responses to TLR stimulation were found to become anti-inflammatory only after the acute phase of malaria [87]. The authors of this study hypothesize that repeated signaling through protozoan-derived ligands tolerates or shunts the common MyD88 TLR signaling pathway such that anti-inflammatory cytokines are preferentially produced, resulting in a refractory state similar to endotoxin tolerance. The same phenomenon is observed during infection with *P. chabaudi*, where a downregulation of proinflammatory cytokine synthesis in response to TLR stimulation occurs in the postacute stage (unpublished data from our laboratory). A representation of the regulation of immune response in rodent malaria is proposed in the Fig. 2 where the role of MyD88 is emphasized. New questions arise from these findings including the role of MyD88 in vivo during infection with *Plasmodium*.

Exploring the role of Myd88/TLRs in rodent malaria

Numerous studies have now carried out on MyD88$^{-/-}$ mice in the context of disease models. Generally, these mice are immuno-compromised in terms of their ability to fight a range of pathogens as illustrated by the following

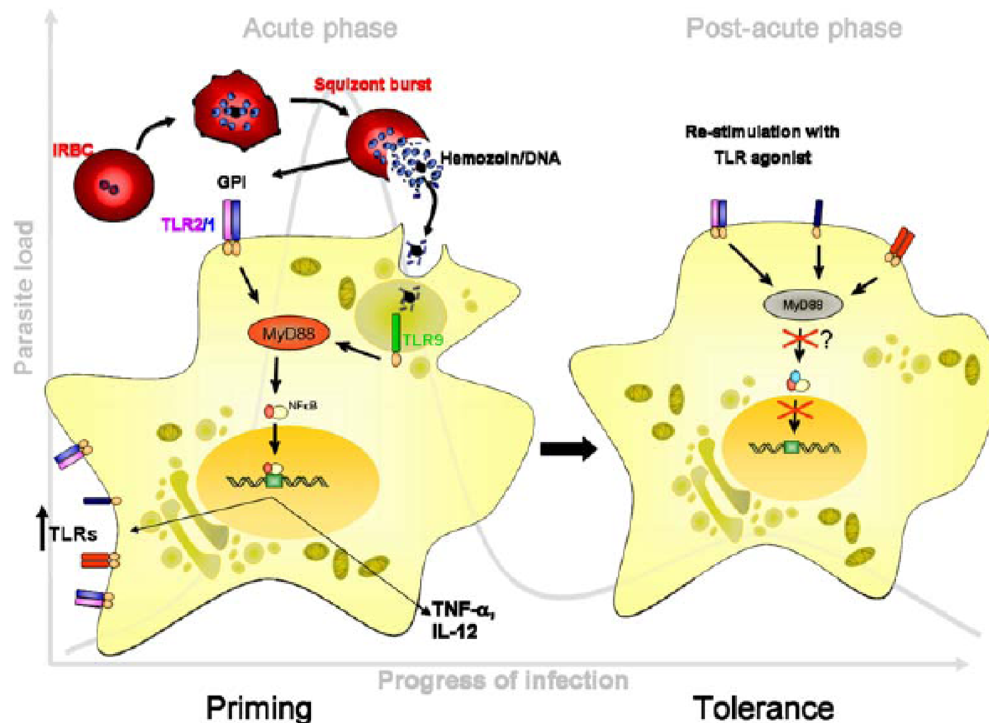

Fig. 2 Possible mechanism of the regulation of immune responses during malaria. The regulation of the immune response during malaria is still unknown. However, different models have been suggested involving TLR pathways. Among them, cell priming following by acquisition of tolerance has been proposed. During the early stages of infection with *Plasmodium falciparum*, cells of the host innate immune system may recognize parasite components like *P. falciparum* GPI and the complex hemozoin/DNA through TLR2 and TLR9, respectively. This priming effect during the early phase of infection involves intense synthesis of proinflammatory cytokines [84] and increases in TLR expression on immune cells [83] and is associated with clinical signs. The over activation of the immune system might be followed by a suppressive effect on TLR signaling in the postacute phase. This theory is supported to a certain extent by the murine study, in which dendritic cell responses to TLR stimulation were found to become anti-inflammatory only during the postacute phase of malaria [87]. It has been proposed that repeated signaling through protozoan-derived ligands may tolerate or shunt the common MyD88 TLR signaling pathway such that anti-inflammatory cytokines are preferentially produced [87]. We hypothesize that MyD88 is a central molecule during malaria, involved in the initial activation of host immune response through TLR pathway and later in the acquisition of tolerance phenomenon

examples: MyD88$^{-/-}$ mice infected with *T. gondii* [88, 89], *T. cruzi* [90], *T. brucei* [61], and *Leishmania* spp. [91, 92] are highly susceptible to infection. When infected with *T. gondii*, all mice die within 10 days of infection. Increased susceptibility is associated with impaired production of the Th1 cytokines IFN-γ and IL-12 and a marked increase in host parasitism [88, 89]. Similar conclusions were reached concerning *T. cruzi* [90] and *T. brucei* [61] infection. The susceptibility of MyD88$^{-/-}$ mice to infection with *L. major* is characterized by large nonhealing lesions, marked parasitism, and the development of a Th2-type immune response [92].

A distinct scenario is observed in the case of rodent malaria. Adachi et al. [93] have investigated the mechanism involved in the regulation of the immune response during infection with the *P. berghei* NK65. They have appointed MyD88 as being responsible for elevated IL-12 production and consequently for the pathology during the infection. However, Myd88 is not involved in the control of the parasite replication.

In a second series of studies, the authors use the *P. berghei* ANKA infection that provides valuable information as an experimental model of cerebral malaria (CM), the lethal complication of malaria caused by *P. falciparum* in humans. Coban et al. [94] showed that MyD88 signaling is involved in the development of CM. Consistent with these findings, they noted a reduction in mortality in the absence of MyD88. Furthermore, they identified TLR2 and TLR9, previously shown to be involved in the recognition GPI anchors and DNA from *Plasmodium*, as responsible in part for the fatal result of the infection. In sharp contrast, Togbe et al. [95] have proposed an opposite model where CM development is independent of MyD88 and TLR signaling. In both studies, the ANKA strain of *P. berghei* was used in the same experimental conditions including the same inoculum and mouse strain. Recently, Lepenies et al. [96] have infected TLR2/4/9-deficient mice (which are MyD88-dependent TLRs) and did not observed differences between triple-knockout mice and wild-type mice regarding survival and pathogenesis [96]. The reason for the discrepancies in these studies concerning the role of MyD88 in CM pathogenesis is unknown.

To determine the role of MyD88 in the inflammatory process during infection with *Plasmodium*, we used the nonlethal *P. chabaudi* strain. In this study, the absence of MyD88 was associated with a reduced production of TNF-α and IFN-γ in infected mice and consequently with attenuated symptoms [97]. However, as previously described, the parasitemia was similar in MyD88$^{-/-}$ and wild-type mice (Fig. 3). The fact that MyD88 signaling pathway is not involved in control of the parasite replication appears to be a rule in the different models of rodent malaria. This contrasts with the function of MyD88 in the infection with *T. cruzi*, *Leishmania*, or *T. gondii*. When we focused our attention on the capacity of MyD88$^{-/-}$ mice to respond to a subsequent infection, we noted the absence of parasite in

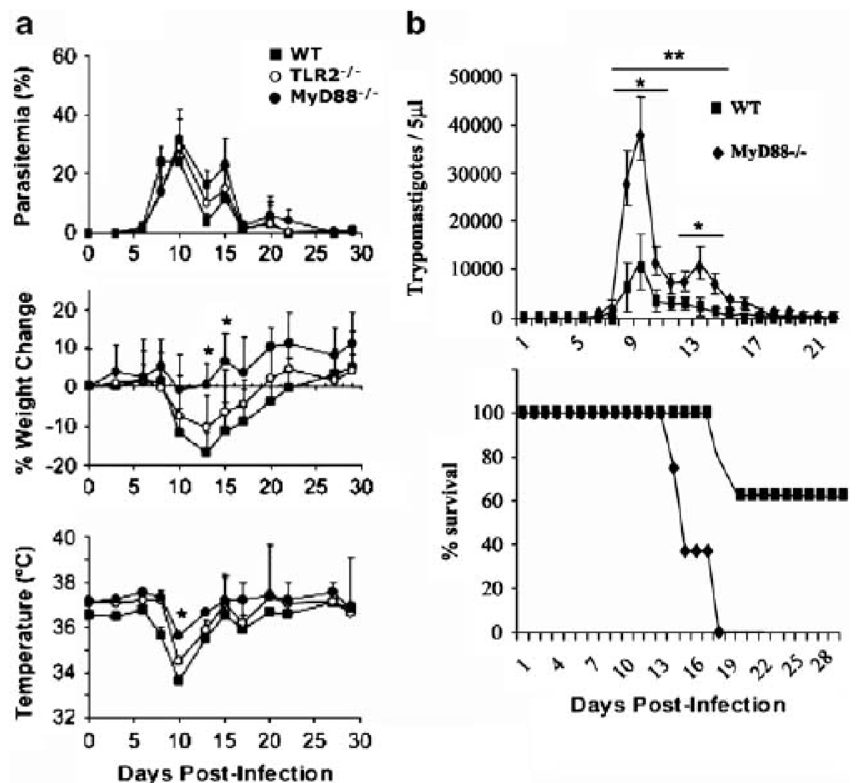

Fig. 3 Contrasting role of MyD88 during infection with *Plasmodium chabaudi* and *Trypanosoma cruzi*. **a** MyD88 is not involved in control of parasitemia during infection with *P. chabaudi*. However, malaria-related symptoms (temperature and body weight) were much less severe in MyD88$^{-/-}$ compared to C57BL/6 (*WT*) or TLR2$^{-/-}$ mice [97]. **b** Enhanced susceptibility of MyD88$^{-/-}$ mice to infection with *T. cruzi* parasites. MyD88$^{-/-}$ mice infected with the Y strain of *T. cruzi* presented higher mortality rate and parasitemia compared to WT mice [90]

blood after a second challenge as in wild-type mice. In agreement with the fact that clearance of parasites is also mediated by antibodies, our data demonstrate that MyD88 is not required for development of acquired immunity and protection in this model.

Concluding remarks

To date, TLRs have been implicated in recognition of every known category of pathogen including protozoan parasites. TLRs and MyD88 signaling pathway activation has been associated with a protective effect during infection with *T. gondii*, *T. cruzi*, and *Leishmania* spp. In this field, malaria represents an exception where activation of MyD88 signaling during the infection appears to be involved in the excessive proinflammatory cytokine production responsible for the symptoms observed. The fact that this adaptor protein plays a more prominent role in pathogenesis, rather than in protection, opens new possibilities regarding the use of TLR-based strategies for immunotherapy aiming to prevent excessive immuno-activation in malaria.

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ARTIGO 3

Malaria primes the innate immune response due to interferon-γ induced enhancement of Toll-like receptor expression and function

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Este artigo apresenta a síntese dos resultados relativos ao objetivo específico 2:

2) Identificar qual TLR reconhece o Plasmodium e ativa as respostas imunes durante a malária humana e murina.

Neste trabalho demonstramos que a malária tanto murina quanto humana causa uma hiper-responsividade celular às ligantes de TLRs. De forma inédita, mostramos que TLR9 é fundamental para reconhecer a presença do *P. falciparum* em humanos e do *P. chabaudi* em camundongos e iniciar as respostas de IL12 em Células Dendríticas (DCs). As DCs ativadas estimulam a produção de IFNγ por células T e NK. IFNγ por sua vez aumenta a expressão e responsividade celular dos TLRs nas células da imunidade inata que passam a produzir níveis extremamente altos de citocinas pró-inflamatórias. Este quadro clínico favorece o choque séptico e os sintomas mais graves da doença.

Malaria primes the innate immune response due to interferon-γ induced enhancement of toll-like receptor expression and function

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Malaria-induced sepsis is associated with an intense proinflammatory cytokinemia for which the underlying mechanisms are poorly understood. It has been demonstrated that experimental infection of humans with *Plasmodium falciparum* primes Toll-like receptor (TLR)-mediated proinflammatory responses. Nevertheless, the relevance of this phenomenon during natural infection and, more importantly, the mechanisms by which malaria mediates TLR hyperresponsiveness are unclear. Here we show that TLR responses are boosted in febrile patients during natural infection with *P. falciparum*. Microarray analyses demonstrated that an extraordinary percentage of the up-regulated genes, including genes involving TLR signaling, had sites for IFN-inducible transcription factors. To further define the mechanism involved in malaria-mediated "priming," we infected mice with *Plasmodium chabaudi*. The human data were remarkably predictive of what we observed in the rodent malaria model. Malaria-induced priming of TLR responses correlated with increased expression of TLR mRNA in a TLR9-, MyD88-, and IFNγ-dependent manner. Acutely infected WT mice were highly susceptible to LPS-induced lethality while TLR9$^{-/-}$, IL12$^{-/-}$ and to a greater extent, IFN$\gamma^{-/-}$ mice were protected. Our data provide unprecedented evidence that TLR9 and MyD88 are essential to initiate IL12 and IFNγ responses and favor host hyperresponsiveness to TLR agonists resulting in overproduction of proinflammatory cytokines and the sepsis-like symptoms of acute malaria.

Malaria constitutes the most devastating global health problem in human history. The pathogenesis of malaria is multifactorial. Serious sequelae can result from 3 primary pathophysiological events: (*i*) red blood cell destruction; (*ii*) adhesion of infected erythrocytes to the capillary veins, especially in the CNS; and (*iii*) an excessive proinflammatory response. The latter is responsible for symptoms such as rigors, headache, chills, spiking fever, sweating, vasodilatation, and hypoglycemia (1, 2). The initial proinflammatory response during malaria appears to play a critical role in the development of cerebral malaria (3), anemia (4), and thus, death.

Toll-like receptors (TLRs) are receptors of the innate immune system that recognize a variety of microbial products such as LPS from Gram-negative bacteria (5). More recently, it was shown that TLRs also recognize molecules derived from protozoan parasites, including *Plasmodium* (6–10). Indeed, there is a growing body of literature that TLRs are central mediators of proinflammatory responses during malaria (11). For example, mice deficient in MyD88, an essential adapter molecule for all of the TLRs except TLR3, displayed impaired synthesis of proinflammatory cytokines and commensurate attenuation of symptoms when infected with different *Plasmodium* strains (12–14).

Despite concerted effort, a consensus on which malarial molecule is responsible for activating cytokine production in immune cells has not been achieved. Two candidates have been proposed: malarial glycosylphosphatidylinositol (GPI) anchors and parasite-derived DNA bound to hemozoin. These putative "malarial toxins" have been shown to activate TLR2 and TLR9, respectively (8, 10). As human polymorphisms that affect the outcome of malaria have been described for *TLR2*, *TLR9* (15, 16), and *MAL*/*TIRAP* (17), it seems likely that TLRs are involved in innate immune responses to malaria and possible that TLRs 2 and 9 are the main receptors involved.

One approach to define a role for a specific TLR in disease is to examine affected patients for the development of tolerance through a defined signal transduction pathway. LPS-challenged humans, for example, are hyporesponsive to LPS-mediated responses when subsequently re-challenged (18). Cells from patients with Gram-negative bacterial septicemia are similarly hyporesponsive to LPS (19), indicating that the TLR4 pathway has entered into a state of innate immune "tolerance" (20). Tolerance almost certainly exists in malaria, as patients from areas where reinfection occurs almost daily often do not exhibit the signs and symptoms of disease despite the presence of blood parasites (21, 22).

McCall et al. studied TLR function in peripheral blood mononuclear cells (PBMC) from individuals experimentally infected with malaria (23). Their results suggested that as a result of malaria infection, the innate immune response was enhanced to TLR1/TLR2 and TLR4 agonists. This finding was most surprising because it is markedly dissimilar to numerous reports of immune hyporesponsiveness (often referred to as "immune paralysis") during bacterial sepsis even though this syndrome, in many respects,

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mimics malaria. However, due to the nature of human experimental infection, subjects in this study were minimally symptomatic with very low parasitemia. This suggested to us that the effects they described were confined to the very earliest stages of malaria infection and that tolerance had not yet developed at the time that patient blood was collected for study. Furthermore, no obvious mechanism of the priming observed was delineated.

It was for this reason that we attempted to identify TLR usage during acute natural malaria with the idea that naturally infected patients would, in time, develop specific tolerance to the appropriate TLR ligand after experiencing high levels of parasitemia for a period of several days. In fact, we found that acute infection with *Plasmodium falciparum* in humans resulted in enhanced activation of innate immune cells to TLR agonists. This phenomenon was reproduced in *Plasmodium chabaudi AS* infected mice and occurred in a TLR9/IL 12/IFNγ-dependent manner. The mechanism by which malaria infection primes the innate immune responses of the host was found to be that TLR9 activation during *Plasmodium* infection initiates endogenous IL 12 and IFNγ production, which in turn enhances TLR expression and "primes" associated signaling pathways. Our data indicate that this augmented TLR expression leads to a stage of hyperresponsiveness to TLR agonists, and, as confirmed in mice, dramatically enhances the deleterious effect of an endotoxin challenge, as might occur in humans during coinfection with Gram-negative pathogens such as *Salmonella*.

Results

Increased TLR Responses During Acute *P. falciparum* Infection. PBMCs were isolated from *P. falciparum* naturally infected subjects to investigate TLR responses during acute malaria infection. PBMCs obtained from control subjects living in the same endemic area were analyzed in parallel (Fig. 1). With the sole exception of the TLR3 ligand, pI:C, increases in all TLR responses were observed in PBMCs from individuals acutely infected compared to healthy subjects (Fig. 1). Together, these results suggested that *P. falciparum* infection primes cells to subsequent TLR stimulation.

Chemotherapy Reverses Hyperresponsiveness of TLR Responses. Plasma samples from subjects with acute *P. falciparum* infection collected before and 3–4 weeks after curative mefloquine chemotherapy were used for evaluation of cytokines. *P. falciparum* infection led to a significant enhancement of systemic levels of proinflammatory cytokines. All patients reported greatly improved sense of well being and the absence of symptoms after therapy. The clinical characteristics of these patients are listed in Table S1. Coincident with this clinical improvement, cytokine levels were significantly diminished after chemotherapy (Fig. 2*A*). After curative chemotherapy, cytokine levels produced in response to TLR stimulation were back to baseline levels (Fig. 2*B*).

TLR/IFN Pathways Are Boosted During Acute Malaria Infection. To define the mechanism by which malaria mediates up-regulation of proinflammatory cytokines, we profiled mRNA obtained from infected individuals before and after curative chemotherapy. Fig. 2*C* and Table S2 show a panel of select genes up-regulated as a result of *P. falciparum* infection. Although neither TLR3 nor TLR9 expression was dramatically altered, TLR7 expression was enhanced approximately 2.6-fold ($P < 0.01$, data not shown). Statistically significant changes were also observed with TLRs 1, 2, 4, and 8 ($P < 0.05$). Genes involved in TLR signaling pathways consistently showed changes during *P. falciparum* infection (Fig. 2*C*). Expression of CD36, a coreceptor for TLR2 associated with the recognition of malaria parasites and subsequent induction of proinflammatory cytokines (24), was also up-regulated. In addition, we observed enhanced expression of various elements of IFN signaling pathways (Table S2). An analysis of the promoters of the up-regulated genes revealed that a large number of these genes had sites for IFN-inducible transcription factors (Table S3). The transcriptional changes of select genes in TLR or related signal transduction pathways were validated by qPCR (Fig. 2*D*). To determine if the up-regulation of TLR mRNA results in increased protein expression, we assessed the surface expression of TLR2 and TLR4 by flow cytometry. Expression of TLR2 and TLR4 were significantly augmented in $CD11c^+/CD14^+$ monocytes (Fig. 3 and S1).

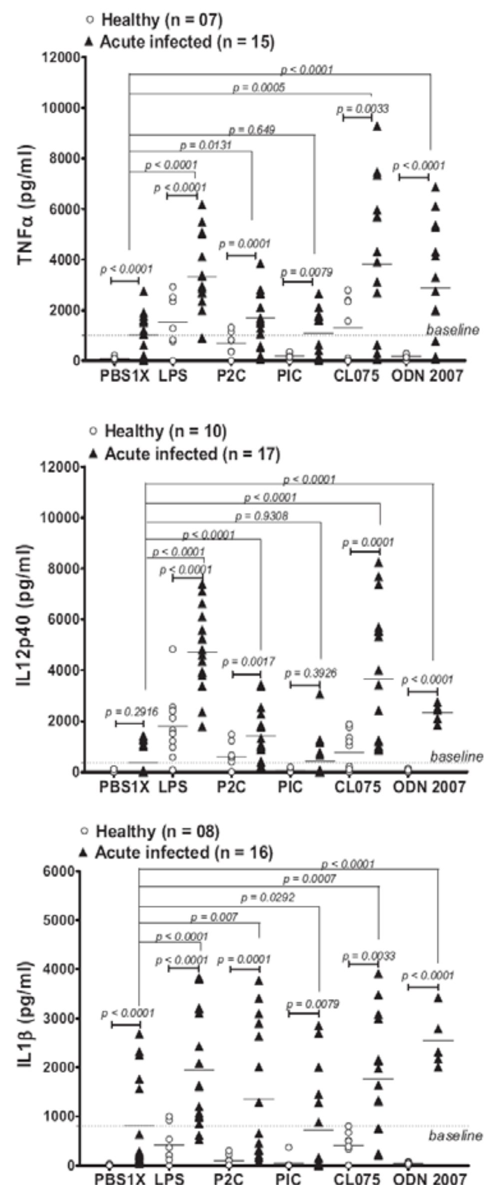

Fig. 1. Hyperresponsiveness of PBMCs from symptomatic malaria patients. ELISA analysis of cytokine levels in PBMC supernatants from uninfected health controls (open circles) or *P. falciparum* acutely infected subjects (closed triangles) after 20 h of stimulation with LPS (100 ng/ml), Pam2cysk4 (4 nM), Poly:IC (100 μg/ml), CL075 (100 ng/ml), and ODN 2007 (10 μM). All stimulations were performed with 2 other 10-fold different concentrations of TLR ligand, with similar results. Significant differences are indicated with *p*-values using unpaired *t* test with Welch correction or Mann-Whitney test when a normality test failed.

Increased TLR Responses During Acute *P. chabaudi* Infection. Next, we evaluated the response of mouse spleen cells to TLR ligands during

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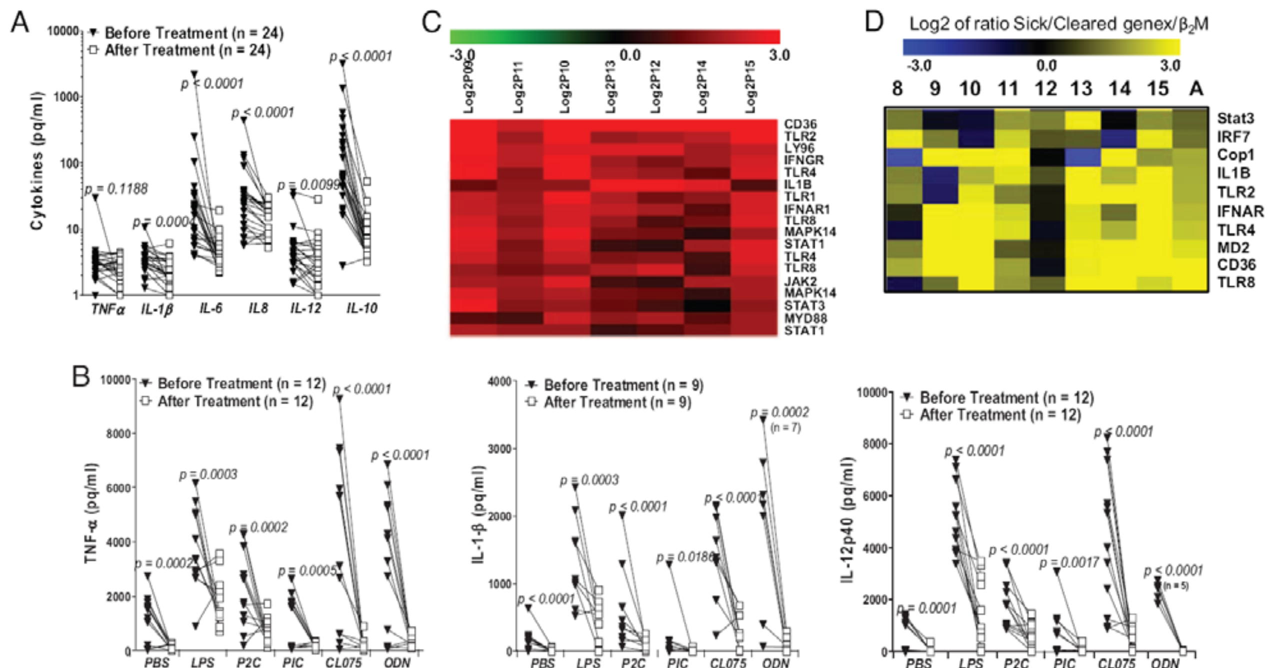

Fig. 2. Cytokine response in symptomatic malaria patients is reversed after curative chemotherapy. (*A*) Levels of 6 cytokines (TNFα, IL1β, IL6, IL8, IL10, and IL12p70) were determined in the plasma of *P. falciparum* infected subjects before treatment (closed triangles) or 30 days after treatment (open boxes) using CBA. (*B*) PBMC isolated from *P. falciparum* infected individuals before (closed triangles) or after treatment (open boxes) were cultured in the presence of the indicated stimuli for 20 h. Levels of TNF-α, IL-1β, and IL12p40 were measured in culture supernatants by ELISA. Significant differences are indicated with *p*-values using paired *t* test or Willcoxon matched-pairs signed-ranks test when a normality test failed. (*C*) Results of the clusterization. Each row represents a gene and each column the Log2 of the ratio of gene expression level: "Before treatment"/"After treatment" for the gene in question. (*D*) Validation of the results by qPCR is shown for some of the genes in the Fig. 1*C*. Each row represents a gene tested and each column the log2 of the ratio of the quantity of cDNA "Before treatment"/"After treatment" standardized by the β2microglobuline cDNA.

experimental infection with *P. chabaudi* AS. In this model, mice are injected i.p. with 10^5 infected red blood cells (iRBCs). Although animals exhibit signs of disease, lethal infection is uncommon. The parasitemia course in WT mice was typical for experimental infection with *P. chabaudi* (Fig. 4*A*) (25). Severe symptoms were concomitant with parasitemia and the peak of cytokine production in serum (Fig. S2). Splenic cells were harvested before and at several weeks post infection and stimulated with TLR ligands or malaria extracts. We observed a significant increase in IFNγ production (approximately 20-fold) by splenocytes isolated from mice during acute *P. chabaudi* infection (7 days post infection) when compared to control mice (Fig. 4*B*). During the following weeks, IFNγ levels produced in response to TLR agonists were comparable to those found in control mice, although during the second week after infection a drop in IFNγ responses was observed. Taken together, our results show that rodent malaria recapitulates the innate immune response in human disease, leading to proinflammatory priming of TLR responses.

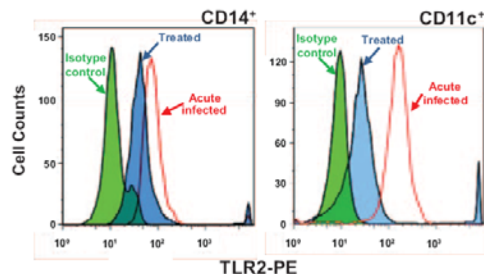

Fig. 3. Increased expression of TLR2 and TLR4 in monocytic cells from symptomatic malaria patients. PBMC were isolated from acute infected individuals before and 30 days after treatment and analyzed ex vivo through flow cytometry. The expression of TLR2 and TLR4 was evaluated in CD14+ and CD11c+ cells. Representative histogram showing fluorescence intensity of TLR2 in CD14+ (*Left*) and CD11c+ (*Right*) cells.

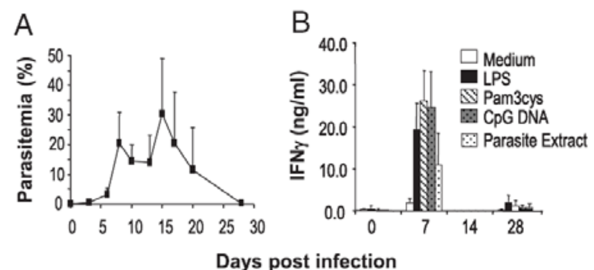

Fig. 4. Hyperresponsiveness of spleen cells from mice undergoing acute malaria. C57BL/6 mice were challenged with 10^5 iRBCs and followed every 3 days for (*A*) parasitemia and (*B*) levels of IFN measured in culture supernatants from mouse spleen cells harvested at various weeks post infection and stimulated with LPS (1 g/ml), Pam3cysk4ser (1 g/ml), CpG DNA (1 g/ml), or malaria extract (100 g/ml) for 48 h. As a control for cell viability, cells were stimulated with the mitogen Concanavalin A (5 μg/ml). The levels of IFNγ were measured in culture supernatants 48 sh post stimulation. The results are averages of 5 animals from a representative out of 2 experiments that yield similar results.

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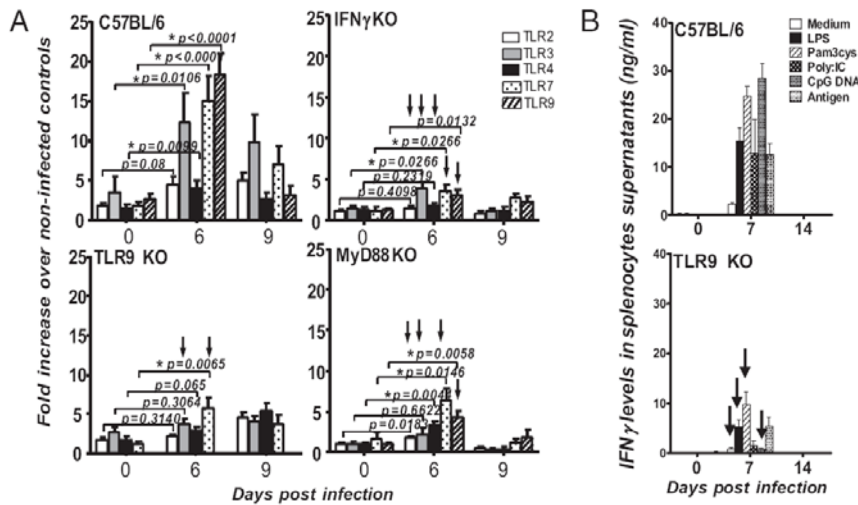

Fig. 5. Over expression of TLRs during acute rodent malaria. (*A*) Total RNA was isolated from spleens of WT, TLR9$^{-/-}$, MyD88$^{-/-}$, and IFNγ$^{-/-}$ mice before and after 6 and 9 days of infection with *P. chabaudi*. TLRs mRNA levels were measured by quantitative Real time PCR. The relative level of mRNA was determined by the comparative threshold cycle method, whereby data for each sample were normalized to β-actin and expressed as a fold change compared with uninfected controls. Bars are means + SEM from 4 animals per group performed in duplicate. Arrows indicate where differences were statistically significant ($P < 0.05$) compared to mRNA levels found in WT mice. (*B*). Spleen cells were harvested from WT, TLR9$^{-/-}$ and IFNγ$^{-/-}$ mice before and after 7 and 14 days of infection and cultivated in the presence of the indicated stimuli. IFNγ levels were measured in supernatants 48 h later. Results are means + SEM of twelve animals from 3 independent experiments. Arrows indicate levels of cytokines which differed statistically ($P < 0.05$) from those observed in WT mice.

MyD88 and IFNγ-Dependent Up-Regulation of TLR mRNA Correlates with *P. chabaudi* Mediated-Priming of TLR Responses. One explanation for the enhanced response to TLR ligands during infection would be that levels of receptor expression are increased. We therefore evaluated the levels of TLR mRNA in spleens of WT C57BL/6, TLR9$^{-/-}$, MyD88$^{-/-}$, and IFNγ$^{-/-}$ mice during *P. chabaudi* infection. Significant up-regulation of all of the TLRs tested was observed in WT mice at 6 days post infection and was clearly trending back to baseline by day 9. Up-regulation of TLR expression was significantly diminished in TLR9$^{-/-}$, MyD88$^{-/-}$, and IFNγ$^{-/-}$ mice (Fig. 5*A*). As expected, 7 days post infection, splenocytes from WT mice produced large amounts of IFNγ while cells from infected TLR9$^{-/-}$ mice produced significantly lower levels of this (Fig. 5*B*) and other cytokines (Fig. S3) in response to TLR ligands.

TLR9, IL12, IFNγ, and T Cells Mediate Hyperresponsiveness and Susceptibility to Endotoxin Shock During Malaria. We have gained further insights into the role of TLR9 in priming innate immune responses during malaria. We show that in vitro stimulation of bone marrow derived dendritic cells (BMDCs) with *P. chabaudi* iRBCs induced IL12 production in a manner that depends on TLR9 (Fig. S4*A*). To assess the mechanism and relevance of TLR hyperresponsiveness during acute malaria, we infected WT, TLR9$^{-/-}$, IL12$^{-/-}$, IFNγ$^{-/-}$ and RAG$^{-/-}$ mice with *P. chabaudi* and challenged them with LPS 7 days postinfection. WT mice produce high levels of proinflammatory cytokines (Fig. S4) and became very susceptible to i.v. injection of a sub lethal LPS challenge (i.e., 10 μg) (Table 1). TLR2$^{-/-}$ mice were as susceptible as WT (data not shown) while TLR9$^{-/-}$ mice produce significant lower levels of proinflammatory cytokines (Fig. S4*B*) and become more resistant to LPS challenge (Table 1). IL12$^{-/-}$ mice showed impaired production of proinflammatory cytokines (Fig. S4*B*) and full resistance to low doses of LPS, while IFNγ$^{-/-}$ mice were completely resistant to even higher LPS dose (i.e., 100 μg) despite the fact that these mice faced higher parasitemia (Table 1). RAG$^{-/-}$ mice showed intermediate resistance to low and high doses of LPS. Together, these results show that the IL12/IFNγ axis is crucial for malaria induced-priming of TLR responses and that TLR9 have an impact on this phenomenon.

Discussion

The major findings of this study can be summarized as follows: augmented TLR responses in patients naturally infected with *P. falciparum* were associated both with cytokinemia and clinical symptoms of malaria. The vast majority of the highly inducible genes involved in the innate immune response, including the TLRs, appear to be IFN inducible. We hypothesize that augmented expression of genes in the TLR pathway favor the recognition of *Plasmodium* by phagocytes. Finally, the state of being acutely infected with malaria heightens the innate immune response to challenge with unrelated microbial products in an MyD88-, TLR9-, IL12-, and IFNγ-dependent manner.

The manner in which the innate immune system is activated in malaria is difficult to precisely define. We are still not certain what ligand or ligands are responsible for stimulating cytokine production, although it is intriguing that a large number of TLR components are regulated downstream from an IFN response. Overall, the innate immune response to acute malaria results in an immunological state that is notably enhanced. Such a "primed" immune system could be expected to prevent super infection with bacteria or viruses and might be crucial for host survival. On the other hand,

Table 1. TLR9$^{-/-}$, IL12$^{-/-}$ IFNγ$^{-/-}$ and RAG$^{-/-}$ mice undergoing acute malaria display increased in vivo resistance to low LPS doses

Mouse	Condition	LPS dose μg/mouse	Number of dead/tested mice	Mortality, %	Mean parasitemia, %	*p* value chi-square test
C57BL/6	Control	1 10 100	0/3 0/15 0/9	0 0 0	0 0 0	
	Infected	1 10 100	0/6 31/34 22/23	0 91 95.6	9 9 9	
TLR9$^{-/-}$	Control	10 100	0/9 0/5	0 0	0 0	
	Infected	10 100	10/16 8/8	62 100	12 15	$P = 0.00186$
IL12$^{-/-}$	Control	10 100	0/8 0/3	0 0	0 0	
	Infected	10 100	0/16 2/6	0 33.3	19 22	$P < 0.0001$ $P = 0.0003$
IFNγ$^{-/-}$	Control	10 100	0/4 0/4	0 0	0 0	
	Infected	10 100	0/10 0/9	0 0	18 13	$P < 0.0001$ $P < 0.0001$
RAG$^{-/-}$	Control	10 100	0/6 0/3	0 0	0 0	
	Infected	10 100	2/10 3/5	20 60	8 12	$P < 0.0001$ $P = 0.0195$

a highly deleterious hypercytokinemia would be expected to ensue should a robust bacterial or viral invasion occur.

McCall and colleagues recently reported that adult individuals experimentally infected with *P. falciparum* were primed for responses to some, but not all, TLR ligands. These authors found an enhanced response to LPS (TLR4) and Pam3CysK4 (TLR2/TLR1) (23). Our results are notably different because the enhanced innate immune response was far more pronounced, and we found an enhanced response to all TLR ligands except pI:C. Although McCall et al. made an effort to correlate TLR2/4 expression with the priming they observed; they did not see enhanced expression in TLR2/4 in monocytes. The most likely reason for the limited degree of priming and the lack of enhanced TLR expression was due to the differences in the total body burden of parasites and the duration of parasitemia. It is useful to recall that the experimental volunteers were treated as soon as they become symptomatic and/or parasitemic, while our patients often did not present at our malaria clinic until they had had fevers and rigors for more than a week. As the average experimentally infected subject was aparasitemic for approximately 9 days (23), we infer that our patients were infected for a period of no more than 2 weeks before study.

While virtually every cytokine response we tested was enhanced in malaria-infected individuals, this was not the case for the anti-inflammatory cytokine, IL10. IL10 levels were greatly increased in the plasma of malaria patients. However, when PBMCs from infected patients were stimulated with TLR ligands, we often observed either no change in the IL-10 response or a suppression of IL-10 release by PBMCs (data not shown). Therefore, it can be concluded that the priming of cells for enhanced TLR responses is not coordinated throughout the entire transcriptome, but specifically applies to a defined group of important proinflammatory genes.

The in vivo consequences of malaria-induced priming appear to be important and applicable to infection with other *Plasmodium* species. Thus, *P. chabaudi*-infected mice had evidence of priming and developed a lethal hypersensitivity to very small amounts of LPS in a TLR9-, IL12-, and IFNγ-dependent fashion. Indeed, these data are some of the best to date that malarial DNA recognition might be pathophysiologically significant by engaging TLR9, as we previously proposed (10). As IFNγ is not produced in abundance by phagocytes, and is expressed in mouse lymphocytes (26), the mechanism appears to involve cross talk between the innate and the acquired arms of the immune system.

IFNγ has been shown not only to increase TLR expression but also to prime cells to LPS responses in a number of experimental infections (27–30) and has also been pointed as crucial to prime macrophages to release high amounts of TNF upon LPS administration and contribute to LPS induced septic shock (31). We (12) and others (14) have shown that TLR9 accounts for a considerable amount of IFNγ produced during malaria. Furthermore, production of IL12, a potent IFNγ inducer, is diminished in BMDCs from $TLR9^{-/-}$ mice upon in vitro stimulation with *P. chabaudi* iRBCs. Thus, it is reasonable to assume that the mechanism by which TLR9 mediates priming of host innate responses during malaria is by mediating parasite recognition and initiating IFNγ production. This assumption is strongly supported by findings in the *Propionibacterium* model in which TLR9-mediated production of IFNγ and IL12 is required to induce priming (32). Thus, we believe that the initial activation of innate immune responses is initiated by activation of TLR9 and induction of IL12 by innate immune cells (e.g., DCs and macrophages) resulting in production of IFNγ by NK and T cells (33, 34). In turn, the produced IFNγ will prime host cells to overexpress TLRs and become hyperresponsive to TLR agonists (Fig. S4C).

One can speculate on the consequences for the human host caused by malarial priming. The enhanced ability to respond to microbial ligands during immune surveillance probably protects the host from bacterial invasion. The areas of the world with the highest incidence and prevalence of malaria also have a high incidence of invasive bacterial infections, including *Salmonella*, Pneumococcus, and Meningococcus (35). In general, the innate immune response represents the classic "two-edged sword." Priming means that the innate immune system has an enhanced capacity to be over activated during secondary infection and initiate the septic shock syndrome. Coinfection with bacteria is not only common, but, as we might have predicted from these results, patients with malaria and bacteremia die at 3 times the frequency of individuals with malaria alone (36). In addition to the ability of bacterial products to activate a hyper immune response during coinfection, the likelihood that parasite and/or endogenous TLR agonists act as second stimuli for cells primed during malaria should not be disregarded. To begin, different *P. falciparum* derived TLR agonists, such as GPI anchors (8) and DNA bound to hemozoin (10), might activate an enhanced innate immune response and result in unchecked inflammation. Moreover, host endogenous ligands that are weak TLR agonists have been described (37). Such endogenous molecules might, under the right circumstances (e.g., parasite-induced apoptosis), exacerbate the proinflammatory state of patients with malaria, even though under ordinary circumstances, they lack potency. Thus, it is our hypothesis that proinflammatory priming during malaria favors activation of TLRs by components from host and/or microbial origins and has the capability to induce injurious inflammatory states, including cerebral malaria.

The observation that the innate immune system is primed during febrile malaria could not have been predicted intuitively. In bacterial sepsis, most immune responses appear to be strongly suppressed, thus leading to an immune state that is often described as "immunoparalysis" (38). Although immunosuppressive therapy was once thought to be highly protective during septic shock (39) and the use of high dose steroids was commonplace, it is now recognized that this therapy increases mortality (40). By the time a patient with bacterial sepsis is diagnosed, the innate immune system is globally and profoundly immunosuppressed. Hence, additional anti-inflammatory therapy is unlikely to alter the course of events. Just the opposite appears to occur in acute malaria. Although anti-inflammatory approaches to life-threatening malaria have never been proven to be beneficial, it seems that the potential for targeted immunosuppressive therapy should not be ignored. By defining the innate immune response during the severest forms of malaria, as we have done here in patients with moderate illness, such strategies could be rationally designed and tested. Clearly, a better understanding of the innate immune response is critical if novel therapies are to be rationally designed.

Materials and Methods

Reagents. Unless stated elsewhere, all reagents were from Sigma-Aldrich. CL075 was obtained from Invivogen; ODN 7909 was synthesized by ALPHA DNA as phosphorothioate-linked ODNs; Pam2cysk4 was from EMC Microcollection; CpG ODN 2007 was from the Coley Pharmaceutical Group. LPS was repurified by phenol chloroform extraction as described (41). MAbs to CD11c and TNFα were from BD PharMingen. MAbs to TLR2 and TLR4 were from eBiosciences. RPMI and DMEM were from Gibco. Cytokine ELISA kits were from R&D Systems.

Subjects. The study was approved by the Ethic and Research Council of the Rene Rachou Institute and the Brazilian Council of Ethics and Research (approval number 10567). Patients with acute febrile *P. falciparum* malaria ($n = 57$) were seen in the outpatient malaria clinic in the Tropical Medicine Research Center in Porto Velho, Brazil, an endemic malaria region in the Amazon basin. Informed consent was obtained before enrollment. All patients gave a history of recent fever and constitutional signs; none presented with severe anemia or cerebral malaria (see Table S1). Average ages of patients and a group of controls were 29.4 ± 12.8 and 31.2 ± 7.36 respectively. Up to 100 cc of blood was obtained immediately after confirmation of *P. falciparum* infection by a standard peripheral smear and 3–4 weeks after mefloquine therapy. PCR was used to confirm infection and cure, as well as to rule out coinfection with *P. vivax* (42). Each patient served as his or her own control; additional control individuals ($n = 16$) included noninfected subjects living in Porto Velho and malaria naïve individuals living in Belo Horizonte, Brazil, where malaria is not endemic.

PBMC Stimulation Assays. PBMCs were isolated from undiluted whole blood on Ficoll-paque Plus (GE Healthcare) per the manufacturer's recommendations. Cells were plated into 96-well cell culture plates at a final density of 2×10^5 in DMEM containing 10% FCS (Gibco) and 10 μg ciprofloxacin (Cellofarm) per ml and stimulated for 20 h as indicated.

Microarray Experiments and Data Analysis. Information on microarray experiments and data analysis can be found in *SI Text*.

Analysis of Cell Surface Expression of TLRs. PBMCs from acutely infected patients ($n = 11$) were stained with combinations of the following mAbs: CD11c-FITC (Serotec), CD14-FITC, (BD Biosciences), TLR2-PE, TLR4-PE (eBiosciences). Cells were gated by forward or side scatter to separate lymphocytes from monocytes and assessed for fluorescence using CellQuest software (BD Biosciences). Data were analyzed using Flowjo software (TreeStar).

Rodent Model of Malaria and Knockout Mice. The *Plasmodium chabaudi chabaudi AS* strain was used for experimental infections. Mice were infected by i.p. (i.p.) injection of 10^5 infected erythrocytes (iRBCs) and checked daily; moribund animals were scored as dead and euthanized. Laboratory values that reflect malaria-associated pathology were determined every 2–3 days throughout the infection (25). C57BL/6 as well as $RAG^{-/-}$ and $IFN\gamma^{-/-}$ mice were originally purchased from Jackson Laboratories. $MyD88^{-/-}$, $TLR2^{-/-}$, $TLR9^{-/-}$ were a gift of S. Akira (Osaka University, Japan). Knockout mice were 8–12 weeks old and were all backcrossed for at least 8 generations onto a C57BL/6 background. Mice were bred under pathogen-free conditions in the animal house of René Rachou Institute-Fundaçao Oswaldo Cruz.

Mouse Splenocyte Stimulation Assays. Cytokine measurements from stimulated mouse splenocytes during *P. chabaudi* infection were performed with groups of 4 to 8 C57BL/6 mice and the corresponding number of knockout animals, as indicated in the figure legends. Each experiment was repeated 2–3 times. Each animal was analyzed individually. Mice were infected i.p. with 10^5 iRBCs. Spleens were aseptically removed and macerated through a nylon mesh over the course of the experimental infection. After RBC lysis, splenocytes were resuspended in RPMI, 10% FCS (Gibco), 1% gentamicin (Schering Plough) at a density of 2.5×10^6 cells per ml. Splenocytes were subsequently cultured in 48-well plates for 48 h in a final volume of 500 μl in the presence of TLRs ligands.

Cytokine Measurements. Except where noted, all measurements of cytokines were performed using commercially available ELISA kits (R&D Systems). Serum cytokines from *P. falciparum* infected-patients were quantified with the CBA inflammation kit (Becton-Dickinson).

Real Time PCR Analysis (qPCR). Total RNA was isolated from mouse spleens over the course of infection, as described. Sequences of primers are listed in Table S4. The relative level of gene expression was determined by the comparative threshold cycle (Ct) method using the formula $2^{-\Delta\Delta Ct}$ whereby data for each sample were normalized to β-actin mRNA levels and expressed as a fold change compared with uninfected controls.

Statistical Analysis. All data were analyzed using Graphpad Instat 4.0 Software. Cytokine measurements from human PBMCs were analyzed using two-tailed student's *t* test. Mann-Whitney testing was used for non-parametric analysis when data did not fit a Gaussian distribution. A P value ≤ 0.05 was considered to be statistically significant.

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Supporting Information

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SI Materials and Methods

Microarray Experiments. A total of 23 patients were enrolled for RNA profiling; 2 were ultimately disqualified for study because of treatment failure or PCR-documented coinfection with *Plasmodium vivax*. Total RNA was extracted from PBMCs using an RNeasy kit (Qiagen) following the manufacturer's recommendations. RNA integrity and purity was analyzed with an Experion Automated Electrophoresis System (Bio-Rad) and a ND-1000 spectrophotometer (NanoDrop Technologies Inc.). The first 7 specimens were amplified using the senseAMP kit from Genisphere and arrayed using an operon custom microarray chip (designed by Drs. B. Seed and M. Freeman, Harvard University, Cambridge, MA) containing approximatley 17,000 unique cDNA at the Massachusetts General Hospital microarray facility.

The gene expression profiling of the remaining samples was performed using Illumina HumanWG-6 v2.0 Expression BeadChips (Illumina Inc.), which contains approximatley 47,000 transcripts, at the SCIBLU Genomics DNA Microarray Resource Centre at Lund University. Three hundred nanogram of total RNA was reverse transcribed and subsequently in vitro transcribed to cRNA using the Illumina TotalPrep RNA Amplification Kit (Illumina Inc.). The cRNA (1.5 μg) was mixed with hybridization buffer and added to the BeadChips for hybridization at 58 °C for 18 h. BeadChips were then washed, blocked, and stained with streptavidin-Cy3, before they were washed again and dried by centrifugation.

Microarray Data Analysis. For the Operon arrays, simple filtering, normalization and averaging were carried out using BASE (BioArray Software Environment) (https://base.mgh.harvard.edu/). Tiger Multi Experiment Viewer (TMEV) was used for cluster analysis. The *p*-values were based on permutation with a standard Bonferroni correction. Selected genes were analyzed with KMC algorithm (K median).

For the analysis of Illumina arrays, raw signal intensities were normalized using cubic spline normalization method and log transformed (log2). Genes were filtered to include only genes with signal intensity greater than the average from the negative controls in at least one of the samples with a detection p value less than 0.01. Differences in gene expression between the 2 conditions were considered significant if $P < 0.01$ with a paired t test with Benjamini-Hochberg correction for multiple testing and a fold change greater than 1.7.

The biological function of the genes up-regulated in one or both data sets during disease was analyzed with Onto Express (http://vortex.cs.wayne.edu/projects.htm). The potential utilization of transcription factor binding sites was further analyzed using the web-based online tool from Advanced Biomedical Computer Center ABCC (http://grid.abcc.ncifcrf.gov).

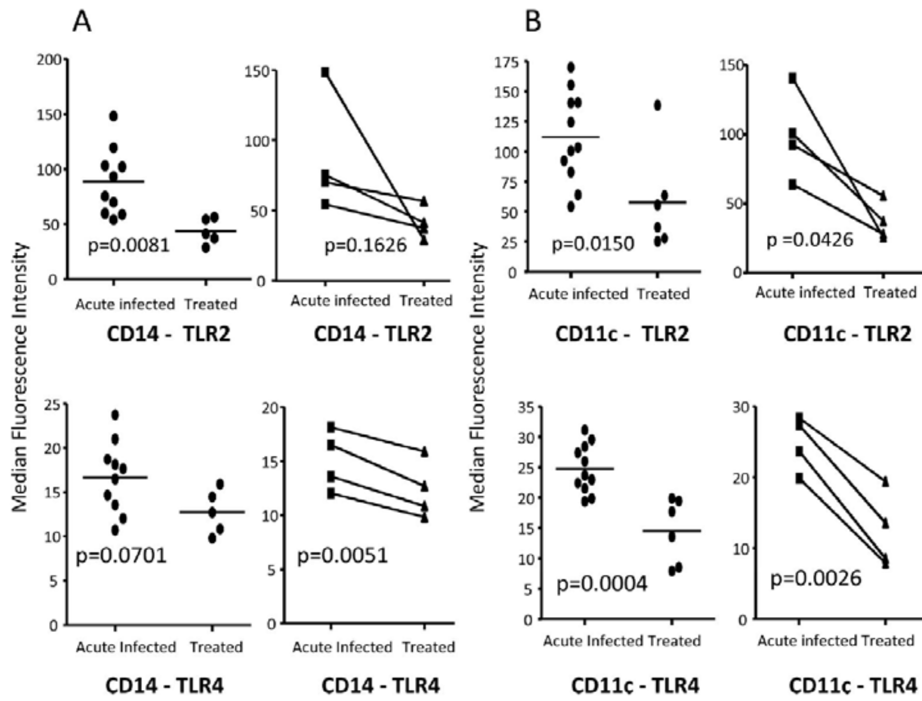

Fig. S1. Paired (*Right*) and not paired (*Left*) scatter plot, respectively, of TLR2 and TLR4 MFI in CD14⁺ (*A*) and CD11c⁺ (*B*) cells from patients before and after treatment. Significant differences are indicated with *p*-values using paired *t* test or unpaired *t* test.

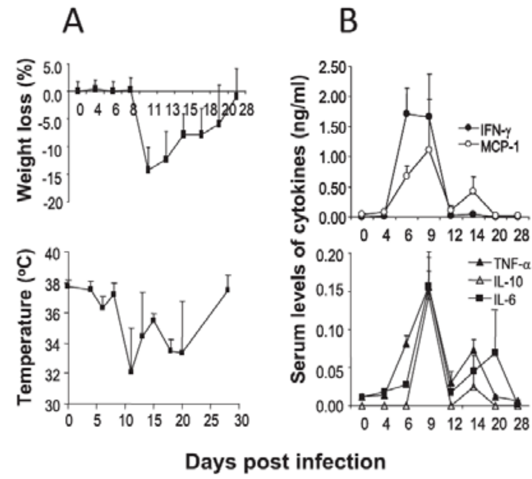

Fig. S2. Severe symptoms associated with peak of cytokine production during *P. chabaudi* infection in mice. (*A*) Body weight and body temperature and (*B*) serum levels of cytokines (i.e. IFN, MCP-1, TNF, IL10, and IL6) measured in C57BL/6 mice infected with *P. chabaudi* at various days post infection. The results are averages of 5 animals from a representative out of 2 experiments that yield similar results.

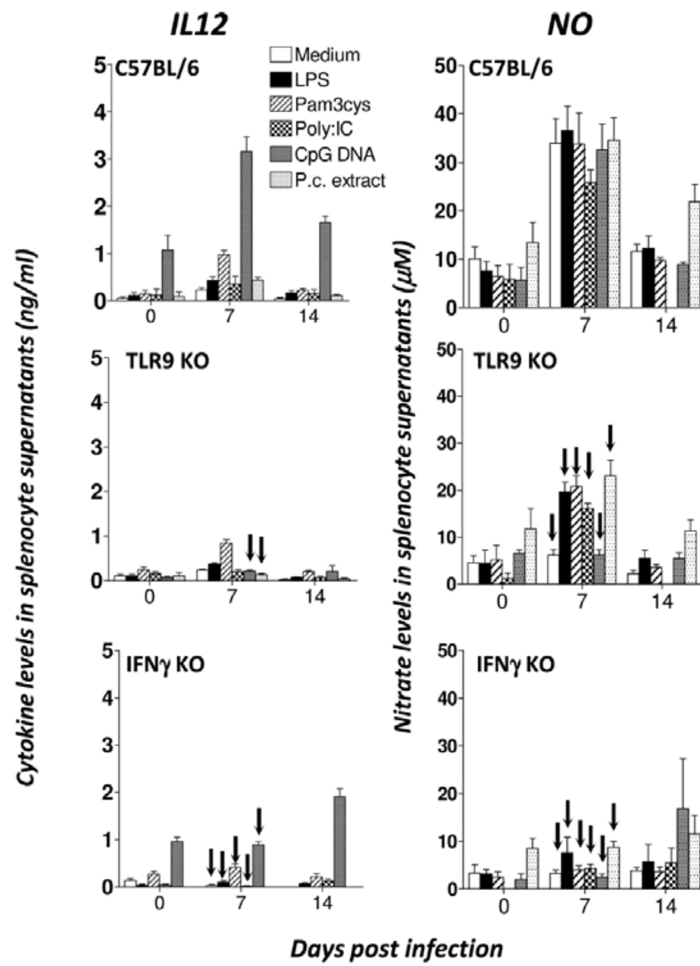

Fig. S3. IL-12 and NO levels in splenocytes supernatants from C57BL/6, TLR9$^{-/-}$ and IFNγ$^{-/-}$ mice before and after 7 and 14 days post infection cultivated in the presence of the indicated stimuli. Results are means + SEM of 12 animals from 3 independent experiments. Arrows indicate levels of cytokines which differed statistically ($P < 0.05$) from those observed in WT. mice.

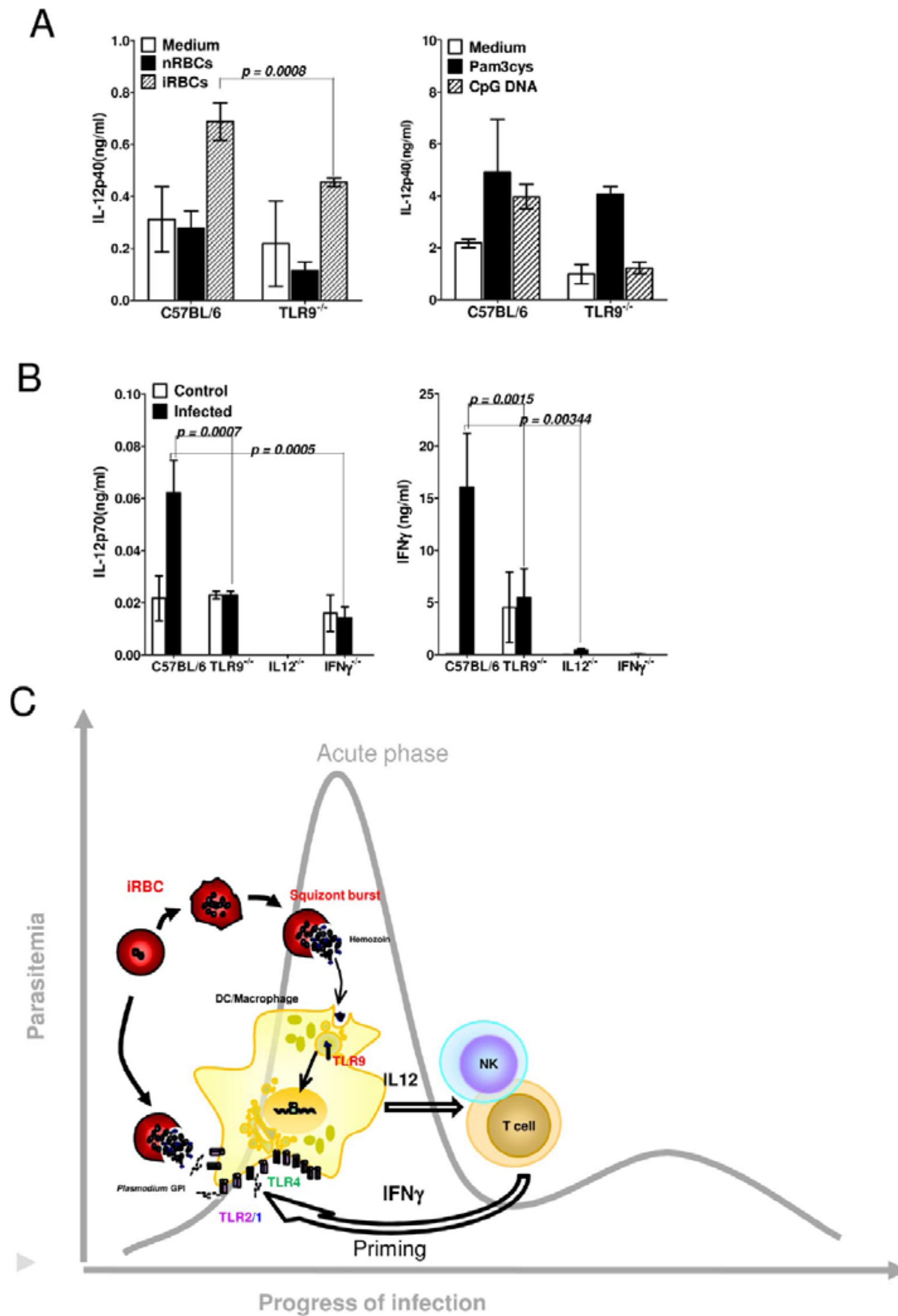

Fig. S4. (*A*) BMDCs from TLR9$^{-/-}$ mice have impaired IL12 production upon stimulation with *P. chabaudi* iRBCs. BMDCs (2×10^5) from WT and TLR9$^{-/-}$ mice were incubated with *P. chabaudi* iRBCs (10:1) for 12 h when compared to WT mice. Levels of IL12 were measured in culture supernatants by ELISA. The results are averages of 4 animals from a representative out of 2 experiments that yield similar results. (*B*) Levels of cytokine in sera of C57BL/6, TLR9$^{-/-}$, IL-12$^{-/-}$, or IFNγ$^{-/-}$ mice inoculated with *P. chabaudi* and challenged with LPS. Mice were infected with 10^5 *P. chabaudi*-iRBCs. Non-infected red blood cells (nRBCs) were used as control. At day 7 postinfection mice were challenged with 10 μg of *E. coli* LPS. Cytokine levels were assessed in sera by CBA 9 h after LPS challenge. (*C*) Theoretical scheme of the role of TLR9/IL12/IFNγ axis in mediating pro-inflammatory priming during malaria.

Table S2. Main cellular pathways triggered during *P. falciparum* malaria

Database Name	Pathway Name	p-value	Up-regulated genes	
			Operon	*Illumina 6 v2*
KEGG	**Antigen processing and presentation**	4.6E-5	PA28, MHCII, CTSB/L/S, HSP70, HSP90, MHCI, ERp57, CALR, CANX, GILT, CTSB	CTSB/L/S, KIR3DL1, KIR3DL2, KIR3DL3, KIR3DL4, KLRC3, KLRD1, KLRC4, KLRK1, PSME2, PA28, MHCII, CD8A, CD8B
KEGG	**Toll-like receptor signaling pathway**	1.2E-2	IκBα, MYD88, MAPK14, IL1β, TLR1, LY96, TLR2, TLR4, TLR8, p38, TANK, IRAKM	MyD88, TRAM, MAPK14, LY96, TLR1, TLR6, TLR7, TLR8, p38, IKKα, IKKβ
KEGG	**Cytokine-cytokine receptor interaction**	3.51E-2	CCR2, IL1β, IFNAR1, IFNGR2, TNFSF10, TNFSF12, TNFSF13, TNFSF13B, EDAR	BLR1, CCL5, CCL28, CCR2, CCR6, IL23A, IL11RA, CSF2RA, IL2RB, IL7R, IL15RA, IL17R, IFNAR1, IFNGR2, IL10RB, TNFSF10, TNFSF12, SF25, SF1A, LTB, LTBR, TNFSF5, SF5, TNFSF13, TNFSF13B, EDAR
KEGG	**IFN signaling pathway**	4.2E-2	IFNAR1, IFNα/βR, IFNGR2, JAK2, STAT1	IFNAR1, IFNα/βR, IFNGR2, JAK2

Table S3. IFNγ-inducible genes are largely induced during *P. falciparum* malaria

Array	Transcription site	Number of genes	% of genes	Site	Factor	Probability
Operon	Gamma-IRE CS	272	100	CWKKANNY	Unknown	3.91E-03
	GAS/SIE/APRE-3	271	100	TTNNNNNAA	STAT	3.91E-03
	GAS/SIE/APRE-2	270	99	TTNNNNAA	STAT	3.91E-03
	GAS/SIE/APRE-4	269	99	TTNNNNNNAA	STAT	3.91E-03
	GAS/SIE/APRE-1	235	86	TTNCNNNAA	STAT	9.77E-04
Illumina	Gamma-IRE CS	472	100	CWKKANNY	Unknown	3.91E-03
	GAS/SIE/APRE-3	459	97.2	TTNNNNNAA	STAT	3.91E-03
	GAS/SIE/APRE-2	468	99	TTNNNNAA	STAT	3.91E-03
	GAS/SIE/APRE-4	456	96	TTNNNNNNAA	STAT	3.91E-03
	GAS/SIE/APRE-1	355	75.2	TTNCNNNAA	STAT	9.77E-04

Table S4. Sequences of primers used in qPCR reactions

Primer	Forward (5'- 3')	Reverse (5'- 3')
TLR2	CGTTGTTCCCTGTGTTGCT	AAAGTGGTTGTCGCCTGCT
TLR3	TTGCGTTGCGAAGTGAAG	TAAAAAGAGCGAGGGGACAG
TLR4	TTCACCTCTGCCTTCACTACA	GGGACTTCTCAACCTTCTCAA
TLR7	GCTGTGTGGTTTGTCTGGTG	CCCCTTTATCTTTGCTTTCC
TLR9	GAAAGCATCAACCACACCAA	ACAAGTCCACAAAGCGAAGG
B-actin	GGATGCAGAAGGAGATTACTG	CGATCCACACAGAGTACTTG

ARTIGO 4

Therapeutic targeting of nucleic acid sensing Toll-like receptors prevents Cerebral Malaria

Bernardo S. Franklin, Sally Ishizaka, Fabian Gusovsky, Marco Antônio Ataide, Natalia Oliveira, Rosane B. Oliveira, Douglas T. Golenbock and Ricardo T. Gazzinelli.

O objetivo 3 deu origem a esse artigo que está em fase de submissão à revista Science.

Iniciamos uma colaboração com a EISAI Research Institute que desenvolveu um antagonista de TLR9 denominado AT9. Neste trabalho demonstramos os resultados dos testes do AT9 que bloqueia a ativação de TLR9 na malária. Mostramos que AT9 inibe a resposta de células à estimulação com CpG ODN (um ligante sintético de TLR9) *in vitro*. O tratamento oral de camundongos com AT9 inibe a resposta de TLR9 *in vivo* e *ex vivo* e protegeu camundongos da malária cerebral (CM) por *P. berghei* ANKA. Camundongos tratados com AT9 não apresentaram as lesões cerebrais e disfunções neurológicas característicos da CM por *P. berghei* e foram protegidos dos efeitos do "priming" causado pela malária. Mostramos então que TLR9 possui um papel essencial em mediar os sintomas da malária cerebral e que o bloqueio da ativação de TLR9 por AT9 é uma promissora estratégia terapêutica contra a malária.

Therapeutic targeting of nucleic acid sensing Toll-like receptors prevents cerebral malaria

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Toll-like receptors (TLRs) and their outputs, pro-inflammatory cytokines, have been implicated in the pathogenesis of malaria. Interference with TLR function is therefore likely to render a better outcome by preventing excessive release of pro-inflammatory mediators. Herein, we describe the protective effect and mechanism of action of AT9, a synthetic antagonist of nucleic-acid sensing TLRs, on Plasmodium berghei ANKA induced cerebral malaria (CM). In vitro, AT9 inhibited the activation of human and mouse TLR7/8/9 in a dose dependent manner. Furthermore, therapy with AT9 diminished the in vivo cytokine responses to a TLR9 agonist or Plasmodium infection and prevented severe signs of CM, such as limb paralysis, brain vascular leak and death. Therefore, AT9 is an anti-inflammatory drug with potential use to prevent symptoms and improve malaria outcome.

Malaria is still an important global concern affecting millions of people and putting 40% of world population at risk (1). Nevertheless, a welcome recent trend is the formation of major global governmental and non-governmental initiatives and partnerships that hold great promise for the control of this disease that has for centuries plagued developing countries. Thanks to an extensive collaboration between the Walter Reed Army Institute of Research and the drug company GlaxoSmithKline, a recombinant protein that fuses a part of the *Plasmodium falciparum* circumsporozoite (CS) protein to the hepatitis B surface antigen, designated as RTS,S vaccine, has achieved phase III clinical trial status (2). Although the news is covered with enthusiasm, the expectation is that RTS,S will offer at best partial protection, maybe 30%, against infection. Some indicators predict it might diminish levels of severe malaria by as much as 50% which may be enough to give infants and small children a better chance of surviving the scourge (1).

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will still be vulnerable to the disease. Thus, we continue to rely on the development of new effective therapeutic compounds for weakening symptoms and lethality in cases where the infection nevertheless took hold.

The urgent need of such therapeutic approaches is, in our opinion, as important as the design of a protective vaccine to prevent infection. Our point of view is supported by reports of people living in areas where transmission is holoendemic. Sub-Saharan Africa is one of those areas where people are almost continuously infected by *P. falciparum*, nevertheless the majority of infected adults rarely experience overt disease (3, 4). They go about their routines of school, work, and household chores feeling essentially healthy despite a parasite load in their blood that would almost universally prove lethal to a malaria-naïve visitor. This vigor in the face of infection suggests that the most severe clinical manifestations of malaria are mainly caused by an exaggerated host immune response to *Plasmodium* infection rather than the presence of blood parasites. Indeed, there is currently consensus that malaria is a disease where a strong pro-inflammatory response is responsible for the severity of clinical manifestations (5-7). Patients afflicted by Plasmodium usually experience extremely high systemic cytokinemia, high fever, paroxysms, renal failure and ultimately death. In this regard, malaria there is striking similarities between malaria and bacterial sepsis (8, 9).

The Toll-like receptors (TLRs) constitute a family of innate immune receptors that are critical for pro-inflammatory cytokine production during microbial challenge (10). Since its discovery in the early 90's, it has accumulated a relatively large bulk of evidence that TLRs are implicated in the pathogenic basis of multiple immune mediated inflammatory disorders (IMIDs), such as systemic lupus erythematosus and rheumatoid arthritis. There is now sufficient validation around certain TLRs to justify them as therapeutic targets [reviewed in ref (11)]. Indeed, the anti-malarial drug, hydroxychloroquine have been efficiently used to treat IMIDs long before its inhibitory effect on TLR7/9 activation was recognized (12).

TLRs have also been shown to play an important role in malaria pathogenesis. This is clearly illustrated in studies using mice genetically engineered not to express the myeloid differentiation primary-response gene 88 (MyD88) (13) an essential intracellular adaptor for signaling by all TLRs, but TLR3 (14). While the lack of functional MyD88 did not affect control of parasite replication, it resulted in impaired cytokine production, and attenuation of pathology during acute malaria in mice (15-17). Indications of TLR involvement in the pathogenesis of human malaria, although more complex, have been also revealed by different studies. First, it has been demonstrated that *P. falciparum* derived glycosylphosphatidylinositol (GPI) anchors and genomic DNA carried by hemozoin are potent stimulators of TLR2/TLR4 and TLR9, respectively (18, 19). In addition, analysis of human genome showed polymorphisms in TLRs (20, 21) or TLR adaptor proteins (22), to be associated with malaria outcome, although the mechanistic implications of TLRs in malaria pathogenesis was not revealed.

Studies performed in our laboratory (23) and elsewhere (24, 25) have recently provided new insights for understanding the mechanism by which TLRs are involved on the pathogenesis of human malaria. We established that an IFN-mediated pro-inflammatory priming associated with hyper-responsiveness of TLRs and cytokinemia is a hallmark of symptomatic episodes of *P. falciparum* malaria (23). The data obtained from *P. falciparum* infected

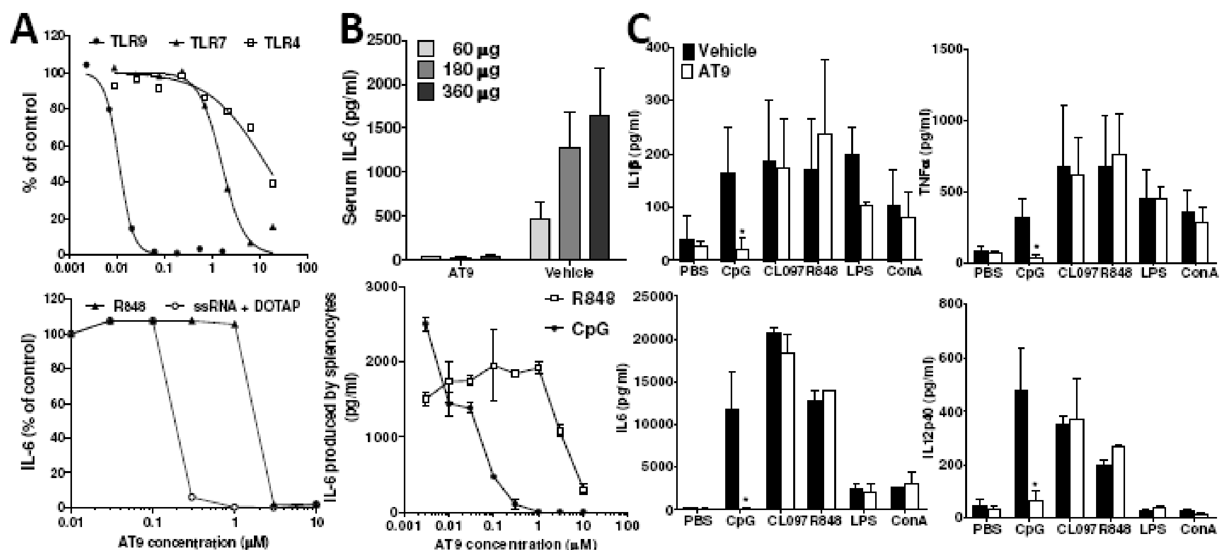
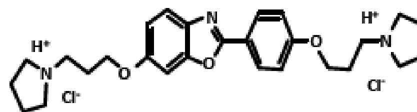


Figure 1 – AT9 prevents TLR7 and TLR9 activation by nucleic acids. (A) (Top panel) HEK293 cells stably carrying plasmids for TLR4/MD2, TLR7, or TLR9 and the NFκB reporter gene ELAM-1-luciferase were stimulated with the appropriate ligand (LPS with soluble CD14 (10 nM), R848 or oligo 2006) overnight. Next, Steady-Glo reagent (Promega Inc., Madison, WI) was added to the wells and the amount of luciferase activity in each sample was quantified in a Wallac Envision counter. (Botton panel) Ficoll-separated mononuclear cells were isolated from healthy volunteer donors, washed, and plated with stimulatory oligonucleotide 2216 in complete RPMI for 72 hours. IL6 in supernatant was quantified by ELISA (R&D Systems) and expressed as percentage of levels found in non-stimulated cells supernatants. (B) (Top panel) BALB/c mice were predosed with 60 mg/kg of AT9 1.5 hours before challenge with the indicated μg of Oligo 1668 (Oligos, Etc., Wilsonville, OR). At 2 hours post-challenge serum was collected and assayed for IL-6 by ELISA. (Bottom panel) Spleen cells from mice were incubated with CpG ODN 1401 (1 μg/ml) or Resiquimod (1 μM) for 72 hours in the presence of increasing concentrarions of AT9. (C) Mice were orally treated with 20 mg/kg/day of AT9 during 5 days. After 2 hours of the last dose, spleen cells were harvested and challenged with indicated stimuli. Cytokine levels in culture supernatants were analyzed by Searchlight multiplex.

individuals were remarkably predictive of what we observed in the *P. chabaudi* rodent malaria model (23, 26) and thus, the *in vivo* consequences of malaria-induced priming appear to be relevant and applicable to infection with different *Plasmodium* species. More precisely, we demonstrated that activation of TLR9 during rodent malaria induces the production of IL-12, by dendritic cells (DCs), which in turn initiates IFNγ responses in T cells. IFNγ then primes innate immune cells by up-regulating TLR expression and function ensuing in a highly deleterious cytokinemia. We therefore hypothesize that inhibition of TLR9 activation, by antibodies, peptides, or small molecules will in all likelihood render a better clinical outcome by preventing the pro-inflammatory priming and excessive production of inflammatory mediators in malaria patients.

With this in mind, we tested a new compound named anti-TLR9 (AT9) developed by the Eisai Research Institute, which acts as an antagonist for the nucleic acid sensing-TLRs. AT9 is a small water soluble aromatic organic compound. Its bioactive structure is composed of Benzoxazole with two sided pyrrolidin rings (see formula below). Lamphier and colleagues (2009) have demonstrated that AT9 is highly effective *in vivo* to suppress the response to



oligonucleotides (ODNs) containing immunostimulatory unmethylated CpG motifs. In addition, they demonstrated that once incubated with cells, AT9 rapidly translocates to endosomal compartments where it works as a quencher, sequestering nucleic acids and preventing its association with nucleic acid sensing TLRs (manuscript submitted for publication).

To test the effect of AT9 on TLR activation, we stimulated HEK293 cells stably transfected with TLR4/MD2, TLR7, or TLR9, and the NFκB reporter gene ELAM-1-luciferase (27) with each corresponding ligand (LPS, R848 or oligo 2006, respectively) in the presence of increasing concentrations of AT9. Measurement of ELAM-1-luciferase activity showed that AT9 inhibited TLR9 activation in a dose dependent manner. Higher concentrations of AT9, however, were able to inhibit TLR7/8 activation by the imidazoquinoline compound R848. Incubation of cells with AT9 showed only a small effect on TLR4 activation (Fig 1A). Treatment of human PBMCs with AT9 also diminished IL6 production in response to the TLR9 agonist (CpG ODN) (data not shown), or single stranded RNA (ssRNA), which induces the activation of NFκB via human TLR8 (28, 29), and to a lesser extent to R848, which activates human TLR7 and 8 (30) (Fig 1A – bottom panel).

To test the *in vivo* efficacy of AT9 on inhibition of TLR9, C57BL/6 mice were orally treated with 60 mg/kg of AT9 1.5 hours before challenge with increasing concentrations of CpG ODN 1668. Oral treatment with AT9 revealed a significant impairment of IL-6 production, in sera 2 hours after challenge (Fig 1B). Furthermore, consistent with what we observed for human cells, incubation of mouse spleen cells with increasing concentrations of AT9 diminished in vitro IL-6 production in response to CpG ODN 1668 and R848 in a dose dependent manner. To test the *in vivo* specific activity of lower concentrations of AT9 on TLR activation, BALB/c mice were orally treated with 20 mg/kg/day of AT9 during 5 days. Two hours after the last dose, the spleen cells were harvested and cultured in the presence of the indicated TLR ligands or Concanavalin A. Multiplex analysis of various cytokines showed that 20 mg/kg/day of AT9 caused target inhibition of responses to the TLR9 agonist (CpG DNA), but not other synthetic non-nucleic acid agonists of TLR7 (i.e. CL097 or R848) or LPS (Fig 1C).

Due to its ability in blocking activation of nucleotide-sensing TLRs, especially TLR9, we decided to evaluate

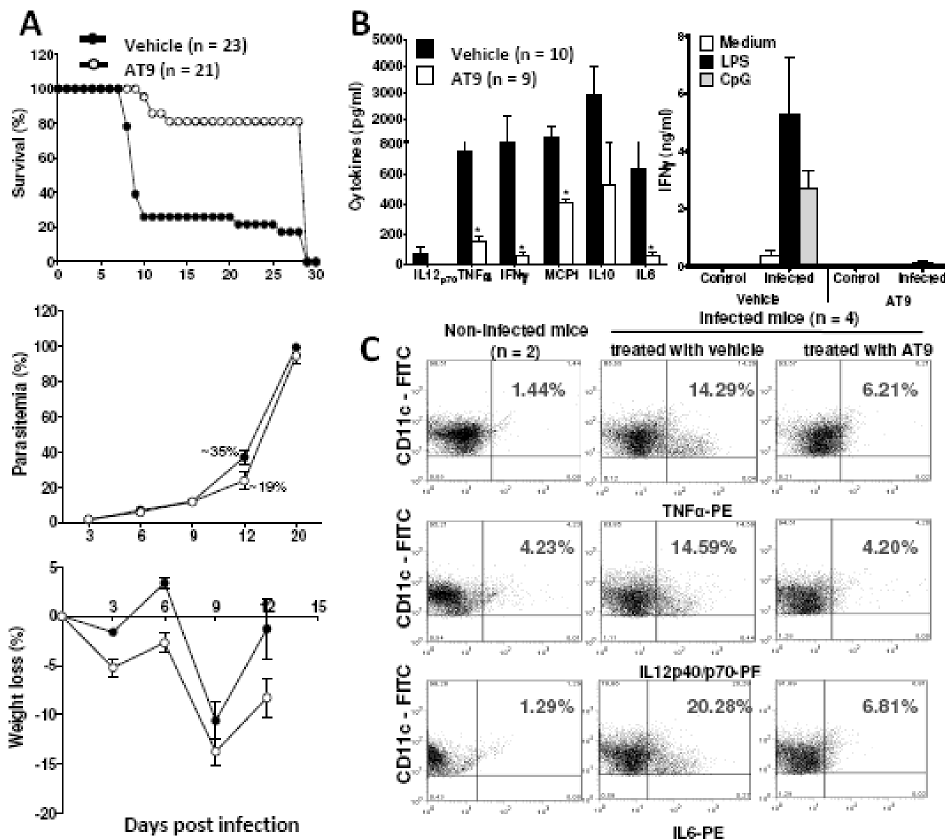


Figure 2 - AT9 protects mice against *P. berghei ANKA* mediated CM. C57BL/6 mice were treated with 120 mg/kg/day of AT9-02 one day before and during 12 days post infection with 105 PbA infected erythrocytes (iRBCs). Mice treated with vehicle (acidified water) were infected and used as control group. (A) Survival, parasitemia and body weight were compared between groups of mice at various days post infection. (B) Cytokine levels were assessed in the sera (10 mice per group) or in supernatants of splenocytes cultured in the presence of LPS (1 μg/ml) or CpG ODN (1 μg/ml). Results are from a sum of 4 different experiments. Unpaired two-tailed student's t or Mann-Whitney test were used to compare means between cytokine levels detected in vehicle or treated group of mice. A P value < 0.05 was considered to be statistically significant. (C) Analysis of intracellular staining of cytokines in CD11c+ splenic DCs from vehicle or AT9 treated mice at 8 days post infection with 105 PbA iRBCs.

the effect ER-820466 on malaria pathogenesis. We first evaluated the efficacy of AT9 treatment on *P. chabaudi* infection. Although not usually lethal, this murine model of malaria primes TLR pro-inflammatory responses rendering mice extremely susceptible to low doses of LPS (23). Treatment with 20 or 60 mg/kg/day of AT9 had no effect on parasitemia, weight loss or body temperature during *P. chabaudi* infection (Supplementary Fig 1A). Nevertheless, treatment with AT9 at 60 mg/kg/day diminished the production of pro-inflammatory cytokines produced by spleen cells (Supplementary Fig. 1B) as well as systemic cytokinemia. It also inhibited TLR responsiveness during acute malaria (Supplementary Fig 2). When given at higher concentrations (120 mg/kg/day), AT9 protected infected mice from LPS induced shock (Supplementary Fig 3B). It is important to recall that mice treated with 120 mg/kg/day of AT9 presented an even higher resistance (86% survival) to LPS induced shock than mice TLR9-/- mice (38% survival) as compared to vehicle mice (less than 10% survival) (23). Based on our in vitro experiments (Fig 1), it is conceivable that in vivo 120 mg/kg/day of AT9 is able to inhibit activation of TLR7/9.

If this is the case, these results provides evidence that along with TLR9, TLR7 and 8 are also activated during malarial priming of TLR responses. Our main hypothesis is that TLR hyper-responsiveness could be highly deleterious, and possibly responsible for lethality, especially in cases where bacterial co-morbidities take place (23, 24, 31). Moreover, based on the results presented here, we propose that hyper-responsiveness of TLRs would favor the direct activation of innate immune cells by TLR agonists derived from Plasmodium parasites, which are otherwise not so potent as the ones found in bacteria (13). Thus, the parasite components released during rupture of infected red blood cells, would be in part responsible for cytokinemia and some of sign/symptoms observed during acute episodes of malaria, as is the case of paroxysm.

Cerebral malaria (CM) is the most severe outcome of *P. falciparum* infection in humans, which is also recapitulated in the rodent malaria model by infection of C57BL/6 mice with *P. berghei ANKA* (PbA) (32, 33). The pathogenic basis of CM is not entirely known. However, there is consensus that the host immune response is largely responsible for the development of CM, as the density of parasitemia does not correlate well with clinical disease (34). Furthermore, although controversial, a role for TLRs in CM pathogenesis has also been suggested (16). To test if blockade of nucleic-acid sensing TLRs signaling has any impact on CM, we treated PbA infected C57BL/6 mice with 120 mg/kg/day of AT9 or vehicle. Mice were treated one day before until day 12 post-infection. Strikingly, treatment with AT9 had a pronounced effect enhancing survival after infection with PbA (Fig 2A). Most mice treated with vehicle produced significantly higher levels of pro-inflammatory cytokines (Fig 2B), and succumbed to PbA mediated CM by day 8 - 12 post-infection. No change in parasitemia was observed in mice treated with AT9 (Fig 2A), further indicating that the therapeutic effect of AT9 on malaria is most likely due to its ability to block nucleic acid sensing TLR7 and 9. Importantly, PbA mediated priming of TLR responses was also diminished by AT9 therapy (Fig 2B). Nucleic acid sensing TLRs (such as TLR9 and 7) are highly expressed on dendritic cells (DCs), which have been shown to be activated by *Plasmodium* products and play a critical role in malaria pathogenesis (17, 35-38). Exposure of

of bone marrow derived dendritic cells (BMDCs) to erythrocytes infected with *P. chabaudi* elicited the production of IL-12 (39) which was ablated in DCs from TLR9$^{-/-}$ mice (23). We next sought to identify the effect of AT9 on cytokines produced by CD11c+ splenic DCs employing the flow cytometry (FACS) assay for intracellular cytokine staining. We found that in PbA infected mice, the frequency of CD11c^{+} splenic DCs producing TNFα, IL12p40/p70 and IL6 was significantly increased at day 8 post-infection. The production of all three cytokines was mostly abolished in DCs from PbA infected mice treated with AT9 (Fig. 2C). Maturation of DCs, assessed by evaluation of CD40, CD80 and CD86 expression on CD11c+ cells, was not affected by treatment with AT9 (data not shown).

Brain seizures with severe debilitation are a hallmark of CM. We therefore performed neurological examinations in each mouse during acute PbA infection. We show that mice treated with AT9 attain better clinical outcome; symptoms, when present, were drastically attenuated when compared to vehicle treated mice (Fig. 3A). With malarial infection comes a cyclical fever and concurrent TNFα release. As intra-erythrocytic parasites mature, they express virulence-associated genes that remodel the erythrocyte leading to sequestration within a variety of vascular beds, including those of the brain. Parasite sequestration is exacerbated by cytokine mediated up-regulation of host endothelial receptors that bind to parasitized erythrocytes (i.e., ICAM-1, VCAM) (40). TNFα has been shown to affect blood brain barrier (BBB) permeability leading to leak and infiltration of mononuclear cells contributing to CM (41). Due to its effect on cytokine production on PbA infection (as shown in Fig. 2B and C), we next tested the effect of AT9 on the integrity of BBB during PbA infection. First, we studied the cerebral vascular leak after intravenously injection of Evans blue in mice with end-stage CM. Mice treated with vehicle displayed a distinct vascular leak as shown by a massive blue staining of the brain, which was absent in AT9 treated mice (Fig. 3B). The morphological correlate of the vascular leak was investigated in brain tissue sections. Consistent with the macroscopic observation, brain vessels of infected mice treated with vehicle displayed typical microvascular damage with sequestration of iRBCs including the presence of hemozoin, mononuclear cell adhesion on the endothelium and hemorrhage into the parenchyma, which were much less frequent in AT9 treated mice. In general, PbA infected mice treated with AT9 presented significant lower numbers of brain intravascular inflammatory foci (Fig. 3C).

The role of TLRs in rodent CM is controversial. The reason for the discrepant results is likely to be related to variations on genetic background of knockout mice, which were not fully backcrossed into C57BL/6 background, and possibly variations in the PbA strains used in different studies (16, 42). In this sense, the use of the AT9 compound was highly appropriate, since we used very well defined C57BL/6 inbred mice that received either the vehicle alone or drug and the results were clear-cut. In addition, to support our hypothesis about the mechanism of action that AT9 therapy protects against PbA mediated CM, we infected the "three deficient" (3d) mouse strain, which is a C57BL/6 mouse with a single mutation in the UN93B1 gene. UNC93B1 is an endoplasmic reticulum-resident membrane protein, responsible for trafficking the nucleic-acid sensing TLRs from ER to endosomal compartment. As a consequence of non-functional UNC93B1, mice are not responsive to TLR3/7/9 agonists (21). Although no difference was observed on blood parasite load, a significant number of mice lacking functional UNC93B1 escaped from CM and early

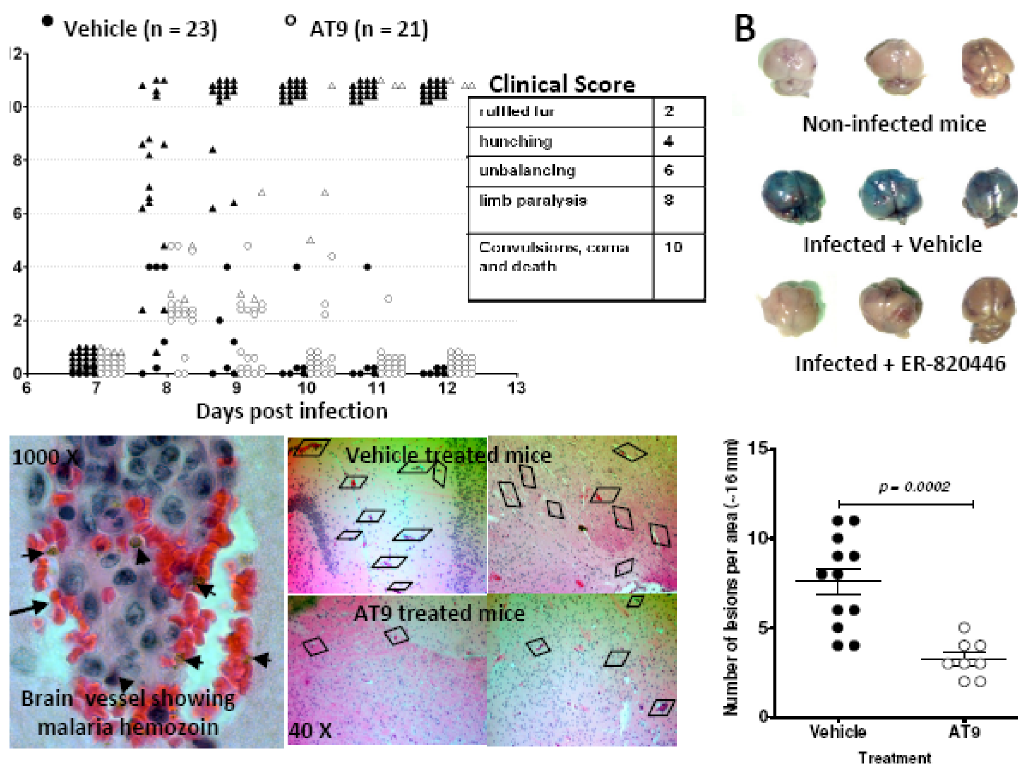


Figure 3 – AT9 improves malaria clinical outcome by preventing cerebral vascular leak. (A) Each of the classical CM symptoms (ruffled fur, abnormal posture, unbalancing, limb paralysis, convulsion, coma and death) was given a score (0, 2, 4, 8 or 10). Mice were then graphically ranked based on symptoms presented at each time point. (B) After 9 days of infection mice were injected intravenously with 0.2 ml of 1% Evans blue (Sigma-Aldrich), shortly before the death of vehicle treated mice (120 mg/kg/day). One hour later, mice were sacrificed, and the coloration of brain was assessed. Naïve mice were also injected with Evans blue and used as control. (C) Brains from vehicle or ER820446 treated mice were harvested at day 9 post infection, fixed in formalin and brain sections were stained with Hematoxylin & Eosin (HE). Brain lesions were identified over oil immersion 1000X magnification (left panel) and counted over 40X magnification (right panels) on an optical microscope. Statistical analysis were performed using unpaired two-tailed student's t test. A P value < 0.05 was considered to be statistically significant.

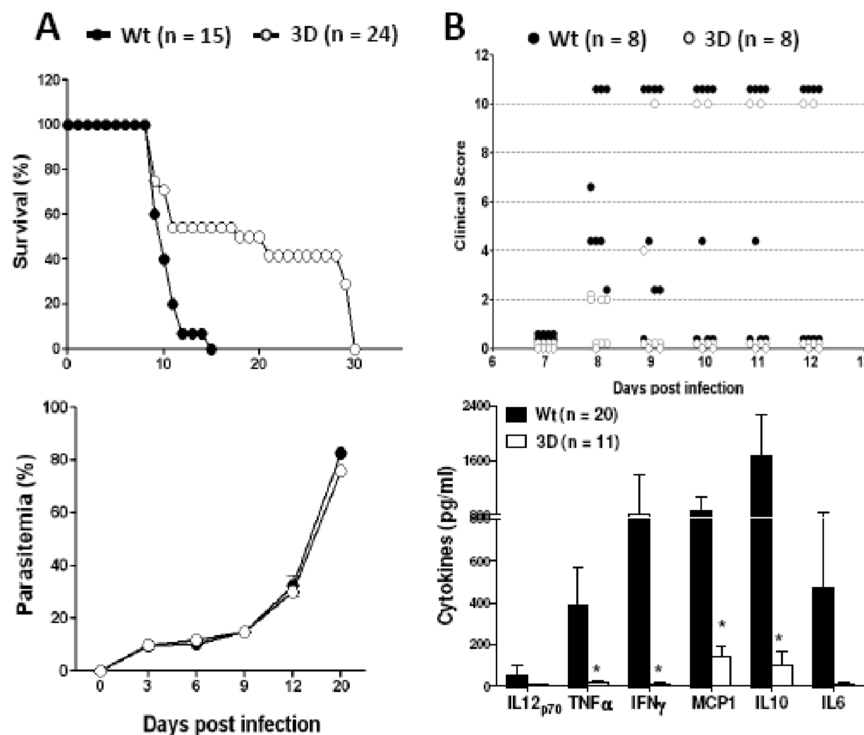


Figure 4 – TLR7/9 are key mediators of CM. Wt mice or mice with disruption in UNC93B1 (3D) were infected with 105 *P. berghei ANKA* iRBCs. Survival and parasitemia were compared between groups of mice at various days post infection. Cytokine levels in sera were assessed at 9 days post infection by CBA.

between 8 and 12 days post infection (Fig 4). Similar results were observed in $TLR9^{-/-}$ mice, which were backcrossed into C57BL/6 genetic background, for at least 13 generations (Supporting Fig. S3). Together, these findings provide very strong evidence that the nucleic acid sensing TLRs, in particular TLR9, are crucial for pathogenesis of PbA induced-CM, and provides valuable insights into how AT9 is acting in vivo to prevent malaria pathogenesis.

The top priority in current malaria research is the development of affordable strategies for disease prevention. Therefore, an understanding of the complexity of malaria pathogenesis is essential to the successful development of new strategies to control disease severity, including the development of therapeutic drugs and prophylactic vaccines. While acquired immunity following malarial infection has been thoroughly studied, innate immunity has not been subjected to similar scrutiny, let alone it was considered promising for therapeutic targets for infectious and inflammatory diseases. The results presented here, support that TLRs have a pivotal role in mediating release of inflammatory mediators and pathogenesis of malaria, but not in control of parasitemia. As in many diseases associated with sepsis, the innate immune system appears to have a central role in causing its most deleterious sequelae during acute malaria episodes. Therefore, AT9, a small molecule that acts as an antagonist for nucleic acid sensing TLRs seems to be a real alternative to prevent severe and lethal malaria. The development of new therapeutic strategies for malaria prevention is welcome especially after recent reports that Artemisinin-based therapies are failing a growing number of patients (43, 44).

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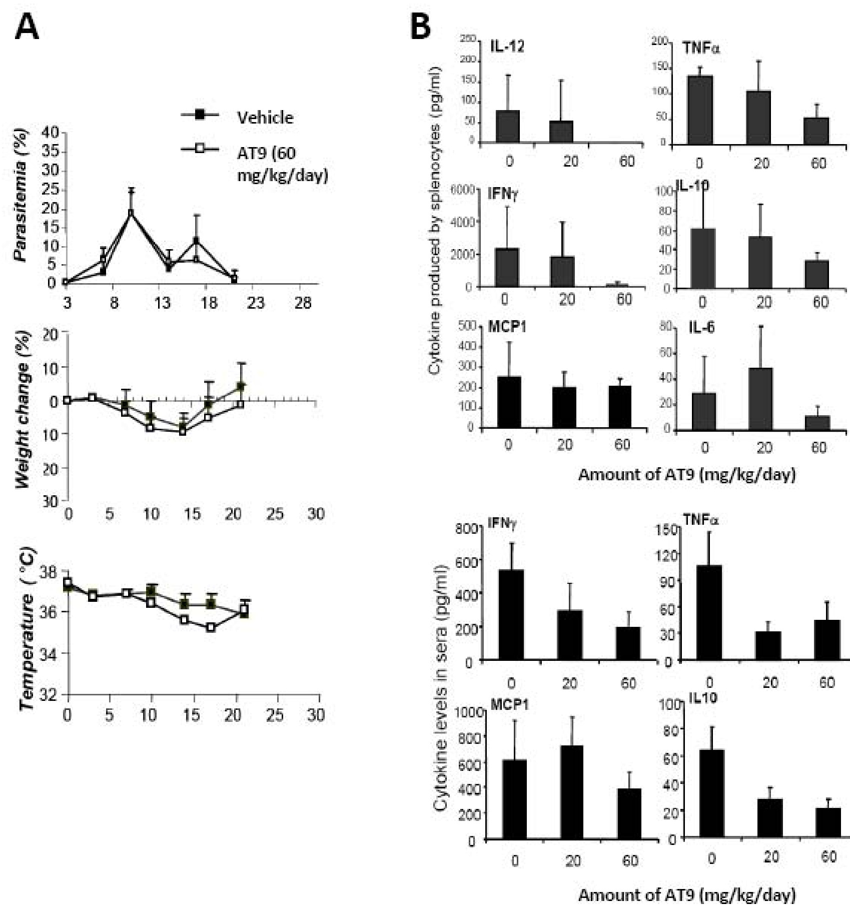
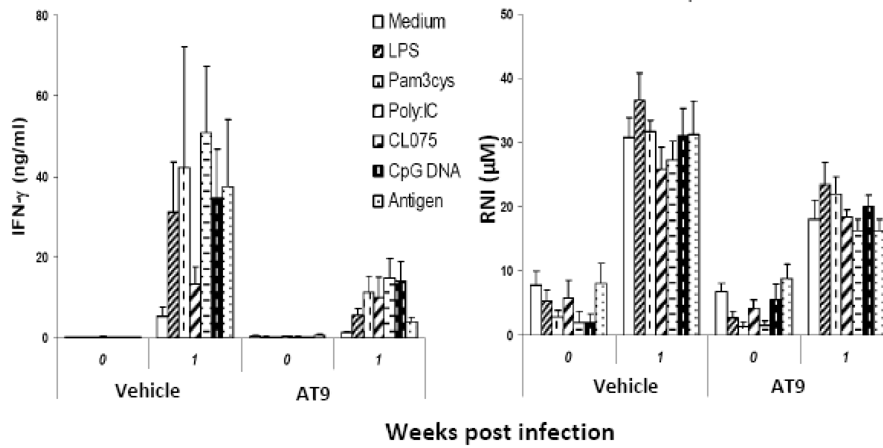
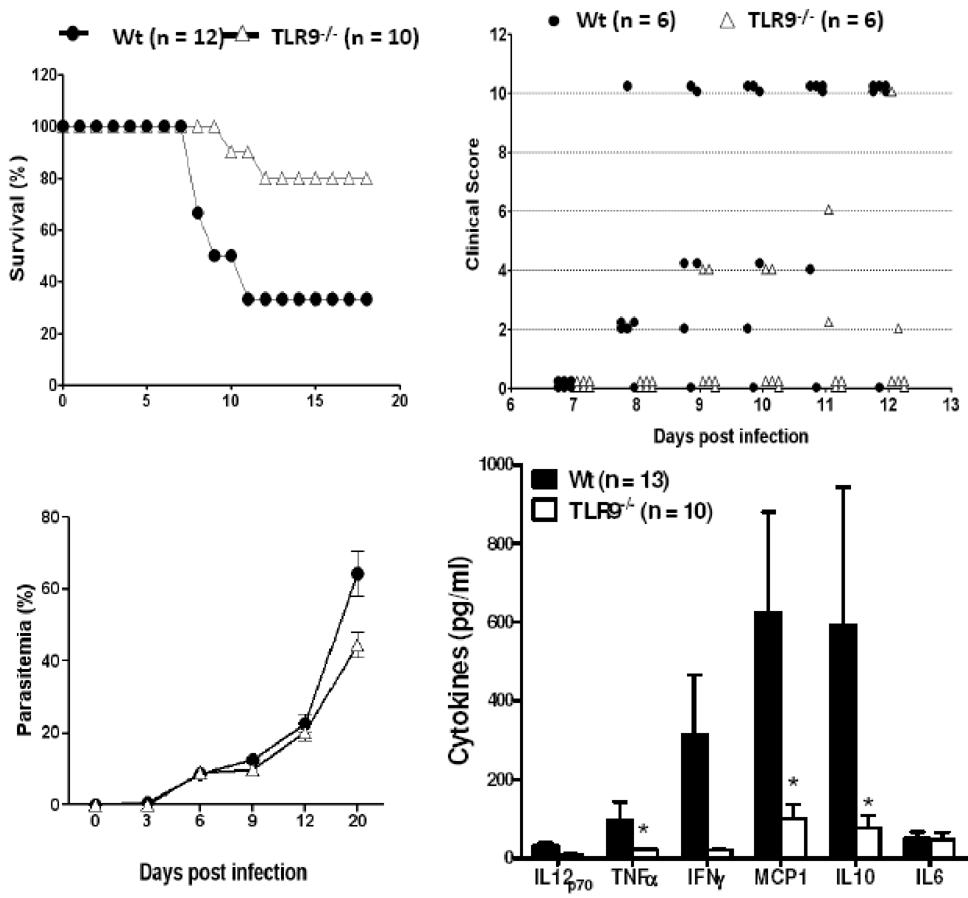


Supporting Figure S1 – Effect of ER820446 treatment on *P. chabaudi* infection. C57BL/6 mice were treated with 20 or 60 mg/kg/day of AT9 or vehicle one day before and during 20 days post-infection with 10^5 *P. chabaudi* iRBCs. (A) Parasitemia (top panels), body weight (middle panel), and temperature (bottom panel) were compared every three days between groups of mice at various days post infection. The results are averages of eight animals from one representative out of three experiments that yielded similar results. (B) Levels of cytokines produced by spleen cells (top panels) or in sera (bottom panels) from AT9 treated mice were measured by Cytometryx Bead Array (CBA) after 7 days post infection.



Mice	LPS dose (i.v)	Treatment	Number of mice dead/tested	Mean parasitemia (%)	Mortality (%)
C57BL/6 control	10µg	Vehicle	0/7	-	0
C57BL/6 infected	10µg	Vehicle	14/15	15.5	93.3
C57BL/6 control	10µg	AT9 (120 mg/kg/day)	0/7	-	0
C57BL/6 infected	10µg	AT9 (120 mg/kg/day)	1/7	15.5	14.28

Supporting Figure S2- AT9 reduces priming TLR pro-inflammatory responses. C57BL/6 mice were treated with 60 mg/kg/day of AT9 or vehicle one day before and during 6 days post infection with 10^5 *P. chabaudi* iRBCs. (A) At 7 days post infection spleens were harvested and cultured in the presence of LPS (1 µg/ml), Pam3cysK4 (1 µg/ml), Poly:IC (100 µg/ml), CL075 (100 ng/ml), CpG ODN (1 µg/ml) or *P. chabaudi* extract (100 µg/ml) for 48 hours. Cytokine levels or Reative Nitrogen intermediates (RNI) were assessed in culture supernatans by ELISA. (B) Mice were treated with 120 mg/kg/day one day before and during 6 days post infection with 10^5 *P. chabaudi* iRBCs. Mice were challenged i.v. at 7 days post-infection with a low dose (10 µg) of LPS. Survival was monitored after 12 hours post LPS challenge.



Supporting Figure S3 – TLR9 is a key mediator of CM. Mice with disruption in TLR9 were infected with 10⁵ *P. berghei* ANKA iRBCs. (A) Comparison of survival and parasitemia between Wt or TLR9-/- of mice at various days post infection. (B) Cytokine levels measured in sera at 9 days post-infection.

Material and Methods (Supporting information)

Mice: C57BL/6 mice (originally purchased from Jackson Laboratories) 6–8 weeks of age (Animal Unit, University of Massachusetts) were caged in groups of four animals. The animals were housed under standard conditions and had free access to a standard diet and tap water. Mice with deficiency to UNC93B1 (3D mice) were a gift of Bruce Beutler (The Scripps Research Institute, CA, US) and TLR9$^{-/-}$ mice were a gift of S. Akira (Osaka University, Japan). All animal work was approved by the appropriate Institutional Animal Care and Use Committee.

Drug and treatment: The compound AT9-02 (EISAI) was dissolved in acid water and its concentrations were adjusted so that the final dose in mg/kg body weight was given in 0.1 ml. Mice treated with vehicle (acid water) were used as control group. The animals were treated through oral gavage once daily at the dose stated for several days post-infection. The drug was administered 24 h prior to infection.

***In vitro* assays:** AT9 was assayed for suppression of freshly isolated human PBMCs or BALB/c mouse spleen IL-6 production in response to stimulation by oligonucleotide 1668, R848 or ssRNA. The compound was added to dissociated cells (5 x 10^5 per well in complete RPMI/10% FBS in a 96 well plate) before addition of stimulants. Cells were stimulated for 72 hrs and supernatant removed for ELISA analysis of IL6 (Pestka Biomedical Laboratories and Quantikine Colorimetric ELISA kits, R&D Systems, Inc.).

Infection and examination: The *Plasmodium chabaudi* AS strain and the *Plasmodium berghei* ANKA (PbA, Swiss Tropical Institute, Basel) were used. The parasites kept in liquid nitrogen were thawed and passaged into C57BL/6 mice as parasite donors as described before (23). For experimental infection parasited erythrocytes (iRBCs) were collected from donors in heparinized tubes by retro-orbital bleeding and 1 x 10^5 iRBCs were injected intra-peritoneally into new mice. These mice were carefully observed daily and parasitemia was estimated by counting Giemsa stained thin blood smears. For infections with the *P. berghei* model, neurological examination was performed by two independent observers using different parameters which included ruffled fur, abnormal postural responses, reduced reflexes and reduced grip strength, coma or convulsions. Mice that presented complete disability in all parameters or died between days 7 – 12 post infection were considering as having CM. Mice were treated in accordance with procedures approved by the Animal Ethics Committee of the University of Massachusetts. Brains were removed and used for histological analysis after Hematoxilin & Eosin (HE) staining.

Cytokine analysis: Cytokines were assessed in the sera or in supernatants of cultured splenocytes from C57BL/6 mice infected i.p. with 10^5 *P. chabaudi* or *P. berghei* iRBCs, treated or not with AT9-02. Each experiment was repeated 2–3 times. Each animal was analyzed individually. Spleens were aseptically removed and macerated through a nylon mesh over the course of the experimental infection. After RBC lysis, splenocytes were resuspended in RPMI, 10% FCS (Gibco), 1% gentamicin (Schering Plough) at a density of 2.5 x 106 cells per ml. Splenocytes were subsequently cultured in 48-well plates for 48 h in a final volume of 1 ml in the presence of Concanavalin A (Sigma), higly purified LPS (Sigma Aldrich) or CpG ODN (Invivogen). Measurements of cytokines were performed using commercially available ELISA kits (R&D Systems). Serum cytokines from *P. chabaudi* infected-mice were quantified with the CBA inflammation kit (Becton-Dickinson).

Intracellular staining of cytokines: After 8 days of infection with 10^5 PbA iRBCs spleens from mice treated or not with AT9 were harvested and CD11c+ splenic DCs were isolated using CD11c microbeads (Miltenyi Biotec) according to manufactures instructions. Cells (5 x 10E5) were Fc blocked with 2.4G2 mAb (BD Pharmingen San Diego, CA) and labeled with FITC-conjugated anti-CD11c, PE-conjugated anti mouse CD40, CD86 and CD80 mAbs (BD Pharmingen). A nonrelated IgG mAb was used as a control for staining specificity. For intracellular analysis of cytokines cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Pharmingen) and stained for intracellular TNFα, IL12p40/p70 or IL6 levels using PE-conjugated anti mouse mAbs (BD Pharmingen). Data were analyzed using Flowjo software (TreeStar).

Statistical Analysis: All data were analyzed using Graphpad Instat 4.0 Software. Unless stated elsewhere, all comparisons were performed using two-tailed student's t test. Mann-Whitney testing was used for non-parametric analysis when data did not fit a Gaussian distribution. A P value ≤ 0.05 was considered to be statistically significant.

6 Considerações Finais

6 CONSIDERAÇÕES FINAIS

Como parte do nosso primeiro trabalho (Franklin *et al.*, *Microbes and Infection*, 2007) identificamos um papel dos TLRs na patogênese da malária. Mais especificamente, mostramos que os TLRs participam da ativação inicial do sistema imune inato do hospedeiro e contribuem para a produção de citocinas pró-inflamatórias. No entanto, a ativação destes receptores não é necessária para o controle da parasitemia e o desenvolvimento de uma imunidade protetora e memória imunológica contra o parasito. Acreditamos que na ausência de MyD88 e dos TLRs o sistema imune inato produza quantidades de citocinas pró-inflamatórias que, apesar de menores, são suficientes para o controle da replicação do parasito sem gerar os sintomas resultantes de uma inflamação sistêmica.

Mostramos ainda que as células dendríticas (DCs) desempenham um importante papel na ativação da resposta de células T e na produção de citocinas. O bloqueio da ativação das células T pelas DCs com o uso de anticorpos específicos revelou um importante papel destas duas populações celulares na patogênese da doença. Finalmente, mostramos que a função das DCs na produção de citocinas que medeiam a patogênese da malária é dependente da função dos TLRs, já que células dendríticas de animais MyD88$^{-/-}$ apresentaram níveis muito menores de citocinas pró-inflamatórias comparados aos níveis produzidos por células de animais selvagens (Wt) durante a infecção aguda por *Plasmodium*.

Um papel dos TLRs na patogênese da malária também foi observado por Nakanishi e sua equipe utilizando o modelo murino com *P. berghei* NK65 (Adachi *et al.* 2001). Estes autores mostraram que animais Myd88$^{-/-}$ não apresentaram a patologia hepática característica (apoptose e necrose de hepatócitos e denso infiltrado de linfócitos) deste modelo de malária, sugerindo que a ativação dos TLRs estaria

envolvida na patogenia da doença. A resistência de animais Myd88$^{-/-}$ foi associada a uma menor produção de IL12 em comparação com camundongos Wt. De fato, animais IL12$^{-/-}$ também se mostraram resistentes à patogenia mediada pela infecção por *P. berghei*.

A malária Cerebral (CM) é certamente uma das complicações mais graves da infecção aguda por *P. falciparum* em humanos e por *P. berghei* ANKA em camundongos. Utilizando o modelo de *P. berghei* ANKA Akira e sua equipe mostraram que animais Myd88$^{-/-}$ são mais resistentes a CM mediada do que camundongos Wt (Coban *et al.* 2007).

Apesar de estes estudos evidenciarem o envolvimento de Myd88 na patogênese da malária e serem de grande importância para o entendimento dos mecanismos imunológicos envolvidos nesta doença, ainda não foi identificado o TLR, dependente de MyD88, que reconheceria o parasito e iniciaria as respostas imunes durante a infecção.

A busca por um TLR diretamente envolvido na malária tem sido foco de vários estudos em camundongos (Adachi *et al.* 2001; Coban *et al.* 2007; Togbe *et al.* 2007; Lepenies *et al.* 2008) e seres humanos (Mockenhaupt *et al.* 2006; Mockenhaupt *et al.* 2006; Ockenhouse *et al.* 2006; Khor *et al.* 2007). Além disso, foi demonstrada a presença de ligantes de TLR em *Plasmodium:* âncoras de Glicofosfatidil-inositol (GPI) que ativam TLR2 (Krishnegowda *et al.* 2005) e DNA do parasito que, quando carreado para o endosoma celular pela hemozoína, ativa TLR9 (Parroche *et al.* 2007). Entretanto, até então, permanece desconhecido qual(ais) é(são) o(s) TLR(s) responsável(is) pelo reconhecimento do parasito e ativação da resposta imune do hospedeiro. Muito menos são conhecidas quais as células da imunidade inata que reconhecem o parasito ou mesmo quais moléculas do parasito ativam estes TLRs.

Em nosso terceiro trabalho, (Franklin *et al. Proceedings of the National Academy of Sciences PNAS*, 2009) identificamos TLR9 como responsável por reconhecer a presença do parasito e iniciar as respostas inflamatórias. TLR9 é ativado nas primeiras fases da infecção, possivelmente através do reconhecimento de sequências de DNA do parasito, e inicia a resposta de IL12 em DCs. Evidenciando, mais uma vez, o papel destas células na patogenia da malária. DCs de animais deficientes em TLR9 produziram níveis menores de IL12 em resposta a hemácias infectadas com *P. chabaudi in vitro*. IL-12 é uma potente indutora de IFNγ por linfócitos T e células NK. O IFNγ produzido, por sua vez, atua nas células do sistema imune inato aumentando a expressão de vários genes pró-inflamatórios, incluindo os de TLRs, primando as células no caso de um subseqüente encontro com ligantes de TLR ou outros receptores da imunidade inata (Figura 2).

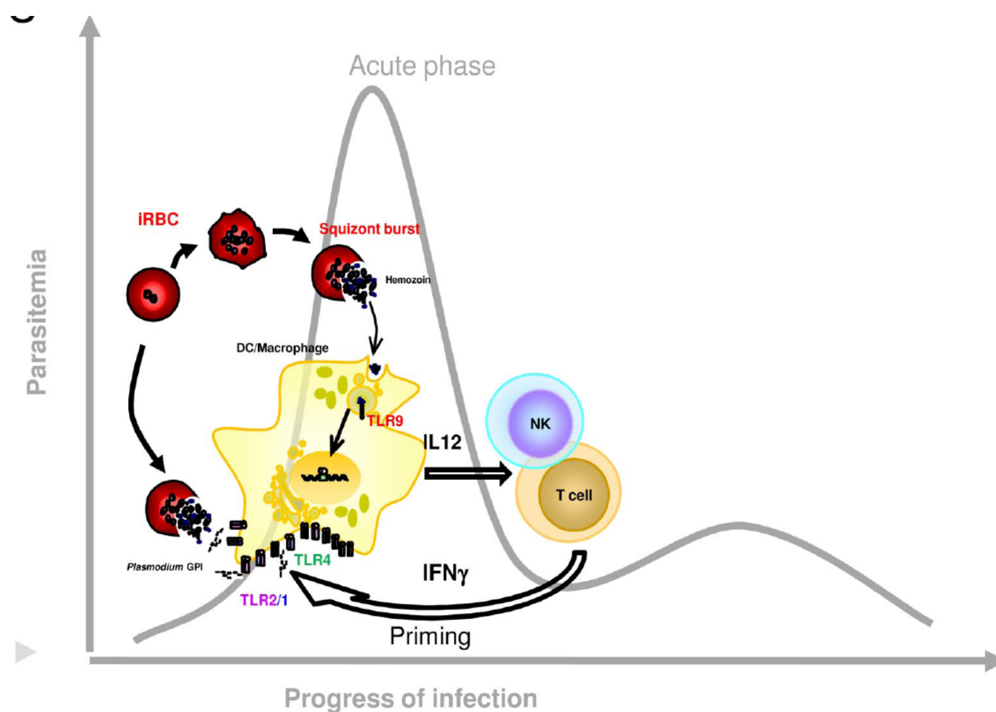


Figura 2 - Esquema da ativação de TLR9 durante a malária. A ativação de TLR9 pelo reconhecimento de moléculas do parasito (provavelmente DNA associado à hemozoína) leva à produção de IL12, uma potente indutora de IFNγ em linfócitos T e células NK. O IFNγ produzido atua nas células

do sistema imune aumentando a expressão e responsividade dos TLRs levando a uma hiper-ativação sistêmica do sistema imune inato. Fonte: *Franklin et al., (2009)* (Franklin *et al.* 2009).

Na busca de um receptor TLR responsável pelo reconhecimento do *Plasmodium* descobrimos um importante mecanismo envolvido na patogênese da malária. Mostramos que a malária prima o sistema imune inato do hospedeiro tornando-o hiper-responsivo no caso de uma segunda ativação mediada por encontros subseqüentes com ligantes de TLRs. A esse fenômeno dá-se o nome de "*priming*". Mostramos ainda que TLR9 e IFNγ são essenciais para o *priming*. De fato, IFNγ é conhecido por primar células do sistema imune, principalmente macrófagos, para responder à estímulos subsequentes. Usando um modelo que também causa *priming* das respostas do sistema imune inato, Kalis et al (2005) mostraram que a produção de IFNγ, resultante da ativação de TLR9, está envolvida na hiper-responsividade celular causada pela infecção por *Propionibacterium* (Kalis *et al.* 2005).

Estes achados são de extrema importância para o entendimento dos processos patológicos envolvidos na malária, principalmente se levarmos em consideração o fato de que as áreas do mundo com a maior incidência e prevalência para malária também possuem alta incidência para infecções bacterianas invasivas, tais como *Salmonella sp.*, *Pneumococcus sp.* e *Meningococcus sp.*(Bronzan *et al.* 2007). Há 10 anos já era observado que infecções bacterianas aumentam a gravidade e a mortalidade por malária (Berkley *et al.* 1999).

Além de uma co-infecção por bactéria ou outro patógeno exógeno, não se pode desconsiderar a ocorrência de um agente endógeno como o segundo estímulo para um sistema imune primado pela malária. Já foi demonstrado que a malária causa enfraquecimento e permeabilização das barreiras mucosas podendo expor o sistema imune à flora intestinal (Wilairatana *et al.* 1997). O contato de um sistema imune já

primado com moléculas provenientes da flora intestinal pode estar envolvido no choque séptico e na inflamação sistêmica observados na malária.

Finalmente, em nosso mais recente trabalho, submetido à revista Science, mostramos que a ativação de TLR9 pode ser impedida terapeuticamente, através do uso de um antagonista sintético, denominado ER-820446, para evitar a hiperativação do sistema imune e a manifestação dos sintomas mais graves da malária. Neste trabalho, células humanas e murinas incubadas com este antagonista produziram níveis significativamente menores de citocinas em resposta a ligantes de TLR9 *in vitro* e *ex vivo*. Quando administrado oralmente em camundongos, o composto sintético foi capaz de reduzir os níveis séricos de citocinas e a hiper-responsividade celular observada na infecção aguda por *P. chabaudi*. Devido ao seu efeito anti-inflamatório, decidimos testar este antagonista na malária cerebral induzida pelo *P. berghei ANKA*. De maneira surpreendente, animais tratados com ER-820446 produziram níveis de citocinas pro-inflamatórias significativamente menores do que animais tratados com veículo, não apresentaram lesões cerebrais características da infecção por *P. berghei* e foram protegidos da malária cerebral causada por esta espécie de *Plasmodium*.

Por muito tempo a imunidade inata foi tida como inespecífica e não tão desenvolvida quanto a imunidade adquirida. Muito menos foi considerada como alvo quimioterápico no tratamento de doenças. Os dados mostrados nesse trabalho são inéditos e de extrema importância não só para o tratamento da malária, mas para o entendimento dos mecanismos imunológicos envolvidos na patogenia desta doença.

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