ÉRIKA MACHADO DE SALLES

**Efeitos da sinalização purinérgica durante a infecção aguda e crônica pelo
Plasmodium chabaudi AS**

Tese apresentada ao Programa de Pós-Graduação em Imunologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **175** nas fls. **114** do livro **02** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a) **Maria Regina D'Império Lima**, Coordenador (a) da Linha de pesquisa "*Avaliação da resposta imune na malária murina causada pelo Plasmodium Chobaudi*" do qual participam o(s) aluno(s) **Érika Machado de Salles**, **Maria Nogueira de Menezes** e a pesquisadora **Sheyla Inés Castillo Méndez**, está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)** em **14.12.2011**, com validade de **4 anos**.

São Paulo, 15 de dezembro de 2011.

Prof. Dr. WOTHAN TAVARES DE LIMA
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Prof. Dr. ARIEL MARIANO SILBER
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CEUA – ICB/USP

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Certificamos que o projeto intitulado "*Avaliação da resposta imunológica desenvolvida na malária experimental*", registrado sob o protocolo nº **29/2016**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de *Pesquisa Científica*, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle e Experimentação Animal (CONCEA). Ante esta conformidade, o referido projeto foi avaliado e aprovado em **31/05/2016** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Ciências Biomédicas da Universidade de São Paulo (CEUA-ICB/USP), outorgando esta licença de uso de animais com validade de **4 ano(s)** a partir da data de aprovação.

- Investigador Principal: **Dr.(a.) Maria Regina D'Imperio Lima**

- Departamento: *Imunologia*

- Membros da Equipe: *Alexandra dos Anjos Cassado (Pós-doutorando), Maria Nogueira de Menezes, Érika Machado de Salles*

Ao final do período outorgado por esta licença, o pesquisador responsável deverá encaminhar a esta comissão, até o último dia de validade da atual proposta, *relatório final* de acordo com a Resolução Normativa CONCEA nº 30/2016 - Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA), conforme modelo constante no endereço eletrônico www.icb.usp.br/ceua. Havendo interesse na renovação do projeto, a solicitação deverá ser protocolada pela Secretaria da CEUA-ICB/USP até o último dia de validade da atual proposta. Após esta data uma nova proposta deverá ser encaminhada.

CERTIFICATE

We hereby certify that the project entitled "*Evaluation of immune response developed in experimental malaria*", protocol nº **29/2016**, which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human), for *Scientific Research Purposes*, is in accordance with the provisions of the Law nº 11.794 passed on October 8th, 2008, Decree nº 6899 passed on July 15th, 2009, and the rules issued by the National Council for Control and Animal Experimentation (CONCEA). According to this legislation, the project was evaluated and approved on **5/31/2016** by the ETHICS COMMITTEE ON ANIMAL USE, Institute of Biomedical Sciences, University of Sao Paulo (CEUA-ICB/USP), and the license for animal use is valid for **4 year(s)** from the date of approval.

- Principal Investigator: **Dr.(a.) Maria Regina D'Imperio Lima**

- Team members: *Alexandra dos Anjos Cassado (Postdoctoral Researcher), Maria Nogueira de Menezes, Érika Machado de Salles*

At the end of the period granted by this license, the Principal Investigator must submit a final report of the project to this committee, according to the Rule nº 30 and the Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA) issued by the CONCEA. If a renewal of the project is intended, the request must be submitted to the CEUA-ICB/USP secretary before the expiration of the current proposal. After this date, a new proposal must be prepared.

Espécie/Species	Linhagem/Strain	Sexo/Gender	Idade-Peso/ Age-Weight	Total
<i>Mus musculus</i>	P2X7KO	Fêmea/female	4 semanas/weeks	400
	C57bl/6	Fêmea/female	4 semanas/weeks	1075
	C57bl/6	Macho/Male	4 semanas/weeks	230
	CD4KO	Fêmea/female	4 semanas/weeks	160
	FoxP3-GFP	Fêmea/female	4 semanas/weeks	250
	Myd88KO	Macho/Male	4 semanas/weeks	150

São Paulo, 03 de junho de 2016.

Prof. Dr. Anderson de Sá Nunes
Coordenador CEUA-ICB/USP

Eliane Aparecida Gomes de M. Nascimento
Secretária CEUA-ICB/USP

Dedico esta tese:

Aos meus pais, Rozeval e Elizabeth, pela compreensão e apoio em todos os momentos

Ao meu irmão, Rafael, por toda amizade e carinho

*Ao meu amor Eduardo, pelo companheirismo e dedicação, pelo incentivo e por
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RESUMO

Salles, EM. Efeitos da sinalização purinérgica durante a infecção aguda e crônica pelo *Plasmodium chabaudi* AS. [Tese (Doutorado em Imunologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

A malária, uma doença causada por um parasito protozoário do gênero *Plasmodium*, permanece um sério problema de saúde em países subdesenvolvidos. O estágio sanguíneo da infecção é responsável por todos os sintomas associados com a malária que estão diretamente relacionados com a excessiva ativação do sistema imune. Recentemente, tem sido mostrado que receptores imunes inatos são capazes de detectar sinais de dano, tais como a adenosina trifosfato (ATP). O receptor ionotrópico P2X7 detecta altas concentrações de ATP extracelular e pode contribuir na ativação da resposta imune contra o *Plasmodium*. Para avaliar se o receptor P2X7 tem participação na proteção contra o *Plasmodium chabaudi* AS, analisamos a progressão da doença em camundongos C57BL/6 (B6) e P2X7^{-/-}. A parasitemia e os parâmetros clínicos (anemia, perda de peso e temperatura) foi semelhante em ambos os grupos até o dia 7 pós infecção (p.i.), mas após este período os camundongos P2X7^{-/-} tiveram dificuldade de controlar a parasitemia e restaurar os parâmetros clínicos. Devido a possibilidade do eixo ATP-P2X7 proporcionar a amplificação dos sinais do TCR durante a sinapse imune, focamos nossas análises nas células T CD4⁺ que têm um importante papel no desenvolvimento da imunidade protetora contra a malária. O ineficiente controle da parasitemia durante o período agudo e crônico em camundongos P2X7^{-/-} foi associado com a baixa produção de IFN γ . A expressão do fator transcricional T-bet, em células Th1 efectoras e de memória efectora induzidas pela infecção, foi criticamente dependente do receptor P2X7 em células T CD4⁺. Além disso, as células T CD4⁺P2X7^{-/-} transferidas para camundongos CD4^{-/-} infectados apresentaram maior diferenciação para células T foliculares (Tfh) que Th1 em comparação com as células T CD4⁺P2X7⁺ de camundongos B6. Este estudo fornece uma nova visão na imunologia da malária, mostrando que o equilíbrio mediado pelo receptor P2X7 entre os fatores de transcrição Bcl-6 e T-bet ajusta a imunidade celular e humoral durante a infecção.

Palavra-chave: *Plasmodium chabaudi*. Receptor P2X7. ATP. Célula Th1. IFN γ . Célula Tfh

ABSTRACT

Salles, EM. Effects of purinergic signaling during acute and chronic infections by *Plasmodium chabaudi* AS. [Ph. D. Thesis (Immunology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

Malaria, a disease caused by the protozoan parasite *Plasmodium*, remains a serious healthcare problem in developing countries. The blood stage of infection is responsible for all symptoms associated with malaria, which are mostly related to excessive activation of the immune system. Recently, it has been shown that innate immune receptors are able to detect signals as adenosine triphosphate (ATP). P2X7 receptor detects high levels of extracellular ATP and therefore could contribute to activate the immune response against *Plasmodium*. To evaluate whether the P2X7 receptor contributes to protection against blood stages of *Plasmodium chabaudi* AS, the disease progression was analyzed in C57BL/6 (B6) and P2X7^{-/-} mice. The parasitemia and clinical parameters (i.e. anemia, weight loss and hypothermia) developed similarly in both female groups up to day 7 p.i., but after this period the P2X7^{-/-} mice had difficulty in controlling the parasitemia and restoring the clinical parameters. Because the sensing of environment extracellular ATP by the P2X7 receptor is thought to amplify the TCR signal during the immune synapsis, we focused our analysis on CD4⁺ T cells that have a central role in the development of protective immunity against *Plasmodium chabaudi* malaria. The inefficient parasite control in acutely and chronically infected P2X7^{-/-} mice was associated with low production of IFN γ . The expression of T-bet transcriptional factor in Th1 effector/effector memory cells induced by infection critically depended on the P2X7 signaling in CD4⁺ T cells. Furthermore, P2X7^{-/-} CD4⁺ T cells transferred to infected CD4^{-/-} mice, in comparison to B6 CD4⁺P2X7⁺ T cells, showed a bias towards the follicular T (Tfh) helper cell lineage over Th1 memory cell lineage. This study provides a new insight into immunology in malaria by showing that the balance between T-bet and Bcl-6 transcriptional factors tunes the cellular and humoral immunity during the infection.

Keywords: *Plasmodium chabaudi*. P2X7 receptor. ATP. Th1 cell. IFN γ . Tfh cell

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LISTA DE ABREVIATURAS E SÍMBOLOS

- **ADP** – Adenosina difosfato
- **AHR** - Receptor aril hidrocarboneto
- **AMP** - 2-amino-2-metil-1-propanol
- **AMP** – Adenosina monofosfato
- **ANOVA** – Análise de variância
- **APC**- Célula apresentadora de antígeno
- **APC-Cy7**- Aloficocianina-Cy7
- **ARNT** - translocador nuclear do receptor aril hidrocarboneto
- **ATP** – Adenosina Trifosfato
- **Bcl-6** - Proteína 6 do linfoma de células B
- **Blimp-1** – Proteína de maturação de linfócitos B
- **BSA** - Albumina sérica Bovina
- **CCR7** - Receptor de quimiocina C-C tipo 7
- **CD** – Grupos de diferenciação
- **CFSE** – do inglês, *Carboxifluorescein Diacetate Succinimidil Ester*
- **CTCF** – Cálculo corrigido da fluorescência da célula total
- **CXCR5** - Receptor de quimiocina C-X-C tipo 5
- **DAMP** – Via molecular associada ao dano
- **DNA** – Ácido Desoxirribonucléico
- **EB** - Brometo de Etídeo
- **ELISA** – do inglês, *enzyme-linked immunosorbent assay*
- **ELISPOT** – do inglês, *Enzyme-Linked ImmunoSpot*

- **eNOS** – síntese de óxido nítrico endotelial
- **EP** – Eritrócito parasitado
- **FITC**- Fluoresceína
- **GPI** - glicosil-fosfatidil-inositol
- **Hb** – Hemoglobina
- **HIF** – Fator Indutor de hipoxia
- **i.p** - intraperitoneal
- **ICAM 1** – Molécula de adesão intercellular 1
- **ICOS** - coestimulador de células T induzível
- **IFN** – Interferon
- **Ig** - Imunoglobulina
- **IL**- Interleucina
- **iRBC** – Eritrócito parasitado
- **mAb** – Anticorpo monoclonal
- **MAPK** – Proteína quinase ativada por mitógeno
- **MFI** – Média de intensidade de fluorescência
- **MHC** – Complexo principal de Histocompatibilidade
- **NFAT** – Fator nuclear de células T ativadas
- **NK** – do inglês, *Natural Killer cell*
- **NKT** –do inglês, *Natural Killer T cell*
- **NLRP3** – do inglês, *NOD-like receptor family, pyrin domain containing 3*
- **ROS** – Reativos do óxido nítrico
- **nRBC** – Eritrócito não parasitado
- **p.i.** – pós infecção
- **PAMP** – Padrão molecular Associado a Patógenos

- **PBS** – Salina Tamponada com fosfato
- **PD1** - Proteína de morte celular programada
- **PE**- Ficoeritrina (Phycoerythrin)
- **PE-Cy7** - Ficoeritrina-Cy7
- **PerCp** - *Complexo proteína clorofila peridina*
- **PI** – Iodeto de propídio
- **PS** – Fosfatidilserina
- **PS** – Fosfatidilserina
- **RLU** – Unidade relativa de luz
- **RPMI** – Meio McCoy de cultura modificado
- **SPF** – do inglês, *specific pathogen-free*
- **STAT** – Transdutores de sinal e ativadores de transcrição
- **T_{CM}** – Célula T de memória central
- **TCR** – Receptor de célula T
- **T_E** – Célula T efetora
- **T_{EM}** – Célula T de memória efetora
- **Th** – Célula T auxiliar
- **Tfh** – Células T auxiliares do folículo
- **TGFβ** – Fator transformador de crescimento beta
- **TLR** – Receptor tipo Toll
- **TMB** – Tetrametilbenzidina
- **TNFα** – Fator de Necrose Tumoral α
- **TUNEL** – do inglês, *Terminal deoxynucleotidyl transferase dUTP nick end labeling*
- **WHO** – do inglês, *World Health Organization*

α – alpha

β – beta

γ – gama

$\text{--}/\text{--}$ – nocautes

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1 INTRODUÇÃO

1.1 Aspectos gerais da malária

1.1.1 Epidemiologia e agente etiológico

A malária permanece um sério problema de saúde pública em regiões tropicais e sub-tropicais do mundo (**Figura 1**). A Organização Mundial de Saúde (OMS) estimou 214 milhões de casos de malária com 438.000 mortes no ano de 2015, com 88% dos casos encontrados na África sub-Saariana (World Health Organization - WHO, 2015). Segundo dados estimados pela OMS, o número de casos de malária obteve um declínio de 18% entre os anos 2000 e 2015, e a incidência de malária, que leva em consideração o crescimento populacional, obteve uma queda de 37% no mesmo período. Porém, a malária ainda é considerada uma das principais causas de morte na região sub-Saariana, correspondendo à 10% dos casos de morte de crianças menores de cinco anos na região (WHO, 2015). No Brasil, a área endêmica de malária encontra-se na Região da Amazônia legal, que corresponde aos estados do Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Paraná, Rondônia, Roraima e Tocantins. Em 2015, foram confirmados no Brasil 143.648 casos de malária quase que totalmente restritos à Região Amazônica (Ministério da Saúde, Brasil, 2015). Apesar dos poucos casos de malária em região extra-Amazônica, o controle da doença é necessário diante do fluxo migratório em áreas suscetíveis e alta letalidade em casos de diagnóstico tardio e manejo clínico inadequado.

A malária é uma doença infecciosa aguda ou crônica causada por protozoários do gênero *Plasmodium*. Há cinco espécies de protozoários que podem causar a malária humana: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* e *Plasmodium knowlesi*, um parasita de macaco que tem sido implicado em casos humanos (Daneshvar et al., 2009). O *P. Ovale* é encontrado principalmente em regiões do continente africano, enquanto que o *P. Knowlesi* ocorre apenas no sudeste asiático. No Brasil, três espécies estão associadas à malária humana: *Plasmodium vivax*, *Plasmodium falciparum* e *Plasmodium malariae* (Ministério da Saúde, Brasil, 2015), sendo que há maior prevalência de infecções com o *P. vivax* e menor prevalência de *P. malariae*. Há também a presença de formas mistas, que é a infecção do paciente por mais de uma espécie de plasmódio.

Figura 1 – Casos de malária no mundo, 2013

Fonte: World Malaria Report, WHO, 2014

1.1.2 Ciclo do *plasmódio* e aspectos clínicos da doença

A infecção humana tem início quando os esporozoítos são introduzidos na pele através da picada de um mosquito *anopheles* fêmea. Os esporozoítos atingem a corrente sanguínea e migram para o fígado, onde passam por uma fase assintomática de divisão rápida dentro de hepatócitos, dando início ao ciclo pré-eritrocítico, clinicamente silencioso. Os parasitos multiplicam-se e dão origem a milhares de novos parasitos (merozoítos). Os merozoítos, envoltos por uma estrutura que contém parte da membrana do hepatócito (merossomas), caem diretamente na corrente sanguínea (Sturm et al., 2006). No sangue, os merozoítos infectam eritrócitos maduros com alta taxa de expansão (Miller et al., 2014) (**Figura 2**). Dentro dos eritrócitos, os merozoítos se desenvolvem em trofozoítos que se multiplicam e maturam até a formação de esquizontes. Após a maturação, há o rompimento dos eritrócitos infectados com a liberação de merozoítos na corrente sanguínea que irão infectar novos eritrócitos. Além disso, alguns merozoítos são diferenciados em gametócitos que, quando sugados pelo mosquito vetor através da picada do hospedeiro infectado, perpetua o ciclo sexual no inseto. O ciclo eritrocítico é repetido a cada 48 horas nas infecções por *P. vivax* e *P. falciparum* (Crutcher, Hoffman, 1996) e a cada 72 horas nas infecções por *P. malariae*.

Os aspectos clínicos da malária caracterizam-se principalmente por febre elevada, sudorese profunda e calafrios. A anemia intensa, a malária cerebral, a síndrome respiratória, a acidose metabólica e o choque sistêmico são síndromes associadas às formas graves da doença (Bartoloni, Zammarchi, 2012). Durante o período de replicação assexuada do *P. falciparum*, alguns eritrócitos infectados aderem ao endotélio vascular devido à expressão de moléculas específicas que possibilitam essa adesão. Esse fenômeno pode facilitar a evasão do parasito dos fagócitos esplênicos, mas tem consequências patológicas importantes nos órgãos afetados. Assim, a adesão dos eritrócitos infectados no endotélio vascular cerebral está implicada na patogênese da malária cerebral (**Figura 2**). Além disso, mulheres grávidas infectadas podem desenvolver a malária placentária com o sequestro de eritrócitos infectados no endotélio da placenta. Um fenômeno semelhante também tem sido descrito na síndrome respiratória com a adesão de eritrócitos nos vasos que irrigam o tecido pulmonar.

Figura 2 – Ciclo de vida do *Plasmodium* e patologia da malária

Fonte: Schofield and Grau, 2005.

1.2 Resposta imune inata contra o *Plasmodium*

Durante a fase aguda da malária, observa-se uma intensa ativação do sistema imune caracterizada por esplenomegalia e hipergamaglobulinemia (Greenwood, 1974; Ingram-Johnson, 1925). O baço tem um papel importante na malária como sítio de remoção de eritrócitos parasitados e geração de imunidade (Chotivanich et al., 2002; Engwerda et al., 2005). Além disso, observa-se uma hiperativação sistêmica caracterizada pelos elevados níveis de intermediários reativos do óxido nítrico (ROS) (Nussler et al., 1994), interferon- γ (IFN γ) (Rhodes-Feuillette et al., 1985), fator de necrose tumoral- α (TNF α) (Kwiatkowski et al., 1990) e interleucina (IL) -1, -6 e -8 (Urquhart, 1994). Tanto o TNF α quanto a IL-1 β são considerados importantes mediadores da febre e participam da aderência de eritrócitos infectados ao endotélio vascular (Pober et al., 1986). A aderência dos eritrócitos infectados nas vênulas capilares e pós-capilares acontece devido a ação do TNF α na indução de moléculas de adesão intercelular (ICAM-1) no endotélio vascular, contribuindo para o desenvolvimento da malária cerebral (Wassmer et al., 2011). Os eritrócitos infectados aderidos ao endotélio obstruem o fluxo sanguíneo, causam hipóxia no cérebro e aumentam a inflamação (Turner, 1997). Além da malária cerebral, outras complicações podem ser encontradas durante a infecção. A retirada de eritrócitos não infectados, devido à alterações na membrana, parece estar relacionada à anemia grave em humanos (Looareesuwan et al., 1991).

Dentre os produtos imunogênicos gerados durante infecção pelo *Plasmodium* podemos destacar o glicosil-fosfatidil-inositol (GPI) e a hemozoína. Estas moléculas são capazes de ativar receptores do tipo Toll (TLRs) em macrófagos e células dendríticas e induzir a produção de citocinas pró-inflamatórias, tais como o TNF- α , IL-1 β e IL-12. De forma mais detalhada, moléculas ancoradas ao GPI de *P. falciparum* induzem a produção de citocinas pró-inflamatórias por macrófagos através da interação com o complexo TLR2/TLR1 (Ropert et al., 2008). A hemozoína, que é formada e mantida no vacúolo digestivo do *Plasmodium* como um produto do catabolismo da hemoglobina, pode se ligar ao DNA do plasmódio e ativar células dendríticas por meio do TLR9 (Coban et al., 2005; Parroche et al., 2007). O acúmulo de hemozoína dentro de células fagocíticas pode ser usado no diagnóstico da malária e tem sido correlacionado com a severidade da doença (Nguyen et al., 1995). O sistema imune também é capaz de reconhecer moléculas liberadas quando

há dano celular ou injúria tecidual. Dentre os sinais de dano podemos destacar a adenosina-5'-trifosfato (ATP). No início da infecção, podemos postular que a ruptura dos eritrócitos infectados com o *Plasmodium* possa desencadear uma resposta inflamatória que poderia ser induzida tanto por fatores derivados do parasito como por sinais de dano do hospedeiro. Os eritrócitos infectados aumentam a quantidade de ATP intracelular e liberação de ATP por canais na membrana (Akkaya et al., 2009). Além disso, o aumento de Ca^{++} no *P. falciparum*, induzido pelo ATP extracelular e receptores purinérgicos no *Plasmodium*, pode ajudar na invasão do parasito a novos eritrócitos (Levano-Garcia et al., 2010). Dentro dos eritrócitos, o *Plasmodium* também é capaz de aumentar a expressão de transportadores de solutos orgânicos e canais de íons do eritrócito (Merckx et al., 2009). O transporte de proteínas e íons permite ao parasita responder a mudanças nas concentrações externas de íons, nutrientes, eliminar o produto de degradação metabólica e regular o volume da célula (Desai, 2012).

1.3 Interação entre imunidade inata e adquirida contra o Plasmodium

A modulação da resposta imune a fim de proporcionar a eliminação do parasito sem desencadear patologia severa parece ser um ponto crucial para a boa evolução da doença. A produção de IFN γ é importante para o controle da parasitemia (Ângulo, Fresno, 2002). Além das células T CD4 $^{+}$, outras células tais como: natural killer (NK), T natural killer (NKT), T $\gamma\delta$ (McCall, Sauerwein, 2010) e T CD8 $^{+}$ também são capazes de produzir IFN γ . No entanto, a rápida produção de altas quantidades de IFN γ pode levar a uma excessiva reação inflamatória e agravamento da patologia. Portanto, a produção de citocinas anti-inflamatórias, tais como IL-10 e o fator transformador de crescimento β (TGF β), são necessárias para contra balancear a forte resposta pró-inflamatória durante a fase aguda da infecção. Alguns fatores associados à gravidade da doença, como a anemia severa, têm sido relacionados com quantidade insuficiente de IL-10 e altas concentrações de TNF α (Kurtzhals et al., 1998). Um outro trabalho realizado em Vietnã associou a malária severa com altas concentrações de citocinas inflamatórias e baixas quantidades de IL-10 no plasma (Day et al., 1999). E em Gana, um estudo de correlação entre produção de citocinas pró e anti-inflamatórias e a imunidade clínica

na malária mostrou que altos níveis séricos de IFN γ , IL-12 ou TNF α com TGF β estavam associados com menor parasitemia mas com febre alta (Dodoo et al., 2002). Em conjunto, podemos concluir que a resposta imune inicial contra o *Plasmodium* depende de um balanço entre citocinas pró-inflamatórias e anti-inflamatórias, enquanto o hospedeiro desenvolve uma resposta imune adaptativa para a eliminação do *Plasmodium*.

1.4 Resposta imune adquirida contra o *Plasmodium*

A imunidade adquirida ao *Plasmodium* é comumente evidenciada em regiões endêmicas, onde há prevalência de reinfeção. Contudo, a imunidade desaparece após o tratamento prolongado com quimioterápicos ou após o afastamento durante alguns meses da zona endêmica. Além disso, a imunidade adquirida é específica à espécie que infectou o indivíduo, que ainda permanece susceptível à infecção por outras espécies do *Plasmodium* (Borrmann et al., 2011). A imunidade adquirida, aparentemente, pode contribuir em três vias: reduzindo a probabilidade de doença clínica, aumentando a velocidade de eliminação do parasito e aumentando a tolerância a infecções sub-patentes (Doolan et al., 2009). No início da infecção, a falta de especificidade dos anticorpos produzidos contra o *Plasmodium*, juntamente com a geração de autoanticorpos, parece contribuir para retardar o desenvolvimento de imunidade contra a malária (Boonpucknavig et al., 1984; Daniel-Ribeiro et al., 1989). Em indivíduos imunes, a produção de anticorpos específicos contra o *Plasmodium* é essencial para a proteção. Experimentos de transferência passiva de soro de adultos protegidos para indivíduos não imunes evidenciam claramente o papel protetor dos anticorpos (Sabchareon et al., 1991). A proteção parece estar particularmente associada aos isótipos de IgG opsonizantes (Bouharoun-Tayoun et al., 1990). A geração de memória imunológica é dependente da manutenção de células T CD4⁺, CD8⁺ e B específicas ao antígeno. Em infecções crônicas, células de memória podem ser continuamente geradas e expandidas (Snyder et al., 2008). Durante o estágio sanguíneo do *Plasmodium*, células T CD4⁺ e B possuem papel fundamental no controle da infecção (Mueller et al., 2013) e a presença de células T CD4⁺ específicas ao *Plasmodium* correlaciona com a imunidade clínica (Pombo et al., 2002; Reece et al., 2004).

A fim de corroborar e complementar os resultados encontrados na malária

humana, a utilização de roedores infectados com o *Plasmodium* tem sido extensamente utilizada como modelo experimental. Entre os diferentes modelos, a infecção pelo *Plasmodium chabaudi* parece ser aquela que mais se aproxima da doença humana causada pelo *P. falciparum* (Stephens et al., 2012). A infecção pelo *P. chabaudi* é o principal modelo utilizado para compreender a resposta imune contra o plasmódio. Na interface entre a imunidade inata e adquirida, observa-se uma forte ativação policlonal dos linfócitos T e B, caracterizada pela grande produção de moléculas efetoras, tais como IFN γ e IgG2a (Falanga et al., 1987; D'Império Lima et al., 1991). Com a progressão da doença, uma resposta imune específica é gerada após a eliminação de grande parte dos linfócitos T e B por apoptose (Helmby et al., 2000). Assim como na malária humana, a resistência ao *P. chabaudi* é mediada por linfócitos B, T CD4 $^{+}$ e CD8 $^{+}$ (Podoba et al., 1991). A imunidade humoral é indispensável e a proteção pode ser conferida pela transferência de anticorpos específicos (Cavinato et al., 2001; Falanga et al., 1984). A colaboração T-B através da molécula CD28 e a produção de IL-21 são essenciais para a produção de anticorpos específicos, assim como para desenvolvimento de imunidade protetora contra o *P. chabaudi* (Elias et al., 2005; Freitas do Rosário et al., 2008; Pérez-Mazliah et al., 2015). Os mecanismos pelos quais os anticorpos são efetivos incluem o bloqueio da invasão de eritrócitos pelos merozoítos (Blackman et al., 1990) e a ligação de anticorpos a moléculas do parasito presentes na superfície de eritrócitos (Bull et al., 1998). Além da produção de anticorpos, a produção de IFN γ pelos linfócitos T CD4 $^{+}$ é crítica para a geração de imunidade protetora contra *Plasmodium* (Xu et al., 2002). Outras citocinas também são importantes durante a infecção com o *Plasmodium*. A produção de IL10 regula a resposta inflamatória contra o parasito (Linke et al., 1996) e controla a patologia associada com a doença (Li et al., 1999). Em 2003, Li e colaboradores mostraram que camundongos deficientes de IL10 infectados com o *P. chabaudi* tratados com anticorpos anti-TNF α melhoram a patologia e exacerbam a doença quando tratados com anticorpos anti-TGF β . Além disso, a geração de células T IL10 $^{+}$ IFN γ $^{+}$ CD4 $^{+}$ protetoras contra o *P. chabaudi* é dependente de IL-27 (Freitas do Rosário et al., 2012).

1.4.1 Subpopulações de linfócitos T CD4⁺

A diferenciação de células T CD4⁺ em subpopulações distintas ocorre por meio de mecanismos de sinalização intracelular após a interação do complexo principal de histocompatibilidade de classe II (MHC II) com o peptídeo ligado e o receptor de células T (TCR), coestimulação e liberação de citocinas. A produção de IL-2 via transdutores de sinal e ativadores de transcrição 5 (STAT5) suprime a ligação de STAT3 ao locus do gene da proteína 6 do linfoma de células B (Bcl-6) e promove a transcrição da proteína de maturação de linfócitos B (Blimp1) (Johnston et al., 2012; Oestreich et al., 2012). A transcrição de Blimp1 possibilita o aumento do fator de transcrição T-bet e, consequentemente, a diferenciação de células Th1. Por outro lado, a sinalização de IL-6 e IL-21 via o fator de transcrição STAT3 potencializa a expressão de Bcl-6 e diferenciação de células Th foliculares (Tfh) (Eto et al., 2011; Karnowski et al., 2012; Linterman et al., 2010; Nurieva et al., 2008; Ray et al., 2014). Portanto, os fatores de transcrição Bcl-6 e Blimp1 são reguladores antagônicos da diferenciação de células Tfh. Além disso, a diferenciação de células Tfh requer um contato inicial com células dendríticas (Choi et al., 2011) e interação prolongada com células B do centro germinativo para reforçar o seu fenótipo (Crotty, 2014). As células Tfh são necessárias para o estabelecimento de memória de células B do centro germinativo dependentes de T e células secretoras de anticorpos de alta afinidade (Crotty, 2011; Wang et al., 2012). Além do aumento da transcrição de Bcl-6, as células Tfh também são caracterizadas pela alta expressão da proteína de morte celular programada (PD1) e o coestimulador de células T induzível (ICOS), expressão do receptor de quimiocina C-X-C tipo 5 (CXCR5), secreção de IL-21 e baixa expressão do receptor de quimiocina C-C tipo 7 (CCR7) (Tangye et al., 2013). A expressão de CXCR5 possibilita a migração de células Tfh para dentro do folículo linfóide para ajudar células B a formarem o centro germinativo (Haynes et al., 2007).

Em modelo murino de malária pelo *Plasmodium berghei*, a infecção induz alta frequência de precursores de células Tfh mas resulta em pouca diferenciação de tais células (Ryg-Cornejo et al., 2016). Além disso, o bloqueio de TNF α , IFN γ ou deleção de T-bet restaura a diferenciação de células Tfh e formação do centro germinativo (Ryg-Cornejo et al., 2016). Embora o IFN γ reduza a diferenciação de células Tfh, sua produção é necessária para o controle da infecção em modelo de roedores (Meding et al., 1990; Sauvaget et al., 1978; Simon-Haarhaus et al., 1991; Su,

Stevenson, 2000). Células T CD4⁺ produtoras de IFN γ e células T CD4⁺ de memória efetora são capazes de conferir proteção parcial durante infecção com *P. chabaudi* (Stephens, Langhorne, 2010). Além disso, o *priming* do IFN γ parece ser importante na manutenção da imunidade protetora contra o *P. chabaudi* (da Silva et al., 2013). Em conjunto, o IFN γ é um potente indutor da imunidade celular na malária (Meding et al., 1990; Su, Stevenson, 2000), troca de isotipo de anticorpos IgG e promove a ativação de fagócitos (Schroder et al., 2004). Dadas as evidências da ação de células Th1 e Tfh na imunidade contra a malária, faz-se necessário mais estudos a cerca dos mecanismos envolvidos na geração e manutenção de sub-populações de células T CD4⁺ durante a infecção. A percepção de sinais liberados durante a infecção é um ponto chave na diferenciação de linfócitos T CD4⁺. Além das citocinas liberadas, os linfócitos T CD4⁺ também são capazes de reconhecer moléculas provenientes de dano celular tais como o ATP, que podem contribuir para o processo de ativação celular. Portanto, o reconhecimento imune de ácidos nucleicos é campo novo de estudo que pode contribuir para compreensão de novos mecanismos responsáveis pela ativação, diferenciação e morte de linfócitos na presença de patógenos.

1.5 Reconhecimento imune de ácidos nucleicos e seus metabólitos

Durante a última década houve um rápido progresso na descoberta de componentes de patógenos que são reconhecidos pelo sistema imune e seu papel crítico na defesa contra a infecção. A descoberta dos TLRs, em meados de 1990, nos mostrou que o reconhecimento do sistema imune inato possui certa especificidade a componentes patogênicos que ficaram conhecidos como vias moleculares associadas a patógenos (PAMPs). Hoje sabemos que além dos PAMPs, o organismo também é capaz de reconhecer sinais derivados de danos no hospedeiro. Estes sinais, chamados de vias moleculares associadas ao dano (DAMPs), podem ser liberados por meio de canais da membrana, exocitose vesicular, morte celular ou injúria tecidual em condições patogênicas, não patogênicas e fisiológicas. Estas moléculas são reconhecidas por receptores de reconhecimento de padrão tanto por células do sistema imune inato quanto adaptativo. Dentre os DAMPs conhecidos, o ATP é um sinal de dano agudo e atua

como um mediador da inflamação (Di Virgilio et al., 2003, 2009). Portanto, em condições patogênicas com grande injúria tecidual e morte celular, o ATP pode ter um importante papel na resposta imune. Como os nucleotídeos são quimioatraentes, eles têm sido chamados de sinais “find-me” que atraem fagócitos (Chekeni et al., 2010), ativam plaquetas e estimulam a vasodilatação dependente da síntese de óxido nítrico endotelial (eNOS).

Uma vez liberado no meio extracelular, o ATP pode se ligar a moléculas chamadas de receptores purinérgicos. Os receptores purinérgicos são divididos em duas classes: P1 (receptores de adenosina) e P2 (receptores de nucleotídeos extracelulares). Há quatro tipos existentes de receptores P1 (A₁, A_{2A}, A_{2B} e A₃) (Fredholm et al., 2001). Os receptores P2 são divididos em duas subclasses: P2X e P2Y. Os receptores P2Y são acoplados à proteína G incluem 8 membros: P2Y_{1,2,4,6,11,12,13,14}. Os receptores P2X (P2X₁₋₇) proporcionam a abertura de canais de íons ativados pelo ATP extracelular e são seletivos para cátions monovalentes e divalentes (Na⁺, K⁺ e Ca²⁺) (North, 2002) (**Figura 3**). Os receptores P2X estão presentes em muitas células do sistema imune, tais como macrófagos, monócitos, neutrófilos, mastócitos, células dendríticas, linfócitos T e células NK. Essas moléculas têm sido consideradas como importante componentes da resposta imune inata contra infecções, podendo induzir a morte de patógenos em macrófagos e células epiteliais infectadas.

Figura 3 – Estrutura dos receptores purinérgicos P2X, P2Y e P1

Fonte: Abbracchio et al., 2009

Os receptores P2X7 foram inicialmente descritos como receptores P2Z presentes nas células do sistema imune, a partir do fenômeno de indução, por ATP extracelular, de permeabilização das membranas plasmáticas de macrófagos e mastócitos (Steinberg et al., 1990). O papel fisiológico dos receptores P2X7 ainda permanece pouco entendido. A sua participação em mecanismos de morte celular tem sido proposta baseando-se principalmente no fenômeno da permeabilização e na indução de apoptose em alguns tipos de células como timócitos e macrófagos (Bulanova et al., 2005; Mantuano-Barradas et al., 2003). Estudos recentes sugerem que através do reconhecimento pelo receptor P2X7, a molécula de ATP ativa o complexo inflamossomo- proteína 3 contendo domínios NATCH, LRR e pyrin (NALP3), um conjunto de moléculas da imunidade inata que controla as caspases inflamatórias e induz a produção de IL-1 β e IL-18 (Agostini et al., 2004; Dinarello, 2006). Alguns trabalhos mostram que o ATP em doses milimolares estimula a produção de citocinas pró-inflamatórias através de receptores P2X7 (Bours et al., 2006). Mecanismos envolvidos na produção de IL-1 β mediada por ATP têm sido extensivamente estudados (Karmakar et al., 2016; Mariathasan et al., 2006; Stoffels et al., 2015). O ATP extracelular parece ser um sinal crucial para desencadear a síntese e liberação de IL-1 β , após estímulos inflamatórios tal como aquele conferido por lipopolissacarídeos. Certos patógenos intracelulares parecem utilizar mecanismos envolvendo receptores de nucleotídeos em suas estratégias de escape. Alguns patógenos podem proteger células infectadas contra apoptose dependente do receptor P2X7 devido à produção de ATPases que consomem o ATP extracelular (Yilmaz et al., 2008). A formação extracelular de adenosina é resultado de uma cascata enzimática consistida de NTPDases (CD39) e ecto-5'-nucleotidases (Ecto5'NTase, CD73). O CD39 hidroliza ATP em adenosina difosfato (ADP) e adenosina monofosfato (AMP) (Dwyer et al., 2007), enquanto o CD73 hidroliza ADP/AMP para adenosina (Colgan et al., 2006). A adenosina extracelular se liga a receptores P1, que são expressos constitutivamente na superfície de células do sistema imune (Ishii, Akira, 2008). A adenosina, atuando sobre receptores P1, subclasse A2a, inibe a produção de IL-12, TNF- α e NO (Levy et al., 2006; Ohta, Sitkovsky, 2001) e aumenta a secreção de citocinas anti-inflamatórias tais como IL-10.

1.6 O receptor P2X7 em linfócitos T CD4⁺

Inúmeras evidências sugerem que os sinais de dano têm um papel importante na regulação da imunidade inata e adaptativa (Matzinger, 2002). A ativação do receptor P2X7 em células T pelo ATP extracelular induz mudanças no volume das células e composição da membrana. Após a interação do ATP ao receptor P2X7, as células T encolhem e incham rapidamente e expõem fosfatidilserina (PS) na membrana celular (Taylor et al., 2008). Em 2004, Courageot e colaboradores mostraram que o influxo de cálcio e íons de sódio, proporcionado pela ativação de receptores P2X7, era responsável pela inibição de aminofosfolípídeos translocases e exposição de PS em timócitos. A ativação prolongada de receptores P2X7 pode desencadear a exposição de PS e morte de linfócitos T. No entanto, a exposição de PS após estimulação com ATP é reversível se o ATP for removido dentro dos primeiros 30 minutos de exposição (Mackenzie et al., 2005). Além disso, em concentrações acima de 100 μ M de ATP, o receptor P2X7 pode formar um poro na membrana da célula que permite a passagem de solutos de até 900 Da (Steinberg et al., 1990). No entanto, ainda há dúvidas se o receptor P2X7 é capaz de mediar a dilatação do canal de íons ou proteínas associadas ao receptor P2X7, tais como a panexina 1. Porém, se sabe que a inibição da panexina 1 reduz significativamente a morte de células T induzida por ATP (Shoji et al., 2014). Além disso, subpopulações de linfócitos T expressam diferentes quantidades de receptores P2X7 na superfície e altas quantidades do receptor estão associadas com maior sensibilidade à morte (Aswad et al., 2006).

A ativação do receptor P2X7 também está associada com uma rápida mudança na superfície de células T. Essa mudança ocorre em decorrência da ativação de metaloproteinases que catalisam a clivagem de diversas proteínas de superfície, tais como CD62L, CD27 e receptor de IL-6 (Gu et al., 1998; Rissiek et al., 2015). Além da ativação parácrina, o receptor P2X7 pode ser ativado por uma liberação autócrina de ATP, após o estímulo do receptor do linfócito T (TCR). A abertura de canais dependente do receptor P2X7 pode sustentar a sinalização da proteína quinase ativada por mitógeno (MAPK) e, conseqüentemente, estimular a produção de IL-2 e a proliferação celular. Todos os receptores P2X, com a exceção do P2X5, podem facilitar a entrada de Ca^{2+} em resposta ao estímulo pelo ATP extracelular, desta forma sugerindo que estes receptores podem regular a ativação

de linfócitos T. A liberação de ATP mediada pelo receptor P2X7 e a ativação autócrina de receptores P2X1 e P2X4 têm funções importantes na amplificação de sinais do TCR e comunicação intracelular nas sinapses imunológicas formadas entre células T e células apresentadoras de antígenos (Woehrle et al., 2010) (**Figura 4**). Em conjunto, há inúmeros indícios mostrando a importância da sinalização purinérgica para o desenvolvimento, ativação e morte de linfócitos T. A regulação purinérgica é uma importante área de pesquisa, com enorme potencial para o desenvolvimento de novas estratégias terapêuticas para o tratamento de diversas doenças infecciosas. O estudo dessa via de sinalização pode contribuir para a compreensão de mecanismos purinérgicos responsáveis pela geração da resposta imune, particularmente em processos infecciosos que envolvem extenso dano celular.

Figura 4 – Sinalização purinérgica na ativação de células T

Fonte: Junger et al., 2011

2.1 Objetivo geral

O objetivo central do presente trabalho foi avaliar a participação do receptor P2X7 em células T CD4⁺ durante o desenvolvimento da malária causada pelo *P. chabaudi* AS, a fim de compreendermos mecanismos direcionados ao desenvolvimento da resposta imune efetora na presença de DAMPs e antígenos.

2.2 Objetivos específicos

Objetivo 1: Avaliar a participação de receptores P2X7 na ativação e geração de subpopulações de células T CD4⁺ durante a fase aguda e crônica da infecção com o *P. chabaudi*.

Objetivo 2: Avaliar a participação de receptores P2X7 na produção de IFN γ por células T CD4⁺ e anticorpos específicos contra o plasmódio.

Objetivo 3: Averiguar se o receptor P2X7 possui importância na geração de células T CD4⁺ de memória na malária.

3.1 Camundongos e infecção

Camundongos C57BL/6 (B6), P2X7^{-/-} e CD4^{-/-} fêmeas, com 6 a 8 semanas de idade, foram fornecidos pelo biotério do Departamento de Imunologia da Universidade de São Paulo (Originalmente da The Jackson Laboratory) e mantidos em condição SPF (do inglês *specific pathogen-free*). O *P. chabaudi* cepa AS foi mantido como previamente descrito (Podoba, Stevenson, 1991). Os camundongos foram mantidos em ciclos invertido de claro e escuro por 15 dias antes da infecção (Cavinato et al., 2001). Camundongos foram inoculados via intraperitoneal (i.p.) com 1×10^6 eritrócitos parasitados (iRBCs).

3.2 Comitê de Ética

Todos os procedimentos experimentais estão de acordo com os regulamentos nacionais de diretrizes éticas para a experimentação e bem-estar dos camundongos do Conselho Nacional de Saúde e Experimentação Animal do Colégio Brasileiro (COBEA). Os protocolos foram aprovados pelo Comitê de saúde animal da USP, com os números de licenciamento: 050/2009 e 175/2011.

3.3 Parasitemia e Análise clínica

As parasitemias foram monitoradas por meio de esfregaços sanguíneos corados com Giemsa. O peso corporal foi determinado em balança analítica (Sartorius, USA) e o resultado apresentado como a porcentagem de variação de peso do dia 0. A temperatura axial foi medida com termômetro digital (Kent Scientific Co., USA). A concentração de hemoglobina foi avaliada com um kit de hemoglobina (Doles Inc., Brasil).

3.4 Suspensão de Células

As células do baço e sangue foram lavadas e mantidas em RPMI 1640 gelado e suplementado com penicilina (100 U/ml), estreptomicina (100 µg/ml), 2-mercaptoetanol (50 µM), L-glutamina (2 mM), piruvato de sódio (1 mM) e 3-20% de soro fetal bovino inativado pelo calor. Todos os suplementos foram adquiridos da Life Technologies. Leucócitos foram obtidos em gradiente de Percoll 70% (GE Health Care, USA). Para o ensaio de fluxo de cálcio, células T CD4⁺ foram

separadas magneticamente (Colunas LS – Miltenyi Biotec, Germany) por seleção positiva usando microbeads anti-CD4 com autoMACs (Miltenyi Biotec, Germany). Para a transferência adotiva, células T CD4⁺ foram separadas magneticamente (Colunas LS – Miltenyi Biotec, Germany) por seleção negativa usando microbeads anti-CD19, -IA^b and CD8 com autoMAC (Miltenyi Biotec, Germany) e separadas por sorting no aparelho FACs Aria (BD Bioscience).

3.5 Análise fenotípica das células por citometria de fluxo

As células (1×10^6) foram marcadas com anticorpos monoclonais (mAbs) anti-CD4 (H129.19 or GK1.5), CD19 (ID3), CD138 (281-2), CD44 (IM7), CD62L (MEL-14), CD178 (ICOS) (7E.17G9), CD127 (A7R34), CD95 (Fas) (Jo2), GL7 (GL7), CD38 (90), PD1 (J43) and CXCR5 (2G8), conjugados à fluoresceína (FITC), ficoeritrina (PE), alofocianina (APC), complexo proteína clorofila peridina (PerCP), ficoeritrina-Cy7(PE-Cy7), alofocianina-Cy7 (APC-Cy7), Pacific Blue e AmCyan obtidos da BD Pharmingen. Para a marcação intracelular, mAbs anti Bcl-6 conjugados com PE (K112-91, BD Pharmingen) e anti-T-bet conjugados com PerCP (eBio4B10, eBioscience) foram utilizados de acordo com as instruções do fabricante. A marcação de anexina foi realizada com tampão apropriado (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1,8 mM CaCl₂ [pH 7,4]). As amostras foram analisadas em citômetro de fluxo FACSCanto e os dados foram analisados utilizando-se o programa FlowJo v.7.2.2 (Tree Star Inc., USA).

3.6 Ensaio de permeabilização celular

Células do sangue e baço (1×10^6) foram marcadas com mAb anti-CD4 (GK1.5) conjugados com APC (BD Pharmingen). Em alguns experimentos, células marcadas foram pré-aquecidas (37 °C) em phosphate-buffered saline (PBS) com 3% de albumina de soro bovino (BSA) (Sigma-Aldrich) e então incubadas com 25-500 µM de ATP (Amersham Bioscience, USA) ou o sobrenadante de iRBC lisados ou somente meio por 15 minutos. Os eritrócitos não infectados (nRBCs) e iRBCs (2×10^8) foram lisados com 200 µl de tampão de lise (40mM NH₄Cl, 4,2 mM Tris [pH7,4]) por 5 minutos em 4 °C. Células do baço (1×10^6) foram incubadas com 200 µl do sobrenadante dos RBCs diluídos 1:5 em meio RPMI gelado com 1% de soro fetal

bovino. O brometo de etídio (EB) (2,5 μ M) (Sigma-Aldrich) foi adicionado e as amostras foram imediatamente analisadas por citômetro de fluxo.

3.7 Determinação do ATP no soro

O ATP foi quantificado usando kit de ensaio para detecção do ATP por bioluminescência (Sigma-Aldrich). O soro foi diluído 1:1 com o reagente luciferase. A unidade relativa de luz (RLUs) em cada amostra foi determinada em um luminômetro com temperatura controlada (Berthold, USA).

3.8 Ensaio de proliferação celular

A proliferação de células T CD4⁺ foi medida como previamente descrito (Elias et al., 2005). Em resumo, 3×10^7 células esplênicas foram incubadas por 20 minutos em 37 °C com 5 μ M 5,6-carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, USA) em PBS com 0,1% de BSA (Sigma-Aldrich). 1×10^6 células foram cultivadas com iRBC (4×10^6) ou somente meio por 72h em 37 °C com 5% de CO₂, marcados com mAb para CD4 conjugados com PECy7 e analisados por citometria de fluxo.

3.9 Detecção de citocinas

Para detecção de citocina intracelular, células do baço (1×10^6) foram colocadas em cultura com StopGolgi de acordo com as instruções do fabricante na presença de iRBC ou somente meio por 10 horas em 37 °C com 5% de CO₂. As células foram lavadas, marcadas com CD4 conjugado com APC e fixadas com tampão Citofix/Cytoperm. Para a marcação intracelular, utilizamos o mAb anti-IFN γ (XMG-1.2) conjugado com Pacific Blue e o anti-IL-10 (JESS-16E3) conjugado com APC em tampão Perm/Wash, e analisados por citometria de fluxo. Todos os reagentes foram adquiridos da BD Bioscience. O ensaio de secreção da citocina IL-2 foi realizado de acordo com as instruções do fabricante (BD Bioscience). O ensaio utiliza um mAb bi-funcional capaz de se ligar ao CD45 e IL-2. As células (2×10^6) foram incubadas com o mAb bi-funcional por 45 minutos em 37 °C com 5% de CO₂. O IL-2 ligado a superfície da célula foi detectado com mAb anti-IL-2 conjugados com PE por citometria de fluxo. Para a detecção de citocinas no sobrenadante, 1×10^6

células do baço foram cultivadas com 3×10^6 iRBCs ou somente meio por 72 horas em 37 °C com 5% de CO₂. A concentração de citocinas foi determinada usando o kit OptEIA INF γ (BD Bioscience) e ELISA de IL-21 para camundongo (eBioscience).

3.10 Detecção por ELISA de anticorpos específicos no soro

Anticorpos séricos anti-*P. chabaudi* foram detectados por ELISA. Inicialmente, placas de ELISA (Costar) foram sensibilizadas com 50 μ L de extrato total de *P. chabaudi* (8 μ g/ mL) diluídos em tampão carbonato/bicarbonato 0,05 M pH 9,6 e incubadas durante a noite, a 4 °C. Após lavagens sucessivas com PBS contendo 0,05% de tween 20 (Sigma-Aldrich), as placas foram saturadas por 60 minutos com 200 μ L de PBS-tween contendo 1% de albumina de soro bovino (BSA, Sigma-Aldrich). Após as lavagens, foi adicionado em cada poço, 100 μ L de diluições decrescentes de cada amostra feitas com PBS-tween contendo 1% de BSA. Após 1 hora de incubação a temperatura ambiente, as placas foram lavadas e a presença de anticorpos específicos contra o parasita, revelada adicionando-se anticorpos de cabra anti-IgM, anti-IgG, anti-IgG1 ou anti-IgG2a de camundongos conjugados à peroxidase (Southern Biotechnology Associates), diluídos 1:2000 em PBS-tween com 1% de BSA. Por fim, as placas foram novamente lavadas e 100 μ L de Tetrametilbenzidina (TMB) foi adicionado em cada poço. A reação foi bloqueada com ácido cítrico 0,2 M e a leitura, realizada através de um espectrofotômetro Spectra Max 190 (Molecular Devices), utilizando o comprimento de onda 450 nm.

3.11 ELISPOT para células secretoras de anticorpos

O ensaio de ELISPOT (ELISA SPOT assay) foi realizado como descrito previamente (D'Império Lima et al., 1991), no qual microplacas de 96 poços (Costar) adsorvidas overnight (4 °C) com o anticorpo goat anti-mouse Ig total (10 μ g/mL) foi bloqueada com gelatina 1% (Merck) diluída em PBS 1X, por 120 minutos. Células do baço diluídas (10^6 até 5×10^2 células/poço) foram cultivadas em meio RPMI 1640 com 1% SFB, por 6 horas, 37 °C em 5% CO₂. Os “spots” foram revelados adicionando os anticorpos goat anti-mouse IgM, IgG2c ou IgG1 biotinilados e, posteriormente, adicionando a estreptoavidina conjugada à fosfatase alcalina (todos os anticorpos e conjugados foram Southern Biotechnology Associates). 5-Bromo-

cloro-3-indolil fosfato (Sigma-Aldrich) diluído em tampão 2-amino-2-metil-1-propanol (Parroche et al.) (Merck) foi utilizado como substrato. E, a partir das diluições (número de células plaqueadas vs “spots”) e o total do número de esplenócitos plaqueados, o número de células secretoras de Ig por baço foi calculado.

3.12 Ensaio de fluxo de Cálcio

Células T CD4 (1×10^7) foram incubadas com uma mistura de 4 μ M de Fura-3AM (Molecular Probes, USA) e 0,7 mg/ml de Probenicid (Sigma-Aldrich) em 37 °C por 30 minutos. Após a lavagem, 1×10^5 células foram analisadas em um microscópio de fluorescência (Microscópio invertido Nikon, Japão) para determinar a intensidade de fluorescência. O cálcio intracelular foi determinado pelo cálculo corrigido da fluorescência da célula total (CTCF). CTCF= densidade integrada – (área da célula selecionada x média de fluorescência do *background* da imagem).

3.13 Ensaio de TUNEL

Os baços foram coletados e congelados em Tissue-Tek O.C.T. (Sakura Finetek, USA). A fragmentação do DNA in situ em cortes de 8 μ m foi analisada usando o sistema de TUNEL DeadEnd™ por fluorescência de acordo com o protocolo do manufaturador (Promega, USA).

3.14 Fragmentação do DNA

As células do baço foram marcadas com mAb anti-CD4 (H129.19) conjugado com APC e fixadas com etanol 70%. Após a lavagem, as células foram incubadas com um tampão de extração do DNA (0.2 M Na₂HPO₄ and 0.1% Triton x-100, Sigma-Aldrich) em 24 °C por 5 minutos, centrifugadas e incubadas com uma solução marcadora de DNA (20 μ g/ml de PI-Sigma Aldrich). 50 μ g de RNase (Invitrogen) foi adicionado em cada amostra e as células foram incubadas em 24 °C por 30 minutos no escuro. A incorporação do PI foi determinada por citômetro de fluxo.

3.15 Análise de histologia e imunofluorescência

Para a análise histológica, os baços foram primeiramente fixados em formal

tamponado por 12 horas e depois colocados em parafina. Os cortes do baço (5 μm) foram marcados com eosina e hematoxilina usando o procedimento padrão. Para a análise de imunofluorescência, os baços foram coletados e imediatamente colocados em Tissue-Tek O.C.T. (Sakura Finetek, USA) e congelados. Os cortes de 8 μm foram fixados em acetona e bloqueados com 3% BSA e Fc Block (1:100) (BD Bioscience) em 24 °C por 1 hora. Os cortes foram marcados com mAb anti-CD4 (RM4-5) conjugado com Alexa 700 (eBioscience), anti-CD19 (ID3) conjugado com APC, GL-7-Biotina (GL7) e estreptavidina conjugada com FITC (BD Bioscience) em 24 °C por 2 horas. Após a lavagem, as células foram marcadas com DAPI (Molecular Probes) a lâmina foi montada com o meio Vectashield (Vector, USA). Os cortes foram analisados por microscopia confocal (Zeiss LSM 780, Alemanha).

3.16 Transferência Adotiva

As células T CD4 ($1,5 \times 10^6$) do baço de camundongos B6 ou P2X7^{-/-} não infectados foram transferidas, por injeção retro-orbital, para camundongos CD4^{-/-}. Uma semana após a transferência das células, os camundongos CD4^{-/-} foram infectados com 1×10^6 de iRBCs.

3.17 Análise estatística

A análise estatística foi realizada com o teste “Two-way ANOVA”, seguido por análise do Teste de Bonferroni. Todas as análises estatísticas foram realizadas no programa Prisma 4 (Graph Pad Software) e as diferenças entre os três grupos foram consideradas significativas para valores de $p < 0,05$ (5%).

4.1 O receptor P2X7 contribui para a proteção contra o *Plasmodium chabaudi*

Para avaliarmos a importância do receptor P2X7 na proteção contra o *Plasmodium chabaudi*, analisamos a parasitemia e as manifestações clínicas da doença em camundongos B6 e P2X7^{-/-} durante a fase aguda e crônica da infecção. Realizamos uma cinética de parasitemia, temperatura, peso corporal e Hemoglobina (Hb) durante 18 dias de infecção (**Figura 5A**). Até o 7º dia de infecção, onde há o pico de parasitemia em camundongos B6, não observamos diferenças significativas entre os grupos. No entanto, camundongos P2X7^{-/-} tiveram dificuldade em controlar a parasitemia e recuperar o peso após o 8º dia de infecção. Além disso, observamos que camundongos P2X7^{-/-} tiveram dificuldade em controlar a temperatura entre os dias 10 e 12 de infecção. A hemoglobina dos camundongos P2X7^{-/-} não foi controlada no 18º dia de infecção, enquanto os camundongos B6 já apresentavam níveis normais.

Com o objetivo de analisarmos a parasitemia residual durante a fase crônica da infecção, realizamos a transferência do sangue de camundongos B6 e P2X7^{-/-} nos dias 30 e 50 p.i. para camundongos RAG^{-/-} (**Figura 5B**). Verificamos maior parasitemia em camundongos RAG^{-/-} que receberam o sangue de camundongos P2X7^{-/-} dos dias 30 e 50 p.i. em comparação com sangue de camundongos B6.

Figura 5 - Parasitemia e parâmetros clínicos durante a fase aguda e crônica da infecção em camundongos B6 e P2X7^{-/-}

(A e B) Camundongos B6 e P2X7^{-/-} fêmeas foram infectados com 1×10^6 iRBCs. Os dados são representativos de 3 experimentos realizados em separado. (A) Parasitemia, concentração de hemoglobina (Hb) no soro, peso corporal e temperatura foram monitorados diariamente. O peso corporal é apresentado de acordo com a variação do peso estabelecido no dia 0 de infecção. Cada curva representa um único camundongo; (1-4) camundongos B6 e (5-8) camundongos P2X7^{-/-}. (B) Parasitemias em camundongos RAG^{-/-} transferidos com 50 μ l de sangue provenientes de camundongos B6 e P2X7^{-/-} nos dias 30 e 50 p.i. foram monitorados a cada dois dias pós-transferência (p.t.). Cada curva representa um único camundongo; (1-3) Sangue de camundongos B6 no dia 30 p.i. $\Rightarrow$ camundongos RAG^{-/-}; (4-7) Sangue de camundongos B6 no dia 50 p.i. $\Rightarrow$ camundongos RAG^{-/-}; (8-10) Sangue de camundongos P2X7^{-/-} no dia 30 p.i. $\Rightarrow$ camundongos RAG^{-/-} e (4-7) Sangue de camundongos P2X7^{-/-} no dia 50 p.i. $\Rightarrow$ camundongos RAG^{-/-}.

4.2 O receptor P2X7 contribui para a ativação de células T CD4⁺ e produção de anticorpos durante a fase aguda da malária

Durante o mestrado, iniciamos este trabalho com a hipótese que o ATP liberado durante o rompimento dos eritrócitos poderia ativar o receptor P2X7 em linfócitos (Salles, 2011). Avaliamos a quantidade de ATP no soro e a incorporação do EB em células T CD4⁺ antes e após o rompimento do eritrócito parasitado durante a fase aguda da infecção. Após o rompimento dos eritrócitos, os camundongos apresentaram maior quantidade de ATP no soro e permeabilização dependente do ATP de células T CD4⁺ do sangue (**Apêndice A – Figura A, Apêndice A – Figura B**). Além disso, também já tínhamos observado que o sobrenadante de eritrócitos parasitados lisados era capaz de permeabilizar linfócitos T CD4⁺ do baço de camundongos B6 durante a fase aguda da infecção (**Apêndice A – Figura C**). Sabendo que as células T CD4⁺ possuem um papel protetor durante a malária (Podoba, Stevenson, 1991; Meding, Langhorne, 1991), nosso próximo passo foi observar a participação do receptor P2X7 na proliferação e produção de IFN γ pelas células T CD4⁺. A análise foi realizada nos dias 4 e 7 p.i., pois neste período há intensa proliferação de células T CD4⁺ e secreção de IFN γ (Muxel et al., 2011). De acordo com os resultados encontrados, a habilidade de células T CD4⁺ do quarto dia de infecção de proliferar espontaneamente *in vitro* ou após estímulo com iRBCs foi significativamente menor na ausência do receptor P2X7 (**Figura 6A**). Além disso, a produção de IFN γ pelas células T CD4⁺ de camundongos P2X7^{-/-} estimuladas com iRBC foi menor em comparação com as células T CD4⁺ de camundongos B6 (**Figura 6B**). Também analisamos a frequência de células T IFN γ ⁺IL-10⁺CD4⁺ e não observamos diferença entre os grupos (**Figura 6B**). A seguir, observamos que a perda do receptor P2X7 drasticamente afetou a expansão da população de células T CD4⁺ esplênicas durante a infecção aguda (**Figura 6C**). No dia 7 de infecção, somente camundongos B6 apresentaram um aumento no número de células T IFN γ ⁺CD4⁺ por baço (Figura 6D). Além disso, não observamos produção de IL-10 pelas células T CD4⁺ de ambos os grupos de camundongos infectados (**Figura 6D**).

Figura 6 –Proliferação e produção de IFN γ pelas células T CD4 $^{+}$ durante a fase aguda da infecção em camundongos B6 e P2X7 $^{-/-}$

(A-D) Camundongos B6 e P2X7 $^{-/-}$ fêmeas foram analisados nos dias 4 e 7 p.i. com 1×10^6 iRBCs. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média $\pm$ desvio padrão (n=3). Diferenças significativas com $p < 0,05$ foram indicadas por: (*) entre grupos infectados e controles, (**) entre grupos B6 e P2X7 $^{-/-}$ e (-) entre grupos

indicados. (A) Ensaio de proliferação de células T CD4⁺ realizado com esplenócitos estimulados com iRBC (1 esplenócito/3 iRBCs) ou somente mantidos com meio de cultura. O histograma mostra as células T CD4⁺ marcadas com CFSE. A porcentagem de células CFSE^{lo}CD4⁺ são apresentadas no gráfico de barras. (B) O gráfico de contorno mostra as células T CD4⁺ produtoras de IFN γ e IL-10 na cultura de células descrita em A. A porcentagem de células IFN γ ⁺IL-10⁻CD4⁺ e IFN γ ⁺IL-10⁺CD4⁺ são apresentadas no gráfico de barras. (C) O número de células TCD4⁺ por baço foi determinado por citometria de fluxo. (D) O número de células IFN γ ⁺CD4⁺ e IL-10⁺CD4⁺ foi determinado por citometria de fluxo.

4.3 A resposta de células Th1 de memória é reduzida em camundongos P2X7^{-/-} durante a infecção crônica com o *Plasmodium chabaudi*

As mais altas parasitemias observadas em camundongos P2X7^{-/-} cronicamente infectados em comparação com camundongos B6 sugerem que o receptor P2X7 contribui para o desenvolvimento da imunidade adquirida contra o *P. chabaudi*. Devido ao importante papel das células Th1 na completa eliminação do parasito (da Silva et al., 2013; Freitas do Rosario et al., 2008), analisamos a resposta de células T CD4⁺ esplênicas em camundongos B6 e P2X7^{-/-} durante a fase crônica da infecção. Primeiramente, avaliamos a permeabilização das células ao ATP por meio da marcação de EB mediante a formação do poro dependente do receptor P2X7. As células T CD4⁺ dos dias 20 e 30 p.i. apresentaram maior permeabilização com concentrações de ATP acima de 100 μ M em comparação com o controle (Figura 7A). No entanto, no dia 50 de infecção, a permeabilização das células T CD4⁺ retorna às frequências encontradas no controle (**Figura 7A**). A seguir, analisamos o número de células T CD4⁺ de ambos os grupos durante a fase crônica da infecção e, diferente do encontrado na fase aguda, observamos maior número de células em camundongos P2X7^{-/-} que em camundongos B6 (**Figura 7B**). Além disso, a análise de subpopulações de células T CD4⁺, como previamente definido por (Stephens, Langhorne, 2010) (**Figura 7C**), revelaram maior número de células T efetoras (T_E), T de memória efetora (T_{EM}) e T de memória central (T_{CM}) em camundongos P2X7^{-/-} (**Figura 7D**). No entanto, a perda do receptor P2X7 comprometeu a produção de IFN γ em células T CD4⁺, sem afetar na secreção de IL-10 (**Figura 7D**), como observado durante a infecção aguda. De acordo com os resultados da Figura 3D, também observamos menor quantidade de IFN γ , mas não de IL-10, no sobrenadante de esplenócitos de camundongos P2X7^{-/-} do dia 30 de infecção e estimulados com iRBC (**Figura 7F**). Além disso, o estímulo de ATP na cultura de esplenócitos de camundongos B6 aumentou a produção de IFN γ

dependente do receptor P2X7 e inibiu a produção de IL-10 independente do receptor P2X7.

Figura 7 – Número de células T CD4⁺ e produção de IFN γ durante a fase crônica da infecção em camundongos B6 e P2X7^{-/-}

(A-F) Camundongos B6 e P2X7^{-/-} fêmeas foram analisados nos dias 20, 30 e 50 p.i. com 1×10^6 iRBCs. Camundongos não infectados foram utilizados como controle (dia 0).

Os dados são representativos de 3 experimentos realizados em separado com média $\pm$ desvio padrão (n=3). Diferenças significativas com $p < 0,05$ foram indicadas por: (*) entre grupos infectados e controles, (**) entre grupos B6 e P2X7^{-/-} e (-) entre grupos indicados. (A) Esplenócitos estimulados com 25-500 μ M de ATP ou sem estímulo. A porcentagem de células T CD4⁺ marcadas com BE foram determinadas por citometria de fluxo. A barra cinza horizontal representa o intervalo de confiança obtido com células de camundongos P2X7^{-/-}. (B) O número de células TCD4⁺ por baço foi determinado por citometria de fluxo. (C) Estratégia de análise usada para definir subpopulações de células T CD4⁺. (D) O número de células CD4⁺ T_E (CD44^{hi}ILR7 α ⁻), T_{EM} (CD44^{hi}ILR7 α ⁺CD62L^{lo}) e T_{CM} (CD44^{hi}ILR7 α ⁺CD62L^{hi}) foi determinado por citometria de fluxo. (E) Os esplenócitos foram estimulados com iRBC (1 esplenócito/3 iRBCs) ou somente mantidos com meio de cultura. A porcentagem de células IFN γ ⁺IL-10⁻CD4⁺, IFN γ ⁺IL-10⁺CD4⁺ e IFN γ ⁻IL-10⁺CD4⁺ foram determinadas por citometria de fluxo. (F) As concentrações de IFN γ e IL-10 foram determinadas por ELISA no sobrenadante da cultura de esplenócitos descritas em E, estimulados com 25-500 μ M de ATP ou sem estímulo.

4.4 O receptor P2X7 contribui para o aumento de expressão de T-bet em células T CD4⁺ durante a infecção aguda e crônica com o *Plasmodium chabaudi*

O próximo experimento teve como objetivo determinar se o receptor P2X7 é necessário para induzir, em células T CD4⁺ esplênicas, o fator de transcrição T-bet, crítico para a diferenciação de células Th1 (Lugo-Villarino et al., 2003). Após análises realizadas nos dias 7, 20 e 30 p.i., observamos que a expressão de T-bet foi aumentada somente em células T CD4⁺ de camundongos B6 em comparação com o controle não infectado, enquanto as células T CD4⁺ de camundongos P2X7^{-/-} não apresentaram aumento (**Figura 8A**). No dia 30 de infecção, o aumento da expressão de T-bet foi observado em células T CD4⁺ T_{E/EM} e T_{CM} (**Figura 8B**).

Figura 8 – Expressão de T-bet em células T CD4⁺ durante a fase crônica da infecção em camundongos B6 e P2X7^{-/-}

(A-B) Camundongos B6 e P2X7^{-/-} fêmeas foram analisados nos dias 7, 20 e 30 p.i. com 1×10^6 iRBCs. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média $\pm$ desvio padrão (n=3). Diferenças significativas com $p < 0,05$ foram indicadas por: (*) entre grupos infectados e controles e (-) entre grupos indicados. (A) O histograma apresenta a expressão de T-bet em células T CD4⁺. A média da intensidade de fluorescência (Amaral et al.) da expressão de T-bet são apresentadas no gráfico de dispersão. (B) O gráfico de contorno mostra células CD4⁺ T_{E/EM} (CD44⁺CD62L^{lo}), T_{CM} (CD44⁺CD62L^{hi}) e Naive (CD44⁻CD62L^{hi}). A porcentagem de cada subpopulação de células T CD4⁺ são mostradas. O histograma apresenta a expressão de T-bet em subpopulações de células T CD4⁺. MFI da expressão de T-bet são apresentados nos gráficos de dispersão.

4.5 A perda do receptor P2X7 contribui para o aumento da resposta de anticorpos durante a infecção crônica com o *Plasmodium chabaudi*

Uma notável observação em camundongos P2X7^{-/-} cronicamente infectados foi a hiperplasia de folículos linfoides secundários esplênicos (**Figura 9A**). A análise de folículos do baço de camundongos no dia 20 de infecção, por microscopia confocal, revelou grande quantidade de células T CD4⁺ em extensiva área do centro germinativo marcado com GL-7 (**Figura 9B**). Consistentemente, nos dias 20 e 30 p.i., camundongos P2X7^{-/-} apresentaram maior número de células B por baço, incluindo células B do centro germinativo (Fas⁺GL7⁺CD19⁺) (**Figura 9C**). Devido ao essencial papel que células Tfh possuem na maturação da afinidade de anticorpos (Breitfeld et al., 2000; Schaerli et al., 2000), focamos na análise de tais células em camundongos B6 e P2X7^{-/-} durante a fase crônica de infecção. A perda do receptor P2X7 possibilitou um aumento no número de células Tfh que foi identificada pela expressão de CXCR5, PD1, ICOS e Bcl-6 (**Figura 9D**). Os esplenócitos de camundongos P2X7^{-/-} do dia 20 de infecção que foram estimulados com iRBC produziram maior quantidade de IL-21, uma citocina característica de geração e manutenção de células Tfh (Nurieva et al., 2008). Além disso, as concentrações de anticorpos IgM contra o parasito no dia 30 p.i. e IgG2a no dia 50 p.i. foram maiores em camundongos P2X7^{-/-} que em camundongos B6 (**Figura 9F**). Também analisamos a concentração de IgG1 contra o parasito e baixos níveis foram encontrados em ambos os grupos (dados não mostrados).

Figura 9 – Desenvolvimento de folículos linfoides secundários em camundongos B6 e P2X7^{-/-} durante a fase crônica de infecção

(A-F) Camundongos B6 e P2X7^{-/-} fêmeas foram analisados nos dias 20, 30 e 50 p.i. com 1 x 10⁶ iRBCs. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média ± desvio padrão (n=3). Diferenças significativas com p<0,05 foram indicadas por: (*) entre grupos infectados e controles, (**) entre grupos B6 e P2X7^{-/-} e (-) entre grupos indicados. (A) O tecido corado com hematoxilina e eosina mostra a hiperplasia folicular esplênica em camundongos P2X7^{-/-} (aumento de 40x; as barras de escala correspondem à 200 µm). (#) Média das áreas dos folículos linfoides. (B) Imagem de imunofluorescência por microscópio confocal (aumento de 100x; as barras de escala correspondem à 400 µm) de tecido esplênico. Os cortes de tecido foram marcados com CD19 (verde), CD4 (vermelho) e GL7 (azul). (C) Número de células CD19⁺ e Fas⁺GL7⁺CD19⁺ foram determinadas por citometria de fluxo. (D) O gráfico de contorno mostra a expressão de PD1, ICOS e Bcl-6 *versus* CXCR5 em células T CD4⁺. O número de células T PD1^{hi}CXCR5⁺CD4⁺, ICOS⁺CXCR5⁺CD4⁺ e Bcl-6⁺CXCR5⁺CD4⁺ é mostrado no

gráfico de colunas. (E) A concentração de IL-21 foi determinada por ELISA no sobrenadante de cultura de esplenócitos estimulados com iRBC (1 esplenócito/3 iRBCs) ou somente mantidos com meio de cultura. (F) A concentração de anticorpos IgM e IgG2a contra o parasito foram determinadas por ELISA.

4.6 O receptor P2X7 medeia a morte de células Tfh durante a infecção crônica com o *Plasmodium chabaudi*

Uma explicação para a diferença no número de células Tfh entre os grupos poderia ser que a sinalização do receptor P2X7 promove a diferenciação de células Th1 ao invés da diferenciação de células Tfh por ampliar a expressão de T-bet. Uma outra possibilidade, que pode coexistir com a hipótese anterior, é que o receptor P2X7 poderia mediar a morte de células Tfh esplênicas como descrito nas placas de Peyer (Proietti et al., 2014). Portanto, para investigarmos a hipótese da morte dependente do receptor P2X7, nós realizamos um ensaio de marcação de Nicks por dUTP e deoxinucleotidil terminal transferase (TUNEL). O ensaio por TUNEL revelou células positivas, principalmente no baço de camundongos B6 no dia 20 de infecção (**Figura 10A**), formando agregados de células TUNEL⁺ nos folículos linfoides. Além disso, observamos uma baixa porcentagem de núcleos hipodiplóides em células T CD4⁺ de camundongos P2X7^{-/-} no dia 20 de infecção (**Figura 10B**). Sabendo que a exposição de fosfatidilserina (PS) antecede a morte de células T mediadas pelo receptor P2X7 (Taylor et al., 2008), a marcação de anexina V foi comparada em células Tfh (PD1⁺CXCR5⁺CD4⁺) e não-Tfh (PD1⁻CD4⁺). Em camundongos B6, muitas células Tfh apresentaram marcação de anexina V, considerando que grande parte das células não-Tfh não apresentaram marcação (**Figura 10C**). Observamos menor quantidade de marcação de anexina V nas células T CD4⁺ de camundongos P2X7^{-/-} que em camundongos B6. Tendo como base a marcação do Acqua Live/Dead, muitas células Tfh anexina⁺ mantiveram a integridade da membrana (**Figura 10D**). Além disso, quando estimuladas com 100-500 μ M de ATP, a formação do poro mediada pelo receptor P2X7 foi observada principalmente em células Tfh quando comparada com células não-Tfh (**Figura 10E**).

Figura 10 – Formação do poro associado ao receptor P2X7 e morte de células Tfh em camundongos B6 e P2X7^{-/-} durante a fase crônica de infecção

(A-F) Camundongos B6 e P2X7^{-/-} fêmeas foram analisados no dia 20 p.i. com 1×10^6 iRBCs. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média $\pm$ desvio padrão (n=3-5). Diferenças significativas com $p < 0,05$ foram indicadas por: (*) entre grupos infectados e controles e P2X7^{-/-} e (-) entre grupos indicados. (A) Imagem de imunofluorescência (aumento de 40x; as barras de escala correspondem à 200 μm) de cortes do baço foram obtidas usando o ensaio de TUNEL. (B) O histograma mostra

a incorporação de iodeto de propídio (PI) em células T CD4⁺. A porcentagem de núcleos hipodiplóides é mostrada no gráfico de colunas. (C) Estratégia de análise usada para definir células PD1⁺CXCR5⁺CD4⁺ e PD1⁻CD4⁺. Os histogramas mostram a marcação de anexina V em subpopulações de células T CD4⁺. O MFI da marcação de anexina V é mostrado no gráfico de colunas. (D) O gráfico de contorno mostra células PD1⁺CXCR5⁺CD4⁺ e PD1⁻CD4⁺ marcadas com anexina V e Acqua Live/Dead. (E) Os esplenócitos foram estimulados com 100-500 μ M de ATP ou sem estímulo. O histograma mostra células PD1⁺CXCR5⁺CD4⁺ e PD1⁻CD4⁺ marcadas com EB. A porcentagem de células T CD4⁺ marcadas com EB é mostrada no gráfico de linhas. A barra horizontal cinza representa o intervalo de confiança obtido com células de camundongos P2X7^{-/-}.

4.7 A deficiência do receptor P2X7 em células T CD4⁺ dificulta a diferenciação de células Th1 e aumenta as células Tfh

Para verificar se a expressão do receptor P2X7 em células T CD4⁺ é necessária para a predominância de células Th1 em comparação com o número de células Tfh durante a infecção com o *P. chabaudi*, células T CD4⁺ esplênicas foram separadas de camundongos doadores B6 e P2X7^{-/-} e transferidas para camundongos recipientes CD4^{-/-} (**Figura 11A**). O tratamento com doses subcurativas de cloroquina proporcionou o controle das parasitemias a níveis comparáveis entre os grupos (**Figura 11B**). Um aumento similar em número de células T CD4⁺ por baço foi observada nos camundongos com 30 dias de infecção (**Figura 11C**). Além disso, células T CD4⁺ de camundongos P2X7^{-/-} geraram menor número de células T_E, T_{EM} e T_{CM} que as células T CD4⁺ de camundongos B6 (**Figura 11D**). Para evidenciar o papel da sinalização pelo receptor P2X7 na ativação de células T CD4⁺ durante a fase aguda da infecção, observamos os níveis de cálcio citosólico. As células T CD4⁺ de camundongos B6 apresentaram maiores níveis de cálcio quando comparadas com células de camundongos P2X7^{-/-} (**Figura 12A**). Além disso, as produções de IL-2 e IFN γ corroboraram com os níveis de cálcio e foram menores nas células T CD4⁺ de camundongos P2X7^{-/-} (**Figura 12B e 12C**). Também observamos maior expressão de T-bet nas células T CD4⁺ de camundongos B6 nos dias 7 e 30 de infecção que em células T CD4⁺ de camundongos P2X7^{-/-} (**Figura 12D**). De acordo com a hipótese que o a sinalização pelo receptor P2X7 dirige o balanço de células Th1 e Tfh durante a infecção com o *P. chabaudi*, a população de células Tfh foi significativamente aumentada na ausência do receptor P2X7 (**Figura 12E**).

Figura 11 – Parasitemia e número de células T CD4⁺ em camundongos CD4^{-/-} transferidos com células T CD4⁺ de camundongos B6 e P2X7^{-/-}

(A-D) Células T CD4⁺ de camundongos B6 e P2X7^{-/-} foram transferidas para camundongos CD4^{-/-} e infectados com 1 x 10⁶ iRBCs 7 dias depois. Células T CD4⁺ foram analisadas nos dias 7 e 30 de infecção. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média ± desvio padrão (n=3-5). Diferenças significativas com p<0,05 foram indicadas por: (*) entre grupos infectados e controles, (**) entre grupos B6 e P2X7^{-/-} e (-) entre grupos indicados. (A) Ilustração esquemática do protocolo experimental. (B) Curvas de parasitemia. As setas indicam os dias de tratamento com cloroquina. (C) O número de células T CD4⁺ por baço foram determinadas por citometria de fluxo. (D) número de células CD4⁺ T_E (CD44^{hi}ILR7α⁻), T_{EM} (CD44^{hi}ILR7α⁺CD62L^{lo}) e T_{CM} (CD44^{hi}ILR7α⁺CD62L^{hi}) foi determinado por citometria de fluxo.

Figura 12 – Células Th1 e Tfh esplênicas em camundongos CD4^{-/-} transferidos com células T CD4⁺ de camundongos B6 e P2X7^{-/-}

(A-E) Células T CD4⁺ de camundongos B6 e P2X7^{-/-} foram transferidos para camundongos CD4^{-/-} e infectados com 1 x 10⁶ iRBCs 7 dias depois. Células T CD4⁺ foram analisadas nos dias 7 e 30 de infecção. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média ± desvio padrão (n=3-5). Diferenças significativas com p<0,05 foram indicadas por: (*) entre grupos infectados e controles, (**) entre

grupos B6 e P2X7^{-/-} e (-) entre grupos indicados. (A) Imagem de imunofluorescência (aumento de 100x; as barras de escala correspondem a 100 µm) mostrando os níveis de cálcio citosólico em células T CD4⁺ purificadas. (#) Média corrigida da fluorescência total da célula (CTCF) de células T CD4⁺. (B) Gráfico de pontos mostra as células T CD4⁺ secretando IL-2. O número de células T CD4⁺ por baço é mostrado no gráfico de colunas. (C) Gráfico de contorno mostra as células T CD4⁺ produtoras de INFγ. O número de células T IFNγ⁺CD4⁺ por baço são apresentadas no gráfico de colunas. (D) O histograma mostra a expressão de T-bet em células T CD4⁺. O MFI da expressão de T-bet é mostrado no gráfico de colunas. (E) O gráfico de contorno mostra a expressão de PD1, ICOS e Bcl-6 *versus* CXCR5 em células T CD4⁺. O número de células T PD1⁺CXCR5⁺CD4⁺, ICOS⁺CXCR5⁺CD4⁺ e Bcl-6⁺CXCR5⁺CD4⁺ é mostrado no gráfico de colunas.

4.8 A ausência do receptor P2X7 em células T CD4⁺ compromete a habilidade de transferir proteção para camundongos CD4^{-/-}

Para avaliar se a expressão do receptor P2X7 em células T CD4⁺ é importante para a proteção contra o *P. chabaudi*, células T CD4⁺ do baço de camundongos B6 e P2X7^{-/-} obtidas no dia 20 de infecção foram transferidas para camundongos CD4^{-/-} e infectados com o parasito (**Figura 13A**). Os camundongos CD4^{-/-} que foram transferidos com células T CD4⁺ de camundongos P2X7^{-/-} no dia 20 de infecção apresentaram o primeiro e o segundo pico significativamente maiores que dos animais transferidos com células T CD4⁺ de camundongos B6 no dia 20 de infecção (**Figura 13B**). De fato, o segundo pico de parasitemia do grupo que recebeu células de camundongos P2X7^{-/-} é comparável ao grupo controle que recebeu células de camundongos B6 não infectados. Tanto o grupo controle quanto o grupo que recebeu células de camundongos P2X7^{-/-} falharam em controlar as parasitemias recrudescentes.

Figura 13 – Parasitemia em camundongos $CD4^{-/-}$ transferidos com células T $CD4^{+}$ de camundongos B6 e $P2X7^{-/-}$ e infectados com o *Plasmodium chabaudi*

(A-B) Células T $CD4^{+}$ de camundongos B6 e $P2X7^{-/-}$ no dia 20 de infecção foram transferidos para camundongos $CD4^{-/-}$ e infectados com 1×10^4 iRBCs no mesmo dia. Camundongos $CD4^{-/-}$ transferidos com células T $CD4^{+}$ de camundongos B6 não infectados foram usados como controle (Dia 0). Os dados são representativos de 2 experimentos realizados em separado com média $\pm$ desvio padrão ($n=3-6$). Diferenças significativas com $p<0,05$ foram indicadas por: (*) entre grupos do dia 0 e 20 de infecção e (**) entre grupos B6 e $P2X7^{-/-}$ do dia 20 de infecção. (A) Ilustração esquemática do protocolo experimental. (B) Curvas de parasitemia.

O receptor P2X7 tem sido associado tanto na proteção quanto na exacerbação de doenças infecciosas por promover a ativação e morte de macrófagos infectados em condições com dano tecidual ou celular (Amaral et al., 2014; Chaves et al., 2014; Correa et al., 2010; Morandini et al., 2014). Durante o ciclo eritrocítico do *P. chabaudi*, o dano celular promovido pelo rompimento sincrônico dos eritrócitos infectados é capaz de liberar quantidade de ATP suficiente para ativar receptores P2X7 em células T CD4⁺ (Salles, 2011), críticas na geração de imunidade protetora contra o *Plasmodium* (Xu et al., 2002). Neste trabalho, analisamos o envolvimento do receptor purinérgico P2X7 na ativação de linfócitos T CD4⁺ e na patogênese da malária. Ao utilizarmos camundongos P2X7^{-/-} infectados com o *P. chabaudi*, a ausência do receptor resultou em maior dificuldade de controlar a parasitemia aguda e reestabelecer os parâmetros clínicos da doença (concentração de Hb, peso corporal e temperatura). A diminuição da resposta de células T CD4⁺ no baço de camundongos P2X7^{-/-} pode estar diretamente relacionada com a dificuldade em controlar a infecção. O receptor P2X7 pode atuar como um fator co-estimulador, durante a ativação de uma célula T em contato com a célula apresentadora de antígeno, por promover a liberação local de ATP dentro da sinapse imunológica e induzir a ativação de outros canais P2X (Rissiek et al., 2015; Schenk, 2008). Além disso, a ativação do receptor P2X7 em células T amplifica o fraco sinal do TCR, expressão de genes e função efetora da célula T, além de inibir a diferenciação e função de células T reguladoras (Feske et al., 2012). É importante ressaltar que o receptor P2X7 expresso nas células T parece sentir o ATP extracelular liberado de maneira parácrina no ambiente (Junger, 2011). Esta hipótese baseia-se na observação de que, diferente dos receptores P2X1 e P2X4 que se colocizam na sinapse imune, o receptor P2X7 permanece uniformemente distribuído em toda a superfície da célula durante a interação da célula T com a célula dendrítica. Um outro mecanismo, pelo qual a sinalização do receptor P2X7 em fagócitos poderia estimular a resposta de linfócitos contra a malária, seria a formação do inflamassoma NLRP3 e, em consequência, a produção de citocinas pró-inflamatórias. Porém, foi demonstrado recentemente que o receptor P2X7 é necessário para a ativação de caspase-1 durante a infecção com o *Plasmodium chabaudi*, mas a produção de baixos níveis de IL-1 β em camundongos infectados

ocorre independentemente da ativação do inflamassoma NLRP3 (Ataide et al., 2014). Além disso, este estudo mostra que camundongos B6, ASC^{-/-} e Casp1^{-/-} infectados com o *P. chabaudi* possuem parasitemia similar e resolvem completamente a infecção aguda.

O eixo ATP-P2X7 pode estar envolvido na resposta de células T CD4⁺ na malária, em particular por causa da massiva destruição de iRBCs que pode liberar grande quantidade de ATP na corrente sanguínea e polpa vermelha do baço. De acordo com os nossos dados, embora o receptor P2X7 seja expresso em diversas células imunes incluindo monócitos, macrófagos, células dendríticas e células T (Di Virgilio et al., 2001), a presença do receptor P2X7 em células T CD4⁺ é crítico para a diferenciação de células Th1 produtoras de IFN γ durante a infecção com o *P. chabaudi*. A produção de IFN γ pelas células Th1 é importante para o controle da parasitemia e eliminação do parasito (Su, Stevenson, 2000, 2002; van der Heyde et al., 1997) e o receptor P2X7 parece contribuir para o aumento da transcrição de T-bet em células T CD4⁺ e diferenciação de células Th1 produtoras de IFN γ . Estes achados explicam a contribuição do receptor P2X7 no controle de parasitemias subpatentes, que também parece depender das células Th1 (da Silva et al., 2013; Freitas do Rosario et al., 2012; Stephens, Langhorne, 2010). As células de memória efetora Th1 são reduzidas gradualmente durante a infecção crônica com o *P. chabaudi* (Freitas do Rosário et al., 2008), e o *priming* induzido pelo IFN γ é crucial para manter uma imunidade protetora de longa duração (da Silva et al., 2013). A produção de IFN γ e os anticorpos de baixa afinidade participam do controle da fase aguda da infecção (Mota et al., 1998; Su e Stevenson et al., 2000, 2002). Para que ocorra a produção de anticorpos, as células B entram em contato com células pré-Tfh (Castillo Méndez, 2011), uma população conhecida por estar envolvida na geração de plasmablastos extrafoliculares de vida curta (Ma et al., 2012). Aqui, mostramos que o receptor P2X7 controla o número de células Tfh durante a infecção com o *P. chabaudi*. Recentemente, foi demonstrado que o receptor P2X7 também controla o número de células Tfh nas placas de Peyer e promove o mutualismo da microbiota do hospedeiro (Proietti et al., 2014). Embora as células Tfh sejam críticas para a geração de uma resposta de anticorpo, a desregulação na quantidade e função de tais células poderia gerar vários problemas no sistema imune. Uma hipótese seria o aumento de doenças autoimunes. Uma linhagem de camundongos

que carrega uma mutação com perda de função no gene roquin, uma ubiquitina ligase E3 que impede a diferenciação e a ativação de células Tfh auto-reativas, possui grande quantidade de células Tfh no folículo e desenvolve uma síndrome como o lúpus eritematoso sistêmico e diabetes autoimune; essas células expressam altos níveis de ICOS, CXCR5, CD200 e PD1 e produzem grande quantidade de IL-21 (Vinuesa et al., 2005). A expressão de ICOS em células T CD4⁺ parece estar mais relacionada com a afinidade de interação com células B e com o controle de células Th1 do que com a diferenciação de células Tfh (Wikenheiser et al., 2016). A expansão de células Tfh em pacientes com lúpus eritematoso sistêmico correlaciona com títulos de auto-anticorpos e dano tecidual (Simpson et al., 2010). Por outro lado, o defeito na geração de células Tfh está associado com diversos defeitos na imunidade humoral protetora (Crotty, 2014; Tangye et al., 2012) e com a redução de doenças autoimunes (Linterman et al., 2009).

Neste trabalho, nós demonstramos que o receptor P2X7 tem um papel crítico na diferenciação de células T CD4⁺ que são necessárias para a proteção contra o *P. chabaudi*. É provável que esta via de sinalização seja importante para a geração de subpopulações de células T CD4⁺ em condições inflamatórias (patológicas ou estéreis) onde há dano tecidual. Como exemplo, em transplantes de coração, a expressão do receptor P2X7 é aumentada em linfócitos infiltrados no enxerto, e o uso de antagonistas do receptor P2X7 inibe a produção de IFN γ , ativação de células T CD4⁺ e diferenciação de células Th1/Th17 (Vergani et al., 2013). A diferenciação de subpopulações de células T CD4⁺ parece estar diretamente relacionada com proteínas que auxiliam a entrada de Ca²⁺ e, consequentemente, translocação do fator nuclear de células T ativadas (NFAT) para o núcleo. A ativação do receptor P2X7 pelo ATP induz o influxo de Ca²⁺ e a ativação de moléculas da cascata de sinalização tais como a calcineurina que, por sua vez, ativa o NFAT no citoplasma. O NFAT ativado é transportado para o núcleo onde proporciona um aumento na expressão de IL-2 (Adinolfi et al., 2005; Woehrle et al., 2010). A seguir, a IL-2 ativa o fator de transcrição Blimp-1 que é responsável pelo silenciamento do gene da *Il-2*, desta forma provendo um mecanismo de regulação negativa (Martins et al., 2008). O sinal da IL-2 junto com a ativação persistente do TCR com o antígeno - MHC pode induzir a expressão do receptor de morte Fas (também conhecido como CD95) e Fas ligante (também conhecido como CD95L) em células T e promover a apoptose

destas células (Lenardo et al., 1991). Além da participação do receptor P2X7 no aumento de Ca^{2+} intracelular, também observamos menor proliferação de células T CD4^+ na ausência do receptor P2X7 que pode ser uma consequência da menor produção de IL-2 em camundongos $\text{P2X7}^{-/-}$. A sinalização por IL-2 também influencia a diferenciação de células Th1 por induzir a transcrição de STAT5 em células T CD4^+ que aumenta a expressão da cadeia $\beta 2$ do receptor de IL-12, T-bet e Blimp-1 (Gong, Malek, 2007; Liao et al., 2011). O $\text{IFN}\alpha$ também é capaz de ativar T-bet e o receptor de IL-12 de cadeia $\beta 2$ em células T *naïves* (Hibbert et al., 2003). Porém, ainda não sabemos se existe uma conexão entre a expressão do receptor de $\text{IFN}\alpha$ em células T CD4^+ e o receptor P2X7. Outra citocina importante na ativação de fatores nucleares responsáveis pela diferenciação celular é a IL-27. Na malária, o receptor de IL-27 controla o estabelecimento de T CD4^+ de memória e afeta a reativação de tais células em infecções secundária (Gwyer et al., 2014). Além disso, a ativação de STAT3 desencadeada pela IL-27 promove a transcrição dos fatores de transcrição de c-Maf e o receptor aril hidrocarboneto (AHR), que ativam os promotores dos genes *Il-10* e *Il-21* (Mascanfroni et al., 2015). O receptor P2X7, como um modulador chave da glicose aeróbica, é capaz de aumentar a expressão do fator indutor de hipóxia (HIF)-1 α em células T em condições inflamatórias com grande quantidade de ATP extracelular (Amoroso et al., 2012). Desta forma, o HIF-1 α desfaz a interação do translocador nuclear do receptor aril hidrocarboneto (ARNT) com o AHR (Semenza, 2012), o que resulta em ubiquitinação e degradação da proteína e inibição da expressão de IL-10 e IL-21 (Mascanfroni et al., 2015). Pode ser que em camundongos $\text{P2X7}^{-/-}$ infectados com o *P. chabaudi*, haja um aumento de IL-27 sem a ativação de HIF-1 α e, portanto, maior produção de IL-21 favorecendo a manutenção de células Tfh. Outros dois mecanismos moleculares não exclusivos podem ser responsáveis pelo domínio de células Th1 sobre células Tfh mediado pelo receptor P2X7. Primeiramente, a sinalização via o receptor P2X7 aumenta a transcrição de T-bet sob o controle do programa de células Th1 e, portanto, inibe a transcrição de Bcl-6 sob o controle do programa de células Tfh. Além disso, células T CD4^+ não Tfh (ou seja, Th1, Th2, Th17 ou outras subpopulações) expressam altos níveis de Blimp-1, um antagonista de Bcl-6 e *vice versa* (Johnston et al., 2009). Segundo, a sinalização via o receptor P2X7 induz a morte de células Tfh esplênicas. Células Tfh possuem alta expressão do gene *P2rx7*, quando comparadas com

células Th1 (Iyer et al., 2013). Também já foi mostrado que nas placas de Peyer de camundongos e tonsilas humanas, células Tfh respondem ao ATP extracelular e medeiam a morte da célula pelo receptor P2X7 (Proietti et al., 2014). De forma semelhante, em nosso estudo, a perda do receptor P2X7 significativamente reduziu a apoptose de células T CD4⁺ durante a infecção com o *P. chabaudi*, assim como diminuiu a exposição de PS em células Tfh. De acordo com o papel do receptor P2X7 em controlar o número de células Tfh, pacientes com tuberculose carregando um simples polimorfismo do P2rx7 com perda de função apresentaram maiores respostas com anticorpos IgG contra a micobactéria que grupos controles (Wu et al., 2015). Em nosso modelo, também observamos maior quantidade de anticorpos contra o *P. chabaudi* em camundongos que não possuíam o receptor P2X7.

Uma importante observação a cerca da regulação da resposta imune pelo receptor P2X7 é que diferentes subpopulações diferem na sensibilidade ao ATP e que estas diferenças correlacionam com a expressão do receptor na superfície da célula. Além disso, concentrações de ATP acima de 100 μ M são capazes de ativar o receptor P2X7 e induzir a abertura do poro, enquanto baixas concentrações podem induzir a abertura de canais de íons por meio da ligação de receptores P2X (Di Virgilio et al., 2001). Estudos mostram que a ativação prolongada do receptor P2X7 com altas concentrações de ATP podem induzir apoptose (Aswad, Dennert, 2006) e com baixas concentrações induzir a ativação celular (Adinolfi et al., 2005). Outro fator importante a ser considerado é a isoforma da proteína. O receptor P2X7 possui 11 variantes identificadas como P2X7RA-K, tendo o P2X7RA como a forma completa do receptor (Sluyter, Stokes, 2011). Como exemplo, a isoforma P2X7RB estimula a ativação de NFATc1 e, conseqüentemente, a proliferação celular (Sluyter, Stokes, 2011). Portanto, o tipo de isoforma pode interferir na função do receptor P2X7 em cada subpopulação celular. A morte de células T dependente do receptor P2X7 possivelmente ocorre devido a massiva despolarização de membrana e permeabilização da célula por um longo período. Como relatado neste trabalho, a sensibilidade de células Tfh ao ATP e a apoptose de células no baço dependentes do receptor P2X7 pode ser devido a uma maior expressão do receptor em células Tfh e maior quantidade de ATP extracelular dentro dos centros germinativos. A hipótese de uma seleção de células Tfh dentro do centro germinativo por meio de sinais de dano possui grande importância na compreensão da resposta imune

adquirida. No entanto, precisamos compreender a fonte e mecanismos de liberação destes sinais de dano no meio extracelular. Eritrócitos humanos infectados com o *Plasmodium falciparum* liberam grande quantidade de ATP ao meio extracelular (Akkaya et al., 2009), porém não sabemos se este ATP consegue atingir células do centro germinativo do baço. Além dos eritrócitos infectados, células T também são fontes de ATP extracelular que é necessário para a ativação de receptores P2X7 durante a malária aguda. No entanto, o ATP extracelular pode ser regulado por ectonucleotidases e rapidamente hidrolisado em ADP, AMP e adenosina.

A análise detalhada do papel do receptor P2X7 na resposta imune contra o *P. chabaudi* nos permitiu revelar a importância desta molécula para populações de células Th1 e Tfh. Ao promover a resposta de células Th1 inflamatórias e controlar a geração de anticorpos de alta afinidade, o sistema imune pode-se tornar mais efetivo na proteção contra infecções que causam extensivo dano tecidual e, ao mesmo tempo, reduzir o risco de produção de autoanticorpos (**Figura 14**). Porém, em condições particulares, a intensa sinalização via receptor P2X7 poderia induzir uma resposta de células Th1 exacerbada, impedindo o desenvolvimento de anticorpos protetores, gerando uma doença severa. Este trabalho abre a possibilidade de manipulação do eixo ATP-P2X7, por meio de agonistas e antagonistas do receptor P2X7, para alterar o balanço de células Th1/Tfh e melhorar as condições patológicas ou protocolos de imunização.

Figura 14 – Ilustração esquemática que mostra o papel do eixo ATP-P2X7 na geração da resposta imune em condições patológicas onde há dano tecidual

O ATP liberado em condições patológicas ativa receptores P2X7 em células $CD4^+$ e proporciona aumento da resposta pró-inflamatória. A ativação do receptor P2X7 com altas concentrações de ATP induz a formação de um poro e, portanto, intenso influxo de Ca^{2+} , produção de IL-2 e proliferação celular. Neste contexto inflamatório, o ATP proporciona maior geração células Th1 produtoras de IFN γ e controla a geração de células Tfh. As células Tfh são sensíveis ao ATP extracelular e são mais suscetíveis a morte após exposição prolongada.

Este trabalho sugere que o reconhecimento do ATP pelo receptor P2X7 contribui para a ativação do sistema imune durante a infecção com o *Plasmodium chabaudi* e, conseqüentemente, controle da parasitemia. Nossos resultados mostraram que a expressão do receptor P2X7 em células T CD4⁺ é importante para a diferenciação de células Th1, contribuindo para a produção de IFN γ e o controle do parasito. Por outro lado, encontramos um aumento no número de células Tfh, na formação dos centros germinativos e produção de anticorpos contra o parasito na ausência do receptor P2X7. Estes achados ressaltam a importância da participação dos sinais de dano na geração de subpopulações de células Th1 e Tfh para o controle de patógenos.

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APÊNDICE A – Liberação de ATP no soro e permeabilização de células T CD4⁺ do sangue e baço de camundongos B6 e P2X7^{-/-} durante a fase aguda e infecção com o *Plasmodium chabaudi*.

(B-D) Camundongos B6 e P2X7^{-/-} fêmeas foram analisados nos dias 4, 5 e 7 p.i. com 1×10^6 iRBCs. Camundongos não infectados foram utilizados como controle (dia 0). Os dados são representativos de 3 experimentos realizados em separado com média $\pm$ desvio padrão (n=3). Diferenças significativas com $p < 0,05$ foram indicadas por: (*) entre grupos infectados e controles, (**) entre grupos B6 e P2X7^{-/-} e (-) entre grupos indicados. (A) O ATP foi quantificado por bioluminescência no soro de camundongos B6 nos dias 5 p.i., antes (9 horas, $6,5 \pm 0,5\%$ iRBC, >95% de trofozoítos e esquizontes) e após a ruptura de eritrócitos (14 horas, $12,0 \pm 1,2\%$ iRBC, >95% de formas em anel). (B) Permeabilidade ao BE em células T CD4⁺ do sangue nos dias 4 e 5 p.i. antes e após a ruptura dos eritrócitos. (C) Esplenócitos foram estimulados com o sobrenadante de eritrócitos parasitados ($37,0 \pm 0,3\%$ iRBCs > 95% de trofozoítos e esquizontes). A porcentagem de células T CD4⁺ marcadas com BE foram determinadas por citometria de fluxo.

APÊNDICE B – In vivo approaches reveal a key role for DCs in CD4⁺ T cell activation and parasite clearance during the acute phase of experimental blood-stage malária. **Plos Pathogens, 2015**

RESEARCH ARTICLE

In Vivo Approaches Reveal a Key Role for DCs in CD4+ T Cell Activation and Parasite Clearance during the Acute Phase of Experimental Blood-Stage Malaria

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Abstract

Dendritic cells (DCs) are phagocytes that are highly specialized for antigen presentation. Heterogeneous populations of macrophages and DCs form a phagocyte network inside the red pulp (RP) of the spleen, which is a major site for the control of blood-borne infections such as malaria. However, the dynamics of splenic DCs during *Plasmodium* infections are poorly understood, limiting our knowledge regarding their protective role in malaria. Here, we used *in vivo* experimental approaches that enabled us to deplete or visualize DCs in order to clarify these issues. To elucidate the roles of DCs and marginal zone macrophages in the protection against blood-stage malaria, we infected DTx (diphtheria toxin)-treated C57BL/6.CD11c-DTR mice, as well as C57BL/6 mice treated with low doses of clodronate liposomes (CLiP), with *Plasmodium chabaudi* AS (*Pc*) parasites. The first evidence suggesting that DCs could contribute directly to parasite clearance was an early effect of the DTx treatment, but not of the CLiP treatment, in parasitemia control. DCs were also required for CD4+ T cell responses during infection. The phagocytosis of infected red blood cells (iRBCs) by splenic DCs was analyzed by confocal intravital microscopy, as well as by flow cytometry and immunofluorescence, at three distinct phases of *Pc* malaria: at the first encounter, at pre-crisis concomitant with parasitemia growth and at crisis when the parasitemia decline coincides with spleen closure. *In vivo* and *ex vivo* imaging of the spleen revealed that DCs actively phagocytize iRBCs and interact with CD4+ T cells both in T cell-rich areas and in the RP. Subcapsular RP DCs were highly efficient in the recognition and capture of iRBCs during pre-crisis, while complete DC maturation was only achieved during

crisis. These findings indicate that, beyond their classical role in antigen presentation, DCs also contribute to the direct elimination of iRBCs during acute *Plasmodium* infection.

Author Summary

Malaria is a significant health issue, particularly in the tropical and subtropical regions of the world. The red pulp (RP) of the spleen is a major site for the control of blood-borne infections such as malaria. Macrophages and dendritic cells (DCs) form a complex phagocyte network inside the splenic RP. DCs are usually thought of as highly efficient antigen-presenting cells that play an essential role in the activation of adaptive immunity. However, the direct role of DCs in the clearance of pathogens is still unclear. To clarify these issues, we took advantage of *in vivo* experimental approaches that enabled us to deplete or visualize DCs. The depletion of phagocytes demonstrated that DCs are key participants in the protection against blood stages of experimental malaria. Using confocal intravital microscopy, we observed that splenic RP DCs efficiently recognized and phagocytized infected erythrocytes during acute infection. We also showed that splenic DCs were crucial for the CD4⁺ T cell response to infection, but full DC maturation was achieved only after the peak of parasitemia. This study help to elucidate the protective mechanisms against *Plasmodium* parasites, and it shows that *in vivo* imaging is a reliable tool to evaluate iRBC phagocytosis during experimental malaria.

Introduction

The spleen is a primary site for the control of blood-borne infectious diseases in humans and rodents [1], [2]. Although splenic phagocytic activity has been well documented *in vitro* [3]–[5] and *ex vivo* [3], [6]–[8], few studies have reported on the *in vivo* three-dimensional (3D) interactions between splenic phagocytes and pathogens [9]. Addressing this issue is particularly important in the case of malaria, a disease characterized by splenic involvement that is critical for controlling blood-stage *Plasmodium* parasites [10]. In recent years, confocal intravital microscopy (CIVM) [11] has been used to study host-pathogen interactions during infectious diseases caused by viruses [12], [13], bacteria [14] and protozoan parasites [15]. For example, CIVM revealed important aspects of the *Plasmodium* life cycle [16], [17]. Other works described *Plasmodium*-induced immune responses inside the placenta [18] and the dermis using fluorescent stereomicroscopy [19]. A single publication reported the movements of *Plasmodium*-infected red blood cells (iRBCs) inside the spleen [20]. However, no *in vivo* study has addressed the interactions between blood-stage *Plasmodium* parasites and the splenic immune system.

Splenectomized patients with acute *Plasmodium falciparum* infections have an impaired ability to remove parasites from circulation [21], similar to splenectomized mice infected with the blood-stages of *Plasmodium chabaudi* (Pc) [22]. In humans and mice, the phagocytosis of iRBCs or free merozoites by splenic phagocytes begins soon after infection and helps to control the parasitemia and induce the lymphocyte response [23], [24]. This occurs primarily inside the red pulp (RP) and the marginal zone (MZ) of the spleen [23], [24], where a complex phagocyte network is formed by heterogeneous populations of macrophages and dendritic cells (DCs) [25], [26]. In an effort to characterize the role of splenic phagocytes in Pc malaria, a recent study identified migrating monocytes as major participants in the clearance of iRBCs [8]. However, previous studies that quantified the *ex vivo* phagocytosis of iRBCs by flow cytometry

reported low percentages of splenic phagocytes containing *Pc* remnants [8], [27]. This observation is not fully compatible with the notion that the role of the spleen is of the utmost importance in parasite control.

DCs are phagocytes that are highly specialized in presenting antigens to T cells [28]. Splenic DCs are efficient antigen presenting cells (APCs) during the massive T and B cell responses to acute *Pc* malaria [29]–[31]. Within the first week of *Pc* infection, splenic DCs up-regulate the expression of major histocompatibility complex (MHC) and costimulatory molecules, secrete pro-inflammatory cytokines, and stimulate T cell proliferation and IFN- γ production [32]–[34]. Nevertheless, it is still unclear whether DCs are unique in their ability to initiate CD4⁺ T cell responses to *Pc* blood-stages in the spleen, as observed in *Plasmodium berghei* (*Pb*) malaria [35]. Moreover, many details concerning the dynamics of splenic DCs in malaria remain unknown, limiting our understanding of the involvement of these cells in the protective immune response. After taking on antigens, immature DCs lose the ability to phagocytize and migrate towards T cell-rich areas to initiate the adaptive immune response [28]. Thus, it would be expected that DCs leave the RP soon after phagocytizing iRBCs or free merozoites and no longer contribute to parasite clearance, although this is as yet only a supposition.

In this study, we took advantage of experimental approaches that enabled us to deplete or visualize splenic DCs *in vivo* to clarify these issues. The *in vivo* depletion of phagocytes clearly demonstrated that DCs are key participants in the early control of the blood stage of infection with *Pc* and *Plasmodium yoelii* (*Py*) iRBCs, as well as the blood stage of infection with *Pb* sporozoites. The phagocytosis of *Pc* iRBCs by splenic DCs was analyzed by CIVM, as well as by flow cytometry and immunofluorescence, in three distinct situations: at the first encounter, at a pre-crisis phase concomitant with parasitemia growth and at a crisis phase, when parasitemia has dramatically dropped and changes in the splenic architecture have culminated in spleen closure [36]. CIVM allowed us to visualize the phagocytosis of *Pc* iRBCs by the RP DC network, the movement dynamics and morphological changes of DCs and the interaction between DCs and CD4⁺ T cells at the different phases of acute *Pc* malaria. To our knowledge, this is the first description of the *in vivo* interaction between *Plasmodium* iRBCs and the splenic immune system.

Results

DCs are required for parasitemia control and splenic CD4⁺ T cell activation during the blood stage of experimental malaria

To evaluate whether DCs are important for the early control of blood-stage *Pc* malaria, C57BL/6. CD11c-DTR (B6.CD11c-DTR) mice were treated with diphtheria toxin (DTx). The great majority of splenic CD11c⁺I-A⁺ cells were eliminated in DTx-treated B6.CD11c-DTR mice (Fig. 1A). No effect was observed on F4/80⁺ RP macrophages, but the already small population of MARCO/MOMA-1⁺ MZ macrophages was depleted (S1 Fig.). Starting in the earliest days of infection, DTx-treated B6.CD11c-DTR mice had higher parasitemia (Fig. 1B) and weight loss (Fig. 1C) in comparison to their PBS-treated counterparts, leading to an accumulated mortality of 75% of mice on day 15 p.i. (Fig. 1D). On day 4 p.i., DTx-treated B6.CD11c-DTR mice had reduced numbers of CD4⁺ T cells per spleen (Fig. 1E). DTx treatment also completely abrogated the CD4⁺ T cell proliferation and IFN- γ production *in vitro* in response to iRBCs (Fig. 1F). None of these effects were observed in DTx-treated C57BL/6 (B6) mice (Figs. 1 and S1). Furthermore, the selective elimination of MZ macrophages by treating B6 mice with a low dose of clodronate liposomes (ClLip) did not affect the course of parasitemia, IFN- γ production by splenic CD4⁺ T cells or mouse survival (S2 Fig.). Similarly to what was observed for the *Pc* parasite, DTx treatment in B6.CD11c-DTR mice exacerbated *Py* malaria from the beginning of infection

Figure 1. Effects of DC depletion on acute *Pc* malaria. (A–F) B6 and B6.CD11c-DTR mice were treated with either DTx to deplete CD11c⁺ cells or PBS as a control. The mice were i.p. infected with 1×10^6 *Pc* iRBCs 24 h later. (A) Representative contour plots obtained 24 h after treatment by flow cytometry confirm the efficiency of DTx-induced depletion of splenic CD11c⁺I-A⁺ cells in B6.CD11c-DTR mice. Data show the percentages of CD11c⁺I-A⁺ cells in the splenocyte population. (B) Parasitemia curves are shown (means $\pm$ SD). (C) Variations in body weight relative to day 0 are shown (means $\pm$ SD). (D) Survival curves are shown. (E) Data show the numbers of CD3⁺CD4⁺ T cells per spleen at days 0 and 4 p.i. (means $\pm$ SD). (F) Data show the percentages of proliferating CFSE^{low}CD4⁺ T cells and IFN- γ concentrations in the supernatants of spleen cell cultures stimulated for 72 h with iRBCs (means $\pm$ SD). In B–D, significant differences ($p < 0.05$) between the indicated groups are designated by *. In E and F, significant differences ($p < 0.05$) between all other groups are designated by *. In A–F, one representative experiment out of three ($n = 5$) is shown.

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(S3A–S3C Fig.). The role of DCs in the early control of parasitemia was also evaluated in B6 and B6.CD11c-DTR mice that were treated with DTx on day 2 p.i. with *Pb* sporozoites. DTx-treated B6.CD11c-DTR mice presented with higher parasitemias (S3D–S3E Fig.). In this case, however, DTx treatment prolonged the survival of infected B6.CD11c-DTR mice by protecting them from cerebral malaria (S3F Fig.).

Splenic DCs rapidly phagocytize iRBCs in recently infected mice

To investigate whether splenic DCs phagocytize iRBCs in recently infected mice, we analyzed the interaction between YFP⁺ cells and mCherry-*Pc* iRBCs in the subcapsular RP of C57BL/6. CD11c-YFP (B6.CD11c-YFP) mice using CIVM [26]. Mice were infected by i.v. administration of mature iRBCs (>95% late trophozoites/schizonts), as these cells are known to be recognized and phagocytized by DCs [37]. In naïve mice, YFP⁺ cells were non-motile and actively extended protrusions and dendrites (S1 Video). At 15 min p.i., mCherry-*Pc* iRBCs were present in the subcapsular RP (Fig. 2A, S2 Video). CIVM 3D animations showed mCherry-*Pc* iRBC remnants

Figure 2. In vivo analysis of iRBC uptake by subcapsular RP DCs soon after *Pc* infection. (A-D) B6. CD11c-YFP mice were i.v. infected with 1×10^8 mature mCherry-*Pc* iRBCs. Spleens were analyzed by CIVM after 15 min and in non-infected controls. **(A)** Serial snapshots taken at 15 min p.i. (0 min) show the subcapsular RP. The upper panels show the amplification of a few cells co-expressing YFP (green) and mCherry (red). In the image on the right, a region showing contact between YFP+ cells and mCherry-*Pc* iRBCs is magnified. **(B)** CIVM 3D animation reveals that YFP+ cells contain mCherry-*Pc* iRBC remnants (yellow spots of merged mCherry/YFP-3D signal). **(C)** Percentage of mCherry+ cells in the YFP+ cell population is shown (mean $\pm$ SEM). **(D)** YFP+ cell volume and sphericity are shown for naïve mice (-) and recently infected mice (+*Pc*). Black, red and blue dots are from three different experiments. Horizontal lines represent mean values and SEM. **(E-H)** B6.CD11c-YFP mice were i.v. infected with 1×10^8 mature CMTPX-*Pc* iRBCs. After 15 min,

mice were injected i.v. with a fluorescent anti-F4/80 mAb and the spleens were analyzed by CIVM. **(E)** Snapshots taken 30 min later show YFP⁺ cells (green), iRBCs (red), F4/80⁺ cells (blue) and merged F4/80⁺YFP⁺ cells (white) in the subcapsular RP. **(F)** Percentage of F4/80⁺ cells in the YFP⁺ cell population is shown (mean $\pm$ SEM). **(G)** Percentages of CMTX⁺ cells in the F4/80⁺YFP⁺ and F4/80⁻YFP⁺ cell subsets are shown (means $\pm$ SEM). **(H)** The relative proportions of F4/80⁺ and F4/80⁻ cells in the CMTX⁺YFP⁺ cell population were calculated from the data obtained in **F** and **G** (means $\pm$ SEM). In **A**, **B** and **E**, the scale bars correspond to 50 μ m. One representative experiment out of three (n = 2) is shown. In **C**, **D**, **F**, **G** and **H**, data were calculated using Imaris software. Data from three experiments (n = 2) are shown.

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inside YFP⁺ cells (yellow spots of merged mCherry/YFP-3D signal; [Fig. 2B](#), [S3 Video](#)). At this time, 16% of YFP⁺ cells contained mCherry-*Pc* fragments ([Fig. 2C](#)). We also observed several mCherry-*Pc* iRBCs trapped by YFP⁺ cells without visible signs of internalization ([Fig. 2A](#), [S4 Video](#)). Thus, a substantial proportion of subcapsular RP YFP⁺ cells trapped or internalized iRBCs soon after *Pc* infection. These cells were not activated, as indicated by small YFP⁺ cell volume and sphericity ([Fig. 2D](#)).

The phagocytic activity of splenic DCs from recently infected B6 mice was also analyzed *ex vivo* by immunofluorescence and flow cytometry. Immunofluorescence revealed approximately 5% CD11c pixels that were colocalized with GFP pixels in those spleens ([Fig. 3A and 3B](#)). The majority of GFP-*Pc* iRBCs were trapped inside the RP and MZ ([Fig. 3B](#)). Nearly 2% of CD11c⁺ cells internalized Cell Tracer Violet (CTV)-*Pc* parasites (4×10^4 CTV⁺CD11c⁺ cells/spleen), as revealed by flow cytometry ([Fig. 3C](#)). Comparable data were obtained with Green Fluorescent Protein (GFP)-*Pc* iRBCs ([S1 Table](#)). This phagocytic activity was not restricted to a DC subtype, as subsets of CD11c⁺ cells co-expressing CD11b, CD8, B220 or CD4 were CTV⁺ ([S4A Fig.](#)). Considering the numbers of cells per spleen, CD11b⁺CD11c⁺ cells were responsible for most of the parasite clearance carried out by CD11c⁺ cells in recently infected mice ([S4B Fig.](#)).

Although 61% of YFP⁺ cells in recently infected B6.CD11c-YFP mice had a DC phenotype, expressing CD11c and MHC class II (I-A) but not F4/80, 20% displayed the phenotype of F4/80⁺ RP macrophages ([S5 Fig.](#)). Therefore, we also analyzed the phagocytic activity of the YFP⁺ cell subsets by CIVM and flow cytometry. With injection of a fluorescent anti-F4/80 mAb into mice, CIVM revealed that 17% of cells in the subcapsular RP YFP⁺ cell population were F4/80⁺ soon after infection ([Fig. 2E and 2F](#), [S5 Video](#)). Approximately 15% of F4/80⁺YFP⁺ and F4/80⁻YFP⁺ cells internalized Cell Tracker Red CMTX (CMTX)-*Pc* parasites ([Fig. 2G](#)), but only 20% of the CMTX⁺YFP⁺ cells were F4/80⁺ ([Fig. 2H](#)). Flow cytometry analysis of the YFP⁺ cell subsets showed that a proportion of CD11c⁺ and F4/80⁺ cells was CTV⁺ in B6.CD11c-YFP mice that were recently infected with CTV-*Pc* iRBCs ([Fig. 3D](#)). The CD11c⁺ cells made up 63% of the CTV⁺YFP⁺ cell population (4.5×10^4 CTV⁺CD11c⁺YFP⁺ cells/spleen), while 37% of CTV⁺YFP⁺ cells expressed F4/80 (2.5×10^4 CTV⁺F4/80⁺YFP⁺ cells/spleen) ([Fig. 3E and 3F](#)).

Splenic DCs interact with CD4⁺ T cells in CD4⁺ T cell-rich areas and the RP during early *Pc* malaria

Next, we evaluated the dynamics of splenic DCs during early *Pc* malaria. At 12 h p.i., the subcapsular RP YFP⁺ cells from B6.CD11c-YFP mice displayed higher speed and displacement ([Fig. 4A](#)). This enhanced motility of YFP⁺ cells correlated with their migration towards CD4⁺ T cell-rich areas. This was evident in immunofluorescences, at 2 h and 24 h p.i., by the presence of yellow areas of merged FITC/PE signal ([Fig. 4B](#)) and higher percentages of CD11c-CD4 pixel colocalization ([Fig. 4C](#)). We also adoptively transferred CD4⁺ T cells expressing Cyan Fluorescent Protein (CFP) into B6.CD11c-YFP mice to evaluate the interaction of subcapsular RP DCs with CD4⁺ T cells during early *Pc* malaria. In naïve mice, most CFP⁺CD4⁺ cells made transient contacts with YFP⁺ cells ([Fig. 4D](#), [S6 Video](#)), and CFP⁺CD4⁺ cells were actively

Figure 3. Ex vivo analysis of iRBC uptake by splenic DCs soon after *Pc* infection. (A–C) B6 mice were i.v. infected with 1×10^8 purified mature CTV-*Pc* iRBCs (flow cytometry) or GFP-*Pc* iRBCs (immunofluorescence). Spleens were analyzed after 15 min and in non-infected controls. (A) A representative immunofluorescence image (10x magnification) shows the spleen of an infected B6 mouse. The staining of MOMA-1⁺ metallophilic macrophages and CD19⁺ B cells delineates the RP/MZ from the white pulp (WP). The lower panel details a merged GFP⁺CD11c⁺ cell in the RP. (B) Percentage of CD11c pixels colocalized with GFP pixels and percentages of GFP pixel distribution in the splenic RP/MZ and WP were obtained from immunofluorescence images (means $\pm$ SD). (C) Representative contour plots obtained by flow cytometry show CTV staining in the CD11c⁺ cells of naïve mice (-) and recently infected mice. CD11c⁺ cells in the CD3⁺CD19⁺DX5⁺Ter119⁺ cell population were analyzed, while excluding T cells, B cells, NK cells and RBCs. Data in contour plots show the percentages of CTV⁺ cells in the CD11c⁺ cell population. The numbers of total and CTV⁺CD11c⁺ cells per spleen in recently infected mice were calculated from the data obtained in contour plots (means $\pm$ SD). (D–F) B6.CD11c-YFP mice were i.v. infected with 1×10^8 purified mature CTV-*Pc* iRBCs. Spleens were analyzed by flow cytometry after 15 min and in non-infected controls. (D) Representative contour plots show CTV staining in the CD11c⁺YFP⁺ and F4/80⁺YFP⁺ cells. Data show the percentages of CTV⁺ cells in each population. (E) The numbers of total and CTV⁺CD11c⁺YFP⁺ and F4/80⁺YFP⁺ cells per spleen were calculated from the data obtained in D (means $\pm$ SD). (F) The relative proportions of CD11c⁺ and F4/80⁺ cells in the CTV⁺YFP⁺ cell population were calculated from the data obtained in E (means $\pm$ SD). In A, the scale bars correspond to 50 μ m. In B, data were calculated using FIJI software. In B and E, significant differences ($p < 0.05$) between the indicated groups are designated by *. In A–F, one representative experiment out of three ($n = 3$ –4) is shown.

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moving inside spleen (Fig. 4E). At 24 h p.i., CFP⁺CD4⁺ cells contacted YFP⁺ cells more stably (Fig. 4D, S7 Video), as indicated by a decrease in CFP⁺CD4⁺ cell speed and an increase in arrest coefficient (Fig. 4E).

Splenic DCs from the pre-crisis show intense phagocytic activity

To investigate whether splenic DCs have a direct role in parasite clearance during pre-crisis, we analyzed the interactions between splenic DCs and iRBCs after five days of infection *in vivo* and *ex vivo*. This possibility was suggested by our data showing that, on day 5 p.i., splenic DCs had an enhanced expression of the phagocytic receptor FcγRI (S6A–S6B Fig.). Notably, we visualized many mCherry-*Pc* iRBCs inside the subcapsular RP, and YFP⁺ cells displayed intense

Figure 4. Analysis of the interactions between splenic DCs and CD4⁺ T cells after *Pc* infection. (A) B6. CD11c-YFP mice were i.p. infected with 1×10^6 mCherry-*Pc* iRBCs. Spleens were analyzed by CIVM after 2 h and 12 h and in non-infected controls. Speed and displacement of YFP⁺ cells are shown. Black, red and blue dots are from three different experiments. Horizontal lines represent mean values and SEM. (B and C) B6 mice were i.p. infected with 1×10^6 mCherry-*Pc* iRBCs. Spleens were analyzed after 2 h or 24 h and in non-infected controls. (B) Representative immunofluorescence images (10x magnification) show CD11c⁺ cells and CD4⁺ cells in DAPI-stained tissue sections. (C) Percentages of CD11c pixels colocalized with GFP pixels in the immunofluorescence images are shown (means $\pm$ SD). (D and E) B6. CD11c-YFP mice were adoptively transferred with 5×10^6 CFP⁺CD4⁺ T cells and i.p. infected with 1×10^6 mCherry-*Pc* iRBCs. Spleens were analyzed by CIVM after 24 h and in non-infected controls. (D) Snapshots show the subcapsular RP with CFP⁺CD4⁺ cell tracking. (E) Speed and arrest coefficients of CFP⁺CD4⁺ cells are shown. Black, red

and blue dots are from three different experiments. Horizontal lines represent mean values and SEM. In **B** and **D**, the scale bars correspond to 100 and 50 μm , respectively. In **A**, **C** and **E**, significant differences ($p < 0.05$) between the indicated groups are designated by *. In **A** and **E**, data were calculated using Imaris software. Data from three experiments ($n = 2$) are shown. In **C**, data were calculated from eight images (two images per mouse) using FIJI software. In **B** and **C**, one representative experiment out of three ($n = 4$) is shown. In **D**, one representative experiment out of three ($n = 2$) is shown.

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phagocytic activity (Fig. 5A, S8 Video). The presence of intense vacuolization in these DCs was also clear, and we observed some YFP⁺ cells (containing iRBC remnants from previous internalization events) phagocytizing mCherry-*Pc* iRBCs (Fig. 5A, S9 Video). CIVM 3D animations confirmed the internalization of mCherry-*Pc* parasites by YFP⁺ cells (Fig. 5B, S10 Video). This phenomenon was observed in 45% of the YFP⁺ cells (Fig. 5C). At five days p.i., YFP⁺ cells were activated and displayed higher cell volume and lower cell sphericity than those from recently infected mice (Fig. 5D; S1 Table). On day 5 p.i., the CD11c⁺ cells also expressed higher levels of MHC class II, CD80 and CD86 compared to those from naïve mice (S6C–S6D Fig.).

Immunofluorescence corroborated the significant role of splenic DCs in the widespread iRBC phagocytosis observed during pre-crisis. The percentages of CD11c pixels that colocalized with GFP pixels reached up to 40% in spleens from B6 mice on day 5 p.i. (Fig. 6A and 6B). Flow cytometry confirmed that splenic DCs were able to phagocytize iRBCs during pre-crisis. When mature CTV-*Pc* iRBCs were i.v. injected into B6 mice on day 5 p.i., approximately 4% of splenic DCs were CTV⁺ (1.4×10^5 CTV⁺CD11c⁺ cells/spleen) (Fig. 6C and 6D). Phagocytic activity was not restricted to a particular DC subtype, as a proportion of all subsets studied internalized iRBCs during pre-crisis (S4A Fig.). However, CTV⁺ CD11b⁺CD11c⁺ and CD8⁺CD11c⁺ cell numbers were significantly higher per spleen than those of other DC subsets (S4B Fig.). In addition, on day 5 p.i., 10% of CD11c⁺ cells from mice infected with GFP-*Pc* iRBCs were GFP⁺ (4×10^5 CTV⁺CD11c⁺ cells/spleen) (Fig. 6E and 6F). Comparatively, we observed substantially higher activation and phagocytic activity both *in vivo* and *ex vivo* in the splenic DCs during pre-crisis (S1 Table). Furthermore, a significantly higher frequency of iRBC uptake was detected using CIVM in comparison with flow cytometry.

Notably, flow cytometry analysis of splenic YFP⁺ cells from B6.CD11c-YFP mice during pre-crisis showed a sharp reduction in the percentages of F4/80⁺ cells so that the great majority of the YFP⁺ cell population presented with a classical DC phenotype (S5 Fig.). Moreover, a large fraction of CD11c⁺YFP⁺ cells in these mice expressed higher levels of MHC class II molecules in comparison to those in recently infected B6.CD11c-YFP mice. This observation was confirmed by CIVM, which revealed a reduction of F4/80⁺YFP⁺ cells in the subcapsular RP of B6.CD11c-YFP mice on day 5 p.i. (Fig. 5E and 5F, S11 Video). Due to the incremental number of CD11c⁺ cells in the YFP⁺ cell population, almost all of the phagocytic activity of YFP⁺ cells was imputed to DCs during pre-crisis, as observed by CIVM (Fig. 5G and 5H) and by flow cytometry (Fig. 6G, 6H and 6I).

Pc phagocytosis by splenic DCs is no longer observed during crisis

During the crisis phase of acute *Pc* malaria, profound modifications in the splenic architecture occur, resulting in RP closure [36]. Therefore, we extended our study into this phase of the disease. CIVM revealed only occasional mCherry-*Pc* iRBCs trapped by subcapsular RP YFP⁺ cells in B6.CD11c-YFP mice on day 8 p.i. (Fig. 7A and 7B, S12 Video), and yellow spots of merged mCherry/YFP-3D signal were infrequent (Fig. 7C). At that same time point, YFP⁺ cell volumes were smaller than during pre-crisis (Fig. 7D, S1 Table). YFP⁺ cell sphericity was reduced in mice on days 5 and 8 p.i. compared with naïve mice (Fig. 7D, S1 Table). Flow cytometry also revealed poor phagocytosis by splenic DCs, a process that was investigated both when mice

Figure 5. In vivo analysis of iRBC uptake by subcapsular RP DCs during pre-crisis. (A-D) B6.CD11c-YFP mice were i.p. infected with 1×10^6 mCherry-Pc iRBCs. Spleens were analyzed by CIVM after five days, at a time of day when mature parasite stages predominated. (A) Serial snapshots show the subcapsular RP and, in the right panel, a detailed image of a YFP⁺ cell (green) upon phagocytosis of an mCherry-Pc iRBC (red). (B) CIVM 3D animation shows the presence of mCherry-Pc iRBC remnants (yellow spots of merged mCherry/YFP-3D signal) inside the YFP⁺ cells. (C) Percentage of mCherry⁺ cells in the YFP⁺ cell population is shown (mean $\pm$ SEM). (D) YFP⁺ cell volume and sphericity are shown. Black, red and blue dots are from three different experiments. Horizontal lines represent mean values and SEM. (E-H) B6.CD11c-YFP mice were i.p. infected with 1×10^6 Pc iRBCs. At five days p.i., mice were i.v. infected with 1×10^8 mature CMTPIX-Pc iRBCs. After 15 min, mice were injected i.v. with a fluorescent anti-F4/80 mAb and the spleens were analyzed by CIVM. (E) Snapshots taken 30 min later show YFP⁺ cells (green), iRBCs (red), F4/80⁺ cells

(blue) and merged F4/80⁺YFP⁺ cells (white) in the subcapsular RP. **(F)** Percentage of F4/80⁺ cells in the YFP⁺ cell population is shown (mean $\pm$ SEM). **(G)** Percentages of CMTXP⁺ cells in the F4/80⁺YFP⁺ and F4/80⁻YFP⁺ cell subsets are shown (means $\pm$ SEM). **(H)** The relative proportions of F4/80⁺ and F4/80⁻ cells in the CMTXP⁺YFP⁺ cell population were calculated from the data obtained in **F** and **G** (means $\pm$ SEM). In **A**, **B** and **E**, the scale bars correspond to 50 μ m. One representative experiment out of three ($n = 2$) is shown. Data were calculated using Imaris software. In **C**, **D**, **F**, **G** and **H**, data were calculated using Imaris software. Data from three experiments ($n = 2$) are shown. In **G**, significant differences ($p < 0.05$) between the indicated groups are designated by *.

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Figure 6. Ex vivo analysis of iRBC uptake by splenic DCs during pre-crisis. **(A and B)** B6 mice were i.p. infected with 1×10^6 GFP-Pc iRBCs. Spleens were analyzed after five days, at a time of day when mature parasite stages predominated. **(A)** A representative immunofluorescence image (10x magnification) represents the spleens of GFP-Pc-infected mice. The lower panel details a merged GFP/CD11c⁺ cell. **(B)** Percentage of CD11c pixels colocalized with GFP pixels in the immunofluorescence images is shown (mean $\pm$ SD). **(C and D)** B6 mice were i.p. infected with 1×10^6 Pc iRBCs. At five days p.i., half of the B6 mice were i.v. re-infected with 1×10^8 mature CTV-Pc iRBCs. Spleens were analyzed by flow cytometry after 15 min. **(C)** Representative contour plots show CTV staining in the CD11c⁺ cells of Pc-infected mice that were re-infected or not with CTV-Pc iRBCs. CD11c⁺ cells in the CD3⁺CD19⁻DX5⁺Ter119⁻ cell population were analyzed. Data show the percentages of CTV⁺ cells in the CD11c⁺ cell population. **(D)** Numbers of total and CTV⁺CD11c⁺ cells per spleen in re-infected mice were calculated from the data obtained in **C** (means $\pm$ SD). **(E and F)** B6 mice were i.p. infected with 1×10^6 Pc iRBCs or GFP-Pc iRBCs. Spleens were analyzed after five days, at a time of day when mature parasite stages predominated. **(E)** Representative contour plots obtained by flow cytometry show GFP staining in the CD11c⁺ cells of mice that were infected with Pc iRBCs or GFP-Pc iRBCs. CD11c⁺ cells in the CD3⁺CD19⁻DX5⁺Ter119⁻ cell population were analyzed. Data show the percentages of GFP⁺ cells in the CD11c⁺ cell population. **(F)** Numbers of total and GFP⁺CD11c⁺ cells per spleen in GFP-Pc-infected mice were calculated from the data obtained in **E**. **(G-I)** B6.CD11c-YFP mice were i.p. infected with 1×10^6 Pc iRBCs. At five days p.i., half of the B6.CD11c-YFP mice were i.v. re-infected with 1×10^8 mature CTV-Pc iRBCs. Spleens were analyzed by flow cytometry after 15 min. **(G)** Representative contour plots show CTV staining in the CD11c⁺YFP⁺ and F4/80⁺YFP⁺ cells. Data show the percentages of CTV⁺ cells in each population. **(H)** The numbers of total and CTV⁺CD11c⁺YFP⁺ and F4/80⁺YFP⁺ cells per spleen were calculated from the data obtained in **G** (means $\pm$ SD). **(I)** The relative proportions of CD11c⁺ and F4/80⁺ cells in the CTV⁺YFP⁺ cell population were calculated from the data obtained in **H** (means $\pm$ SD). In **A**, the scale bars correspond to 50 μ m. In **B**, data were calculated using FIJI software. In **H**, significant differences ($p < 0.05$) between the indicated groups are designated by *. In **A-I**, one representative experiment out of three ($n = 3-4$) is shown.

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Figure 7. In vivo and ex vivo analysis of iRBC uptake by splenic DCs during crisis. (A–D) B6.CD11-YFP mice were i.p. infected with 1×10^6 mCherry-Pc iRBCs. Spleens were analyzed by CIVM after eight days, at a time of day when mature parasite stages predominated. (A) A snapshot shows the subcapsular RP. (B) CIVM 3D animation shows the presence of few mCherry-Pc iRBCs (red) attached to YFP⁺ cells (green). (C) Percentage of mCherry⁺ cells in the YFP⁺ cell population is shown (mean $\pm$ SEM). (D) YFP⁺ cell volume and sphericity are shown. Black, red and blue dots are from three different experiments. Horizontal lines represent mean values and SEM. (E and F) B6 mice were i.p. infected with 1×10^6 Pc iRBCs. At eight days p.i., half of the B6 mice were re-infected i.v. with 1×10^8 purified mature CTV-Pc iRBCs. Spleens were analyzed by flow cytometry after 15 min. (E) Representative contour plots show CTV staining in the CD11c⁺ cells of Pc-infected mice that were re-infected or not with CTV-Pc iRBCs. CD11c⁺ cells in the CD3⁺CD19⁺DX5⁺Ter119⁺ cell population were analyzed. Data show the percentages of CTV⁺ cells in the CD11c⁺ cell population. (F) The numbers of total and CTV⁺CD11c⁺ cells per spleen were calculated from the data obtained in E (means $\pm$ SD). (G and H) B6 mice were i.p. infected with 1×10^6 Pc iRBCs or GFP-Pc iRBCs. Spleens were analyzed after eight days, at a time of day when mature parasite stages predominated. (G) Representative contour plots obtained by flow cytometry show GFP staining in CD11c⁺ cells of mice that were infected with Pc iRBCs or GFP-Pc iRBC. CD11c⁺ cells in the CD3⁺CD19⁺DX5⁺Ter119⁺ cell population were analyzed. Data show the percentages of GFP⁺ cells in the CD11c⁺ cell population. (H) Numbers of total and GFP⁺CD11c⁺ cells per spleen were calculated from the data obtained in G. In A and B, the scale bars correspond to 50 μ m and 30 μ m, respectively. One representative experiment out of three (n = 2) is shown. In C and D, data were obtained using Imaris software. Data from three experiments (n = 2) are shown. In E–H, one representative experiment out of three (n = 5) is shown.

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were re-infected i.v. with mature CTV-Pc iRBCs and when mice were i.p. infected with GFP-Pc iRBCs (Fig. 7E, 7F, 7G and 7H). These data indicate that splenic DCs could be primarily involved in antigen presentation rather than in phagocytosis during crisis, as CD11c⁺ cells expressed high levels of MHC class II and CD80 on day 8 p.i. (S6C–S6D Fig.).

Discussion

The depletion of phagocytes *in vivo* allowed us to clearly demonstrate the key role of DCs in the protection against experimental blood-stage malaria. Abundant CD11c expression is a well-known marker for DCs, which are primary targets of DTx treatment in B6.CD11c-DTR mice [38]. Nevertheless, MZ macrophages are also depleted in DTx-treated B6.CD11c-DTR mice due to ectopic expression of the DTx receptor transgene [39]. The role of DCs was established in our study by comparing the disease progression in DTx-treated B6.CD11c-DTR mice and in B6 mice treated with a low dose of CILip, which selectively depletes MZ macrophages within splenic phagocytes [39], [40]. The significant contribution of DCs in the control of *Pc* malaria was suggested by data showing the worsening of the disease in DTx-treated B6.CD11c-DTR mice, while the elimination of MZ macrophages by the CILip treatment did not alter the course of infection in B6 mice. Our data also showed that splenic DCs are required for CD4⁺ T cell proliferation and IFN- γ production during *Pc* infection. The complete abrogation of these responses in DTx-treated B6.CD11c-DTR mice, but not in CILip-treated B6 mice, demonstrated that other splenic phagocytes such as MZ and RP macrophages did not replace DCs in the initiation of CD4⁺ T cell responses to *Pc* infection.

Our first evidence suggesting that DCs could directly contribute to parasite clearance was the effect of DC depletion on the increase of parasitemia and the reduction of body weight during the first days of blood-stage *Pc* and *Py* malaria. DCs were also required to control the early parasitemia following infection with *Pb* sporozoites. The early protective role of DCs could not be completely attributed to the need for these cells to activate T cells, which take longer to produce IFN- γ and induce antibody secretion during experimental malaria. The splenocytes obtained four and five days after *Pc* infection still require further stimulation with iRBCs *in vitro* to differentiate into effector cells [41], [42], while the *ex vivo* production of IFN- γ and antibodies coincides with the drop of parasitemia a week after infection [42], [43]. Using *in vivo* and *ex vivo* approaches, we unequivocally demonstrated here that the subcapsular RP DCs recognize and phagocytize mature iRBCs during the first encounter and pre-crisis, while spleen closure coincides with limited *Pc* phagocytosis by DCs during crisis. Although the splenic DCs are thought to be a major DC population in intimate contact with the bloodstream, these cells may act together with other DCs outside the spleen to clear *Plasmodium* parasites. This idea is supported by studies in splenectomized mice showing that other reticuloendothelial organs, such as the liver, effectively substitute for the phagocytic functions of the spleen in protecting against *Pc* malaria [22], [44]. In fact, hepatic CD11c⁺ DCs are also capable of internalizing iRBCs in the liver sinusoids during acute *Pc* infection [45].

CIVM allowed us to visualize the interaction between subcapsular RP DCs and iRBCs in great detail. In naïve mice, these cells actively extended protrusions and dendrites, as previously shown [26]. Soon after infection, we observed iRBCs being trapped by DCs that had a non-activated phenotype. The majority of these cells showed a classical DC phenotype, but a proportion of them exhibited strong labeling for F4/80, a marker of RP macrophages that is also expressed by a subset of DCs in the skin [46]. Another study reporting a similar observation concluded that, based on their dendritic morphology, subcapsular RP F4/80⁺YFP⁺ cells represent a subset of peripheral tissue DCs [26]. Although we did not visualize phagocytosis of iRBCs in recently infected mice, the detection of *Pc* remnants inside subcapsular RP DCs suggests that iRBC uptake had occurred. In fact, parasite antigen presentation is likely to occur soon after *Pc* infection, similar to the process observed during *L. monocytogenes* infection [47]. During the first day p.i., subcapsular RP DCs displayed high motility and made stable contacts with CD4⁺ T cells. DCs also migrated rapidly to T cell-rich areas following *Pc* infection, a

process that might involve chemokine signaling as suggested by studies in CCR7-knockout mice [48].

Here, for the first time, we observed the phagocytosis of iRBCs during pre-crisis *in vivo*. This occurred in a large number of subcapsular RP DCs, such that up to half of this population presented with *Pc* remnants. The great majority of these cells had a classical DC phenotype, which was characterized by negative staining for F4/80 and high expression of both MHC class II and costimulatory molecules. It is notable that these cells displayed an activated phenotype. Even if most subcapsular RP DCs during pre-crisis are immature cells that recently migrated to the spleen [49], it is expected that DC activation leads to their maturation and consequent blockade of phagocytic activity, allowing the cellular machinery to be restructured for antigen presentation [28]. In agreement with our data, a previous report determined that the peak of *in vitro* iRBC uptake by splenic DCs occurred at five days p.i., in parallel with the increase in the expression of MHC class II and costimulatory molecules [34]. In both studies, the phagocytic activity was not restricted to a particular DC subset. Our *ex vivo* data implicate CD11b⁺ and CD8⁺ DCs in most of the parasite clearance imputed to splenic DCs in mice both soon after infection and at the pre-crisis phase. Consistent with the immune response to acute *Pc* malaria, the CD11b⁺ and CD8⁺ DC subsets are known to be specifically involved in antigen presentation to CD4⁺ T cells and IL-12 production, respectively [50], [51]. Furthermore, both subsets of DCs are able to induce IFN- γ production by parasite-specific T cells during *Pc* infection [29]. Another important observation during pre-crisis was a sharp decline in the population of F4/80⁺YFP⁺ cells, a phenomenon that also occurred to splenic F4/80⁺ macrophages after the parasitemia peak (unpublished data). Because DCs have a higher turnover than F4/80⁺ macrophages [47], a possible explanation for our results is that a proportion of these phagocytes died after ingesting *Pc* parasites and only DCs were rapidly replaced. This process would substitute F4/80⁺ macrophages, a resident RP population that is primarily required to maintain tissue homeostasis [52], to inflammatory phagocytes. An alternative explanation is the down-regulation of the F4/80 molecule due to macrophage activation as reported during mycobacterial infection [53]. The F4/80⁺YFP⁺ cells could also have migrated to other locations such as the splenic T cell-rich areas.

During crisis, the down-regulation of the phagocytic function of splenic DCs coincided with the period of spleen closure. This was demonstrated here by *in vivo* images showing a few iRBCs in the subcapsular RP at eight days p.i., when parasitemias were even higher than at five days p.i. The decline in iRBC uptake was also associated with the maximum expression of MHC class II and CD80 molecules by splenic DCs, which indicates that complete DC maturation was only achieved during crisis. This idea is corroborated by a previous study that reported a decrease to baseline levels of the *in vitro* uptake of the iRBCs by splenic DCs at day 8 p.i. [34]. Thus, in addition to spleen closure and the subsequent blockade of iRBC entry inside the RP, splenic DCs seem to lose the ability to phagocytize parasites, while concomitantly increasing their ability to present cognate antigens. This is an interesting observation because, during crisis, most of the lymphocytes that are activated during early *Pc* infection undergo apoptosis [54], [55]. Thus, it is possible that mature DCs are required to expand and differentiate the few remaining T cells, giving rise to the memory response to malaria [56], [57].

The quantification of iRBC phagocytosis *ex vivo* by flow cytometry yielded substantially lower percentages of *Pc*⁺ DCs compared with *in vivo* data obtained by CIVM. This discrepancy may result from differences in the fluorescence detection thresholds of CIVM and flow cytometry, the DC subpopulations examined by these techniques (subcapsular RP DCs or total splenic DCs, respectively) or the fluorochrome labeling of the iRBCs (mCherry, GFP, CTV or CMTPIX). Another possible explanation for the low detection of iRBC uptake by flow cytometry is the rapid iRBC degradation or fluorochrome quenching [8], such that *Pc* remnants were

only identified inside DCs shortly after phagocytosis. Previously, low frequencies of iRBC uptake were also detected by flow cytometry in migrating monocytes [8], [27]. Immunofluorescence confirmed that splenic DCs, particularly those localized inside the RP and MZ, play a major role in the clearance of iRBCs during acute *Pc* infection. Although this technique did not efficiently discriminate single cells, the percentages of CD11c-GFP pixel co-localization were comparable to those of *Pc*⁺ DCs obtained by CIVM.

The *in vivo* approaches used in this study indicate that, beyond the classical role of DCs in antigen presentation, these cells also contribute to the direct elimination of iRBCs during acute *Plasmodium* infection. For several days after *Pc* infection, subcapsular RP DCs were highly efficient in the recognition and capture of iRBCs. Complete DC maturation appeared to be achieved only during crisis when restructuring of the spleen might facilitate the development of the acquired immunity. Taking into account the specifics of different parasite-host interactions, we speculate whether our findings in mouse models could be applied to human malaria. The adhesion of *P. falciparum* iRBCs to human monocyte-derived DCs through the scavenger receptor CD36 has been shown to inhibit DC maturation and subsequently reduce their capacity to activate T cells [58]. This observation was interpreted as the impairment of the DC function during *P. falciparum* infection. However, our data showing the induction of FcγRI in splenic DCs during pre-crisis open the possibility that recognition of opsonized iRBCs through this receptor can overcome the down-regulatory activity of CD36 signaling. Thus, the opposite effects of malaria on DC function could be related to the different activation profiles of DCs, which are greatly influenced by the surrounding tissue microenvironment, rather than other factors previously discussed such as different species and strains of hosts and parasites [59]. Together, our data add novel information to this area of immunology and demonstrate that *in vivo* imaging may help to unravel the mechanisms underlying protective immunity against malaria.

Materials and Methods

Mice, parasites and infections

Six- to eight-week-old B6, B6.CD11c-DTR [28], B6.CFP [60] and B6.CD11c-YFP mice [61] were bred under specific pathogen-free conditions at the Animal Facilities of Instituto Gulbenkian de Ciência (IGC), Instituto de Ciências Biomédicas at the Universidade de São Paulo (ICB-USP) or Institut de Transgénose Orléans-Villejuif. *Pc* (AS strain), *Py* (XL strain) and mCherry-*Pc* were maintained previously as described [62], [63]. GFP-*Pc* parasites were selected by treatment with pyrimethamine (Sigma-Aldrich, USA) [64]. The Instituto de Medicina Molecular at the Universidade de Lisboa provided *Anopheles stephensi* mosquitoes infected with *Pb* (ANKA strain). Mice were infected intraperitoneally (i.p.) with 1×10^6 iRBCs (blood from infected mice), and intravenously (i.v.) with 1×10^8 iRBCs or 1×10^3 sporozoites. Purified iRBCs were used where specified. The iRBCs were obtained during a period of the circadian cycle in which mature stages predominated (>95% late trophozoites/schizonts).

Ethics statement

All procedures were in accordance with the national regulations of Conselho Nacional de Saúde and Colégio Brasileiro em Experimentação Animal (COBEA) and Federation of European Laboratory Animal Science Associations (FELASA). The protocols were approved by the Comissão de Ética no Uso de Animais (CEUA) of ICB-USP, São Paulo, Brazil under permit numbers 0036/2007 and 0174/2011, and by FELASA under permit number AO10/2010.

DTx and CLip treatments

To deplete CD11c⁺ cells, B6.CD11c-DTR mice were injected i.p. with a single dose of 2 ng/g body weight of DTx (Sigma-Aldrich) 24 h before iRBC infection or 48 h after sporozoite infection. This dose is half of the one previously established to deplete CD11c⁺ cells [65] and it was used to reduce drug toxicity. To deplete MARCO⁺/MOMA1⁺ cells, B6 mice were injected i.v. with 8.5 µg/g body weight of CLip 24 h before infection [40]. Phosphate buffered saline (PBS) or PBS liposomes (PBSLip) were injected as controls. The procedures to obtain CLip and PBSLip were described elsewhere [66].

CTV or CMTPIX staining of purified iRBCs

Blood from infected B6 mice was resuspended in 1 ml PBS, pipetted over 5 ml of 74% Percoll (GE Healthcare, USA) and centrifuged (2500 x g, acceleration/break 5/0) for 30 min at room temperature (RT). The top cell layers were collected and washed with complete RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 2 mM L-glutamine and 1 mM sodium pyruvate; Life Technologies, USA). Purified iRBCs (>95% purity) were stained with CTV or CMTPIX, following the manufacturer's instructions (Life Technologies).

CIVM analysis

B6.CD11c-YFP mice infected with mCherry-*Pc* iRBCs were deeply anesthetized i.p. with 55 ng/g body weight of ketamine (Imalgene 1000, Merial, USA) and 0.85 ng/g body weight of xylazine (Rompun 2%, Bayer, Germany). Spleens were externalized by a 1 cm incision just below the ribcage. Mice were placed above a metal plate with a coverslip and immobilized without disrupting the vasculature or splenic connective tissue. Live imaging was carried out with an Eclipse Ti microscope (Nikon Instruments Inc., Japan) equipped with an Andor Revolution XD system (Andor Technology, UK), a Yokogawa CSU-X1 spinning disk unit (Andor Technology), a 20x PLAN APO VC objective (Nikon Instruments Inc.) and a 1.5x auxiliary magnification system (Nikon Instruments Inc.). Data were processed with MicroManager 1.2 (General Public License, NIH, USA). For each movie, 28 µm Z-sections with 4 µm Z-steps were acquired for 30 min. Imaris X64 7.0.0. (Andor Technology) was used to edit images and to determine the percentage of mCherry⁺YFP⁺ cells, as well as the CD11c⁺ cell volume and sphericity. In other cases, B6.CD11c-YFP mice were adoptively transferred with 5×10^6 splenic CD4⁺ T cells from B6.CFP mice (purified by FACS sorting using a FACSARIA device; BD Biosciences). These mice were infected as described above and processed 24 h later. Imaris was used to edit images and to determine CD11c⁺ cell speed and displacement, as well as the coefficients of CFP⁺CD4⁺ T cell speed and arrest.

B6.CD11c-YFP mice infected with CMTPIX-*Pc* iRBCs were injected i.v. with PE-conjugated anti-F4/80 mAbs (200 ng/g body weight) and deeply anesthetized to externalize the spleen as described above. Live imaging was carried out with a Zeiss LSM 780-NLO confocal microscope (Zeiss, Germany). Data were processed with Zen 2012 software (Zeiss, Germany). In each movie, 28 µm Z-sections with 2 µm Z-steps were acquired for 30 min. Imaris was used to edit images and to determine the percentages of CMTPIX⁺ cells.

Flow cytometry analysis

Mice were sacrificed and PBS-perfused to remove circulating iRBCs. Spleens were harvested, and the remaining RBCs were lysed with ACK lysis buffer. Splenocytes (1×10^6) were stained with fluorescent monoclonal antibodies (mAbs) against CD3, CD4, CD11c, CD69, CD11b,

CD80, CD86, I-A^b, B220, CD36, CD64 (FcγRI), DX5 and Ter119 (BD Biosciences, USA), F4/80 (eBiosciences, USA), and MOMA-1 and MARCO (Abcam, UK). Cells were analyzed by flow cytometry (FACSCanto; BD Biosciences) with FlowJo 9.5.3. (Tree Star Inc., USA).

Analysis of CD4⁺ T cell proliferation and IFN-γ production

Splenocytes (3×10^7) were resuspended in 1 ml PBS with 0.1% BSA (bovine serum albumin; Sigma-Aldrich) and stained with CFSE (carboxyfluorescein succinimidyl ester; Life Technologies) at a final concentration of 5 μM for 20 min at 37°C. Cells (1×10^6) were cultured in complete RPMI 1640 medium for 72 h at 37°C with 5% CO₂ in the presence of iRBCs (3×10^6). Cells were then stained with fluorescent mAbs against CD3 and CD4, and proliferation was assessed by flow cytometry. IFN-γ was quantified in the supernatants using the OptEIA IFN-γ kit (BD Biosciences).

Immunofluorescence analysis

GFP-*Pc* iRBC-infected B6 mice were sacrificed and PBS-perfused. Spleens were removed and frozen in Tissue-Tek OCT (Sakura Finetek, Japan). Sections 8 μm thick were cut with a CM3050S Cryostat (Leica, USA) and fixed with 1% paraformaldehyde (Alfa Aesar, USA) for 30 min at RT. Sections were incubated with anti-CD16/CD32 mAb (Fc block; BD Biosciences) for 30 min followed by incubation in a humidified dark chamber with fluorescent mAbs against CD11c, CD19, CD3, CD4 (BD Biosciences) and MOMA-1 (Abcam) for 2 h at RT. Sections were then stained for 5 min with 0.5 μg/ml DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich), washed with PBS and mounted with Fluoromount-G (Southern Biotechnologies, USA). Images were acquired with a DMRA2 fluorescence microscope (Leica) and MetaMorph software (Molecular Devices Inc., USA). Image analysis was performed with Photoshop CS4 (Adobe Inc., USA). Percentages of CD11c-GFP/CD11c-CD4 pixel colocalization and of GFP pixel distribution in the spleen were calculated using FIJI for Windows 64-bit (*Colocalization threshold* and *Mixture Modeling Thresholding* plugins, respectively; General Public License, NIH, USA).

Statistical analysis

Results were analyzed with Prism 5 software (Graph Pad) using ANOVA or Student's t-tests. The existence of a normal distribution was confirmed using the Kolmogorov-Smirnov test. Differences were considered statistically significant at $p < 0.05$.

Supporting Information

S1 Fig. Effects of DTx treatment on splenic DCs and MZ macrophages. (A and B) B6 and B6.CD11c-DTR mice were treated with DTx or PBS, and their spleens were analyzed by flow cytometry after 24 h. (A) Representative contour plots show the depletion of CD11c⁺ and MARCO/MOMA-1⁺(CD11b⁺) cells, but not of F4/80⁺ cells, in DTx-treated B6.CD11c-DTR mice. Data show the percentages of CD11c⁺, F4/80⁺ and MARCO/MOMA-1⁺ cells in the splenocyte population. (B) The numbers of CD11c⁺, F4/80⁺ and MARCO/MOMA-1⁺ cells per spleen are shown. In B, significant differences ($p < 0.05$) between all other groups are designated by *. In A and B, one representative experiment out of three ($n = 3$) is shown. (PDF)

S2 Fig. Effects of MZ macrophage depletion on acute *Pc* malaria. (A-D) B6 mice were treated with either a low dose of CLiP to deplete MARCO⁺ and MOMA-1⁺ macrophages or with PBSLiP as controls. The mice were i.p. infected with 1×10^6 *Pc* iRBCs 24 h later. (A)

Representative contour plots obtained 24 h after treatment by flow cytometry confirm the efficiency of CLiP-induced depletion of MARCO⁺ and MOMA-1⁺ cells without affecting CD11c⁺I-A⁺ and F4/80⁺ cells. Data show the percentages of MARCO⁺, MOMA-1⁺, CD11c⁺I-A⁺ and F4/80⁺ cells in the splenocyte population. (B) Parasitemia curves are shown (means ± SD). (C) Survival curves are shown. (D) Data show the percentages of proliferating CFSE^{low}CD4⁺ T cells and IFN-γ concentrations in the supernatants of spleen cell cultures stimulated for 72 h with iRBCs (means ± SD). In A–D, one representative experiment out of three (n = 5) is shown. (PDF)

S3 Fig. Effects of DC depletion on the blood stages of infection with *Py* iRBCs or *Pb* sporozoites. (A–C) B6 and B6.CD11c-DTR mice were treated with either DTx to deplete CD11c⁺ cells or PBS as a control. The mice were i.p. infected with 1×10^6 *Py* iRBCs 24 h later. (A) Parasitemia curves are shown (means ± SD). (B) Variations in body weight relative to day 0 are shown (means ± SD). (C) Survival curves are shown. (D–F) B6 and B6.CD11c-DTR mice were i.v. infected with 1×10^3 *Pb* sporozoites. After 48 h, the mice were treated with DTx to deplete CD11c⁺ cells at the beginning of blood stage. (D) Parasitemia curves are shown (means ± SEM). (E) Variations in body weight relative to day 0 are shown (means ± SEM). (F) Survival curves are shown. In A–F, significant differences ($p < 0.05$) between the indicated groups are designated by *. In A–C, one representative experiment out of three (n = 3–4) is shown. In D–F, data from three experiments (n = 2–3) are shown. (PDF)

S4 Fig. Phagocytosis of iRBCs by splenic DC subsets throughout acute *Pc* malaria. Spleens were analyzed 15 min after i.v. injection of 1×10^8 mature CTV-*Pc* iRBCs (dark line histograms) or PBS (filled histograms) in B6 mice at zero, five or eight days p.i. with 1×10^6 *Pc* iRBCs. (A) Representative histograms obtained by flow cytometry show CTV staining in the splenic DC subsets (CD11b⁺, CD8⁺, B220⁺ or CD4⁺). Data show the percentages of CTV⁺ cells in each subset. (B) Numbers of total and CTV⁺CD11c⁺ cells per spleen were calculated from the data obtained in A. In B, significant differences ($p < 0.05$) between the DC subsets at different days p.i. are designated by *. In A and B, one representative experiment out of three (n = 5) is shown. (PDF)

S5 Fig. Phenotypic analysis of YFP⁺ cells soon after *Pc* infection and during pre-crisis. (A and B) Spleens were analyzed 15 min after i.v. injection of 1×10^8 mature CTV-*Pc* iRBCs in B6.CD11c-YFP mice at zero or five days p.i. with 1×10^6 *Pc* iRBCs. (A) Representative contour plots show the gate strategy for analysis of YFP⁺ cells in naïve mice. Data show the percentages of singlets, leukocytes and YFP⁺ cells in each contour plot. (B) Representative contour plots show CD11c and F4/80 staining in the YFP⁺ cells. Data show the percentages of these cells in the YFP⁺ cell population. Histograms show MHC class II (I-A) staining in CD11c⁺YFP⁺ cells. The fluorescence minus one (FMO) control was obtained in CD11c⁺YFP⁺ cells from a [*Pc* (0) + CTV-*Pc*] mouse (filled histogram). In A and B, one representative experiment out of three (n = 3) is shown. (PDF)

S6 Fig. Expression of activation markers in splenic DCs throughout acute *Pc* malaria. B6 mice were i.p. infected with 1×10^6 *Pc* iRBCs. At zero, five or eight days p.i., spleens were analyzed by flow cytometry. (A) Representative histograms show the expression of CD36 and FcγRI in CD11c⁺ cells. The corresponding FMO control for each marker is represented by the filled histograms. (B) Median fluorescence intensity (MFI) was calculated from the data obtained in A (means ± SD). (C) Representative histograms show the expression of MHC class II

(I-A), CD80 and CD86 molecules in CD11c⁺ cells. The corresponding FMO control for each marker is represented by the filled histograms. (D) MFI was calculated from the data obtained in C (means $\pm$ SD). In B and D, significant differences ($p < 0.05$) between the indicated groups are designated by *. In A-D, one representative experiment out of three ($n = 5$) is shown. (PDF)

S1 Table. Comparative analyses of *in vivo* and *ex vivo* approaches to the study of splenic DCs throughout acute *Pc* malaria. The data compare several parameters in recently infected mice and mice on days 5 p.i. (pre-crisis) and 8 p.i. (crisis). For *in vivo* analyses, the following two experiments were performed: 1) B6.CD11c-YFP mice were i.v. infected with 1×10^8 mature mCherry-*Pc* iRBCs, and spleens were evaluated 15 min later. 2) B6.CD11c-YFP mice were i.p. infected with 1×10^6 mCherry-*Pc* iRBCs, and spleens were analyzed at five or eight days p.i., at a time of day when mature parasite stages predominated. For *ex vivo* analyses, the following two experiments were performed: 1) B6 mice were i.v. infected with 1×10^8 purified mature CTV-*Pc* iRBCs (flow cytometry) or GFP-*Pc* iRBCs (flow cytometry and immunofluorescence), and spleens were evaluated 15 min later. 2) B6 mice were i.p. infected with 1×10^6 GFP-*Pc* iRBCs (flow cytometry and immunofluorescence), and spleens were evaluated after five or eight days p.i., at a time of day when mature parasite stages predominated. Data were compiled from figs. 2, 3, 5 and 6. Significant differences ($p < 0.05$) between the recently infected group and groups on days 5 and 8 p.i. are designated by *. Significant differences ($p < 0.05$) between the groups on days 5 and 8 p.i. are designated by #. (DOCX)

S1 Video. The subcapsular RP DC network in the spleen of a naïve mouse. Time-lapse images show a representative 3D region of the subcapsular RP of the spleen in a naïve B6.CD11c-YFP mouse. The video shows that most YFP⁺ cells (green) are sessile but are still actively making protrusions and extending dendrites. Images were acquired with Z-steps of 4 μ m (total Z = 28 μ m) for 30 min at 1 frame/19 s of live imaging. The video is shown at 10 frames/s with maximum color intensity. (AVI)

S2 Video. The uptake of iRBCs by subcapsular RP DCs soon after *Pc* infection. Time-lapse images show a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse, starting 15 min after i.v. infection with 1×10^8 mature mCherry-*Pc* iRBCs. The video shows mCherry-*Pc* iRBCs (red) moving inside the RP and trapped by YFP⁺ cells (green). The white arrows indicate points of *Pc* trapping by YFP⁺ cells. Images were acquired with Z-steps of 4 μ m (total Z = 28 μ m) for 30 min at 1 frame/17 s of live imaging. The video is shown at 10 frames/s with maximum color intensity. (AVI)

S3 Video. Subcapsular RP DCs containing iRBC remnants soon after *Pc* infection. The animation shows a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse, starting 15 min after i.v. infection with 1×10^8 mature mCherry-*Pc* iRBCs. The images show mCherry-*Pc* iRBC remnants inside YFP⁺ cells that are visualized as yellow spots of merged mCherry/YFP-3D signal. The video is shown at 10 frames/s with maximum color intensity. (AVI)

S4 Video. Trapping of iRBCs by subcapsular RP DCs soon after *Pc* infection. Time-lapse images show details from S2 Video in which a YFP⁺ cell (green) is in close contact with an mCherry-*Pc* iRBC (red). The parasite remained trapped in the YFP⁺ cell membrane

throughout the video and moved from one part of the membrane to the other. The white arrows highlight this interaction between YFP⁺ cell and *Pc* parasite. The video is shown at 10 frames/s of with maximum color intensity.

(AVI)

S5 Video. Trapping of iRBCs by F4/80⁺YFP⁺ and F4/80⁻YFP⁺ cells soon after *Pc* infection.

The animation shows a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse that was injected i.v. with fluorescent anti-F4/80 mAbs, starting 15 min after i.v. infection with 1×10^8 mature CMTPIX-*Pc* iRBCs. The video shows YFP⁺ cells (green), iRBCs (red), F4/80⁺ cells (blue) and merged F4/80⁺YFP⁺ cells (white). The video is shown at 10 frames/s with maximum color intensity.

(AVI)

S6 Video. CD4⁺ T cell / DC dynamics in the spleen of a naïve mouse. Time-lapse images show a representative 3D region of the subcapsular spleen in a B6.CD11c-YFP mouse adoptively transferred with polyclonal CD4⁺ T cells from B6.CFP mice. The video shows actively moving CD4⁺ T cells (blue) making non-stable and sporadic contacts with YFP⁺ cells (green).

Images were acquired with Z-steps of 4 µm (total Z = 28 µm) for 60 min and are shown at 1 frame/15 s of live imaging. The video is shown at 10 frames/s with maximum color intensity.

(AVI)

S7 Video. CD4⁺ T cell / DC dynamics in the spleen of a recently infected mouse. Time-lapse images show a representative 3D region of the subcapsular spleen in a B6.CD11c-YFP mouse adoptively transferred with polyclonal CD4⁺ T cells from B6.CFP mice, starting 24 h after i.v. infection with 1×10^8 mature mCherry-*Pc* iRBCs. The video shows YFP⁺ cells (green) that are slightly more motile, still making protrusions and actively internalizing parasites (red). CD4⁺ T cells (blue) are less motile and are making more stable contacts with YFP⁺ cells. Images were acquired with Z-steps of 4 µm (total Z = 28 µm) for 60 min at 1 frame/15 s of live imaging. The video is shown at 15 frames/s with maximum color intensity.

(AVI)

S8 Video. The uptake of iRBCs by subcapsular RP DCs during pre-crisis. Time-lapse images show a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse, starting 5 days after i.p. infection with 1×10^6 mCherry-*Pc* iRBCs. The video was made at a time of day when mature parasite stages predominated and shows many mCherry-*Pc* iRBCs (red) inside the RP, as well as the intense phagocytic activity of YFP⁺ cells (green). The white arrows indicate points of *Pc* trapping by YFP⁺ cells. Images were acquired with Z-steps of 4 µm (total Z = 28 µm) for 30 min at 1 frame/19 s of live imaging. The video is shown at 15 frames/s with maximum color intensity.

(AVI)

S9 Video. Phagocytosis of an iRBC by a subcapsular RP DC during pre-crisis. Time-lapse images show a detail from [S8 Video](#) in which a YFP⁺ cell (green) containing remnants of previous *Pc* internalizations (yellow spots of merged mCherry/YFP-3D signal) phagocytizes an mCherry-*Pc* iRBC (red). The video is shown at 15 frames/s with maximum color intensity.

(AVI)

S10 Video. Subcapsular RP DCs containing iRBC remnants and in the process of phagocytizing an iRBC during pre-crisis. The animation shows a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse, starting five days after i.p. infection with 1×10^6 mature mCherry-*Pc* iRBCs. The video was made at a time of day when mature parasite stages predominated, mCherry-*Pc* iRBC remnants inside YFP⁺ cells can be observed as yellow

spots of merged mCherry/YFP-3D signal. It was possible to observe in 3D ongoing phagocytosis of an mCherry-*Pc* iRBC by a YFP⁺ cell. The white arrows indicate the ongoing phagocytosis described above. The video is shown at 10 frames/s with maximum color intensity.

(AVI)

S11 Video. Trapping of iRBCs by F4/80⁺YFP⁺ and F4/80⁻YFP⁺ cells during pre-crisis. The animation shows a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse on day 5 p.i. that was injected i.v. with fluorescent anti-F4/80 mAbs, starting 15 min after i.v. injection with 1×10^8 mature CMTPIX-*Pc* iRBCs. The video shows YFP⁺ cells (green), iRBCs (red), few F4/80⁺ cells (blue) and merged F4/80⁺YFP⁺ cells (white). The video is shown at 15 frames/s with maximum color intensity.

(AVI)

S12 Video. Subcapsular RP DCs containing iRBC remnants during crisis. The animation shows a representative 3D region of the subcapsular RP of the spleen in a B6.CD11c-YFP mouse, starting eight days after i.p. infection with 1×10^6 mature mCherry-*Pc* iRBCs. The video was made at a time of day when mature parasite stages predominated. Images show mCherry-*Pc* iRBC remnants inside YFP⁺ cells as yellow spots of merged mCherry/YFP-3D signal. The white arrows indicate the few *Pc* parasites trapped by YFP⁺ cells. The video is shown at 10 frames/s with maximum color intensity.

(AVI)

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Author Contributions

Conceived and designed the experiments: HBdS MRDL CET. Performed the experiments: HBdS RF AdAC EMdS MNdM KRP. Analyzed the data: HBdS. Contributed reagents/materials/analysis tools: JL CRFM SBB IMC JMA BR VMB. Wrote the paper: HBdS MRDL CET.

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APÊNDICE C – Pulmonary infection with hypervirulent *Mycobacteria* reveals a crucial role for the P2X7 receptor in aggressive forms of Tuberculosis. **Plos Pathogens**, 2014

Pulmonary Infection with Hypervirulent Mycobacteria Reveals a Crucial Role for the P2X7 Receptor in Aggressive Forms of Tuberculosis

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Abstract

The purinergic P2X7 receptor (P2X7R) is a sensor of extracellular ATP, a damage-associated molecule that is released from necrotic cells and that induces pro-inflammatory cytokine production and cell death. To investigate whether the innate immune response to damage signals could contribute to the development of pulmonary necrotic lesions in severe forms of tuberculosis, disease progression was examined in C57BL/6 and P2X7R^{-/-} mice that were intratracheally infected with highly virulent mycobacterial strains (*Mycobacterium tuberculosis* strain 1471 of the Beijing genotype family and *Mycobacterium bovis* strain MP287/03). The low-dose infection of C57BL/6 mice with bacteria of these strains caused the rapid development of extensive granulomatous pneumonia with necrotic areas, intense bacillus dissemination and anticipated animal death. In contrast, in P2X7R^{-/-} mice, the lung pathology presented with moderate infiltrates of mononuclear leukocytes without visible signs of necrosis; the disease attenuation was accompanied by a delay in mortality. *In vitro*, the hypervirulent mycobacteria grew rapidly inside macrophages and induced death by a P2X7R-dependent mechanism that facilitated the release of bacilli. Furthermore, these bacteria were resistant to the protective mechanisms elicited in macrophages following extracellular ATP stimulation. Based on this study, we propose that the rapid intracellular growth of hypervirulent mycobacteria results in massive macrophage damage. The ATP released by damaged cells engages P2X7R and accelerates the necrotic death of infected macrophages and the release of bacilli. This vicious cycle exacerbates pneumonia and lung necrosis by promoting widespread cell destruction and bacillus dissemination. These findings suggest the use of drugs that have been designed to inhibit the P2X7R as a new therapeutic approach to treat the aggressive forms of tuberculosis.

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Introduction

Tuberculosis (TB) is a serious public health issue, with nearly 9 million new annual cases of the disease worldwide and 1.3 million deaths per year [1]. A third of the global population is infected with bacteria of the *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis* – *Mtb* and *Mycobacterium bovis* – *Mbv*), although most effectively control the pathogen and remain asymptomatic by establishing equilibrium with the bacilli [2]. Mycobacteria are typically transmitted by aerosols and reach the lungs, where macrophages and other immune cells are recruited during the

early innate response to infection. The latent infection results from the equilibrium between mycobacteria and the host defenses, in which inflammatory cells become organized as primary granulomas.

The reactivation of latent infections in immunocompetent individuals occurs at rates that range from 3% to 10% per lifespan [3]; these rates are dramatically increased by co-infection with the human immunodeficiency virus (HIV) [4]. Accelerations in active TB reactivations have also been described after the administration of tumor necrosis factor (TNF)α/IL-12/IL-23 blockers, which are used to treat inflammatory diseases, such as rheumatoid arthritis

Author Summary

Nearly 9 million new cases of tuberculosis and 1.3 million deaths are reported yearly worldwide. Most individuals infected with tubercle bacilli remain asymptomatic; however, some develop active tuberculosis due to the reactivation of latent infections. Progressive primary tuberculosis is an alternative form of the disease that mostly affects children and immunocompromised individuals. Extensive pneumonia, pulmonary necrosis and bacillus dissemination characterize some of the aggressive forms of tuberculosis. To investigate the molecular mechanisms that underlie severe disease progression, we used experimental models of relatively resistant C57BL/6 mice that were infected with highly virulent strains of *Mycobacterium tuberculosis* or *Mycobacterium bovis*. Two hypervirulent strains (*Mtb* strain 1471 and *Mbv* strain MP287/03) induced extensive pulmonary inflammation and necrosis in mice and promoted bacillus dissemination and animal death. We hypothesized that the innate immune response to endogenous damage signals from necrotic cells could aggravate the disease. We focused our study on the purinergic P2X7 receptor (P2X7R), a sensor of ATP that is released from necrotic cells and that induces pro-inflammatory cytokine production and cell death. Our data provide new insights into the pathogenesis of severe tuberculosis by showing that mice that lack P2X7R have attenuated disease with substantially reduced bacillus dissemination and lung inflammation without evidence of necrosis.

and Crohn's disease [2,5]. Progressive primary TB is an alternative form of the disease that represents less than 10% of active TB cases in normal adults, although it is common in children less than 5 years of age and in immunocompromised individuals [6]. The aggressive forms of mycobacterial infections are characterized by the rapid expansion of primary granulomatous infiltrates in the lungs that results in tuberculous pneumonia and disseminated disease, such as miliary TB pneumonia. The precipitation of TB pathology is hallmarked by the formation of central caseous necrotic lesions filled with extracellular infectious bacilli and cell debris [7]. The factors that determine the transition of mycobacterial infections to active TB and the rapid progression of the disease have not been fully elucidated. The characteristics of the bacteria (i.e., high bacterial virulence or high dose of infection) and the host (i.e., genetic predisposition, immunodeficiency or malnutrition) are thought to contribute to progression of the disease [8].

Mouse TB models have been extremely useful in the characterization of mechanisms that are involved in disease pathogenesis and the generation of protective immunity. However, it is difficult to determine the factors that are responsible for the development of pulmonary necrosis because this process is atypical in mice that are infected with virulent mycobacteria, such as the H37Rv reference strain [7]. Necrotic lesions occur mostly in mice that are deficient in immunologically relevant molecules, such as inducible nitric oxide synthase (iNOS), the T cell receptor (TCR) $\alpha\beta$, interferon (IFN) γ , the TNF receptor (TNFR)-1, IL-6 and IL-12 [9]. Mice deficient in IL-1R1, IL-6, IL-10, CD4, CD8 or γ/δ T cells present with discrete pulmonary intragranulomatous necrosis. Alternatively, artificial conditions are required to induce necrosis in mice, such as intraperitoneal infections with extremely high numbers of bacilli [10] or intranasal infections with a low bacillus inoculum, followed by the administration of lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid

(Poly-IC) [9,11,12]. Moreover, dermal infections of iNOS-deficient mice lead to the formation of non-necrotizing granulomas in the lungs, which exhibit central caseation and hypoxia when IFN γ or TNF α are blocked [13].

Recent epidemiologic studies have shown that *Mtb* strains belonging to the emerging Beijing genotype family, which are associated with high virulence, transmissibility and drug resistance [14], are more likely to cause rapid progression of the primary infection to active TB than the strains belonging to other genotype groups [15]. Highly virulent Beijing strains, in contrast to the less virulent strains of Beijing or non-Beijing *Mtb* genotypes, promote unusual rapid fatal diseases in infected mice that are characterized by a severe pulmonary pathology, with high bacterial burden and extensive lung consolidation and areas of focal necrosis [16,17,18]. Furthermore, infections of mice with few *Mbv* strains are able to cause sudden pneumonia with extensive necrosis that lead to early death [19]. Studies with mice infected with hypervirulent mycobacterial strains suggest a possible method for investigating the specifics of the immunopathogenesis behind the aggressive forms of TB. An elucidation of the molecular mechanisms that are implicated in the development of severe TB is important to elaborate new therapeutic strategies.

Necrotic cell death permits the release of damage-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP). Extracellular ATP (eATP) is a danger signal for cells of the immune system and contributes to inflammatory and reparatory responses [20,21,22]. ATP is also released by activated and apoptotic cells through pannexin-1 hemichannels, which results in autocrine and paracrine cell signaling [23]. Some compelling evidence indicates that eATP levels increase *in vivo* to concentrations greater than 100 μ M during pathological processes [24,25]. Among the purinergic P2 receptors that recognize eATP, the P2X7 receptor (P2X7R) is uniquely able to induce pro-inflammatory cytokine production and cell death [26,27,28]. Therefore, eATP recognition by P2X7R contributes to the development of sterile and infectious inflammation in different experimental models [21,22,28,29,30]. The altered intracellular ionic milieu that results from P2X7R activation presumably triggers the nucleotide-binding domain and leucine-rich repeat-containing gene family pyrin domain-containing 3 protein (NLRP3) inflammasome, which leads to the pro-inflammatory cytokine production and apoptotic cell death [31,32,33]. The prolonged stimulation of P2X7R permits the formation of large transmembrane pores and the flux of molecules between 314 and 900 Da [26]. Depending on the magnitude and duration of the stimulus, the predominance of each of these molecular pathways dictates the development of apoptotic or necrotic cell death [34]. The combined activation of different molecular pathways culminates in pyroptosis, pyronecrosis or damaged mitochondria-dependent necroptosis [35].

Based on these findings, we were determined to investigate whether the recognition of eATP from necrotic cells by P2X7R could elicit an innate immune response and contribute to the development of severe TB. To investigate this possibility, we used murine models of pulmonary TB induced by intratracheal infections with the highly virulent isolates *Mtb* strain 1471 (Beijing genotype) and *Mbv* strain MP287/03. These mycobacterial strains were epidemiologically associated with outbreaks of TB in human and bovine populations, respectively, and showed high virulence in our previous *in vitro* studies [36,37,38]. In C57BL/6 mice that were infected with a low dose (approx. 100 bacilli) of hypervirulent mycobacteria, we reproduced several pathological manifestations of the aggressive forms of human TB, including tuberculous pneumonia with areas of focal necrosis and miliary dissemination to the liver and spleen. We then examined the courses of infection,

lung pathologies and bacterial burdens in P2X7R^{-/-} and C57BL/6 mice. Our data provide new insights into the pathogenesis of severe TB by showing a requirement for P2X7R in the induction of macrophage death and the progression of tuberculous pneumonia, necrotic pulmonary lesions, bacillus dissemination and anticipated animal death.

Results

Severe TB caused by hypervirulent Beijing 1471 *Mtb* and MP287/03 *Mbv* is attenuated in P2X7R-deficient mice

Because ATP released from necrotic cells activates P2X7R and subsequently the innate immune response [23,26,27], we wondered if this molecular pathway may contribute to the development of the aggressive forms of TB that are associated with intense pulmonary necrosis. To investigate this possibility, two hypervirulent *Mtb* and *Mbv* strains were selected from 9 strains (*Mtb* – zt272, 1471 (genotype Beijing), 1777 (genotype LAM) and H37Rv; *Mbv* – AN5, B2, B29, MP287/03 and MP389/03) on the basis of the ability to induce severe TB, i.e., intense pulmonary infiltration with lung necrosis and precocious death of C57BL/6 mice that were infected intratracheally (i.t.) with low doses of bacilli (approx. 100 forms). Beijing 1471 *Mtb* and MP287/03 *Mbv* exhibited higher virulence within each subgroup (*Mtb* and *Mbv*) compared to the other strains (data not shown). The diseases induced by these strains in C57BL/6 and P2X7R^{-/-} mice were compared to those induced by the virulent H37Rv *Mtb* strain, which did not induce pulmonary necrosis in C57BL/6 mice. A previous study in P2X7R^{-/-} mice reported no involvement of the P2X7R during pulmonary H37Rv *Mtb* infection [39], whereas another study described a protective role for this receptor [40].

Our results show that infection with Beijing 1471 *Mtb* killed 90% of C57BL/6 mice until 250 days post-infection (p.i.), whereas MP287/03 *Mbv* induced 100% mortality before day 40 p.i. (Figure 1A). Remarkably, all of the P2X7R^{-/-} mice infected with Beijing 1471 *Mtb* remained alive throughout this period, and the survival time was considerably increased when these animals were infected with MP287/03 *Mbv*. For the H37Rv *Mtb* infection, 100% survival was observed in C57BL/6 and P2X7R^{-/-} groups. The bacillus counts in the lungs of C57BL/6 mice at day 28 p.i. with Beijing 1471 *Mtb* and MP287/03 *Mbv* (CFU Log₁₀ 6.7±0.2 and 8.8±0.2, respectively) were approximately 10-fold and 1,000-fold higher, respectively, than those in mice infected with H37Rv *Mtb* (CFU Log₁₀ 5.8±0.4) (Figure 1B). Furthermore, the P2X7R^{-/-} mice infected with hypervirulent mycobacteria showed a nearly 10-fold reduction of lung bacillary burden compared to the C57BL/6 counterparts. In contrast, similar CFU values were obtained for the lungs of the C57BL/6 and P2X7R^{-/-} mice infected with H37Rv *Mtb*. Moreover, the disease dissemination to the liver and spleen was attenuated in P2X7R^{-/-} mice infected with hypervirulent mycobacteria, whereas no significant difference was found between C57BL/6 and P2X7R^{-/-} mice infected with H37Rv *Mtb*.

P2X7R mediates pneumonia and lung necrosis in mice infected with hypervirulent mycobacteria

To further characterize the effects of P2X7R during severe TB caused by hypervirulent mycobacteria, pneumonia and lung necrosis were evaluated in C57BL/6 and P2X7R^{-/-} mice on day 28 p.i. with the Beijing 1471 *Mtb*, MP287/03 *Mbv* and H37Rv *Mtb* strains. Macroscopically, the lungs had white nodules that were more prominent in C57BL/6 mice infected with hypervirulent mycobacteria (Figure 2A). Furthermore, the lung weights and relative masses were greatest in C57BL/6 mice infected with

MP287/03 *Mbv*, followed by those infected with Beijing 1471 *Mtb* (Figures 2B and 2C). Considerably smaller white nodules were observed in the lungs of P2X7R^{-/-} mice that were infected with either hypervirulent strain, and these lungs were observed to have lower weights and relative masses. The lung weights in C57BL/6 and P2X7R^{-/-} mice infected with H37Rv *Mtb* did not statistically differ from those in non-infected controls.

Microscopically, the lungs of C57BL/6 and P2X7R^{-/-} mice infected with H37Rv *Mtb* presented with incipient granulomas with no visible acid-alcohol resistant bacillus (BAAR) (Figures 3A–B and 3C–D, respectively). Additionally, comparable reductions of nearly 20% in the areas of lung sections covered by intralveolar spaces were observed in C57BL/6 and P2X7R^{-/-} mice infected with H37Rv *Mtb* (Figure 3M). In contrast, extensive cellular infiltrations in the lung tissues and intralveolar spaces, accompanied by granuloma formation with necrotic lesions and BAARs, were observed in C57BL/6 mice infected with Beijing 1471 *Mtb* (Figures 3E and 3F) and MP287/03 *Mbv* (Figures 3I and 3J). Remarkably, reduced areas of inflammation and necrosis with lower numbers of BAARs were observed in the lungs of P2X7R^{-/-} mice infected with both hypervirulent strains (Figures 3G–H and 3K–L). Accordingly, C57BL/6 mice infected with Beijing 1471 *Mtb* and MP287/03 *Mbv* showed sharp decreases of over 80% in the areas of lung sections occupied by intralveolar spaces, a phenomenon that was drastically attenuated in the P2X7R^{-/-} counterparts (Figure 3M).

P2X7R mediates the recruitment of myeloid cells to the lungs in mice infected with hypervirulent mycobacteria

To determine the mechanisms by which the P2X7R detrimentally affects the outcome of severe TB, we next examined whether the lack of P2X7R modifies the pulmonary influx of leukocytes and the cytokine profile. We evaluated IL-1β production because it can be induced following P2X7R activation. We also examined IFNγ and IL-10 production because these cytokines have counterbalancing effects on inflammatory processes. These three cytokines have important roles in pulmonary TB [9,13,41].

In agreement with the macro- and microscopic analysis of the lungs, C57BL/6 mice infected with Beijing 1471 *Mtb* and MP287/03 *Mbv* presented on day 28 p.i. with high numbers of lung infiltrating cells that were significantly reduced in P2X7R^{-/-} counterparts (Figure 4A). For the H37Rv *Mtb* infection, the total cell numbers in the lungs of C57BL/6 mice were similar to those of P2X7R^{-/-} mice and were 2- to 3-fold lower than those induced by hypervirulent strains. Unexpectedly, the composition of cellular infiltrates differed between the two groups of C57BL/6 mice infected with hypervirulent strains. Pulmonary myeloid (CD11b⁺ cells) and lymphoid (CD19⁺, CD4⁺ and CD8⁺ cells) subsets were found in similar proportions in Beijing 1471 *Mtb*-infected C57BL/6 mice. In contrast, a strong predominance of CD11b⁺ cells with virtually no T and B cells was observed in MP287/03 *Mbv*-infected C57BL/6 mice. The absence of P2X7R led to reduced pulmonary infiltration of myeloid cells in both hypervirulent infections and reduced lymphoid cells in the Beijing 1471 *Mtb* infection. However, MP287/03 *Mbv*-infected P2X7R^{-/-} mice presented with higher numbers of CD4⁺ and CD8⁺ cells compared to their C57BL/6 counterparts. C57BL/6 and P2X7R^{-/-} mice infected with H37Rv *Mtb* showed comparable numbers of pulmonary myeloid and lymphoid subsets. The CD11b⁺ population was mostly composed of Ly6C⁺ cells (monocytes) in all of the infected mice, with the exception of MP287/03 *Mbv*-infected C57BL/6 mice, which showed a predominance of Ly6G⁺ cells (neutrophils) (data not shown, manuscript in preparation).

Figure 1. Survival curves and bacillary burdens in the lungs, liver and spleen of C57BL/6 and P2X7R^{-/-} mice infected with hypervirulent mycobacteria. C57BL/6 and P2X7R^{-/-} mice were infected i.t. with approx. 100 bacilli of H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv*. Non-infected mice were used as controls. (A) The infected mice were examined daily to determine the survival curves ($n = 13-19$). (B) The number of CFU/g of tissue was evaluated in the left lung, liver and spleen on day 28 p.i. (means $\pm$ SD, $n = 9$). Significant differences were observed for the indicated groups (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). The data are representative of three separate experiments. doi:10.1371/journal.ppat.1004188.g001

The profiles of cytokines produced by lung infiltrating cells also varied between the two groups of C57BL/6 mice on day 28 p.i. with hypervirulent strains; relatively high or low production levels of pro-inflammatory cytokines were observed during severe TB (Figure 4B). More precisely, cells from Beijing 1471 *Mtb*-infected C57BL/6 mice produced higher levels of IL-1 β and IFN γ . In contrast, cells from MP287/03 *Mbv*-infected mice secreted comparatively lower levels of pro-inflammatory cytokines and higher levels of IL-10, which supports the notion that MP287/03 *Mbv* is a poor inducer of pro-inflammatory cytokines [38]. As observed for the composition of cellular infiltrates, the absence of P2X7R had different effects on the cytokine profiles induced by hypervirulent strains. For the Beijing 1471 *Mtb* infection, cells from P2X7R^{-/-} mice secreted significantly reduced amounts of IL-1 β and IFN γ and increased amounts of IL-10 compared to cells from C57BL/6 mice. For the MP287/03 *Mbv* infection, comparable low levels of IL-1 β were found in cell supernatants from C57BL/6 and P2X7R^{-/-} mice, but the lack of P2X7R resulted in an increase of IFN γ and a decrease of IL-10. The H37Rv *Mtb* infection induced very low production of IL-1 β , IFN γ and IL-10; the amount of

pro-inflammatory cytokines was significantly reduced in the absence P2X7R.

Hypervirulent mycobacteria induce macrophage necrosis by a P2X7R-mediated mechanism

The following experiments were performed to further elucidate the mechanisms that are responsible for the deleterious role of P2X7R in severe TB. The intracellular bacterial growth and dissemination were investigated in bone marrow-derived macrophages (BMDMs) obtained from C57BL/6 and P2X7R^{-/-} mice that were infected with H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* strains. The analysis was performed over a maximum period of 6 days in culture after macrophage differentiation, during which the viability of non-infected BMDMs was maintained above 95%. The intracellular bacterial growth was evaluated at an MOI (multiplicity of infection) of 1, which ensured bacillus multiplication for 6 days p.i. in the absence of spontaneous and bacillus-induced macrophage death. The low MOI was particularly important for hypervirulent mycobacterial strains that grew rapidly and killed the macrophages. The macrophage necrosis induced by the mycobacteria was

Figure 2. Lung gross pathology in C57BL/6 and P2X7R^{-/-} mice on day 28 p.i. with hypervirulent mycobacteria. C57BL/6 and P2X7R^{-/-} mice were infected i.t. with approx. 100 bacilli of H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv*. Non-infected mice were used as controls. (A) Representative images of the right lungs are shown. (B) Right lung weights and (C) relative masses (circles) were evaluated ($n = 9$). The mean values are represented by horizontal lines. The relative lung masses were calculated by the ratios of the mean values of the lung weights in the indicated groups and the control group. Significant differences were observed for the indicated groups (** $p < 0.01$ and *** $p < 0.001$). The data are representative of three separate experiments.

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systematically determined at MOIs of 10 and 20; the best conditions to discriminate C57BL/6 and P2X7R^{-/-} BMDMs are shown.

The hypervirulent mycobacterial strains multiplied inside C57BL/6 BMDMs much faster than did H37Rv *Mtb* and were the most effective strains for inducing cell death (Figure S1A and S1B). In an experimental condition of low bacillus-induced macrophage death, P2X7R did not interfere with intracellular bacterial growth; the growth kinetics of the mycobacterial strains within C57BL/6 and P2X7R^{-/-} BMDMs were similar at an MOI of 1 (Figure S1A). Remarkably, negligible numbers of necrotic P2X7R^{-/-} BMDMs were observed on day 4 p.i. with hypervirulent mycobacteria at an MOI of 10, an experimental condition that induced C57BL/6 BMDM death (Figures 5A and 5B). The kinetics of cellular viability at an MOI of 20 showed that the lack of P2X7R protected the BMDMs from early death, but the Beijing 1471 *Mtb* infection killed C57BL/6 and P2X7R^{-/-} BMDMs on day 6 p.i. (Figure S1B). Our data also show that P2X7R-mediated necrosis of macrophages facilitates the release of hypervirulent mycobacteria; more viable Beijing 1471 *Mtb* and MP287/03 *Mbv* bacilli were detected in the supernatants of C57BL/6 BMDMs compared to those of P2X7R^{-/-} BMDMs (Figure 5C). Supporting the idea that Beijing 1471 *Mtb* and MP287/03 *Mbv* infections induce macrophage necrosis, the majority of dead cells on day 4 p.i. with hypervirulent strains were stained with PI but not with annexin V (Figure S2). Furthermore, in accordance with the *in vivo* data (Figure 4B), P2X7R^{-/-} BMDMs that were infected with Beijing 1471 *Mtb* produced lower levels of IL-1 β than did the counterpart C57BL/6 BMDMs (Figure 5D). MP287/03 *Mbv* was a poor inducer of IL-1 β production in C57BL/6 BMDMs, and this cytokine was not detected in the supernatants from P2X7R^{-/-} BMDMs. The infection of C57BL/6 and P2X7R^{-/-} BMDMs with H37Rv *Mtb* caused negligible macrophage death (Figure 5A), extracellular release of the bacilli (Figure 5C) and IL-1 β production (Figure 5D), which were not influenced by the P2X7R.

For hypervirulent strains but not for H37Rv *Mtb*, eATP stimulation causes a P2X7R-mediated release of viable mycobacteria by infected macrophages

Previous studies show that engagement of the P2X7R promotes phagosome-lysosome fusion and induces apoptosis, thereby causing intracellular mycobacterial killing [42,43,44]. In agreement with the protective role of the P2X7R against mycobacterial infection, macrophages from subjects homozygous for loss-of-function P2X7R polymorphisms exhibit ablated eATP-induced apoptosis and killing of mycobacteria [45,46,47]. As these analyses were performed with BCG, an avirulent *Mbv* strain, the apparent contrast between the protective role of P2X7R reported in these studies and the deleterious role of P2X7R described here may reside in the varying virulence of mycobacteria. If this is the case, eATP stimulation of the P2X7R may have different consequences in macrophages infected with virulent or hypervirulent strains, causing mycobacterial killing or dissemination, respectively. To examine this possibility, the numbers of intracellular and extracellular bacilli were determined in P2X7R^{-/-} and C57BL/6 BMDMs that were infected with H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* strains and then stimulated with eATP. The protocol used in these experiments was similar to those employed in the previous *in vitro* analyses described above [45,46,47].

The activation of H37Rv *Mtb*-infected C57BL/6 BMDMs with 1 and 5 mM eATP led to P2X7R-mediated decreases in the numbers of intracellular bacilli and no increase in the numbers of extracellular bacilli (Figures 6A and 6B). At 1 mM eATP, macrophage activation rather than apoptosis may determine the P2X7R-mediated killing of H37Rv *Mtb*. At this eATP concentration, C57BL/6 BMDMs exhibited a typical morphology of viable macrophages (Figure 6C). In contrast, at 5 mM eATP, the protective effect was associated with a high level of death of H37Rv *Mtb*-infected C57BL/6 BMDMs. Indeed, most C57BL/6 BMDMs presented with a necrotic phenotype at concentrations greater than 3 mM eATP (Figure S3). As expected, P2X7R^{-/-} BMDMs were resistant to eATP-induced death. For hypervirulent

Figure 3. Lung histopathology in C57BL/6 and P2X7R^{-/-} mice on day 28 p.i. with hypervirulent mycobacteria. C57BL/6 and P2X7R^{-/-} mice were infected i.t. with approx. 100 bacilli of H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv*. (A–L) Images show representative lung sections stained with HE (50× magnification; bar scales correspond to 400 μm) and ZN (400× magnification; bar scales correspond to 50 μm) methods. (A and C) H37Rv *Mtb*-infected C57BL/6 and P2X7R^{-/-} mice exhibited incipient granulomas. (B and D) Amplification of the inflamed areas shows no visible BAAR in the mice presented in B and D. (E and I) Beijing 1471 *Mtb*- and MP287/03 *Mbv*-infected C57BL/6 mice displayed extensive lung TB lesions with areas of necrosis. (F and J) Numerous BAARs were observed in the lesions described in E and I. (G and K) Beijing 1471 *Mtb*- and MP287/03 *Mbv*-infected P2X7R^{-/-} mice presented with small lung lesions with no visible necrosis. (H and L) Reduced amounts of BAARs were observed in the lesions described in G and K. (M) Morphometric quantification of lung sections shows a reduction in the intralveolar areas in infected mice compared to non-infected controls (means ± SD, n=3–5). Significant differences were observed for the indicated groups (**p<0.01 and ***p<0.001). The data are representative of three separate experiments. doi:10.1371/journal.ppat.1004188.g003

strains, the effects of eATP stimulation on C57BL/6 BMDMs were completely different than they were for the H37Rv *Mtb* strain. At 1 mM eATP, the numbers of intracellular and extracellular bacilli showed minor or no changes for both the Beijing 1471 *Mtb* and MP287/03 *Mbv* strains (Figures 6A and 6B). At 5 mM eATP, the P2X7R-mediated decrease in the amount of intracellular bacilli was associated with macrophage death (Figure 6C). Most importantly, significantly higher numbers of

viable mycobacteria were detected in the supernatants of C57BL/6 BMDMs infected with the Beijing 1471 *Mtb* and MP287/03 *Mbv* strains (Figure 6B). The release of viable mycobacteria was mediated by the P2X7R, which indicated that macrophages had undergone P2X7R-mediated necrotic death. These results corroborate our supposition that eATP stimulation of infected macrophages has opposite consequences for virulent and hyper-virulent strains. In other words, the heterogeneity of mycobacterial

A

B

Figure 4. Lung infiltrating cells and cytokine production in C57BL/6 and P2X7R^{-/-} mice on day 28 p.i. with hypervirulent mycobacteria. C57BL/6 and P2X7R^{-/-} mice were infected i.t. with approx. 100 bacilli of H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv*. Non-infected mice were used as controls. (A) The numbers of total, CD11b⁺, CD19⁺, CD4⁺ and CD8⁺ cells in the lungs are shown (means $\pm$ SD, $n=3$). (B) IL-1 β , IFN γ and IL-10 were quantified in the supernatants of lung cells after 48 h of culture (means $\pm$ SD, $n=3$). Significant differences were observed for the indicated groups (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$). The data are representative of three separate experiments. doi:10.1371/journal.ppat.1004188.g004

virulence explains the dichotomy concerning the protective and deleterious roles of P2X7R in pulmonary TB.

Discussion

The development of two experimental mouse models of human-like severe TB allowed us to determine the general properties of phylogenetically distant strains of hypervirulent mycobacteria that

are associated with poor prognosis. The ability of Beijing 1471 *Mtb* and MP287/03 *Mbv* to rapidly grow in the lungs, to cause pulmonary necrosis and to disseminate to other organs likely contributed to the worsening of the disease in C57BL/6 mice. These data emphasize the importance of the mycobacterial genetic background in the pathogenesis of severe TB and corroborate previous studies in mice that showed significant variability in the bacillus virulence of *Mtb* strains that were isolated from TB

Figure 5. Induction of necrotic cell death and the release of bacteria and IL-1 β in P2X7R^{-/-} and C57BL/6 BMDMs infected with hypervirulent mycobacteria. C57BL/6 and P2X7R^{-/-} BMDMs were infected with H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* at an MOI of 10. (A) On day 4 p.i., necrotic cells were identified by AO/EthBr incorporation. (B) Images show C57BL/6 and P2X7R^{-/-} BMDMs on day 4 p.i. with Beijing 1471 *Mtb* (200 $\times$ magnification; bar scales correspond to 100 μ m). (C) The release of mycobacteria was examined by CFU quantification in the supernatants of the infected cultures described in A. (D) On day 3 p.i., IL-1 β was quantified in the culture supernatants. Significant differences were observed for the indicated experimental conditions (*** p <0.001). The data represent the means $\pm$ SD of samples in triplicate. The data are representative of three separate experiments. doi:10.1371/journal.ppat.1004188.g005

patients [19,18,48,49] or *Mbv* strains that were isolated from infected cattle [19,48,49]. Additionally, the comparison of the disease progression in C57BL/6 and P2X7R^{-/-} mice that were infected i.t. with a low inoculum (approx. 100 bacilli) of H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* allowed us to describe the crucial role of P2X7R in the aggressive forms of pulmonary TB. These findings provide a new insight into the pathogenesis of severe TB by showing that mice that lack P2X7R have attenuated disease with substantially reduced bacillus burdens in the lungs, livers and spleens. The absence of P2X7R also promoted significant reductions in pneumonia and lung necrosis on day 28 p.i. with Beijing 1471 *Mtb* and MP287/03 *Mbv*, which corroborates our hypothesis of DAMP involvement in the pathogenesis of severe TB. Furthermore, our data are consistent with a previous study in P2X7R^{-/-} mice that reported no role for P2X7R during pulmonary H37Rv *Mtb* infection [39].

P2X7R signaling could detrimentally affect the outcome of severe TB by inducing the production of IL-1 β and exacerbating

the pulmonary influx of pathogen-permissive monocytes and macrophages. A recent study has implicated the recruitment of CD11b⁺F4/80⁺Gr1^{int} myeloid cells to the lungs in the exacerbation of H37Rv *Mtb* infection in mice treated with Poly-IC [12]. Accordingly, our data link the reduction in the pulmonary infiltration of CD11b⁺ myeloid cells with disease attenuation in P2X7R^{-/-} mice that were infected with Beijing 1471 *Mtb* and MP287/03 *Mbv* strains. The lack of P2X7R during the Beijing 1471 *Mtb* infection also resulted in lower IL-1 β production by lung infiltrating cells, which could have been responsible for the secretion of less IFN γ and more IL-10. Previous studies showing the resistance of hypervirulent mycobacteria to IFN γ -mediated intracellular killing [37,38] may explain the ineffectiveness of IL-1 β , a critical cytokine in the protection against H37Rv *Mtb* [41], in preventing the fatal outcome of the disease in Beijing 1471 *Mtb*-infected C57BL/6 mice. Nevertheless, the induction of IL-1 β does not fully explain how P2X7R contributes to exacerbate the disease progression during MP287/03 *Mbv* infection. In this case, severe

Figure 6. Effects of eATP on mycobacterial killing or release by C57BL/6 and P2X7R^{-/-} BMDMs. C57BL/6 and P2X7R^{-/-} BMDMs were infected with H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* at an MOI of 10; treated with 0, 1 or 5 mM eATP for 30 min; and analyzed 6 h later. (A) Mycobacterial killing was evaluated by intracellular CFU quantitation. (B) The release of mycobacteria from necrotic cells was examined by CFU quantification in the culture supernatants. (C) Images show BMDM cultures (200 $\times$ magnification; bar scales correspond to 100 μ m). Significant differences were observed between eATP-treated and non-treated groups and for the indicated experimental conditions (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). The data represent the means $\pm$ SD of samples in triplicate. The data are representative of three separate experiments. doi:10.1371/journal.ppat.1004188.g006

TB occurred concomitantly with a relatively high secretion of IL-10 and low secretion of IL-1 β and IFN γ , which correlated with low numbers of CD4⁺ and CD8⁺ T cells in the lungs. In fact, we

have recently shown that MP287/03 *Mbv*-infected C57BL/6 macrophages are biased towards an M2 immune response profile and induce higher arginase-1 expression and reduced NO

production compared to H37Rv *Mtb*-infected macrophages [38]. The inhibition of innate immune responses was also described for other mycobacterial strains [18,50,51], and it has been considered to be a selective advantage of modern mycobacterial strains that contributes to rapid disease progression and transmission [51]. Furthermore, the lack of the P2X7R during MP287/03 *Mbv* infection did not affect the low production of IL-1 β by lung infiltrating cells, but it restored the CD4⁺ and CD8⁺ T cell responses with the secretion of more IFN γ and less IL-10.

An alternative insight into the mechanism responsible for the prejudicial role of P2X7R during severe TB was provided by the *in vitro* analysis of C57BL/6 and P2X7R^{-/-} macrophages that were infected with the H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* strains. The abilities of hypervirulent mycobacteria to rapidly grow in C57BL/6 mice and to cause pulmonary necrosis were associated with the abilities to multiply vigorously within C57BL/6 macrophages *in vitro* and to induce cell death. These findings show a parallel between the *in vivo* and *in vitro* behavior of the mycobacterial strains as reported in other studies [37,52]. In a recapitulation of the *in vivo* data, only Beijing 1471 *Mtb* elicited the production of high levels of IL-1 β in C57BL/6 macrophages, but both hypervirulent mycobacteria strains induced cell death. The subsequent release of live bacilli and the negative staining for annexin V clearly establish that these infected macrophages undergo necrotic cell death. Therefore, with consideration for the critical role of the NLRP3 inflammasome in *Mtb*-induced necrotic death [53], it is likely that signaling through P2X7R accelerates this process in C57BL/6 macrophages that are infected with Beijing 1471 *Mtb* and MP287/03 *Mbv*. Accordingly, the absence of the P2X7R led to a delay in macrophage death that was accompanied by lower release of hypervirulent mycobacteria. This finding indicates that P2X7R cooperates with mycobacterial components exhibiting membrane-lysing activity, such as the ESAT-6 protein from *Mtb* [54], a phenomenon that may occur through autocrine or paracrine ATP release. The membrane permeabilization that results as a secondary effect of P2X7R-mediated necrotic death might also have facilitated the release of IL-1 β during the Beijing 1471 *Mtb* infection in C57BL/6 macrophages [55], as this response was partially inhibited in P2X7R^{-/-} macrophages. Thus, even if P2X7R-mediated macrophage death is an evolutionarily selected mechanism of host defense, in Beijing 1471 *Mtb* and MP287/03 *Mbv* infections, it allows the bacilli to escape from the innate host defenses and to spread to new cells. A similar behavior was recently described for mycobacteria that possess RD-1 virulence factors, which permit phagolysosomal rupture and allow access to the cytosol [56].

The deleterious role of P2X7R during severe TB implies that hypervirulent mycobacteria are resistant to the protective mechanisms that are elicited in macrophages following eATP stimulation. In avirulent BCG infection, the engagement of the P2X7R promotes phagosome-lysosome fusion and induces macrophage apoptosis, thereby causing mycobacterial death [42,43,44]. Accordingly, macrophages from subjects homozygous for loss-of-function P2X7R polymorphisms exhibit ablated eATP-induced apoptosis and eATP-mediated killing of BCG bacilli [45,46]. In fact, our data clearly establish that eATP stimulation has opposite outcomes in macrophages that are infected with virulent or hypervirulent mycobacteria. Thus, eATP induced the P2X7R-mediated killing of H37Rv *Mtb* bacilli and the P2X7R-mediated release of viable Beijing 1471 *Mtb* and MP287/03 *Mbv* bacilli. The resistance of these hypervirulent mycobacteria to intracellular killing has also been described following macrophage stimulation with IFN γ [37,38] and can be attributed to the ability of pathogenic mycobacteria to block phagosome-lysosome fusion

[57]. The moderate extent of tissue damage with consequently small amounts of eATP release and the susceptibility to eATP-induced intracellular killing explain, respectively, the lack of involvement or the protective role of P2X7R during the H37Rv *Mtb* infection [39,40]. Finally, several epidemiological studies reported that loss-of-function polymorphisms in the human P2X7R gene increase the risk of pulmonary TB [58,59,60,61,62]. However, opinions on this issue have not reached a consensus, and other studies show a lack of association between these parameters [63,64]. In sum, we can envisage that the heterogeneity of the samples analyzed in these studies may account for the failure to reach a consistent conclusion. Namely, the risk of pulmonary TB in individuals with loss-of-function polymorphisms in the P2X7R gene may be increased for mild TB and decreased for severe TB. The protective role of the P2X7R functional deficiency in the aggressive forms of TB could explain why evolutionary pressure has maintained these gene polymorphisms at high rates in the human population.

The data from two mouse models indicated P2X7R as a key molecule in the development of severe TB and suggested that lung necrosis is enhanced by the activation of this receptor. P2X7R appears to have a dual role in the development of aggressive TB. First, it might facilitate the dissemination of hypervirulent mycobacteria by inducing the lysis of infected macrophages. Second, it might contribute to pneumonia and lung necrosis by promoting widespread cell destruction. Based on these results, we propose that the massive destruction of macrophages by hypervirulent mycobacteria with the help of the P2X7R leads to the release of large amounts of ATP (Figure 7). This release triggers a vicious cycle in which eATP exacerbates the pulmonary influx of pathogen-permissive monocytes and macrophages [12] and leads to further cell destruction by a P2X7R-mediated mechanism. This scenario is made worse by the resistance of hypervirulent mycobacteria to the protective mechanisms elicited in macrophages by eATP. The suppressive environment that results from an excess of adenosine, a byproduct of eATP degradation, may also facilitate the survival of hypervirulent mycobacteria [23]. This study provides a perspective for the development of new therapeutic approaches in which drugs designed to inhibit the P2X7R are used to ameliorate the outcomes of aggressive forms of TB.

Materials and Methods

Mice

Male 8- to 10-week-old C57BL/6 and P2X7R^{-/-} (C57BL/6 background) mice (originally from the Jackson Laboratory, USA) were bred under specific pathogen-free conditions at the Isogenic Mice Facility (ICB/USP, Brazil). The P2X7R^{-/-} mice were generated by Pfizer Inc. (USA) and backcrossed to C57BL/6 mice for 7 generations; a single nucleotide polymorphism panel analysis throughout the genome suggested a C57BL/6 genetic background (<http://jaxmice.jax.org/strain/005576.html>). Once infected, the experimental groups were maintained in microisolator cages at the Biosafety Level 3 Mice Facilities (UENF and FCT-USP, Brazil) under controlled temperature and humidity and were fed *ad libitum*.

Ethics statement

All experimental procedures were conducted in accordance with the national regulations on ethical guidelines for mouse experimentation and welfare of the Conselho Nacional de Saúde and Colégio Brasileiro de Experimentação Animal (COBEA, Brazil). The protocols were approved by the Animal Care

Figure 7. Schematic illustration of a hypothetical model to explain the high and low involvement of the P2X7R during severe and mild TB. In severe TB, the rapid intracellular growth of hypervirulent mycobacteria results in massive macrophage damage. The eATP released by damaged cells may engage the P2X7R on their surfaces or on neighboring cells. The autocrine or paracrine P2X7R signaling cooperates with mycobacterial components exhibiting membrane-lysing activity and accelerates the necrotic death of infected macrophages and the spread of bacilli. The resistance of hypervirulent mycobacteria to the protective mechanisms elicited in macrophages by eATP contributes to disease dissemination. The release of large amounts of eATP triggers a vicious cycle that exacerbates the pulmonary recruitment of pathogen-permissive monocytes and macrophages and thereby leads to further intracellular bacillus growth. The suppressive environment that results from an excess of adenosine, a byproduct of eATP degradation, may also facilitate the survival of hypervirulent mycobacteria. According to this model, lung necrosis derives from programmed cell death that is triggered by P2X7R signaling. The modest levels of tissue damage induced by less virulent strains and the susceptibility of these mycobacteria to eATP-induced intracellular killing explain, respectively, the minor role and the protective effect of the P2X7R in the mild forms of TB.

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Committee of ICB-USP and UENF under the permit numbers 0026/2009 and 0020/2006, respectively.

Mycobacteria

The *Mtb* strain of the Beijing genotype (Beijing 1471) was isolated from a TB patient at the St. Petersburg Physiopulmonology Research Institute, Russia [37]. Dr. José Soares Ferreira Neto (Instituto de Medicina Veterinária, USP) and Dr. Philip Suffys (Fundação Oswaldo Cruz, Brazil) kindly provided the bovine *Mbv* isolate (MP287/03 - SB0295 spoligotyping) and the *Mtb* reference strain (H37Rv - ATCC), respectively. Mycobacteria from a single colony-forming unit (CFU) were suspended in Middlebrook 7H9 medium (Difco, BD Biosciences, USA) that was supplemented with 10% albumin-dextrose-catalase (ADC) (Difco) and 0.05% Tween 80 (Sigma-Aldrich, USA), with (for *Mbv*) or without (for *Mtb*) 0.4% sodium pyruvate (Sigma-Aldrich), and were frozen at -80°C in aliquots of 10^8 bacilli/ml. Prior to performing the experiments, the aliquots were thawed, diluted in complete 7H9 medium, sonicated and incubated at 37°C for 7 days. To avoid

bacterial clumps, the aliquots were sonicated for 1 min, homogenized and kept at rest for 10 min at room temperature (RT). The suspensions were collected, and the absence of bacterial clumps was monitored by microscopic examination. The bacilli were quantified by spectrophotometry at 600 nm.

CFU counting

The live bacterial burdens were determined by serial dilutions and the plating of cell or tissue homogenates in Middlebrook 7H10 medium (Difco), which was supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC) (Difco); 0.4% sodium pyruvate was added for the *Mbv* strains. CFU numbers were determined after a 3 week incubation at 37°C .

Mouse infections

Mice were anesthetized by intraperitoneal injections of ketamine (Vetbrands, Brazil; 100 mg/kg) and xylazine (Vetbrands; 15 mg/kg), which were diluted in sterile saline. For each mouse, the trachea was exposed via a small midline incision, and 60 μl of

the bacterial suspension (approx. 100 bacilli) was inoculated intratracheally (i.t.) [65]. The incision was sutured with sterile silk. The inoculum dose was confirmed by a CFU count of lung homogenates at 24 h p.i. and varied $\pm 20\%$ in relation to the dose of infection.

Macroscopic and microscopic analyses of the lungs, liver and spleen

The relative mass (organ weight in infected mice/control mice) of each organ was determined. Tissue samples from the right lung, liver and spleen were fixed with 10% buffered formalin, photographed and embedded in paraffin. Serial 4–5- μm sections were stained with hematoxylin-eosin (HE) dye to analyze the tissue alterations and by the Ziehl-Neelsen (ZN) method to detect BAARs. The samples were examined with a Leica microscope (Germany), and images were captured with a Coolpix P995 Nikon camera (Japan).

Morphometric analysis of lung tissues

The percentages of the lung tissue occupied by intralveolar space were determined as previously reported for intravascular space in the placenta [66]. Briefly, 8 randomly selected images were analyzed in each HE-stained section of lung tissue (50 $\times$ magnification). Using ImageJ software (National Institutes of Health, USA), the images were transformed in gray scale (8-bit) and the areas corresponding to intralveolar space were colored in red. The reduction in the percentages of intralveolar area was calculated by the equation $(x-y)*100/x$, where x and y are, respectively, the percentages of pixels covered by red areas in the images of non-infected and infected mice.

Isolation of lung infiltrating cells

The left lungs were washed with sterile PBS and placed in Petri dishes with RPMI 1640 medium (Gibco, USA). The dissected lung tissue was incubated in medium that contained Liberase (Sigma-Aldrich; 2 $\mu\text{g}/\text{ml}$) and type IV bovine pancreatic DNase (Roche Diagnostic; 1 $\mu\text{g}/\text{ml}$) at 37°C for 45 min. Cells were dispersed with a 10-ml syringe (BD Biosciences) that was fitted with an 18-gauge needle and filtered with a cell strainer (Corning, USA). Red blood cells were depleted with a lysis buffer (0.144 M NH_4Cl , 0.0169 M TRIS base, pH 7.4) at 37°C in a 5% CO_2 atmosphere for 5 min.

Phenotypic analysis of lung infiltrating cells

Lung infiltrating cells (1×10^6) were stained using appropriate combinations of Pacific Blue-, APC-Cy7-, PE-Cy7-, PE- or V500-labeled monoclonal antibodies (mAbs) to CD11b (ICRF44), CD19 (1D3), CD4 (RM4-5) and CD8 (53-6.7) (BD Pharmingen, USA). Cells were fixed with 2% paraformaldehyde and analyzed by flow cytometry (FACSCanto, BD Biosciences) with FlowJo software.

Cytokine quantification

The lung infiltrating cells (5×10^4 cells/well) were suspended in complete RPMI 1640 medium (Gibco) with 1 mM sodium pyruvate, 2 mM glutamine, 0.05% gentamicin and 10% fetal calf serum (FCS) and were cultured in 96 well-plates (Corning) at 37°C in a 5% CO_2 atmosphere for 48 h. Cell culture supernatants were harvested, filter sterilized and stored at -80°C . The levels of IL-1 β , IFN γ and IL-10 in the cell culture supernatants were measured with a Fluorokine kit (R&D System, USA) and a Bioplex system (Bio-Rad, USA).

BMDM generation

BMDMs were generated from mouse femurs as described previously [43], with some modifications. The two femurs were each flushed with 5 ml of complete DMEM-F12 medium (Gibco; 1 mM sodium pyruvate, 2 mM glutamine, 0.05% gentamicin and 10% FCS) and were incubated at 37°C in a 5% CO_2 atmosphere for 12 h. Non-adherent cells were harvested and cultured for an additional 3 days in a cell culture flask (Corning; 75 cm^2) that contained 10 ml of complete DMEM-F12 medium that was supplemented with 20% L929 cell-conditioned medium as a source of macrophage colony-stimulating factor. After 3 days, an additional 10 ml of L929 cell-conditioned medium was added. Four days later, BMDMs were detached from the flasks with 4 ml of 2 mM trypsin in PBS. The viability of BMDMs was higher than 95% over a week period and decreased thereafter.

BMDM infection and eATP stimulation

The *in vitro* experiments were performed in a time window of 6 days after macrophage differentiation. BMDMs (4×10^4 cells) were cultured in 96-well plates in DMEM-F12 medium with 2% FCS at 37°C in a 5% CO_2 atmosphere. To evaluate intracellular bacterial growth, BMDMs were infected at an MOI of 1 for 3 h. After washing 3 times with PBS, the intracellular bacteria were evaluated by performing CFU counts of BMDMs that were lysed with 0.1% saponin (Sigma-Aldrich) for 10 min. To analyze the cytotoxicity induced by the bacteria, BMDMs were infected and cellular viability was determined by acridine orange and ethidium bromide (AO/EthBr; Sigma-Aldrich) incorporation using an inverted NIKON TS-100 fluorescence microscope (Japan). To establish the best experimental condition with which to discriminate the differences between experimental groups, the cellular viabilities were systematically determined at MOIs of 10 and 20. The effects of eATP on bacterial intracellular killing and release were assessed as previously described [45,46,47]. Briefly, BMDMs were infected at an MOI of 10, washed 3 times with PBS and stimulated with 1 or 5 mM eATP (Sigma-Aldrich) for 30 min. The intracellular and extracellular CFU counts were determined after 6 h in culture. The cytotoxicity induced by eATP in non-infected BMDMs was determined after 24 h in culture with 1 or 5 mM eATP. The cells were stained with AO/EthBr and analyzed in an inverted NIKON TS-100 fluorescence microscope.

Annexin V staining

BMDMs (4×10^4 cells) were infected at an MOI of 10, washed 3 times with PBS and cultured for 4 days in 96-well plates in DMEM-F12 medium with 2% FCS at 37°C in a 5% CO_2 atmosphere. Non-infected BMDMs were stimulated with actinomycin D (Sigma-Aldrich; 2.5 $\mu\text{g}/\text{ml}$) for 6 h as a positive control for apoptosis and with eATP (5 mM) for 24 h as a positive control for necrosis. BMDMs were stained with FITC-labeled annexin V [67] diluted in annexin binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2) for 20 min and then stained with propidium iodide (PI; Sigma-Aldrich; 1.6 $\mu\text{g}/\text{ml}$). The samples were analyzed in an inverted NIKON TS-100 fluorescence microscope.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism 4 software (GraphPad, USA), and the differences between the groups were considered significant when $p < 0.05$ (5%). The simultaneous effects of two factors were analyzed by two-way ANOVA and the Bonferroni post-hoc test. One-way ANOVA and the Tukey post-hoc test were used to assess the effects of only one

parameter. Survival curves were analyzed with the log-rank test of the Kaplan-Meier method.

Supporting Information

Figure S1 Kinetics of intracellular bacillus growth and macrophage necrosis in P2X7R^{-/-} and C57BL/6 BMDMs infected with hypervirulent mycobacteria. C57BL/6 and P2X7R^{-/-} BMDMs were infected with H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* at an MOI of 1 (intracellular bacillus growth) or at an MOI of 20 (macrophage necrosis). (A) On days 0, 3 and 6 p.i., intracellular bacillus growth was assessed by CFU quantification. (B) Macrophage necrosis was evaluated by AO/EthBr incorporation on days 0, 2, 4 and 6 p.i. at an MOI of 20. Significant differences were observed between the C57BL/6 and P2X7R^{-/-} BMDMs (***p* < 0.01 and ****p* < 0.001). The data represent the means ± SD of samples in triplicate. The data are representative of three separate experiments. (TIF)

Figure S2 Annexin V and PI staining in P2X7R^{-/-} and C57BL/6 BMDMs infected with hypervirulent mycobacteria. C57BL/6 BMDMs were infected with H37Rv *Mtb*, Beijing 1471 *Mtb* and MP287/03 *Mbv* at an MOI of 10. Non-infected BMDMs were stimulated with actinomycin D (2.5 µg/ml) for 6 h as a positive control for apoptosis and with eATP (5 mM) for 24 h as a positive control for necrosis. Photos show BMDM cultures

(200× magnification; bar scales correspond to 100 µm). The data are representative of three separate experiments. (TIF)

Figure S3 Extracellular ATP-induced necrosis in C57BL/6 and P2X7R^{-/-} BMDMs. C57BL/6 and P2X7R^{-/-} BMDMs were cultured for 24 h with 0, 1, 2, 3 and 5 mM eATP. Macrophage necrosis was determined by AO/EthBr incorporation. Images show viable cells (stained with AO) in green and dead cells with permeabilized membranes (stained with EthBr) in red (200× magnification; bar scales correspond to 100 µm). The data are representative of three separate experiments. (TIF)

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Author Contributions

Conceived and designed the experiments: EPA EBL MRDL. Performed the experiments: EPA MRMDa SCMR VRL FMA CCBB EMS EBL. Analyzed the data: EPA EBL MRDL. Contributed reagents/materials/analysis tools: KRB RCS MHH JMA EBL MRDL. Wrote the paper: EPA EBL MRDL.

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APÊNDICE D – IFN- γ -induced priming maintains long-term strain-transcending immunity against blood-stage *Plasmodium chabaudi* malaria.
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IFN- γ –Induced Priming Maintains Long-Term Strain-Transcending Immunity against Blood-Stage *Plasmodium chabaudi* Malaria

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IFN- γ -Induced Priming Maintains Long-Term Strain-Transcending Immunity against Blood-Stage *Plasmodium chabaudi* Malaria

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The mechanism by which protective immunity to *Plasmodium* is lost in the absence of continued exposure to this parasite has yet to be fully elucidated. It has been recently shown that IFN- γ produced during human and murine acute malaria primes the immune response to TLR agonists. In this study, we investigated whether IFN- γ -induced priming is important to maintain long-term protective immunity against *Plasmodium chabaudi* AS malaria. On day 60 postinfection, C57BL/6 mice still had chronic parasitemia and efficiently controlled homologous and heterologous (AJ strain) challenge. The spleens of chronic mice showed augmented numbers of effector/effector memory (T_{EM}) $CD4^+$ cells, which is associated with increased levels of IFN- γ -induced priming (i.e., high expression of IFN-inducible genes and TLR hyperresponsiveness). After parasite elimination, IFN- γ -induced priming was no longer detected and protective immunity to heterologous challenge was mostly lost with >70% mortality. Spontaneously cured mice had high serum levels of parasite-specific IgG, but effector T/T_{EM} cell numbers, parasite-driven $CD4^+$ T cell proliferation, and IFN- γ production were similar to noninfected controls. Remarkably, the priming of cured mice with low doses of IFN- γ rescued TLR hyperresponsiveness and the capacity to control heterologous challenge, increasing the T_{EM} cell population and restoring the $CD4^+$ T cell responses to parasites. Contribution of TLR signaling to the $CD4^+$ T cell responses in chronic mice was supported by data obtained in mice lacking the MyD88 adaptor. These results indicate that IFN- γ -induced priming is required to maintain protective immunity against *P. chabaudi* and aid in establishing the molecular basis of strain-transcending immunity in human malaria. *The Journal of Immunology*, 2013, 191: 5160–5169.

Malaria remains a major health issue, especially in the tropical and subtropical areas of the world. Despite efforts to develop vaccines and antimalarials, the *Plasmodium* species that cause malaria persist, with an increase in the incidence of the disease in endemic regions, spreading to areas where control or eradication had been achieved previously (1). Parasite resistance to antimalarial drugs, inefficiency of vector control policies, and absence of successful vaccination explain the failure to reduce the risks of infection. A complete understanding of the mechanisms underlying the acquisition of protective im-

munity is crucial to outline new strategies to eradicate malaria. For children living in holoendemic areas, naturally acquired immunity appears to be achieved after successive rounds of infection, protects against clinical manifestations of the disease in a strain-specific way, and does not elicit sterile protection against the parasite (2). In adults, acquired immunity can be achieved after a few rounds of infection and elicits strain-transcending, but still not sterile, clinical protection (3). In any case, protective immunity is usually lost in the absence of continued exposure to the parasites (4). This observation raises two important questions: What are the effector mechanisms of the immune system that are lost after removal of the residual parasitemia and why does this loss occur?

Mouse models of *Plasmodium* infection are widely used to investigate the protective immune response to malaria. Among them, *Plasmodium chabaudi* infection is a feasible model to study strain-specific and strain-transcending immunity because of the variety of well-characterized parasite clones and the similarities to the human disease caused by *Plasmodium falciparum* (5). Reinfection with homologous *P. falciparum* parasites in humans or *P. chabaudi* in mice results in significant parasite control, whereas limited protection to heterologous secondary infections has been observed in both cases (3, 6–8). A polymorphism in the merozoite surface protein-1 (MSP-1) gene mediates, to a large degree, strain-specific immunity in blood-stage *P. chabaudi* malaria (9, 10). This gene is also polymorphic among *P. falciparum* strains, possibly as a result of host immune selection pressure (11). In addition, as observed in *P. falciparum* infection (4), resistance to *P. chabaudi* malaria is optimized by an existing infection (12), particularly for strain-transcending immunity that is maintained by the low levels

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Abbreviations used in this article: AMC, age-matched control; CD62L, L-selectin; DC, dendritic cell; iRBC, infected RBC; KO, knockout; MSP-1, merozoite surface protein-1; ODN, oligodeoxynucleotide; qPCR, quantitative PCR; T_E , effector T; T_{EM} , effector memory T; T_M , memory T.

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of subpatent parasitemia encountered in chronic mice (13). On the basis of this finding, it has been suggested that sustained exposure to malarial Ags is required not only for the generation of memory and effector cells but also for their maintenance (14). In fact, the partial loss of protective immunity to homologous challenge observed after elimination of remnant parasitemia is related to a decline in the CD4⁺ memory T (T_M) cell response to parasites (15).

Although much is known about how innate immune recognition affects adaptive immune responses, the role of adaptive immune cells in shaping the innate immune system is largely unexplored. Dendritic cells (DCs) and macrophages are tightly regulated by cytokines to rapidly respond to infections and also to avoid the undesirable effects of excessive activation. The priming of DCs and macrophages by low concentrations of IFN- γ is a potent mechanism by which the innate immune system is optimized, allowing an increased response to several extracellular stimuli, including TLR agonists (16, 17). IFN- γ -induced priming does not actually activate cells but ensures rapid and strong responses to stimuli, which in excess can eventually cause deleterious consequences. The mechanisms underlying macrophage priming involves a complex network of IFN-inducible genes, whose understanding is still limited (18). Recently, it has been shown that the innate immune system is primed by IFN- γ during acute *P. falciparum* and *P. chabaudi* infections and, in consequence, displays an enhanced response to TLR agonists (19), establishing a crucial role for this cytokine in immunity to malaria. However, the contribution of IFN- γ -induced priming to the maintenance of acquired immunity to *Plasmodium* is still unclear. We wondered whether low levels of IFN- γ produced by effector T (T_E) cells and/or effector memory T (T_{EM}) cells in response to chronic parasitemia in infected individuals could prime the innate immune system and, together with parasite-specific Ab, ensure strain-transcending immunity. To investigate this possibility, we evaluated the correlation between the spleen cell response to TLR agonists (LPS and CpG oligonucleotides) and protection against homologous (AS strain) and heterologous (AJ strain) challenge in C57BL/6 mice infected with *P. chabaudi* AS. Our data indicate that IFN- γ -induced priming is required to maintain long-term strain-transcending protective immunity to *P. chabaudi* malaria, a process that optimizes TLR signaling and guarantees the generation/maintenance of T_{E/EM} cells.

Materials and Methods

Mice and parasites

Six- to 8-wk-old C57BL/6, RAG knockout (KO), and MyD88KO (with a C57BL/6 background) female mice (originally from The Jackson Laboratory) were bred under specific pathogen-free conditions at the Isogenic Mice Facility of the Instituto de Ciências Biomédicas at the Universidade de São Paulo. *P. chabaudi* (PcAS and PcAJ strains) was maintained as described elsewhere (20). Because the schizogonic cycle of these parasites depends on the host circadian rhythm (21), the mice were maintained under an inverted light/dark cycle for at least 15 d before infection to access the period adjacent to erythrocyte invasion.

Infections and clinical analysis

For the primary infections, the mice were inoculated i.p. with 1×10^6 infected RBCs (iRBCs). The mice were challenged i.v. with 1×10^8 iRBCs on days 60 and 200 postinfection (p.i.) and monitored daily to determine survival curves. To evaluate subpatent parasitemia, 100 μ l blood from 60-, 200-, or 260-d infected mice were transferred i.v. into RAGKO mice. Parasitemias were quantified by microscopic examination of Giemsa-stained blood smears. Body weight, temperature, and hemoglobin concentration (Hemoglobin kit; Doles) also were assessed in the infected mice.

Ethics statement

All procedures were in accordance with the national regulations of the Conselho Nacional de Saúde and Colégio Brasileiro em Experimentação Animal, with respect to their ethical guidelines for mouse experimentation

and welfare. The protocols were approved by the Comissão de Ética no Uso de Animais of the Instituto de Ciências Biomédicas at the Universidade de São Paulo (São Paulo, Brazil), with permit numbers 0019/2005 and 0036/2007.

Purification of mature iRBCs

Mature iRBCs were obtained from mice with 40–60% parasitemia and predominantly late trophozoites and schizonts. The pellets from 500 μ l heparinized blood were resuspended in 1 ml PBS, pipetted over 5 ml 74% Percoll (GE Healthcare) and centrifuged ($2500 \times g$, acceleration/break of 5/0) for 30 min at room temperature. The top cell layers were collected and washed three times with complete RPMI 1640 medium (supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME, 2 mM L-glutamine, and 1 mM sodium pyruvate). All supplements were purchased from Life Technologies. This purification technique yielded >95% of the purity of mature iRBCs.

In vivo erythrocyte reinvasion assay

Mice were infected i.v. with 5×10^8 mature iRBCs. Blood samples were collected at 1 h intervals and stained with SYTO16 (Molecular Probes, Life Technologies) as described previously (22). Parasitemias were determined by flow cytometry (FACSCalibur; BD Biosciences) with FlowJo software (Tree Star) starting at 30 min p.i. (t₀), when nearly all parasites were late trophozoites or schizonts. The reinvasion index was calculated as the ratio between the parasitemia percentages at the different time points and the t₀ values.

Parasite-specific ELISA

PcAS-specific IgG1 and IgG2a serum levels were quantified by ELISA as described elsewhere (15). Briefly, 96-well flat-bottom microtest plates (Costar) were coated overnight (4°C) with a total PcAS extract (10 μ g/ml). Plates were saturated with 1% BSA for 1 h. After washing, 100 μ l of the mouse serum samples (diluted from 1/50 to 1/12,800) were added and left for 2 h at room temperature. The assays were developed by adding a goat anti-mouse IgG1 or IgG2a peroxidase-conjugated Abs (Southern Biotechnology Associates) for 1 h. After washing, 100 μ l tetramethylbenzidine (Invitrogen Life Technologies) was added to each well, and 15 min later, the absorbance values were quantified using a Spectra Max 190 spectrophotometer (Molecular Devices) with a 650-nm wavelength filter. The Ab level in each serum sample is expressed as the reciprocal of the end-point titer, which we defined as the lowest dilution that equals the background OD.

Phenotypic analysis of spleen cells

Splenic cell suspensions were incubated in lysis buffer (40 mM NH₄Cl and 4.2 mM Tris [pH 7.4]) for 5 min (4°C) to eliminate RBCs. The splenocytes (1×10^6) were stained using the appropriate combinations of FITC-, PE-, PE-Cy7-, PerCP-, or allophycocyanin-labeled mAbs. CD4⁺ T cells were stained with mAbs against CD4 (H129.19 or GK1.5), CD27 (LG.3A10), CD44 (IM7), L-selectin (CD62L) (MEL-14), and CD127 (IL-7R α , SB/199). DCs, macrophages, monocytes, and neutrophils were stained with mAbs against CD11b (M1/70), CD11c (HL3), I-A^b (AF6), F4/80 (BM8), Ly6C (AL-21), and Ly6G (1A8). All mAbs were purchased from BD Pharmingen with the exception of F4/80 mAb, which was purchased from eBioscience. The cells were analyzed by flow cytometry (FACSCanto; BD Biosciences) with FlowJo software.

CFSE proliferation assay

The proliferative CD4⁺ T cell response to iRBCs was measured as described previously (15). Briefly, 3×10^7 cells/ml (resuspended in PBS with 0.1% BSA) were incubated with CFSE (Molecular Probes) at a final concentration of 5 μ M for 20 min at 37°C. The cells (1×10^6) were then cultured in 96-well plates (Costar) with 3×10^6 iRBCs, 10 μ g/ml of the recombinant 19-kDa fraction of MSP-1 from PcAS (MSP-1₁₉, produced in our molecular biology facility) or medium alone for 72 h at 37°C in a 5% CO₂ atmosphere. After incubation, the cells were stained with an allophycocyanin-labeled mAb against CD4 and analyzed by flow cytometry (FACSCanto) with FlowJo software.

IFN- γ detection

Splenocytes (10^6) were cultured in 96-well plates (Costar) with 3×10^6 iRBCs, 10 μ g/ml MSP-1₁₉, 1 μ g/ml LPS (strain 0111:B4 from *Escherichia coli*; Sigma-Aldrich), 10 μ g/ml CpG oligodeoxynucleotide (ODN) 1826 (Coley Pharmaceutical Group), or medium alone for 72 h at 37°C in a 5% CO₂ atmosphere. IFN- γ was quantified in the cell supernatants using the OptEIA IFN- γ kit (BD Biosciences).

Anti-IFN- γ treatment

Four doses (0.5 mg/mouse) of depleting mAbs against IFN- γ (H22; eBioscience) or Armenian hamster IgG isotype control (eBioscience) were injected i.v. into C57BL/6 mice every 2 d starting 52 d p.i. This depletion strategy was based on a previous study (23).

DC purification

Spleen cells (1×10^8) were incubated with anti-CD11c and anti-*Pan* DC microbeads (Miltenyi Biotec) diluted in PBS with 0.5% BSA and 2 mM EDTA (Invitrogen) for 30 min at 4°C. The cells were then sorted using LS columns (Midi MACS; Miltenyi Biotec). The positive fraction showed >90% CD11c⁺T-A⁺ cells.

Quantitative PCR for IFN-inducible genes

RNA was extracted from DCs (5×10^6) with TRIzol (Invitrogen) (24) and quantified with a NanoDrop device (Eppendorf). cDNA was prepared with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The quantitative PCR (qPCR) reactions were performed in an ABI 7500 Real-Time PCR device (Applied Biosystems) using the SyBR Green kit from Applied Biosystems. The expression of the *stat3*, *irf7*, *ifnar*, *tlr2*, *tlr4*, *tlr9*, *md2*, *cd36*, *il1b*, and β -actin genes was analyzed using the 2 $-\Delta\Delta$ threshold cycle algorithm. The primers used (*stat3*, 5'-GACCCGCCAA-CAAATTAAGA-3' [forward] and 5'-TCGTGGTAAACTGGACACCA-3' [reverse]; *irf7*, 5'-CCAGTTGATCCGCATAAGGT-3' [forward] and 5'-GAGCCAGCATTTTCTCTTG-3' [reverse]; *ifnar*, 5'-GCCCTGCTGATAAGACCAG-3' [forward] and 5'-GTGGGAAGCACATGACAC-3' [reverse]; *tlr2*, 5'-TGCTTTCCTGCTGGAGATT-3' [forward] and 5'-TGTAACGCAACAGCTTCAGG-3' [reverse]; *tlr4*, 5'-GCTTTCACCTC-TGCCTTAC-3' [forward] and 5'-GAAACTGCCATGTTTGAGCA-3' [reverse]; *tlr9*, 5'-TTCCTGCCGTGACTAATCT-3' [forward] and 5'-TGAGGACACACGGGTATGAA-3' [reverse]; *md2*, 5'-CTCCATAGAGT-TGCCGAAGC-3' [forward] and 5'-GCGGTGAATGATGGTGAAAT-3' [reverse]; *cd36*, 5'-TCGGATCTGAAATCGACCTT-3' [forward] and 5'-CACAGGCTTCTCTTCTTG-3' [reverse]; *il1b*, 5'-CAGGCAGGCAG-TATCACTCA-3' [forward] and 5'-TAATGGGAACGTCACACACC-3' [reverse]; and β -actin, 5'-CCTGAACCCTAAGGCCAAC-3' [forward] and 5'-GCCTGGATGGCTACGTACA-3' [reverse]) were designed using the Primer3 software (DOI: <http://frodo.wi.mit.edu/primer3/>) and purchased from Invitrogen.

IFN- γ -induced priming

Four doses (15 ng/kg; 10,000 U/mouse) of rIFN- γ (mouse rIFN- γ expressed in *E. coli*; PeproTech) were injected i.v. into C57BL/6 mice every 2 d starting 192 d p.i. This IFN- γ priming dose was established in a previous study (25).

Statistical analysis

Statistical significance was analyzed with Prism 5 software (GraphPad) using ANOVA and a Tukey's multiple comparison test or Student *t* test where appropriate. The existence of a normal distribution was confirmed using the Kolmogorov-Smirnov test. Differences were considered statistically significant at $p < 0.05$.

Results

The clearance of residual parasitemia leads to partial loss in protective immunity to heterologous PcAJ parasites

The infection of C57BL/6 mice with *PcAS* led to acute parasitemia and its subsequent control (Fig. 1A). After day 30 p.i., parasites were no longer detected in the blood smears, and subpatent parasitemia was assessed by blood transfer to RAGKO mice. Low levels of chronic parasitemia remained up to day 60 p.i., but parasites were not detected in the bloodstream on days 200 and 260 p.i. (Fig. 1B). To evaluate whether these mice maintained protective immunity to homologous and heterologous parasites, they were challenged i.v. with a high inoculum (1×10^8 iRBCs) of *PcAS* or the lethal *PcAJ* strain (10). Although mice on day 60 p.i. (mice_{60d}) efficiently eliminated both parasite challenges and survived, mice on day 200 p.i. (mice_{200d}) developed low parasitemias over the first 3 d following *PcAS* reinfection and failed to control the growth of *PcAJ*, resulting in >70% mortality (Fig. 1C, 1D and 1F, 1G). To evaluate whether long-term acquired immu-

nity requires de novo activation of the immune system, we also analyzed the capacity of mice to inhibit the first round of erythrocyte invasion when challenged i.v. with purified late trophozoites and schizonts (5×10^8 iRBCs). For both *PcAS* and *PcAJ*, the generation of new ring forms was blocked in mice_{60d} (Fig. 1E, 1H). In contrast, the efficiency of erythrocyte invasion by *PcAJ* was similar in mice_{200d} and naive controls. Because mice_{200d} still retained the ability to inhibit erythrocyte invasion by *PcAS*, we concluded that the clearance of chronic parasitemia is associated with the loss of immunological effector mechanisms that are particularly required for protection against heterologous *PcAJ* parasites.

The partial loss in protective immunity to heterologous PcAJ parasites may result from a reduction in CD4⁺ T_E/T_{EM} cell populations

The inactivation of the innate and/or acquired immune systems may account for the decline in protective immunity to heterologous *PcAJ* parasites following clearance of chronic parasitemia. Humoral immunity does not appear to be involved in the partial loss of protective immunity, because parasite-specific IgG1 and IgG2a serum titers were maintained or even increased on day 200 p.i. compared with day 60 p.i. (Fig. 2A). Therefore, we evaluated subsets of CD4⁺ T cells from the spleens of these mice based on a recent study that defined CD4⁺ T_E cells, CD4⁺ T_{EM} cells, and CD4⁺ T_{CM} cells (26), as described in Supplemental Fig. 1. We found a significant increase in the CD4⁺ T_E/T_{EM} cell numbers per spleen in mice_{60d}, whereas these subsets had similar numbers per spleen in mice_{200d} and the age-matched controls (AMCs); only T_{CM} cells were maintained at comparable numbers in mice_{60d} and mice_{200d} (Fig. 2B). Indeed, on day 60 p.i., most CD4⁺ T_M cells had an effector memory phenotype in which CD62L was expressed at low levels. The reduction of the CD4⁺ T_E/T_{EM} cell populations observed after parasite elimination was accompanied by a loss of the ability of CD4⁺ T cells to proliferate and produce IFN- γ in response to iRBCs and to proliferate in response to MSP-1₁₉ (Fig. 2C, 2D). The need for IFN- γ to maintain the protection against heterologous parasites in chronic mice was investigated by treating mice_{60d} with anti-IFN- γ mAbs. A partial loss of the ability to control *PcAJ* challenge was observed in IFN- γ -depleted mice_{d60} when compared with mice_{d60} who were treated with the isotype control (Fig. 2E). This effect was accompanied by a reduction in body temperature (Fig. 2F).

IFN- γ -induced priming of the innate immune system subsides after the elimination of residual parasitemia

The impaired CD4⁺ T cell response to iRBCs may not fully explain the partial loss of protective immunity to heterologous challenge observed after the elimination of chronic parasitemia. This is suggested by the fact that iRBC-stimulated CD4⁺ T cells took a few days to secrete IFN- γ , but chronic and cured mice differed in their ability to promptly inhibit the invasion of new erythrocytes (Fig. 1H). Therefore, we postulated that the clearance of residual parasitemia reduces the extent of IFN- γ -induced priming, a potent mechanism by which the optimization of the innate immune system is achieved (17). Because the molecular pathways underlying IFN- γ -induced priming involve the activation of TLRs and other IFN-inducible genes (18), we compared the expression of *tlr2*, *tlr4*, *tlr9*, *md2*, *cd36*, *il1b*, *stat3*, *irf7*, and *ifnar* mRNAs in splenic DCs. This population is located in the red pulp of the spleen, where it is in direct contact with iRBCs, and also in the white pulp, contributing to both parasite clearance and T cell activation (27, 28). We observed a significant increase in the expression of these mRNAs in DCs from mice_{60d}, with the exception of *ifnar* mRNA, whereas there was no difference between

FIGURE 1. Parasitemias and survival curves of C57BL/6 mice that were primarily infected with *PcAS* and secondarily challenged on days 60 and 200 p.i. with *PcAS* or *PcAJ*. (A) Parasitemia curve for C57BL/6 mice infected i.p. with 1×10^6 iRBCs. (B) Parasitemias for RAGKO mice transferred with 100 μ l blood from C57BL/6 mice on days 60, 200, and 260 p.i. and monitored on days 7 and 14 after blood transfer. (C) Parasitemia curves for AMCs, mice_{60d}, and mice_{200d} challenged i.v. with 1×10^8 *PcAS* iRBCs. (D) Survival curves for mice described in (C). (E) Reinfection index for AMCs, mice_{60d}, and mice_{200d} challenged i.v. with 5×10^8 *PcAS* iRBCs (late trophozoites and schizonts). (F) Parasitemia curves for AMCs, mice_{60d}, and mice_{200d} challenged i.v. with 1×10^8 *PcAJ* iRBCs. (G) Survival curves for the mice described in (F). (H) Reinfection index for AMCs, mice_{60d} and mice_{200d} challenged i.v. with 5×10^8 *PcAJ* iRBCs (late trophozoites and schizonts). (A–H) Data show the mean $\pm$ SD ($n = 4$ –6) of one representative experiment out of three. * $p < 0.05$, significant differences were analyzed between the indicated groups (C–H).

mice_{200d} and the AMCs (Fig. 3A). Because the high expression of TLR mRNAs during acute *PcAS* malaria has been associated with augmented IFN- γ production following stimulation with TLR agonists (19), we then analyzed the spleen cell response to TLR4 and TLR9 agonists (LPS and CpG, respectively). In fact, spleen cells from mice_{60d} produced increased levels of IFN- γ in response to LPS and CpG, whereas there was no significant response for mice_{200d} and the AMCs (Fig. 3B).

MyD88-mediated signaling is required for CD4⁺ T cell responses and the IFN- γ -induced priming of splenic DCs in chronic mice

Our previous results correlate the persistence of strain-transcending immunity to blood-stage *P. chabaudi* malaria with the maintenance of increased CD4⁺ T_E/T_{EM} populations and TLR hyperresponsiveness. Thus, it is reasonable to speculate that TLR signaling contributes to generate and/or maintain CD4⁺ T_E/T_{EM} cells and consequently to increase the IFN- γ -induced priming of splenic DCs. In agreement with this hypothesis, mice lacking the TLR signaling MyD88 adaptor had higher chronic parasitemia than C57BL/6 mice (Fig. 4A). Despite the fact that similar numbers of CD4⁺ T_E cells were found in the spleens of C57BL/6 and MyD88KO mice_{60d}, there was a reduction in the CD4⁺ T_{EM} cell population in the latter group (Fig. 4B). Accordingly, the CD4⁺ T cell responses to parasites were impaired in MyD88KO

mice_{60d}, as shown by the diminished in vitro proliferation and IFN- γ production following iRBC or MSP-1₁₉ stimulation (Fig. 4C, 4D). The absence of the MyD88 adaptor also led to reduced mRNA expression of the IFN-inducible genes *stat3*, *irf7*, and *ifn γ* in splenic DCs (Fig. 4E), indicating that IFN- γ -induced priming also was impaired. Thus, intact TLR signaling machinery appears to be crucial for maintaining CD4⁺ T cell responses, which ultimately leads to IFN- γ -induced priming and optimizes the control of chronic parasitemia.

In vivo priming with IFN- γ restores the protective immunity to heterologous PcAJ parasites in cured mice

Our previous results suggest that the elimination of chronic parasitemia leads to a reduction in the IFN- γ -induced priming of the innate immune system and consequently to defective control of secondary infection, particularly with heterologous *PcAJ* parasites. To investigate whether in vivo priming with IFN- γ can restore the immune system activation status observed in the presence of residual parasitemia, starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four suboptimal (priming) i.v. doses of rIFN- γ (Fig. 5A), as established previously (25). To confirm that this treatment primes the innate immune system, spleen cells from IFN- γ -primed mice_{200d} produced significantly higher levels of IFN- γ in response to LPS and CpG compared with unprimed mice_{200d} (Fig. 5B). Accordingly, IFN- γ -primed mice_{200d}

FIGURE 2. Parasite-specific IgG, CD4⁺ T cell subsets, CD4⁺ T cell responses to iRBCs, and MSP-1₁₉ in chronic and cured C57BL/6 mice. Influence of IFN- γ depletion in parasitemia and mouse temperature. C57BL/6 mice were analyzed on days 60 and 200 p.i. with 1×10^6 iRBCs in comparison with the AMCs. (A) Parasite-specific IgG1 and IgG2a serum titers, as determined by ELISA. (B) Numbers of CD4⁺ T_{CM} (CD3⁺CD4⁺CD44^{hi}IL-7R α ⁺CD62L^{hi}), T_{EM} (CD3⁺CD4⁺CD44^{hi}IL-7R α ⁺CD62L^{lo}), and T_E (CD3⁺CD4⁺CD44^{hi}IL-7R α ⁺CD62L^{lo}) cells per spleen, as determined by flow cytometry. (C) Percentages of proliferating (CFSE^{low}) CD4⁺ T cells stimulated for 72 h with iRBCs (1 T cell/3 iRBCs) or MSP-1₁₉ (10 μ g/ml), as determined by flow cytometry. (D) IFN- γ concentrations in the supernatants of the cell cultures described in (C), as measured by ELISA. (E) Parasitemia curves for mice_{60d} that were treated with anti-IFN- γ mAbs or the isotype control, and subsequently challenged i.v. with 1×10^8 *PcAJ* iRBCs. (F) Body temperatures in mice described in (E). (A–F) Data show the mean $\pm$ SD ($n = 3$ –6) of one representative experiment out of three. * $p < 0.05$, significant differences were analyzed between mice_{60d} and mice_{200d} (A–D). * $p < 0.05$, significant differences were analyzed between anti-IFN- γ -treated mice_{60d} and control IgG-treated mice_{60d} in (E) and (F). ND, Not detected.

showed an improved capacity to control a secondary infection with *PcAS* (Fig. 6A) and *PcAJ* (Fig. 5C). Moreover, in mice_{200d} challenged with *PcAJ*, IFN- γ treatment allowed 100% survival and attenuated the clinical manifestations of the disease (i.e., a reduction

in body temperature and blood hemoglobin concentration) (Fig. 5D–F). Other parameters, such as body weight and hepatic damage, were similar to the AMCs in both groups of challenged mice_{200d} (data not shown). The IFN- γ -primed AMCs presented with an in-

FIGURE 3. IFN-inducible gene expression and the IFN- γ response to TLR agonists in chronic and cured C57BL/6 mice. C57BL/6 mice were analyzed on days 60 and 200 p.i. with 1×10^6 iRBCs in comparison with AMCs. (A) Fold increase over control (0 d) samples of *stat3*, *ifnar*, *irf7*, *tlr2*, *tlr4*, *tlr9*, *md2*, *cd36*, and *il1b* mRNAs, as evaluated by qPCR in splenic DCs and analyzed by the $2^{-\Delta\Delta}$ threshold cycle algorithm. (B) IFN- γ concentrations measured by ELISA in supernatants from spleen cell cultures stimulated with LPS (10 μ g/ml) or CpG ODN 1826 (10 μ g/ml) for 72 h. Data show the mean $\pm$ SD ($n = 5$ –6) of one representative experiment out of three. * $p < 0.05$, significant differences were analyzed between mice_{60d} and mice_{200d} (A, B).

FIGURE 4. Parasitemias, CD4⁺ T cell subsets, and CD4⁺ T cell responses to iRBCs and MSP-119 and IFN-inducible gene expression in C57BL/6 and MyD88KO mice primarily infected with *PcAS*. C57BL/6 and MyD88KO mice infected with 1×10^6 iRBCs were analyzed in comparison with AMCs. (A) Parasitemia curves. (B) Numbers of CD4⁺ T_{CM} (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺CD62L^{hi}CD27⁺), T_{EM} (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺CD62L^{lo}) and T_E (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺CD62L^{lo}) cells per spleen on day 60 p.i., as determined by flow cytometry. (C) Percentages of proliferating (CFSE^{low}) CD4⁺ T cells from the spleens of mice_{60d} stimulated for 72 h with iRBCs (1 T cell/3 iRBCs) or MSP-119 (10 μg/ml), as determined by flow cytometry. (D) IFN-γ concentrations measured by ELISA in the supernatants from the cell cultures described in (C). (E) Fold increase over control (0 d) samples of *stat3*, *irf7*, and *ifnar* mRNAs, as evaluated by qPCR and analyzed by the $2^{-\Delta\Delta}$ threshold cycle algorithm. Data show the mean $\pm$ SD ($n = 4-6$) of one representative experiment out of three. * $p < 0.05$, significant differences were analyzed between the C57BL/6 and MyD88KO mice (A-E). ND, Not detected.

crease in spontaneous and LPS-stimulated IFN-γ production (Fig. 6B), but these mice were not protected against primary infection with *PcAS* (Fig. 6C). These data indicate that IFN-γ-induced priming restores strain-transcending immunity in mice that have been previously exposed to parasites.

In vivo priming with IFN-γ restores the CD4⁺ T cell response to parasites in cured mice

The relevance of TLR signaling in the development of acquired immunity to *P. chabaudi* is suggested by our data showing that enhanced parasitemias in chronic MyD88KO mice are associated with low numbers of T_{EM} cells and reduced iRBC-driven CD4⁺ T cell proliferation and IFN-γ production. Because our previous results show that low doses of IFN-γ restore both TLR hyper-responsiveness and strain-transcending immunity, we sought to investigate whether this treatment also improves CD4⁺ T cell responses in mice_{200d}. Our data show a decrease in CD62L expression in splenic CD4⁺ T_M cells from IFN-γ-primed mice_{200d}, indicating a shift from CD4⁺ T_{CM} cells to CD4⁺ T_{EM} cells (Fig. 7A). This shift resulted from an increase in the T_{EM} cell population and a decrease in the T_{CM} cell population compared with unprimed mice_{200d}, whereas the T_E cell population was not modified (Fig. 7B). This effect was not associated with changes in the population dynamics of splenic phagocytes; IFN-γ-primed mice_{200d} and unprimed mice_{200d} presented with similar numbers per spleen of DCs, red pulp F4/80⁺ macrophages, inflammatory LyC6^{hi} monocytes, and neutrophils (Supplemental Fig. 2). Furthermore, in vivo priming with IFN-γ reestablished the CD4⁺ T cell response to iRBCs and MSP-119 in terms of proliferation (Fig. 7C, 7D) and IFN-γ production (Fig. 7E). The treatment of AMCs with IFN-γ induced a slight increase in spontaneous CD4⁺ T cell proliferation, but it had no effect on the CD4⁺ T cell proliferation and IFN-γ production in response to iRBCs (Supplemental Fig. 3). We conclude that IFN-γ-induced priming restores the ability of CD4⁺ T_M cells to respond to parasites in mice that have already eliminated their chronic parasitemia.

Discussion

In this study, we took advantage of the experimental model of *P. chabaudi* infection to determine the reason why strain-transcending immunity against malaria is mostly lost in the absence of continued exposure to parasites. Our study confirms the notion that a decline in the CD4⁺ T_M cell response to the parasite, but not in parasite-specific IgG, leads to failure to maintain long-term protective

FIGURE 5. TLR responsiveness, parasitemias, survival curves, and clinical manifestations of the disease in cured C57BL/6 mice primed with IFN- γ and challenged with heterologous *PcAJ* parasites. Starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four i.v. doses (15 ng/kg; 10,000 U/mouse) of rIFN- γ and then challenged i.v. with 1×10^8 *PcAJ* iRBCs. IFN- γ -primed mice were compared with unprimed mice and AMCs. **(A)** Experimental design. **(B)** IFN- γ concentrations measured by ELISA in spleen cell supernatants of unchallenged mice stimulated with LPS (10 μ g/ml) or CpG ODN 1826 (10 μ g/ml) for 72 h. **(C)** Parasitemia curves. **(D)** Survival curves. **(E)** Mouse temperatures. **(F)** Hemoglobin concentrations. Data show the mean $\pm$ SD ($n = 4-6$) of one representative experiment out of three. * $p < 0.05$, significant differences were analyzed between IFN- γ -primed and unprimed mice.

immunity to *PcAS* malaria (15). Furthermore, our results show that the immunological effector mechanism that ensures strain-

transcending immunity to *P. chabaudi* acts almost immediately after parasite challenge, inhibiting the first round of erythrocyte

FIGURE 6. Parasitemias and CD4 $^+$ T cell responses in cured C57BL/6 mice and AMCs primed with IFN- γ and challenged with homologous *PcAS* parasites. Starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four i.v. doses (15 ng/kg; 10,000 U/mouse) of rIFN- γ . Noninfected AMCs were treated using the same protocol. IFN- γ -primed mice were compared with unprimed mice. **(A)** Parasitemia curves for mice_{200d} challenged i.v. with 1×10^8 *PcAS* iRBCs. **(B)** LPS-stimulated IFN- γ production in noninfected AMCs. **(C)** Parasitemia curves for AMCs challenged i.p. with 1×10^6 *PcAS* iRBCs. Data show the mean $\pm$ SD ($n = 4-6$) of one representative experiment out of three. * $p < 0.05$, significant differences were analyzed between IFN- γ -primed and unprimed mice (A-C).

FIGURE 7. CD4⁺ T cell subsets and responses to iRBC and MSP-1₁₉ in cured C57BL/6 mice primed with IFN-γ. Starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four i.v. doses (15 ng/kg; 10,000 U/mouse) of rIFN-γ. IFN-γ-primed mice were analyzed on day 200 p.i. in comparison with unprimed mice and AMCs. **(A)** CD62L expression in splenic T_M (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺) cells, as determined by flow cytometry. **(B)** Numbers of CD4⁺ T_{CM} (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺CD62L^{hi}CD27⁺), T_{EM} (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺CD62L^{lo}), and T_E (CD3⁺CD4⁺CD44^{hi}IL-7Rα⁺CD62L^{lo}) cells per spleen. **(C)** Representative histograms of CFSE staining of splenic CD4⁺ T cells stimulated for 72 h with iRBCs (1 T cell/3 iRBCs), as obtained by flow cytometry. **(D)** Percentages of proliferating (CFSE^{low}) CD4⁺ T cells stimulated for 72 h with iRBCs (1 T cell/3 iRBCs) or MSP-1₁₉ (10 μg/ml), as determined by flow cytometry. **(E)** IFN-γ concentrations measured by ELISA in the supernatants of the cell cultures described in (C). Data show the mean ± SD (*n* = 5–6) of one representative experiment out of three. **p* < 0.05, significant differences were analyzed between IFN-γ-primed and unprimed mice (B, D, E).

invasion prior to de novo T cell activation. Theoretically, CD4⁺ T_M cells can activate the innate immune response via IFN-γ or independent of this cytokine, as recently reported for influenza A virus infection (29). Our data corroborate the idea that persistence of the parasite maintains a pool of CD4⁺ T_E/T_{EM} cells that is able to produce low levels of IFN-γ (26). It also shows that the continuous priming of the innate immune system by IFN-γ, a process that is evidenced in chronic mice by the increased expression of IFN-inducible genes in splenic DCs and TLR hyperresponsiveness in splenocytes, allows the control of the heterologous challenge and avoids the development of the clinical manifestations of the disease and consequent animal death. These results may explain why clinical and sterile immunity against malaria are likely to be excluding events.

The IFN-γ-induced enhancement of TLR expression and function was previously shown during acute human and mouse malaria and was considered to be a booster for antimalarial responses when innate immunity plays a major role (19). This study reports a huge IFN-γ response to TLR agonists by splenocytes 1 wk after *P. chabaudi*

infection that subsides within 2 wk; the levels of IFN-γ produced in the fourth week p.i. are comparable with those described in this study in chronic mice. These findings recapitulate the notion that the spleen CD4⁺ T cell response to *P. chabaudi* malaria develops in two phases concomitantly with acute and chronic parasitemias, in which the early phase is intense and short lasting, rapidly providing large amounts of proinflammatory cytokines, and the late phase is characterized by small peaks of IFN-γ production (30). Our data indicate that the continuity of IFN-γ-induced priming of the innate immune system during the late infection is required to control heterologous parasites, against which humoral immunity is not optimized because of MSP-1 gene polymorphisms (9, 10). It is important to note that, in our study, in vivo priming with low doses of IFN-γ protects against parasite challenge in only previously infected mice but not in naive mice, suggesting that cooperation with other immunological effector mechanisms, such as Ab against conserved epitopes in parasite Ags (5), is required to ensure strain-transcending immunity to *P. chabaudi* malaria.

The immunological effector mechanisms enhanced by IFN- γ -induced priming are vast, as indicated by the diversity of the IFN-inducible genes that are highly expressed following macrophage treatment with low doses of IFN- γ (17, 18). Our results that correlate the expression levels of *tlr2*, *tlr4*, *tlr9*, *md2*, and *cd36* mRNAs in splenic DCs from chronic and cured mice with the ability to control heterologous challenge implicate these molecules in the maintenance of strain-transcending immunity to malaria. Considering the existence of several TLR ligands in *Plasmodium*, such as the GPI anchor, which is recognized by the TLR2/TLR1 complex with the contribution of TLR4 (31, 32), and parasite DNA, which is recognized by TLR9 (33), the long-lasting TLR hyperresponsiveness in chronic mice maintains the capacity of macrophages, DCs, and other TLR-bearing cells to recognize iRBCs and merozoites. In fact, it has been shown that DC activation not only requires direct cell-to-cell contact and internalization of iRBCs by DCs but also involves TLR4, TLR9, MyD88, and signaling via NF- κ B (27). Moreover, TLR3 and TLR9 promote bacterial uptake by murine and human macrophages through the induction of a phagocytic gene program, which also is induced by TLR4 and TLR5 (34, 35). The class B scavenger receptor CD36, another molecule that shows enhanced mRNA expression in splenic DCs from chronic mice but not from cured mice, mediates the opsonin-independent phagocytosis of iRBCs by monocytes and DCs (36). The binding of iRBCs to these cells is thought to occur through the recognition of externalized phosphatidylserine or *P. falciparum*-encoded erythrocyte membrane protein 1 (37).

In contrast, the high expression of *stat3* mRNA in splenic DCs from chronic mice indicates that IFN- γ -induced priming of the innate immune system is a fine-tuned process that is regulated during the late infection by feedback inhibitory loops, such as those mediated by IL-10, STAT3, and suppressor of cytokine signaling 1 (18). It has been known for quite a long time that exposure to TLR agonists leads to the development of tolerance through a blockade of the corresponding signal transduction pathways (38–40). Tolerance almost certainly occurs in patients from holoendemic areas of malaria that do not exhibit the signs and symptoms of the disease despite the presence of blood parasites (41, 42). Likewise, body temperature and weight are normalized after the control of acute parasitemia in *PcAS*-infected mice (5). In agreement with the concept that stimulatory and regulatory molecular pathways coexist during *PcAS* malaria, CD4⁺ T_{CM} cells coexpressing IFN- γ and IL-10 have a major role in both protection and the control of the clinical manifestations of the disease (43).

Another important feature of IFN- γ -induced priming observed in this study is its effect on the CD4⁺ T_M cell subsets of cured mice, leading to a shift from CD4⁺ T_{CM} cells to CD4⁺ T_{EM} cells and restoring the proliferative and/or IFN- γ responses to iRBCs and TLR agonists. The need for cognate Ag recognition for the maintenance and survival of CD4⁺ T_M cells has been discussed recently for malaria (14) as well as for other diseases (44, 45). It is possible that the persistence of parasite Ags maintains parasite-specific CD4⁺ T_{CM} cells in cured mice; IFN- γ -induced priming shifts this population to CD4⁺ T_{EM} cells that promptly respond to iRBCs and TLR agonists. Alternatively, the IFN- γ -induced priming may rescue cross-reactive CD4⁺ T_{CM} cells that are driven by Ags from other microbes, as proposed in a model for development of severe *P. falciparum* malaria in nonimmune adults (46). Despite that both mechanisms can contribute to restore the CD4⁺ T_M cell responses following the IFN- γ treatment, the CD4⁺ T cell proliferation and IFN- γ production in response to iRBCs are considerably improved in cured mice but not in AMCs. This finding supports the idea that parasite-specific CD4⁺ T_M cells are main participants in the proliferative and IFN- γ responses that are re-

stored by treating cured mice with IFN- γ . A further indication of the parasite-specific nature of these CD4⁺ T cells is that IFN- γ treatment also increases the proliferative response to MSP-1₁₉ in cured mice. The exact mechanism by which IFN- γ maintains CD4⁺ T_M cells in an effector–memory state is still not well understood. We hypothesize that the reestablishment of the CD4⁺ T_M cell response to iRBCs is, at least in part, an indirect effect of the IFN- γ -induced TLR hyperresponsiveness of DCs. This possibility is supported by our data from MyD88KO mice indicating that TLR signaling also contributes to maintaining the CD4⁺ T_{EM} cell responses during chronic infection, which ultimately leads to IFN- γ -induced priming and helps to control the reminiscent parasitemia. This increased chronic parasitemia in MyD88KO mice did not cause animal death in our study, as reported previously (26, 47).

Altogether, our study shows that sustained IFN- γ -induced priming of the innate immune system is required to maintain host surveillance and consequently strain-transcending immunity to blood-stage *P. chabaudi* malaria. It also suggests that the loss of protection in spontaneously cured mice results from the interruption of a positive cycle in which continuous exposure to parasites maintains the pool of CD4⁺ T_E/T_{EM} cells that are able to produce priming doses of IFN- γ and IFN- γ -induced priming of the innate immune system contributes to sustain the CD4⁺ T_E/T_{EM} cell responses to the parasites. These results may help to explain why the immunity against *Plasmodium* is rapidly lost when the parasites are eliminated from the hosts, providing a molecular basis for strain-transcending immunity in human malaria. The knowledge that IFN- γ -induced priming of the innate immune system contributes to maintain strain-transcending immunity may have important implications in development and boosting immunization strategies against malaria.

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Disclosures

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