MAÍRA FELONATO

O papel das células T reguladoras na paracoccidioidomicose pulmonar de camundongos susceptíveis e resistentes ao *Paracoccidioides brasiliensis*

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.

São Paulo
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Área de concentração:
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Orientadora:
Profa. Dra. Vera Lúcia Garcia Calich

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **76** nas fls. **7** do livro **2** para uso de animais em experimentação, sob a responsabilidade da Profa. Dra. Vera Lúcia Garcia Calich, Coordenadora da Linha de Pesquisa "***Paracoccidioidomicose pulmonar. Fatores do fungo e do hospedeiro que influenciam a resposta imune e a gravidade da doença***" do qual participou(aram) o(s) aluno(s): **Adriana Pina,Laura Raquel Rios Ribeiro e Simone Bernardino** está de acordo com os Princípios Éticos de Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela *COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL* (CEEA) em **23.11.2004.**

São Paulo, 24 de novembro de 2004.

Profa. Dra. Marília C. Leite Seelaender
Coordenadora da CEEA

Prof. Dr. Francisco Carlos Pereira
Secretário da CEEA

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COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

Decl. CEEA.014/2005

DECLARAÇÃO

Em adendo ao Certificado 76/04/CEEA, aprovo a inclusão da aluna: **Maíra Felonato** ao protocolo intitulado: "*Paracoccidioidomicose pulmonar. Fatores do fungo e do hospedeiro que influenciam a resposta imune e a gravidade da doença*", para uso da espécie animal utilizado e métodos semelhantes, mediante solicitação da Profa. Dra. Vera Lúcia Garcia Calich, responsável pela linha de Pesquisa.

São Paulo, 05 de agosto de 2005.

Profa. Dra. Marília Cerqueira L. Seelaender
Coordenadora da CEEA- ICB/USP

Aos meus familiares, em especial ao meu marido Douglas, aos meus pais Suely e Natalino e às minhas irmãs Mariana e Marina com todo meu amor. Obrigada por fazerem parte de minha vida e por acreditarem em mim sempre.
Tudo sempre foi e será por vocês.

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Quero, um dia, dizer às pessoas que nada foi em vão...
que o amor existe, que vale a pena se doar às amizades e às pessoas,
que a vida é bela sim e que eu sempre dei o melhor de mim...
e que valeu a pena.

(Mário Quintana)

RESUMO

FELONATO, M**. O papel das células T reguladoras na paracoccidioidomicose pulmonar de camundongos susceptíveis e resistentes ao *Paracoccidioides brasiliensis***. 2011 150 f. Dissertação (Doutorado em Imunologia) ï Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2011.

O balanço entre imunidade e tolerância é importante para manter a homeostase do organismo, e muitos mecanismos são utilizados para manter a resposta imune sob controle. Um dos mecanismos de tolerância periférica que vem sendo muito estudado é aquele mediado por células T reguladoras (Treg). Estas células são caracterizadas por expressarem grande quantidade do marcador CD25 além do fator de transcrição FoxP3 ñforkhead Foxp3ò. Na paracoccidioidomicose (PCM) os processos de imunossupressão controlados por células Treg são pouco conhecidos, por isso, resolvemos estudar o papel desempenhado por estas células nos fenômenos de resistência e susceptibilidade genética dos hospedeiros contra o fungo. Para isto, estudamos a importância da célula Treg na PCM através da depleção de células CD25 em camundongos A/J e B10.A. Paralelamente um grupo controle recebeu dose equivalente de Ig de rato. Estudamos o efeito da depleção na gravidade da infecção pulmonar, no padrão de citocinas produzido e no afluxo celular após a infecção pelo *Paracoccidioides brasiliensis*. Primeiramente verificamos que camundongos A/J normais e infectados apresentavam maior número e maior atividade de células T supressoras que os camundongos B10.A. Observamos que os camundongos A/J e B10.A tratados apresentaram redução significativa no número de fungos recuperados no pulmão quando comparados aos seus grupos controle. Na décima semana o tratamento não alterou a carga fúngica do fígado dos camundongos A/J, mas reduziu drasticamente a infecção deste órgão nos camundongos B10.A. Quando comparados aos camundongos A/J e B10.A controle, os camundongos depletados apresentaram aumento dos níveis das citocinas com padrão Th1, Th2 e Th17 após 10 semanas de infecção. Na segunda semana pós infecção, os camundongos A/J tratados apresentaram no pulmão número aumentado de linfócitos T, macrófagos e células dendríticas quando comparados aos camundongos A/J controle e ao B10.A tratados. Por outro lado, na décima semana pós infecção os camundongos A/J tratados apresentaram no pulmão número menor de linfócitos T, macrófagos e células dendríticas quando comparado aos camundongos A/J controle. A depleção pouco alterou a composição celular inflamatória dos pulmões de camundongos B10.A. A análise do afluxo celular do fígado mostrou que a depleção de células Treg

diminuiu o número de células T em camundongos A/J e somente diminuiu o número de macrófagos em camundongos B10.A. O tratamento resultou em constante diminuição de células Treg ($CD4^{+}CD25^{+}FoxP3^{+}$) em ambas as linhagens. Dado importante foi à observação de que apenas os camundongos B10.A controle apresentaram alta taxa de mortalidade, demonstrando que a depleção das células Treg leva à melhora do curso da doença tanto de animais suscetíveis como resistentes. Em conclusão, os animais A/J parecem ter apresentado aumento da ativação da resposta imune inata e adaptativa enquanto que animais B10.A parecem ter desenvolvido ativação tardia da resposta adaptativa, com prevalência da produção de citocinas Th17. Nesta linhagem, a ativação da imunidade inata, sem o controle negativo das células Treg, parece ter sido o mecanismo capaz de controlar eficientemente a carga fúngica, sem induzir grande patologia tecidual. Nosso trabalho demonstra que nesse modelo experimental as células Treg por mecanismos distintos, exercem efeito deletério na infecção tanto de camundongos resistentes como os suscetíveis ao *P. brasiliensis.* Além disso, abre novas perspectivas para o entendimento da imunopatologia da PCM.

Palavras chaves: Paracoccidioidomicose, células T reguladoras, citocinas, imunidade adaptativa.

ABSTRACT

FELONATO, M. **The role of regulatory T cells in pulmonary paracoccidioidomycosis of susceptible and resistant mice to *Paracoccidioides brasiliensis*.** 2011. 150 p. Ph. D. thesis (Immunology) ï Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2011.

The balance between immunity and tolerance is important for maintaining homeostasis and several mechanisms are required to control the immune response. The peripheral tolerance mediated by regulatory T cells (Treg) has been extensively studied. These cells are characterized by the expression of a large quantity of CD25 (IL-2 receptor Ŭ-chain) and the transcription factor FoxP3 ñforkhead transcription factor Foxp3ò. In the paracoccidioidomycosis (PCM), the immunosuppressive processes controlled by Treg cells are poorly understood. For this reason, we decided to investigate the role played by Treg cells in the phenomena of resistance and susceptibility of the hosts against *P. brasiliensis* infection. For this, we studied the importance of Treg cells in the severity of PCM through the *in vivo* depletion of $CD25^+$ cells of A/J and B10.A mice. In parallel, control groups received an equivalent dose of normal rat immunoglobulin. We studied the effect of Treg cells depletion on the severity of pulmonary infection, the pattern of cytokines produced and on the inflammatory cell influx to the lungs and liver after infection. We could verify that normal and infected A/J groups presented a higher number of regulatory T cells and increased suppressive activity compared to normal or infected B10.A groups. In both, A/J and B10.A-treated mice a significant reduction in the number of pulmonary fungal cells were observed, when compared with their controls. In addition, at the ten^{th} week of infection, the hepatic fungal loads of A/J mice was not altered, but decreased numbers of fungal cells were detected in the livers of B10.A mice. At this post-infection period, depletion of Treg cells led also to increased levels of Th1, Th2 and Th17 cytokines in both mouse strains. At the second week of infection, the A/J-treated mice presented a higher cell influx of T lymphocytes, macrophages and dendritic cells to the lungs when compared to their control group and B10.A-treated mice. On the other hand, at the chronic phase of infection, depletion of Treg cells resulted in decreased number of pulmonary T lymphocytes, macrophages and dendritic cells in A/J mice, in comparison with their control group. In contrast, anti-CD25-treatment did not induce a significant change in of influx of inflammatory cells to the lung of B10.A mice. The depletion

led to decreased numbers of hepatic T lymphocytes in A/J mice and decreased numbers of macrophages in B10.A mice. Furthermore, the early anti-CD25 treatment resulted in persistent decrease of Treg cells in both mouse strains. Importantly, only B10.A control mice showed higher mortality rates when compared to their treated group. As a whole, our work demonstrates that the depletion of Treg cells improves the course of the infection in both mouse strains. It was also shown that Treg cells depletion led to increased activation of the innate and adaptive immune responses of resistant mice whereas in susceptible mice a more efficient adaptive immune response developed late in infection and appeared to be mediated by a prevalent Th17 immunity. Moreover, in this mouse strain the innate response, without the suppressive effect of Treg cells, exerted an important control of the fungal burden without causing tissue pathology. Thus, in our work we could demonstrate that the Treg cells exert a deleterious effect for resistant and susceptible mice to *P. brasiliensis* infection.

Key words: Paracoccidioidomycosis. Regulatory T cells. Cytokines. Adaptive Immunity.

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SUMÁRIO

1 INTRODUÇÃO

A paracoccidioidomicose (PCM), micose sistêmica descrita por Adolfo Lutz em 1908, é causada pelo fungo dimórfico *Paracoccidioides brasiliensis*. Esta infecção é a mais importante micose sistêmica da América Latina, e a maior incidência ocorre no Brasil, Colômbia, Argentina e Venezuela (FRANCO et al., 1987).

O *P. brasiliensis* é um fungo dimórfico que a 25 ºC apresenta-se na forma miceliana (M) e no organismo humano ou *in vitro* à temperatura de 37º'C encontra-se na forma de levedura (L) (BRUMMER; CASTANEDA; RESTREPO, 1993). A infecção é provavelmente adquirida pela inalação de propágulos de *P. brasiliensis* provenientes do solo. Para que haja o progresso da doença é necessário que os propágulos infecciosos se transformem na forma L, patogênica, que provoca a infecção primária e, em alguns casos, pode disseminar-se para outros órgãos e tecidos tais como o fígado, baço, linfonodos e pele (FRANCO et al., 1987; BORGES-WALMSLEY et al., 2002).

Franco et al. (1987) classificaram a PCM em PCM-infecção e PCM-doença [formas clínicas aguda e sub-aguda (ou tipo juvenil) e crônica (tipo adulta)]. A forma juvenil é menos freqüente e atinge jovens de ambos os sexos. As manifestações clínicas da doença decorrem do rápido e progressivo envolvimento dos órgãos do "sistema mononuclear fagocitário". A forma crônica da PCM atinge preferencialmente adultos do sexo masculino havendo infecção pulmonar, com disseminação para outros órgãos e tecidos (FRANCO et al., 1987). A PCM-doença é menos freqüente nas mulheres do que nos homens provavelmente devido aos hormônios femininos, como o estrógeno, que bloqueiam a transformação da forma M para a forma L (MUCHMORE et al., 1974; LOOSE et al., 1983).

A PCM-infecção não apresenta as manifestações clínicas da doença, atinge indivíduos sadios de ambos os sexos que residem ou residiram em zona endêmica, e induz reação intradérmica positiva para antígenos de *P. brasiliensis* (FRANCO et al., 1987).

Estudos sobre a resposta imune desenvolvida por pacientes com as formas polares da PCM mostraram que a forma benigna é associada com a produção de baixos níveis de anticorpos e positividade para as reações de hipersensibilidade do tipo tardio (HTT), enquanto que a forma disseminada é associada com altos títulos de anticorpos e anergia nas reações de HTT (BRUMMER; CASTANEDA; RESTREPO, 1993).

Leucócitos de pacientes com a forma disseminada da doença quando comparados com os da forma branda produzem altos níveis de citocinas do tipo 2 (IL-4, IL-5, IL-10), e TGF-β; IgG4 e IgE, isótipos regulados por IL-4, são os anticorpos preferencialmente produzidos e há a concomitante síntese de baixos níveis de IFN-γ (BAIDA et al., 1999; BERNARD et al., 2001; OLIVEIRA et al., 2002; MAMONI et al., 2002, 2005). Por outro lado, indivíduos saudáveis, positivos para HTT, apresentam resposta preferencial do tipo Th1, com produção predominante de IFN-γ e TNF-α associados a níveis basais de IL-4, IL-5 e IL-10 (OLIVEIRA et al., 2002).

Em 1985 foi desenvolvido um modelo murino isogênico de PCM, onde camundongos A/J comportaram-se como resistentes e B10.A como susceptíveis à infecção pelo *P. brasiliensis*. A linhagem B10.A mimetiza a doença humana grave e progressiva, desenvolve positividade discreta ou total anergia nas reações de HTT além de ineficiente ativação dos macrófagos. Ao contrário, a linhagem A/J mostra um padrão regressivo ou brando da doença, eficiente ativação dos macrófagos e reações de HTT positivas e persistentes. Linfócitos T CD4+ e T CD8+ do tipo 1, secretores de IFN-γ e IL-2 em concomitância com linfócitos T CD4+ do tipo Th2, secretores de IL-4, 5 e 10, caracterizam a resposta protetora dos camundongos A/J. Ocorre a síntese de todos os isótipos de anticorpos, porém com predomínio de IgG2a. Este quadro caracteriza uma reposta mista do tipo1 e tipo2, porém com predomínio do primeiro. Por outro lado, camundongos B10.A desenvolvem resposta que não pode ser classificada como Th2 típica pois não há a síntese de IL-4. Entretanto, os níveis baixos ou ausentes de IFN-γ se associam a altos níveis de IL-5 e IL-10. Há evidências da participação da IL-4 e do TGF-β na resposta imune devido à síntese elevada de anticorpos específicos dos isótipos IgG1 e IgG2b, respectivamente. (CALICH; SINGER-VERMES; BURGER, 1985; CALICH et al., 1994; CALICH; VAZ; BURGER, 1998; CANO et al., 1995 e 1998; KASHINO et al., 2000; CHIARELLA et al., 2007).

Na imunidade inata, os macrófagos ativados pelo IFN-γ exercem um efeito fungicida sobre conídios de *P. brasiliensis* devido à síntese de óxido nítrico (NO) (GONZALEZ et al., 2000); ao contrário, leveduras fagocitadas por macrófagos não ativados são capazes de se multiplicar dentro dessas células (BRUMMER et al., 1989). O NO pode exercer efeitos positivos ou negativos na imunorregulação do hospedeiro na PCM podendo causar imunossupressão inibindo a linfoproliferação e a produção de TNF-α (BOCCA et al., 1998; NASCIMENTO et al., 2002) e controlando a multiplicação do fungo (NASCIMENTO et al.,

2002). Há uma relação inversa entre a síntese de NO e TNF-α por camundongos susceptíveis e resistentes. Os resistentes produzem altos níveis de TNF-α e concentrações muito baixas de NO, enquanto que os macrófagos de camundongos susceptíveis produzem altos níveis de NO e baixos de TNF-α. Baseado nesses dados pôde-se concluir que o NO é essencial para a resistência, mas a sua superprodução está relacionada com a anergia da imunidade celular e susceptibilidade ao fungo (BOCCA et al., 1998; NASCIMENTO et al., 2002).

Os leucócitos polimorfonucleares (PMN) também parecem proteger camundongos susceptíveis ao *P.brasiliensis*, enquanto que nos camundongos resistentes, a proteção ocorre somente no início da infecção. Em um trabalho recente realizado por Pina et al. (2006), foi observado que a depleção de PMN induzia doença muito grave nos camundongos susceptíveis, associada a elevados níveis de citocinas pró-inflamatórias. Assim, a ativação excessiva do sistema imune pode ser deletéria ao hospedeiro (PINA et al., 2006).

Em 2008 o mesmo grupo demonstrou que macrófagos alveolares de animais susceptíveis são facilmente ativados por IFN-γ e IL-12, produzem altos níveis de NO e têm eficiente atividade fungicida. Por outro lado, os macrófagos de animais resistentes só se ativam com alta concentração de IFN-γ e IL-12 e a sua capacidade fungicida é inibida por TGF-β endógeno (PINA; BERNARDINO; CALICH, 2008).

O estudo das citocinas no curso de PCM pulmonar demonstra que na fase de imunidade adquirida as do tipo 1 ou pró-inflamatórias (IFN-γ, IL-12, IL-2) estão associadas com fenômenos imunológicos imunoprotetores enquanto que IL-4 e IL-10 (principalmente esta última) parecem determinar doenças mais graves e progressivas. Este fato também foi demonstrado em pacientes. A PCM infecção é o pólo Th1 com síntese de IFN-γ, IL-2, IL-12 e imunidade celular desenvolvida. Os pacientes com forma juvenil representam o pólo Th2, pois há a síntese de IL-5, IL-4, IL-10 e TGF-β em níveis bastante elevados além de ausência de imunidade celular. Nos indivíduos com a PCM crônica (que representam cerca de 90% do total de doentes) não há um perfil típico. Parece haver tendência Th2 nos casos mais graves (MAMONI et al., 2005).

Em relação às células T, pouco se sabe sobre seu papel na resposta imune contra o *P. brasiliensis.* Há alguns estudos demonstrando que os linfócitos $TCD4^+$ e $TCD8^+$ participam dos processos da imunoproteção na infecção pelo *P. brasiliensis*. Cano et al. (2000) observaram que as células T $CD8^+$ são protetoras na PCM experimental murina (B10.A e A/J) e a intensidade da ativação dessas células depende do padrão genético do hospedeiro. Em nosso laboratório, Chiarella et al. (2007) demonstraram que a depleção de células T $CD4^+$ não

afeta a doença de camundongos susceptíveis, sugerindo que o controle da doença nestes animais não é efetivado por esta população celular. A anergia de células T $CD4^+$ só é revertida quando camundongos B10.A são tratados com anticorpos monoclonais anti-CD8, sugerindo que uma outra subpopulação celular, T $CD8^+$, atue como imunorreguladora. Camundongos resistentes desenvolvem resposta T $CD4^+$ do tipo Th1 em concomitância com células Th2; apresentam também células T $CD8^+$ que sintetizam grandes quantidades de IFN-γ e são protetoras na PCM pulmonar (CALICH e BLOTTA, 2005; CHIARELLA et al., 2007).

Sabe-se que o balanço entre imunidade e tolerância é importante para manter a homeostase do organismo e que muitos mecanismos são utilizados para manter a resposta imune sob controle, como a anergia de linfócitos T, a apoptose e a ignorância imunológica. Entretanto, um quarto mecanismo de tolerância periférica vem sendo muito estudado e que é mediada por células T reguladoras. Tais células foram primeiramente descritas no início dos anos 70, mas por atuarem através de fatores supressores solúveis hipotéticos que não puderam ser identificados em nível molecular e apresentarem marcadores de membrana que não puderam ser caracterizados, o conceito de células T supressoras permaneceu esquecido por um longo período.

Em 1995, Sakaguchi e seus colaboradores descrevem que aproximadamente 10% das células T $CD4^+$ e 1% das células T CD8+ de animais adultos expressam o receptor da cadeia alfa de IL-2 (CD25) sendo estas células importantes no processo de manutenção da tolerância na periferia. Observaram que após a transferência adotiva de uma população de células T depletadas de células T $CD25^+$, os receptores desenvolviam várias doenças auto-imunes tais como tireoidite, gastrite, diabete e glomerulonefrite. Tal achado permitiu compreender que a anomalia dos mecanismos mediados pelas células T indutoras de tolerância periférica poderia ser uma possível causa do desenvolvimento de várias doenças auto-imunes. (SAKAGUCHI et al., 1995). As células $TCD4^+CD25^+$ foram então denominadas células T reguladoras (Treg) e, desde então, as mesmas têm sido intensivamente estudadas.

Diversos subtipos de células T reguladoras têm sido descritos com base nas diferenças entre marcadores de superfície, produção de citocinas e mecanismos de ação (JONULEIT e SCHMITT, 2003). As células T reguladoras $CD4^+CD25^+$ naturais são originadas no timo e compreendem uma população de células T com propriedades imunossupressoras que reconhecem auto-antígenos e constituem de 5-10% do total de células T $CD4^+$ da periferia

(SAKAGUCHI, 2000, 2005). Assim como a expressão de CD25, são constitutivamente expressos os seguintes marcadores de ativação: GITR "glucocorticoid-induced tumor-necrosis factor receptor-related protein", OX40 (CD134), L-selectina ou CD62-ligante (CD62L) e CTLA-4 ou CD152 "cytotoxic T lymphocyte-associated antigen 4" (SHEVACH et al., 2006). Recentemente foram descritos mais dois marcadores de superfície encontrados nas células T reguladoras. O CD39 (ENTPD1 "ectonucleoside triphosphate diphosphohydrolase-1") e o CD73 "membrane bound ecto-5′-nucleotidase" hidrolizam ATP e ADP a adenosinas que, quando super expressas, tornam-se componente indispensável para a célula T reguladora exercer o seu efeito supressor. Particularmente, o CD39 poderia regular a função supressora da célula T por induzir a produção de adenosina que atua no receptor purinérgico (A2A). (DEAGLIO et al., 2007).

Para melhor distinguir as células T reguladoras das células T ativadas, Yamaguchi e seus colaboradores mostraram que além de todos os marcadores descritos acima, as células Treg apresentam altas quantidades de FR4 "folate receptor 4", um subtipo do receptor da vitamina ácido fólico (B12). Observaram que após a administração do anticorpo monoclonal anti-FR4 o número de células T reguladoras diminuiu, ao mesmo tempo em que foi observado o desenvolvimento de doenças auto-imunes em camundongos jovens (YAMAGUCHI et al., 2007).

Vários marcadores de superfície têm sido utilizados para identificar as células T reguladoras naturais, porém, até o momento, somente o fator de transcrição Foxp3 "transcription factor forkhead Foxp3" é um marcador intracelular exclusivo encontrado nas células Treg (HORI; NOMURA; SAKAGUCHI, 2003; FONTENOT et al., 2005; YI et al., 2006). Além disso, o Foxp3 é um fator de transcrição crucial para o desenvolvimento e funcionalidade das células T reguladoras $CD4^{+}CD25^{+}$ (HORI; NOMURA; SAKAGUCHI, 2003; FONTENOT et al., 2005). Foi descrito que uma mutação do Foxp3 resulta na ausência de células Treg naturais e conseqüente desenvolvimento de doenças auto-imunes (WILDIN e FREITAS, 2005; WILDIN; SMYK-PEARSON; FILIPOVICH, 2002).

Em animais que apresentam perda da função do Foxp3 ("scurfy mice"), observa-se rapidamente o desenvolvimento de uma doença linfoproliferativa fatal, semelhante àquela observada nos animais deficientes de CTLA- 4 ou TGF-β (BRUNKOW et al., 2001). Uma análise mais detalhada revelou que as células T $CD4^{+}CD25^{+}$ destes animais não apresentavam atividade supressora ou células T reguladoras. Entretanto, após a transferência adotiva de

células T $CD4^+CD25^+Foxp3^+$ foi possível observar atividade supressora, mostrando mais uma vez a importância da expressão do Foxp3 na atividade das células T reguladoras (HORI; NOMURA; SAKAGUCHI, 2003).

Vários trabalhos demonstraram que o Foxp3 é exclusivamente expresso em células T $CD4^+CD25^+$ e a expressão ectópica do Foxp3 por células $TCD4^+CD25^-$ confere atividade supressora a estas células (FONTENOT; GAVIN; RUDENSKY, 2003; KHATTRI et al., 2003; HORI; NOMURA; SAKAGUCHI, 2003). Estes dados indicam claramente que o Foxp3 é critico no desenvolvimento e função das células Treg e este dado é diferencial dos outros marcadores associados a estas células tais como CD25 e GITR que são expressos de maneira geral por células T ativadas (RAMSDELL, 2003).

Em recente revisão, descreveu-se a descoberta de 2 novos subtipos de células T reguladoras $FoxP3^+$ derivadas do timo (LOURENÇO e LA CAVA, 2011). Tais células têm sido identificadas com base na expressão de moléculas co-estimulatórias ICOS. O trabalho de Ito e colaboradores mostrou que células com fenótipo $ICOS^+FoxP3^+$ são caracterizadas pela alta capacidade de produzir IL-10, enquanto que as células com fenótipo $ICOS^-FoxP3^+$ são caracterizadas pela alta capacidade de produzir TGF-β. Ambas as subpopulações celulares parecem utilizar os mecanismos dependentes ou independentes do contato célula-célula para o desenvolvimento da supressão imunológica na periferia (ITO et al., 2008).

Outra classe de células reguladoras T $CD4^+$ tem sido descrita por também apresentar propriedades supressoras, mas, ao contrário do que ocorre naturalmente com as células T reguladoras naturais, a supressão é independente do contato célula-célula e principalmente mediado por citocinas supressoras solúveis como a IL-10 e o TGF-β (JONULEIT e SCHMITT, 2003). Essas células T supressoras são secundárias e se desenvolvem a partir de células T $CD4^+CD25^-$ convencionais na periferia. Parece que essas células representam estados alterados de diferenciação em vez de uma única linhagem de células T. Dois tipos de células T reguladoras secundárias têm sido descritas, as Tr1 (RONCAROLO et al., 2001) e as Th3 (WEINER, 2001). As células Tr1 são definidas por sua capacidade de produzir grandes quantidades de IL-10 e níveis baixos a moderados de TGF-β; já as células Th3 produzem preferencialmente TGF-β.

A geração de células Tr1 a partir de células T $CD4^+$ virgens *in vitro* foi descrita pela primeira vez por Groux e seus colaboradores (GROUX et al., 1997). Eles mostraram que células T virgens de camundongos que possuem o TCR transgênico para OVA, após

estímulos com OVA e IL-10, se diferenciavam em um perfil distinto de linfócitos, diferentes do Th1 ou Th2. Estas células Tr1 produzem IL-10, IL-5 e IFN-y e podem ou não produzir TGF-β. Tais células T reguladoras foram caracterizadas em seres humanos e, mais recentemente, foi demonstrado que a produção de IL-10 por células T reguladoras também pode ser induzida pelo estímulo de células T $CD4^+$ em combinação com anticorpos anti-CD3 e anti-CD46 (KEMPER et al., 2003). As células Tr1 pouco proliferam após ativação *in vitro* com estímulos policlonais ou antígeno específico; estudo funcional com células Tr1 revelou que essas células têm propriedades imunossupressoras e podem prevenir o desenvolvimento de respostas auto-imunes mediadas por células T (LEVINGS et al., 2002). As Tr1 podem ainda controlar a ativação de células T virgem e de memória tanto *in vitro* como *in vivo*, e suprimem a resposta imune mediada por subpopulações Th1 e Th2 contra patógenos, tumores e aloantígenos. Além disso, os sobrenadantes de células Tr1 ativadas reduzem fortemente a capacidade de células dendríticas (DC) induzirem a proliferação de célula T aloantígeno específica (LEVINGS et al., 2002; RONCAROLO et al., 2001).

Por outro lado, as células Th3 foram inicialmente identificadas em camundongos após indução de tolerância oral à proteína básica da mielina (MBP) (CHEN et al., 1994). Após o tratamento com MBP, a maioria das células T $CD4^+$ MBP-específicas secretam TGF-β e suprimem a encefalite experimental auto-imune MBP-específica *in vivo* (FUKAURA et al., 1996). Essa supressão foi bloqueada pela injeção de anticorpos anti-TGF-β. Além disso, essas células Th3 suprimem *in vitro* a proliferação e liberação de citocinas por células Th1 MBP-específicas (CHEN et al., 1994). As células Th3 reguladoras são, portanto, um subconjunto de células T reguladoras induzidas por administração oral de antígenos *in vivo*. Tais células auxiliam na produção de IgA e têm propriedades supressivas sobre células Th1 e Th2 (WEINER, 2001).

Recentemente tem sido descrito que os fatores de transcrição Foxo (família Forkhead) são essenciais para o desenvolvimento e função das células T reguladoras $FoxP3^+$, via controle da expressão de genes associados com a função da célula T. Em mamíferos, a subfamília Foxo é composta por 4 membros, Foxo1, Foxo3, Foxo4 e Foxo6. Os membros Foxo 1 e Foxo3 são as isoformas expressas nas células do sistema imune. Após ativação da cascata de sinalização via diferentes moduladores de transcrição, incluindo STAT-3, Runx3 e Smad3, ocorre a fosforilação dos fatores de transcrição Foxo1 e Foxo3 que se ligam a regiões

promotoras do FoxP3 que é fundamental para a função de células T reguladoras FoxP3$^+$ (OHKURA e SAKAGUCHI, 2010).

O fator de transcrição FoxP3 controla diretamente ou indiretamente a expressão de moléculas responsáveis por conferir a função supressora das células T reguladoras. Elegante trabalho desenvolvido por Ohkura e Sakaguchi (2011) descreve que o FoxP3 regula diretamente a expressão dos fatores de transcrição Blimp-1 e IRF-4 que estão relacionados com a expressão das moléculas presentes em células T reguladoras. O fator de transcrição IFR4 participa da ativação dos genes da IL-10, CCR6 e ICOS. Por outro lado, o fator de transcrição Blimp-1, associado com a ativação de IFR4, participa da ativação direta do gene da IL-10 e da ativação indireta dos genes de ICOS e CCR6.

Até o presente momento sabe-se que as células T CD4$^+$CD25$^+$ reguladoras podem ser ativadas por antígenos próprios ou não próprios e, uma vez ativadas, podem suprimir células T de maneira não antígeno-específica. Os efeitos supressivos destas células não são restritos ao sistema imune adaptativo (células T e B), mas podem também influenciar a ativação e função de células do sistema imune inato (monócitos, macrófagos e células dendríticas). As células T reguladoras adaptativas são induzidas por antígenos, desenvolvem-se na periferia e exercem sua função através da secreção de citocinas inibitórias como IL-10, TGF-β e recentemente IL-35 (COLLISON et al., 2007) ou tolerizando as APC por interações célula-célula (MALOY e POWRIE, 2001).

Cada vez mais tem sido observada a participação de células Treg em respostas imunológicas contra patógenos, embora o mecanismo de reconhecimento das estruturas dos microorganismos não esteja ainda esclarecido. *In vitro*, a geração das células T reguladoras induzidas depende da sinalização via TCR e síntese de TGF-β (CHEN et al., 2003), entretanto, *in vivo*, a geração depende da co-estimulação via B7.1/B7.2 (CD80/CD86) (LIANG et al., 2005).

Yu e seus colaboradores mostraram em recente trabalho que apenas a molécula co-estimulatória CD28 é essencial para a geração das células T reguladoras na periferia e que este processo é dependente da citocina IL-2. Tanto CTLA-4 como ICOS são dispensáveis para a geração de células T reguladoras (GUO et al., 2008). Além disso, mostraram que a adição de anticorpo monoclonal anti-CD28 pode restaurar a diferenciação de células T reguladoras na ausência de B7, indicando que somente a sinalização via CD28 é suficiente para este processo.

Em nosso trabalho de mestrado demonstramos que a molécula CD28 é necessária para a função e expansão das células Treg na PCM pulmonar. A ausência da sinalização mediada por CD28 leva a processos de imunidade pouco expandidos, mas bastante eficientes por evitar a concomitante ampliação da imunossupressão mediada por células Treg (FELONATO et al., 2010).

Relevante trabalho mostrou que as células dendríticas derivadas da medula óssea de animais BALB/c são capazes de promover *in vitro* a expansão de células T reguladoras $Foxp3^+$ e que este fenômeno ocorre independente do estado de maturação da célula dendrítica. Entretanto, as células dendríticas imaturas estimularam um aumento significativo no percentual de células T reguladoras quando comparadas com as de outros estágios de maturação e isto foi associado a uma maior produção da citocina IL-2. Esta observação apóia dados anteriores que mostram uma correlação direta entre a produção de IL-2 e a expansão de células T reguladoras $CD4^+CD25^+Foxp3^+$ (MARGUTI et al., 2009).

Recentemente, tem sido demonstrada a relação entre células T reguladoras e receptores da família TLR (toll like receptor). Netea descreveu que animais deficientes de TLR-2 apresentaram diminuição de células T reguladoras $CD4^+CD25^+$ quando comparados com os animais controle; entretanto, nos animais deficientes de TLR-4 tal fenômeno não foi observado (NETEA; VAN der MEER; KULLBERG, 2004). Estes achados sugerem um papel via sinalização de TLR-2 na homeostasia e função das células T reguladoras. Sutmuller e seus colaboradores mostraram que, assim como na deficiência de TLR-2, os animais deficientes de MYD88 apresentaram significante redução de células T reguladoras quando comparados com os animais controle (SUTMULLER et al., 2006).

Nosso grupo demonstrou resultados semelhantes na ausência de TLR-2, onde após a infecção i.t. pelo fungo *Paracoccidioides brasiliensis* os animais deficientes para este receptor apresentaram diminuição das células T reguladoras $CD4^+CD25^+FoxP3^+$ quando comparado aos animais C57Bl/6 (LOURES et al., 2009). Por outro lado, demonstramos também que camundongos C3H/HeJ (TLR-4 deficientes) apresentam aumento no número de células T reguladoras quando comparado aos animais controle C3H/HePas (LOURES et al., 2010). Além disso, após a infecção pelo fungo, camundongos deficientes da molécula adaptadora MYD88 apresentavam diminuição de todas as subpopulações de linfócitos T, inclusive de células T reguladoras quando comparado aos camundongos C57Bl/6 (LOURES et al., 2011).

As células Treg também puderam ser associadas com o desenvolvimento de células Th17. Acredita-se que haja fatores de diferenciação das células Th17 vinculados com a indução das células T reguladoras Foxp3$^+$ e, que assim como as células Treg, as células Th17 estariam envolvidas na resposta auto-imune. Enquanto que a ação de TGF- β induz a diferenciação de células Treg Foxp3$^+$, TGF- β na presença de IL-6 induz a diferenciação das células TCD4$^+$ naives para Th17. De maneira oposta, a citocina IL-27 produzida por células dendríticas ativadas inibe não só a geração de células Th17, mas também inibe a geração de células T reguladoras Foxp3$^+$. Por outro lado, estimula o desenvolvimento de células Treg induzidas (células Tr1) (AWASTHI et al., 2008). O trabalho de Yi Chen et al. (2011) mostra mais uma vez a relação entre células T reguladoras e células Th17. Após a administração da toxina diftérica (DT) isolada da bactéria *Corynebacterium diphtheriae,* observou-se em camundongos FoxP3.luciDTR (camundongos transgênicos que apresentam o receptor da toxina diftérica que está inserido na região promotora do gene FoxP3) diminuição no número de células produtoras de IL-17 quando comparado com os camundongos controle. Além disso, demonstrou-se que após a administração da toxina os camundongos apresentaram redução de 90%-95% de células T reguladoras (YI CHEN et al., 2011).

A ativação não controlada da resposta imune do tipo Th1, Th2 assim como Th17 pode levar a patologia tecidual intensa. Embora mais recentemente descrita e menos estudada, a subpopulação Th17 de linfócitos T CD4$^+$ parece estar bastante envolvida na imunidade contra fungos patogênicos. Como descrito anteriormente, o desenvolvimento de linfócitos Th17 ocorre na presença das citocinas TGF-β e IL-6, e é inibido por citocinas do tipo Th1. Na realidade, o TGF-β é necessário para impedir a diferenciação das células para um padrão Th1 ou Th2 mediada pelos fatores transcrição Tbet e GATA-3, respectivamente, enquanto que na ausência destes fatores de transcrição a IL-6 é suficiente para induzir a polarização Th17 (DAS et al., 2009). Linfócitos Th17 são mantidos pela citocina IL-23, que é estruturalmente relacionada à IL-12. A IL-17 secretada por linfócitos Th17 induz a produção de quimiocinas nos sítios de infecção e causam o recrutamento de neutrófilos. Devido a esta característica peculiar, as células Th17 têm sido consideradas importantes na defesa contra patógenos extracelulares e doenças auto-imunes (BETTELLI; KORN; KUCHROO, 2007; VELDHOEN et al., 2006; MANGAN et al., 2006). Estudos recentes têm demonstrado que o reconhecimento imunológico de *C. albicans* pelo receptor de imunidade inata Dectina-1 (que reconhece β-glucanas) induz a diferenciação de linfócitos T CD4$^+$ para o tipo T-helper 17

(Th17) que secretam a citocina IL-17 e expressam receptores de quimiocinas característicos para o alojamento em tecido mucoso (LEIBUNDGUT-LANDMAN et al., 2007; ACOSTA RODRIGUES et al., 2007). Assim, o processo de ativação de imunidade adaptativa é fortemente regulado pela interação entre receptores de membrana de células da imunidade inata e da presença de células T reguladoras que exercem um importante papel de controle da doença no próprio sítio de ativação da resposta imune.

Há resultados controversos em relação à expressão do TGF-β na superfície das células T reguladoras naturais. Piccirillo et al. (2002) demonstraram que o anticorpo monoclonal anti-TGF-β não é capaz de neutralizar a supressão de células T reguladoras naturais ativadas; no entanto, Nakamura et al. (2001) demonstraram que o anti-TGF-β abole a supressão da proliferação das células T mediada por células T reguladoras naturais (NAKAMURA; KITANI; STROBER, 2001).

Recentemente tem se demonstrado que a interação celular entre as células T reguladoras e as outras populações celulares como a Th17 são importantes para determinar o equilíbrio na resposta imune. (LOCHNER et al., 2008). As células T naive estimuladas com antígeno na presença de TGF-β aumentam a expressão do fator de transcrição FoxP3 e do marcador CD25 e se desenvolvem em T reguladoras. Por outro lado, quando as células T naive são estimuladas na presença de antígeno, IL-6 e TGF-β há o aumento da expressão do fator de transcrição RORγt e diferenciam em células Th17 que produzem a citocina IL-17 (IVANOV et al., 2006). Dados atuais relatam que a diferenciação das células Th pode passar por uma fase intermediária de expressão de FoxP3/RORγt e isto forneceria maior flexibilidade para o desenvolvimento de uma resposta imune adequada (LOCHNER et al., 2008). Verificou-se que as células T $FoxP3^+$ $ROR\gamma t^+$ expressam IL-10 e CCL20 e funcionam como células T reguladoras. Além disso, a proporção entre células expressando $FoxP3^+$ e as células RORγt produtoras de IL-17 permanece constante durante a inflamação e infecção. Este equilíbrio é mantido através da produção de IL-10 que favorece a expressão de FoxP3 e por IL-6 e IL-23 que favorecem a diferenciação Th17.

Em elegante trabalho desenvolvido por Tartar et al. (2010), mostrou-se que as células T duplo-positivas para expressão de $ROR\gamma t^+FoxP3^+$ comportam-se como uma população de células de desenvolvimento intermediário que possibilita o desenvolvimento de ambas, células T reguladoras ou Th17. Além disto, esta população intermediária surge em animais com diabetes antes do processo inflamatório. Expressam também CD62L, importante para a

migração das células para o pâncreas e mTGF-β importante para suprimir as células T efetoras. De maneira importante, estas células são capazes de se diferenciar em populações de células T reguladoras FoxP3^{+}RORγt^{-} ou células Th17 RORγt^{+}FoxP3^{-} (TARTAR et al., 2010). Estas observações mostram a importância da plasticidade na diferenciação e função celular e indicam que tais células duplo-positivas têm a capacidade de migrar para o foco inflamatório para controlar células T efetoras, ampliando assim a sua eficácia no controle da auto-imunidade.

Há evidências crescentes de que células T regulatórias e, em particular, as células T reguladoras naturais CD4^{+}CD25^{+} desempenham um papel fundamental em processos infecciosos. A presença de células T reguladoras induz um controle menos efetivo dos patógenos, pois bloqueia mecanismos efetores da resposta imune, porém a sua ausência ou depleção pode causar patologia tecidual mais grave que aquela induzida pelo próprio microrganismo. A presença de células Treg tem sido associada com muitas patologias infecciosas de natureza crônica, pois facilitariam a manutenção do patógeno, mesmo que em pequeno número. Esta permanência de pequeno número de microrganismos, parece também facilitar a manutenção de memória imunológica em níveis suficientes para proteger contra infecções subseqüentes. Um exemplo descrito é o modelo de infecção por *Leishmania major* em camundongos C57BL/6. Durante a infecção por este protozoário as células T CD4^{+}CD25^{+} acumulam na derme, onde suprimem a capacidade das células T CD4^{+}CD25^{-} de eliminar o parasita do local. Como conseqüência, as células T reguladoras promovem a persistência do patógeno e potencial transmissão para outros hospedeiros. A remoção das células Treg induz a "cura estéril", um estado não compatível com a preservação da imunidade por longo tempo (BELKAID et al., 2002).

Na infecção pelo *Trypanosoma cruzi* o desenvolvimento da resposta imunológica esteve associado com a presença de células T reguladoras. Por estas células apresentarem fenótipo CD4^{+}CD25^{+}Foxp3^{+}GITR^{+} o papel das células T reguladoras foi analisado através do tratamento dos camundongos infectados com anticorpos monoclonais anti-CD25 e anti-GITR e analisados quanto à parasitemia. Inicialmente demonstrou-se que existe uma população de células T reguladoras que migram para o coração dos animais infectados e que, após o tratamento com os anticorpos monoclonais observou-se aumento na mortalidade. Entretanto, o bloqueio causado pelo anti-GITR levou a um aumento do número de células T CD4^{+}, T CD8^{+}, leucócitos expressando CCR5^{+} e a produção de TNF-α, indicando claramente que a

sinalização através de GITR é fundamental para o controle da inflamação cardíaca (MARIANO et al., 2008).

Em modelo de infecção por fungo, descreveu-se que as células T reguladoras são componentes necessários para a manutenção da infecção causada por *C. albicans*; a redução de células T $CD4^+CD25^+$ induz um melhor controle da infecção, mas aumenta a inflamação gastrointestinal. Por outro lado, a transferência de células T reguladoras restaura a resistência à re-infecção (MONTAGNOLI et al., 2002). Na infecção causada por *Pneumocystis carinii* em camundongos deficientes do gene ativador da recombinase-2 ($RAG2^{-/-}$), a transferência de células T $CD4^+CD25^-$ (T "naïve") reduz a carga de fungos nos pulmões, mas desencadeia uma pneumonia letal. Em contraste, a transferência de células T $CD4^+$ $CD25^+$ previne a eliminação do patógeno e o desenvolvimento da doença inflamatória induzida pela transferência de células T "naïve" (HORI; CARVALHO; DEMENGEOT, 2002). Além disso, na doença inflamatória intestinal causada por bactérias comensais, a presença de células T reguladoras promove o controle de inflamação nociva e a deficiência das mesmas leva a uma inflamação maciça (revisado por BELKAID e ROUSE, 2005).

Por outro lado, em modelo de infecção por *Toxoplasma gondii* também foi descrita a contribuição de células T reguladoras na resistência de camundongos BALB/c contra o parasita. Os autores verificaram que após a depleção com anticorpo monoclonal anti-PC61 (anti-$CD25^+$) e subseqüente infecção os camundongos BALB/c apresentavam aumento da parasitemia associado com aumento de citocinas pró inflamatórias (IFN-γ e IL-6) quando comparado com os camundongos que somente foram infectados. Entretanto, camundongos C57Bl/6 que são susceptíveis ao *T. gondii* não apresentavam diferenças entre os grupos e quando comparados com os camundongos BALB/C (MORAMPUDI et al., 2011).

Há poucos relatos na literatura sobre a participação das células T reguladoras na paracoccidioidomicose. Cavassani e seus colaboradores em estudo recente realizado com pacientes apresentando PCM crônica e indivíduos saudáveis demonstraram que o número total de células T $CD4^+CD25^+$ é similar no sangue periférico e nos tecidos de ambos os grupos. No entanto, as células T $CD4^+CD25^+Foxp3^+$ expressando CTLA-4, TNFR, CD103, CD45RO, mTGF-β e os receptores de quimiocinas CCR4 e CCR5 aparecem em número elevado tanto no sangue como nas lesões dos pacientes e apresentam forte atividade supressora. Este trabalho demonstrou, pela primeira vez, que as células T reguladoras

desempenham um importante papel no controle da resposta imunológica local ou sistêmica em pacientes com paracoccidioidomicose (CAVASSINI et al., 2006).

Em processos infecciosos sabe-se que a migração celular é dependente dos receptores de quimiocinas, entre eles os ligantes de CCR5 que são produzidos nos processos inflamatórios causado por fungos, como o *P. brasiliensis.* Desta maneira, Moreira e colaboradores investigaram o papel do CCR5 no controle da PCM experimental. Verificaram que camundongos deficientes de CCR5 são mais eficientes no controle do crescimento fúngico do que no seu grupo controle. Na ausência de CCR5, a porcentagem de células T $CD4^+CD25^+Foxp3^+$ nas lesões era significativamente diminuída. Além disso, a transferência adotiva de células $CD4^+CD25^+$ para os animais deficientes de CCR5 infectados levava a um aumento significativo na carga fúngica. Verificaram também que os linfócitos $TCD4^+$ ou $TCD8^+$ migram para a lesão independente do CCR5, ao contrário das células T reguladoras que mostraram ser dependentes deste receptor. Pelo fato do CCR5 estar expresso em 70% das células T $CD4^+CD25^+$, acredita-se que este receptor possa ser crucial na supressão da resposta imune efetora contra o *P. brasiliensis* (MOREIRA et al., 2008).

Já em pacientes que apresentam a paracoccidioidomicose, as células T reguladoras têm sido relacionadas com a imunossupressão. Assim, Ferreira e colaboradores (2010) demonstraram que pacientes com a doença ativa (pacientes selecionados antes ou durante o primeiro mês de tratamento) apresentavam número maior de células Foxp3+ quando comparado aos pacientes que receberam tratamento (pacientes que apresentam sorologia negativa ao fungo e que não apresentam sinais clínicos da doença), e ao grupo de doadores saudáveis. Estas células apresentavam aumento dos marcadores de superfície característicos de regulação GITR, CD38, CD95L, CTLA-4, LAP e TLR-2 além de maior atividade reguladora quando comparado com as células dos pacientes que receberam tratamento. Além disso, as células dos pacientes com a doença ativa produzem níveis maiores de TGF-β e IL-10 do que as dos pacientes tratados (FERREIRA et al., 2010).

Em resumo, tanto nas respostas imunes contra antígenos, tumores, transplantes e patógenos as células T reguladoras desempenham papel relevante. Controlam a resposta imune excessiva que pode ser lesiva aos tecidos ou desencadear processos auto-imunes, mas podem também levar a infecções mais graves devido à inibição dos fenômenos efetores que atuam na eliminação dos microorganismos.

Assim, a regulação da resposta imune tem que ser bem balanceada e as células T reguladoras exercem um papel fundamental neste processo. Como na paracoccidioidomicose os processos controlados por células T reguladoras são pouco conhecidos, resolvemos estudar o papel desempenhado por estas células nos fenômenos de resistência e susceptibilidade genética dos hospedeiros contra o fungo.

2 JUSTIFICATIVA E OBJETIVO

Na PCM os fenômenos imunoregulatórios são pouco conhecidos. Animais resistentes infectados pela via pulmonar desenvolvem lesões involutivas e doença regressiva restrita aos pulmões. A imunidade inata é pouco eficiente e os macrófagos alveolares de camundongos A/J sintetizam baixos níveis de NO e atividade fungicida deficiente devido à síntese aumentada de TGF-β. Apesar da baixa eficiência da imunidade natural, mais tardiamente (cerca de 2 meses após infecção) desenvolvem-se respostas adaptativas bastante eficientes mediadas por linfócitos T $CD4^+$ e T $CD8^+$ do tipo 1 que têm sua atividade pró-inflamatória balanceada por linfócitos do tipo Th2.

Animais susceptíveis desenvolvem doença progressiva, disseminada e lesões mal organizadas que não restringem o crescimento fúngico. Ao início da doença, camundongos B10.A secretam quantidades apreciáveis de IFN-γ e NO no parênquima pulmonar (CANO, 2000; NASCIMENTO, 2002). A imunidade inata é bastante eficiente e apta a controlar inicialmente o crescimento fúngico. Macrófagos de camundongos B10.A sintetizam altos níveis de IL-12 e NO, entretanto, esta resposta inflamatória exacerbada induz um estado de anergia de células T $CD4^+$. A supressão da imunidade celular não é revertida pela depleção de IL-4 nem de linfócitos T $CD4^+$, demonstrando que a imunidade destes animais não é mediada por linfócitos Th2 (CHIARELLA et al., 2007; ARRUDA et al., 2002 e 2004). É ainda digno de nota o fato de que a inoculação de IL-12 recombinante resulte em número diminuído de fungos nas lesões, mas patologia pulmonar altamente exacerbada. Verificou-se também que linfócitos T $CD8^+$ medeiam a proteção relativa destes animais. Entretanto, o tratamento dos animais com anticorpo monoclonal anti-CD8, leva a reações de HTT positivas associadas à patologia mais grave (CANO et al., 2000).

Nossos dados têm demonstrado que a susceptibilidade genética ao *P. brasiliensis* não pode ser atribuída a uma ativação preferencial de respostas do tipo Th2 e nem a uma baixa reatividade do sistema imune inato ao fungo. Ao contrário, mecanismos de ativação excessiva parecem condicionar infecções mais graves e imunidade adaptativa ausente ou inadequada.

Em conclusão, os fenômenos imunoregulatórios que controlam a PCM de camundongos susceptíveis e resistentes ao fungo são pouco elucidados. Assim, este estudo tem como propósito caracterizar a presença e função de células T reguladoras no pulmão de camundongos susceptíveis (B10.A) e resistentes (A/J) ao *P. brasiliensis* após a infecção intra-traqueal pelo fungo. Com este objetivo, estudaremos o efeito da inibição in vivo das células Treg na gravidade da doença, e nos mecanismos imunológicos associados à mesma.

3 MATERIAL E MÉTODOS

3.1 Animais

Foram utilizados camundongos isogênicos machos das linhagens A/J resistentes e B10.A suscetíveis ao *P. brasiliensis* (CALICH et al., 1985) com idade entre 6 a 8 semanas. Esses animais são criados sob condições SPF no Biotério de camundongos isogênicos do Departamento de Imunologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, e mantidos no biotério de animais de experimentação do referido Departamento.

3.2 Fungo

Foi utilizado o isolado Pb 18 (virulento) do *P. brasiliensis*, (KASHINO et al., 1985; SINGER-VERMES et al., 1989). O fungo foi mantido em meio semi-sólido de Fava Netto (1955) a 36 °C, realizando-se repiques semanais. Suspensões celulares foram obtidas na fase exponencial de crescimento leveduriforme, ou seja, após 1 semana de cultivo, e lavadas 3 vezes em solução salina estéril. A concentração de células fúngicas foi ajustada após contagem em câmara hemocitométrica. A viabilidade da suspensão celular foi avaliada utilizando-se o corante vital Janus Green B, (BERLINER e RECA, 1966) e foi sempre superior a 80%.

3.3 Infecção Intratraqueal (i.t.)

Os animais foram infectados por injeção intratraqueal (i.t) de $1x10^6$ leveduras viáveis de *P. brasiliensis* contidas em PBS. Os animais foram anestesiados por via intraperitoneal e o procedimento foi iniciado quando o animal apresentou-se insensível à dor. Foi realizada uma pequena incisão longitudinal na pele do pescoço e a traquéia foi exposta. O fungo foi administrado e após a inoculação a incisão foi suturada. Em seguida os animais foram colocados sob uma fonte moderada de calor para controlar a hipotermia transitória produzida pela anestesia, até acordarem. Todos os procedimentos cirúrgicos foram executados com suspensão fúngica com viabilidade entre 90 e 95%

3.4 Depleção de linfócitos $CD25^+$

Camundongos A/J e B10.A foram depletados de linfócitos $CD25^+$ através da inoculação de 500µg/0,5 mL i.p. do anticorpo monoclonal PC61 purificado (BIO-EXCEL-USA). Este anticorpo foi aplicado pela via i.p. 3 dias antes da inoculação i.t. de leveduras do Pb 18. Esta depleção foi repetida 3 dias após a infecção. O trabalho de Kohm e colaboradores (2006) mostrou que este protocolo utilizado para a depleção de CD25 é eficaz.

Os animais controle de ambos os grupos foram inoculados com Ig controle de rato nos mesmos dias e na mesma concentração protéica do anti-CD25.

3.5 Avaliação do grau de infecção através da determinação de unidades formadoras de Colônias (UFC)

O grau da infecção em camundongos depletados ou não com o anticorpo anti-CD25 ou Ig de rato foi avaliado 2 e 10 semanas após a infecção. Os animais foram sacrificados e um dos pulmões, um lóbulo do fígado e parte do baço foram separados, pesados, e macerados em 5 mL de PBS com o uso de homogenizadores. O macerado dos órgãos foi submetido à centrifugação e o sedimento final retomado em um volume de 1 mL de PBS. Uma alíquota de 100 µL dessa suspensão e de suas diluições foi semeadas em placas de Petri com meio de cultura adequado.

A incubação foi feita a 35 ^oC e o crescimento das colônias de *P. brasiliensis* foi acompanhado e registrado diariamente por um período médio de 15 dias.

O meio de cultura empregado foi constituído de BHI-ágar suplementado com 5% de "fator de crescimento do fungo" e 4% de soro eqüino (SINGER-VERMES et al., 1992).

3.6 Dosagem de NO

A concentração de NO nos sobrenadantes de órgãos nos tempos de 2 e 10 semanas de infecção foi medida com o reagente de Griess (1% sulfanilamida, 0,1% diidrocloreto de naftiletilenodiamina, 2,5% H_3PO_4). Um volume de 50 µl do sobrenadante foi adicionado a um volume igual do reagente de Griess; depois de incubar em temperatura ambiente por 10 minutos, foi determinada a absorbância em equipamento Labsystems Multiskan MCC/340 em

comprimento de onda de 490 nm. A concentração de NO foi determinada usando curva padrão padronizada com diferentes concentrações de nitrito de sódio (DING; NATHAN; STUEHR, 1988).

3.7 Caracterização das citocinas: ELISA para quantificação de IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IL-23, IFN-γ, TNF-α e TGF-β

A dosagem de citocina foi realizada no sobrenadante dos macerados dos órgãos utilizados na determinação de UFC dos animais. A presença e concentração das citocinas foram analisadas por ELISA de captura utilizando-se pares de AcM para cada citocina murina (Pharmingen ou RD Systems). As concentrações previamente determinadas para cada um dos AcM primário e secundário no ELISA, contra cada citocina, foram previamente determinadas em nosso laboratório.

Foi utilizada metodologia preconizada pelo fornecedor (Pharmingen, BD) e adaptadas para o nosso laboratório como descrito por Cano et al. (1998). As concentrações de cada citocina foram determinadas tendo como base a reta de regressão linear feita para a curva-padrão obtida com o padrão adequado de citocina recombinante.

3.8 Preparo de suspensões de leucócitos infiltrantes de pulmão (LIP) e de fígado

As suspensões celulares de linfócitos infiltrantes de pulmão foram preparadas de acordo com Huffnagle et al. (1991) (HUFFNAGLE; YATES; LIPSCOMB, 1991). Os pulmões foram removidos e digeridos por 60 minutos em meio RPMI-1640 Medium (Sigma) contendo colagenase (1 mg/mL) e DNAse (30 µg/mL). Já as suspensões celulares de linfócitos infiltrantes de fígado foram preparadas de acordo com Mallevaey et al. (2006). O fígado foi primeiro perfundido com PBS e depois removido, digerido e as células separadas através de membrana de 70 µm (*Cell strainers* - BD). Os eritrócitos do pulmão e fígado foram lisados com tampão de lise (cloreto de amônio + TRIS) e em seguida os linfócitos foram isolados por centrifugação em Percoll a 33% (Pharmacia Biotech AB, Uppsala, Sweden). As células foram contadas e a viabilidade determinada pela marcação de azul de Trypan.

3.9 Citometria de fluxo para caracterização de subpopulações celulares

Células infiltrantes dos pulmões (CANO et al., 2000) foram obtidas na segunda e décima semanas após infecção, enquanto que células infiltrantes do fígado foram obtidas após a décima semana de infecção. A concentração celular foi ajustada, e os linfócitos foram adicionados à placas de fundo em U na quantidade de $1x10^6$ céls/poço. Em seguida, as células foram ressuspensas em PBS-azida (0,1%) contendo soro fetal bovino (SFB, 5%). As placas foram centrifugadas, os sobrenadantes foram dispensados e o Ac marcado (25 µL no título adequado) foi adicionado. Após incubação por 20 minutos em geladeira, as células foram lavadas, ressuspensas em PBS-azida e transferidas para tubos de leitura de FACS. Todas as amostras foram mantidas em banho de gelo e protegidas da luz. As células marcadas foram analisadas em citômetro de fluxo FACSCanto e software FlowJo (Tree-Star). Foram usados anticorpos marcados com fluorocromos diferentes (BD Biociences) na combinação adequada para a população celular a ser analisada (marcadores celulares de linfócitos: CD3, CD4, CD8, CD44, CD25, CTLA-4, GITR, CD62L, LAP, CD19 e B220 e marcadores celulares de macrófagos: F4/80, GR1, CD11c, MHC II.

3.10 Citometria de fluxo para caracterização de células T reguladoras $TCD4^+CD25^+$ através da expressão do fator de transcrição FoxP3 e de marcadores de superfície

Após a obtenção de suspensão celular (1 x 10^6 células/mL) do pulmão e fígado, procedeu-se à marcação de proteínas de membrana com anti-CD4 PE-Cy7 (1/100), anti-CD25 Pe-Cy5 (1/200) ou FITC (1/100), CTLA-4 PE (1/100), GITR FITC (1/100) e anti-TGF-β1 de membrana (LAP- latency associated peptide) PERCY (10 µl por amostra) por 30 minutos em temperatura a 4 ^oC. Posteriormente, as células foram lavadas com tampão de lavagem ("*staining buffer"* Kit eBioscience). A seguir, foi adicionado 1 mL/poço do tampão de fixação e permeabilização ("*Fixation/Permeabilization*" Kit, eBioscience) e incubados por 30 minutos a 4ºC. Após este período as células foram lavadas com um tampão de permeabilização específico para esta etapa (*"Permeabilization Buffer"* Kit, eBioscience) e submetidas à marcação intracelular para o fator de transcrição nuclear Foxp3 PE ou APC (1/200). Após a marcação as células foram novamente lavadas, ressuspendidas em tampão (*"Flow Cytometry Staining Buffer"* Kit eBioscience) e analisadas em citometro de fluxo.

Foram feitos os "gates" primeiramente para linfócitos totais, depois para linfócitos T $CD4^+$, linfócitos T $CD4^+CD25^+$ e, finalmente, o gate para linfócitos T $CD4^+CD25^+FoxP3^+$. Desta população foram analisadas as células que expressavam o marcador CTLA-4, GITR e LAP. A aquisição das células (1.000.000 eventos) foi feita pelo FACS Canto (BD Pharmingen®) e a análise utilizou o software FlowJo (Tree-Star).

3.11 Citometria de fluxo para caracterização das citocinas intracelulares produzidas por Células T reguladoras ($CD4^+CD25^+FoxP3^+$)

Para dosagem das citocinas intracelulares, as células dos pulmões foram tratadas com meio de cultura contendo 50 ng/mL de PMA (Phorbol 12-myristate 13-acetato), 500 ng/mL de Ionomicina (Sigma) e Brefeldina (1000X – e-Bioscience) por 6 horas antes da marcação intracelular. Após este período, as proteínas de membrana das células foram marcadas com anti-CD4 Pe-cy7 (1/100) e anti-CD25 FITC (1/100). Em seguida, procedeu-se à marcação intracelular para o fator de transcrição FoxP3 PE ou APC (1/200) e para as citocinas intracelulares IL-10 Pacific Blue (1/100) e TGF-β1 PE (10µl por amostra). Da mesma maneira que para a marcação do fator de transcrição Foxp3, as células foram tratadas com um fixador e permeabilizador ("*Fixation/Permeabilization*" Kit, eBioscience) para alterar a permeabilidade da membrana e o anticorpo pode fazer a marcação intracitoplasmática. Após a marcação as células foram novamente lavadas, ressuspendidas em tampão (*"Flow Cytometry Staining Buffer"* Kit, eBioscience) e levadas ao citômetro de fluxo.

Foram feitos os "gates" primeiramente para linfócitos totais, depois para linfócitos T $CD4^+$, linfócitos T $CD4^+CD25^+$ e finalmente, o gate para linfócitos T $CD4^+CD25^+FoxP3^+$. Desta população foram analisadas as células que apresentavam as citocinas intracelulares IL-10 e TGF-β. A aquisição das células (1.000.000 eventos) foi feita pelo FACS Canto (BD Pharmingen®) e a análise utilizou o software FlowJo (Tree-Star).

3.12 Ensaios *in vitro* da atividade supressora de células CD4+CD25+ obtidas de pulmão de camundongos A/J e B10.A infectados pelo fungo

Para determinação do potencial supressor das células T reguladoras ($CD4^+CD25^+FoxP3^+$) foi utilizado um ensaio de proliferação envolvendo células T respondedoras $CD4^+CD25^-$ obtidas do baço de camundongos A/J e B10.A sadios além das células T reguladoras $CD4^+CD25^+$ obtidas do pulmão dos camundongos A/J e B10.A infectados com *Paracoccidioides brasiliensis*. Para isso realizamos a marcação das células respondedoras $CD4^+CD25^-$ com CFSE (do inglês, carboxy-fluorescein diacetate, succinimidyl ester) (INVITROGEN) seguindo protocolo descrito abaixo.

Para a separação das células T reguladoras $CD4^+CD25^+$ os pulmões dos camundongos A/J e B10.A infectados com o fungo foram digeridos em uma solução contendo 2 mg/mL de colagenase tipo IV (Sigma) e 1mg/mL de desoxiribonuclease (Sigma) e mantidos por agitação à 37 ^oC por 45 minutos. As células T $CD4^+CD25^+$ foram então separadas através de *beads* magnéticas segundo especificações do fabricante (Miltenyi Biotec- MACS).

Os linfócitos T respondedores $CD4^+CD25^-$ do baço de camundongos A/J e B10.A sadios também foram obtidos através do protocolo descrito acima. Além disto, as células T $CD4^+CD25^-$ foram ressuspendidas em 1mL de PBS-BSA (0,1%) estéril contendo 1µl de CFSE, e incubadas a temperatura ambiente no escuro, por 15 minutos. Após este período, as células foram lavadas 2x e ressuspendidas na concentração de 2 x 10^4/mL.

Em paralelo, separamos as células apresentadoras de antígeno (APC) obtidas dos pulmões dos camundongos A/J e B10.A sadios. Os pulmões passaram pelo mesmo protocolo descrito para obtenção das células T reguladoras e T respondedoras. Entretanto, após a primeira etapa de separação de células T $CD4^+$ (separação de células totais exceto T $CD4^+$), as células $TCD4^-$ foram aderidas em placa de plástico de 24 poços por 2 horas, e após este período foi retirado o sobrenadante em conjunto com as células não aderentes. Após desprender as células aderentes à placa, estas foram centrifugadas e irradiadas a 1250 rads (fonte de Césio^{137}).

Os ensaios de proliferação foram realizados em placas de 96 poços em fundo U, contendo, por poço: 2 x 10^4/mL de células T respondedoras ($CD4^+CD25^-$) marcadas com CFSE, 4 x 10^4/mL de células APC e 2 µg/mL de anticorpo purificado anti-CD3 (eBioscience).

As células T supressoras ($CD4^+CD25^+$) foram colocadas na cultura a uma razão de efetora:supressora ($CD25^-/CD25^+$) 1:1 ($2x10^4$:$2x10^4$), 1:0,33 ($2x10^4$:$0,66x10^4$) e 1:0,11 ($2x10^4$:$0,22x10^4$).

Para este ensaio foi preciso realizar alguns grupos experimentais e controles, tais como:

- linfócitos T $CD4^+CD25^-$ marcados com CFSE;
- linfócitos T $CD4^+CD25^-$ marcados com CFSE + α-CD3;
- linfócitos T $CD4^+CD25^-$ marcados com CFSE + APC;
- linfócitos T $CD4^+CD25^-$ marcados com CFSE + α-CD3 + APC;
- linfócitos T $CD4^+CD25^-$ marcados com CFSE + α-CD3 + APC + T $CD4^+CD25^+$

Após 5 dias de incubação o grau de fluorescência das células foi determinado em citômetro de fluxo FACS Canto. Para a análise dos dados de proliferação, foi criado um índice de proliferação calculado da seguinte maneira:

$$IP = \frac{\text{Média geométrica da intensidade de fluorescência dos linfócitos } CD4^+CD25^- \text{ cultivados e não estimulados}}{\text{Média geométrica da intensidade de fluorescência dos linfócitos estimulados na presença ou ausência de células } CD4^+CD25^+}$$

Este índice permite mensurar a queda na intensidade de fluorescência das células marcadas, que será mais alto quanto maior for o número de divisões.

3.13 Determinação do tempo médio de sobrevida

Grupos de 6 camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 foram infectados e a mortalidade acompanhada diariamente.

3.14 Análise histopatológica e morfométrica dos pulmões

Foi examinado um dos pulmões de cada um dos animais A/J e B10.A utilizados na determinação de UFC nos tempos de 2 e 10 semanas pós-infecção. Os órgãos foram preservados em solução de Millonig modificada (CARSON; MARTIN; LYNN, 1973) à temperatura ambiente até o momento do processamento. Posteriormente, já incluídos em

parafina, foram cortados fragmentos na espessura de 4 a 5 μm e submetidos à coloração por hematoxilina-eosina (H&E) para identificação celular e por impregnação pela prata (GROCOTT) para identificação fúngica .

O preparo das lâminas foi feito pelo Setor de Histologia do Departamento de Imunologia do ICB IV- USP e a análise descritiva das lesões foi realizada ao microscópio óptico comum pela Profa. Dra. Katia R.M. Leite do Departamento de Anatomia Patológica da Faculdade de Medicina da Universidade de São Paulo.

Para a análise morfométrica nós utilizamos o microscópio Nikon DXM 1200c com câmera digital (aumento de 10x) e software Nikon NIS Elements AR 2.30. A área da lesão foi calculada em μm^2 analisando 10 campos por amostra de pulmão em um total de 5 camundongos por grupo. Os resultados estão apresentados com média±erro padrão da área total de lesões de cada camundongo.

3.15 Análise estatística

Comparações entre os diferentes tratamentos foram feitas utilizando análise de variância ou teste *t* de Student, dependendo do número de grupos. Para a análise estatística da mortalidade foi utilizado o teste log rank. Foi utilizado o programa Prisma 5 (GraphPad Software). Como nível de significância foi considerado $p<0,05$.

4 RESULTADOS

4.1 Análise da presença de células T reguladoras $CD4^+CD25^+FoxP3^+$ em camundongos A/J e B10.A sadios

Já que nosso estudo teve como foco principal o estudo das células T reguladoras frente à infecção causada pelo *P. brasiliensis* e que esta célula é fundamental para manter a homeostase do organismo, nossa primeira pergunta foi se os camundongos resistentes e susceptíveis ao fungo apresentariam, em condições normais, diferenças entre o número de células T reguladoras. Para isso foram obtidas células dos pulmões destes camundongos, a concentração ajustada para $1x10^6$ cels/mL e as células foram marcadas com anticorpos (anti-CD4, anti-CD25 e Foxp3) na titulação adequada. Para determinação dos níveis intracelulares de FoxP3 as células foram permeabilizadas e o anticorpo pôde fazer a marcação intracitoplasmática. A figura 1A mostra o número de células T $CD4^+CD25^+FoxP3^+$ obtido em cada linhagem. Podemos observar que os camundongos A/J apresentam número basal de células T reguladoras maior quando comparados aos camundongos B10.A. Já a figura 1B mostra que a expressão do marcador FoxP3 também é maior nos camundongos A/J quando comparado com os camundongos B10.A.

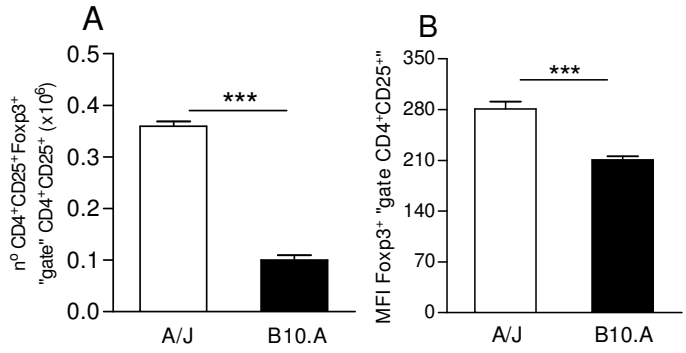

Figura 1: Quantificação de células $CD4^+CD25^+FoxP3^+$ no pulmão de camundongos A/J e B10.A normais. (A) Análise do número absoluto de linfócitos T $CD4^+CD25^+FoxP3^+$, e (B) intensidade média de fluorescência (MFI) dos linfócitos $CD4^+CD25^+FoxP3^+$ dos pulmões. A intensidade média de fluorescência das células que expressam $FoxP3^+$ foi obtida a partir de um *gate* de populações celulares $CD4^+CD25^+$. O valor absoluto de cada sub-população é apresentado como média ±EP. (***P<0,001).

4.2 Caracterização da presença de células T reguladoras $CD4^+CD25^+FoxP3^+$ em camundongos suscetíveis e resistentes após 2 e 10 semanas de infecção

Antes de estudarmos o efeito da depleção das células T reguladoras no curso da infecção desenvolvida por camundongos A/J e B10.A, nos perguntamos como estas células se comportariam frente à infecção com o fungo, já que estas células T reguladoras são fundamentais no controle da resposta imunológica. Assim, grupos de camundongos A/J e B10.A foram infectados com $1x10^6$ células leveduriformes de *P. brasiliensis* e as células infiltrantes dos pulmões foram obtidas na segunda e décima semanas pós-infecção. A concentração foi ajustada a $1x10^6$ cels/mL e as células marcadas com anticorpos (anti-CD4, anti-CD25 e Foxp3) na titulação adequada. A figura 2 mostra a intensidade média de fluorescência de $FoxP3^+$ dentro da população de células $CD4^+CD25^+$ e o número de células T reguladoras.

Observamos que tanto na segunda como na décima semanas de infecção houve diferença estatisticamente significante entre os grupos estudados em relação ao número de células $CD4^+CD25^+FoxP3^+$ (figura 2A) e à expressão de FoxP3, um típico marcador de célula T reguladora (Treg) (figura 2B). Em ambos os períodos de infecção, os camundongos A/J apresentavam maior expressão deste marcador e maior número de células T reguladoras quando comparado com os camundongos B10.A.

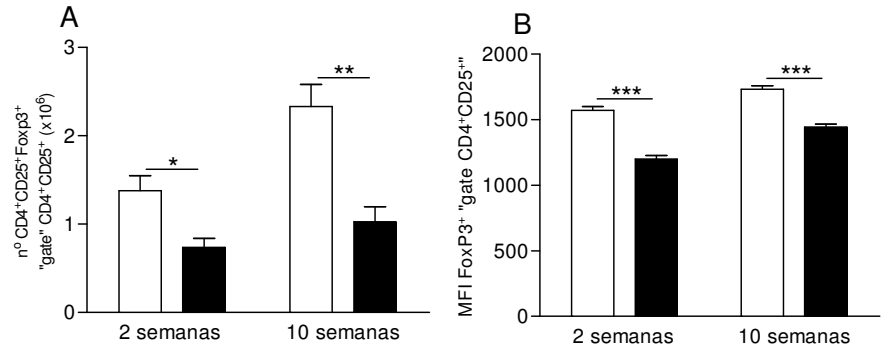

Figura 2: Caracterização do número (A) e intensidade média de fluorescência (B) de linfócitos $CD4^+CD25^+FoxP3^+$ infiltrantes de pulmão de camundongos A/J e B10.A nas segunda e décima semanas de infecção com um $1x10^6$ células leveduriformes do *P. brasiliensis.* A intensidade média de fluorescência das células que expressam $FoxP3^+$ foi obtida a partir de um *gate* de células $CD4^+CD25^+$. O valor absoluto das células T reguladoras é apresentado como média ±EP. (*P<0,05 **P< 0,01 ***P<0,001).

4.3 Análise dos marcadores de superfície e das citocinas intracelulares de células T reguladoras ($CD4^+CD25^+FoxP3^+$) de camundongos suscetíveis e resistentes após 2 e 10 semanas de infecção

Uma vez que mostramos que após a infecção com o fungo as células T reguladoras se expandem e fazem presentes no sítio da infecção tanto na fase inical da doença (2 semanas) como na fase crônica (10 semanas), e além disso, se apresentam em números diferentes em camundongos A/J e B10.A, nos perguntamos quais seriam as características destas células quanto aos marcadores de superfície e citocinas intracelulares característicos de células T reguladoras. Para isto, grupos de camundongos A/J e B10.A foram infectados com $1x10^6$ células do *P. brasiliensis* e as células infiltrantes dos pulmões obtidas na segunda e décima semanas pós-infecção. A concentração foi ajustada a $1x10^6$ cels/mL e as células marcadas com anticorpos (anti-CD4, anti-CD25, anti-LAP, anti-GITR e anti-CTLA-4) na titulação adequada. Para determinação dos parâmetros intracelulares (FoxP3, TGF-β e IL-10) as células foram permeabilizadas e o anticorpo pôde fazer a marcação intracitoplasmática.

Na figura 3A e 3B podemos observar que tanto na segunda como na décima semanas pós infecção, os camundongos A/J apresentavam número de células T reguladoras $CD4^+CD25^+FoxP3^+LAP^+$, $CD4^+CD25^+FoxP3^+CTLA\text{-}4^+$ e $CD4^+CD25^+FoxP3^+GITR^+$ maior que os camundongos B10.A. O mesmo fenômeno pôde ser observado para as citocinas intracelulares (figura 3C), pois os camundongos A/J também apresentavam número maior de células T reguladoras produtoras de TGF-β e IL-10 quando comparados aos camundongos B10.A.

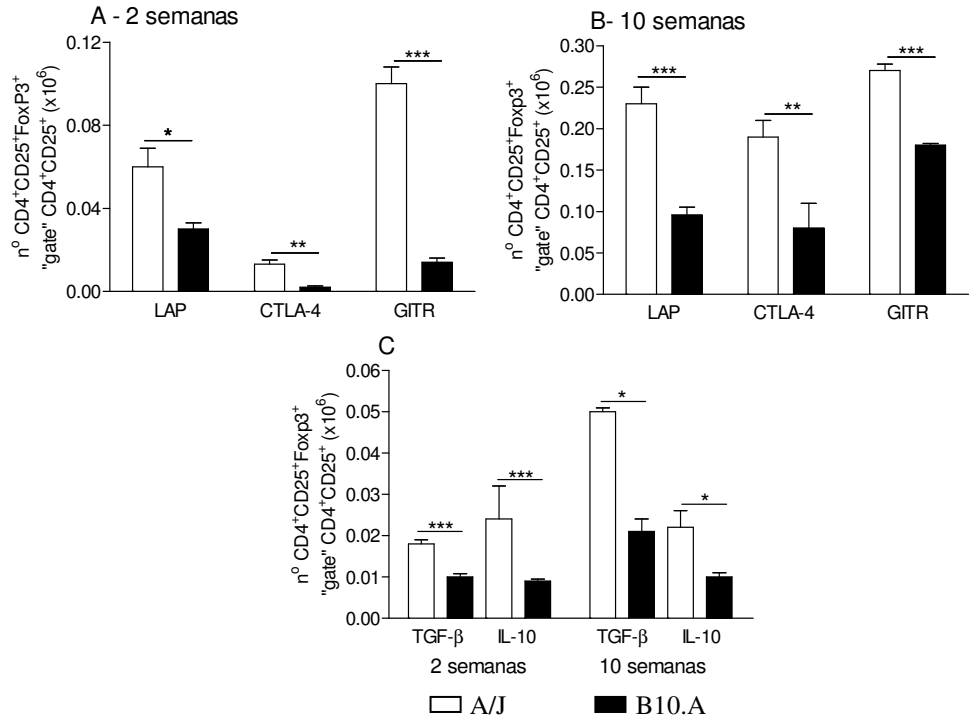

Figura 3: Caracterização da expressão de TGF-β de membrana (LAP), CTLA-4 e GITR de membrana, e IL-10 e TGF-β intracelular em células Treg ($CD4^+CD25^+FoxP3^+$) de pulmões. Camundongos A/J e B10.A foram infectados com $1x10^6$ células leveduriformes do *P. brasiliensis* e linfócitos infiltrantes de pulmão obtidos nas segunda e décima semanas pós infecção. A expressão dos marcadores de membrana e as citocinas intracelulares foram obtidas a partir de *gate* de células T reguladoras $CD4^+CD25^+FoxP3^+$. Os resultados são apresentados como média do número absoluto de células ±EP. (*P<0,05 **P<0,01 ***P<0,001).

4.4 Avaliação da capacidade supressora de células T $CD4^+CD25^+$ de camundongos suscetíveis e resistentes

A caracterização das células T reguladoras no curso da infecção pelo fungo demonstrou que além de aparecerem em números distintos, os marcadores de superfície assim como as citocinas intracelulares eram diferencialmente expressas entre camundongos resistentes e susceptíveis ao fungo. Assim, nos perguntamos se estas células apresentavam também efeitos supressores distintos sobre a linfoproliferação policlonal de linfócitos normais.

Para a análise da função supressora das células T reguladoras, as células T respondedoras $CD4^+CD25^-$ obtidas do baço dos camundongos A/J e B10.A sadios foram colocadas em cultura na ausência ou presença de diferentes proporções de células T reguladoras $CD4^+CD25^+$ obtidas de pulmões de camundongos A/J e B10.A infectados. As células respondedoras foram marcadas com CFSE, estimuladas ou não com anticorpo anti-CD3 e APCs irradiadas e a proliferação foi avaliada através da perda do corante CFSE após as divisões celulares.

As figuras 4A e 4B mostram os resultados obtidos com células T reguladoras isoladas dos pulmões nas segunda e décima semanas após a infecção. Podemos verificar que nos dois períodos de infecção as células T $CD4^+CD25^-$ do baço dos camundongos A/J e B10.A não infectados com o fungo proliferam após estímulo com anti-CD3 e APC (S.I "Sistema Indicador" de proliferação).

Por outro lado, quando adicionamos às culturas as células T $CD4^+CD25^+$ obtidas dos pulmões dos camundongos infectados observamos redução significativa da proliferação de ambas as linhagens quando comparamos com o sistema indicador que não possuía células T $CD4^+CD25^+$. Além disso, observamos que a redução na proliferação é dose-dependente com células de ambos os camundongos, A/J e B10.A.

Vale ressaltar, que nos dois períodos de infecção a redução da proliferação foi mais marcante com as células T $CD4^+CD25^+$ da linhagem resistente, o que indica, que as células T reguladoras dos camundongos A/J apresentavam maior capacidade intrínsica de suprimir a resposta imune do que as células T reguladoras dos animais B10.A. Além disso, esta diferença entre as linhagens é mais marcante na segunda do que na décima semana pós infecção.

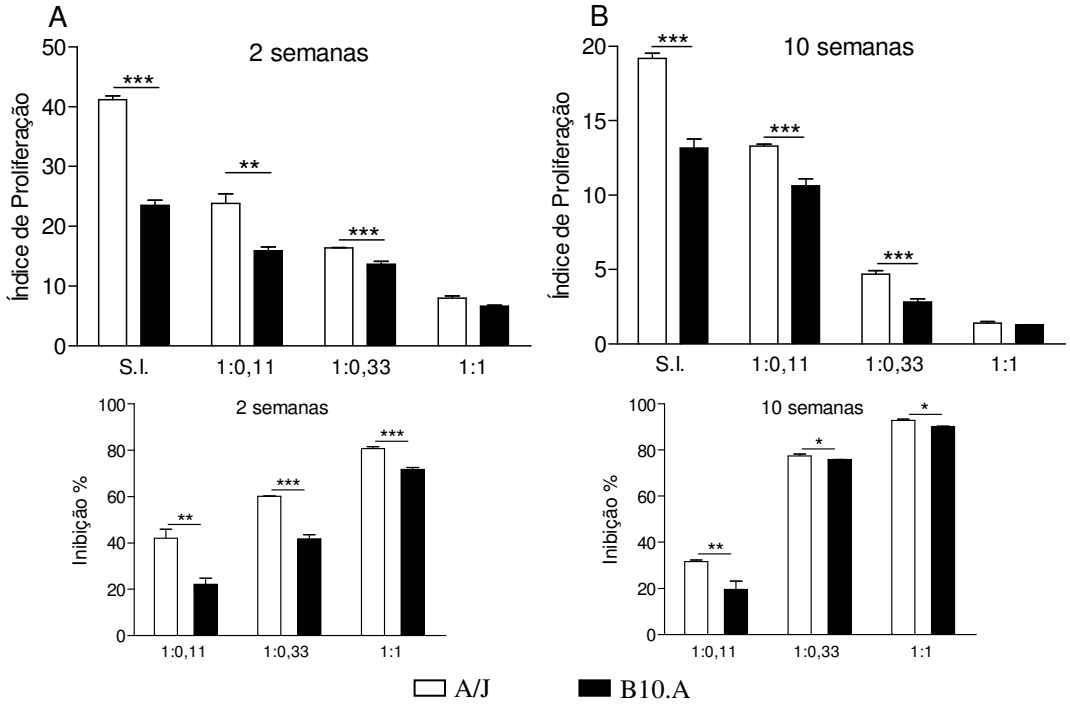

Figura 4: Avaliação da atividade supressora das células T reguladoras CD4+CD25+. As células T respondedoras $CD4^+CD25^-$ obtidas do baço dos camundongos A/J e B10.A sadios, separadas por *beads* magnéticas; foram então estimuladas ou não com anticorpo anti-CD3, APCs normais irradiadas e co-cultivadas ou não com células Treg $CD4^+CD25^+$ obtidas dos pulmões dos camundongos A/J e B10.A infectados, também separadas por *beads*. A diluição do CFSE nas células T respondedoras ($CD4^+CD25^-$) foi avaliada por citometria de fluxo após 5 dias de cultura. O valor do Índice de Proliferação de cada população é apresentado como média ± EP. (*P<0,05 **P<0,01 ***P<0,001).

4.5 Efeito da depleção de células Treg na carga fúngica tecidual de 2 e 10 semanas após a infecção com *P. brasiliensis*

Estudamos o efeito da depleção das células $CD25^+$ no número de fungos recuperados nas segunda e décima semanas após a infecção com $1x10^6$ células leveduriformes de *Paracoccidioides brasiliensis*. Para isto, grupos de camundongos susceptíveis e resistentes foram depletados de linfócitos $CD25^+$ através da inoculação de 500 µg/0,5 mL i.p. do anticorpo monoclonal PC61 purificado. Este anticorpo foi aplicado pela via i.p. 3 dias antes da inoculação i.t. de leveduras do Pb 18. Esta depleção foi repetida 3 dias após a infecção. Paralelamente outros camundongos foram injetados com Ig de rato no mesmo dia e na mesma concentração do PC61 e usados como grupos controle. Os resultados a seguir mostram a média de UFC/g de tecido (log_{10}). Todos os dados aqui apresentados, são resultados de três experimentos independentes com resultados equivalentes (n=6).

Na figura 5 podemos observar os resultados obtidos após 2 e 10 semanas de infecção. A figura 5A mostra que 2 semanas após a infecção não houve diferença de carga fúngica do pulmão entre as linhagens tratadas com Ig de rato (grupos controle). Por outro lado, após a depleção com anticorpo anti-CD25 observamos que tanto os camundongos resistentes (A/J) como os camundongos suscetíveis (B10.A) apresentaram diminuição no número de fungos recuperados quando comparados aos grupos controle. Entretanto, podemos observar que nos camundongos A/J a depleção parece ter ocasionado uma diminuição maior na carga fúngica do que aquela observada em camundongos B10.A.

Ao analisarmos na décima semana pós infecção o pulmão dos camundongos B10.A tratados com Ig de rato (grupo controle) observamos maior número de fungos recuperados quando comparado aos camundongos A/J do mesmo grupo. Por outro lado, verificamos que a depleção com anticorpo anti-CD25 induziu em ambas as linhagens diminuição no número de fungos recuperados quando comparado aos grupos tratados com Ig normal (figura 5A).

Já a figura 5B mostra a disseminação do fungo para o fígado. Podemos observar que no fígado dos camundongos A/J e B10.A tratados com Ig existe um maior número de fungos

recuperados do que nos camundongos depletados. De maneira interessante, pode-se observar que os camundongos A/J tratados com Ig apresentam número de fungos recuperados maior que os camundongos B10.A também tratados com Ig.

Da mesma maneira analisamos a disseminação do fungo para o fígado em camundongos B10.A e A/J no período de 10 semanas de infecção. Podemos observar que neste período ocorre um maior crescimento do fungo no fígado de camundongos B10.A controle do que nos camundongos A/J do mesmo grupo. Entretanto, após a depleção com anticorpo anti-CD25, apenas a linhagem de camundongos B10.A apresentou diminuição no número de fungos recuperados quando comparado ao grupo controle tratado com Ig. Entre os grupos de camundongos A/J não observamos diferenças significantes (figura 5B).

No baço, apesar de ter ocorrido disseminação, não observamos na segunda semana pós infecção diferenças estatisticamente significantes entre os grupos (figura 5C).

Por outro lado, também observamos efeito do tratamento com anticorpo monoclonal anti-CD25 no crescimento do fungo. Os camundongos A/J e B10.A depletados apresentaram número de fungos recuperados menor que aqueles obtidos dos camundongos controle (figura 5C).

Nossos dados demonstram que a depleção de células T $CD25^+$ ocasionou diminuição significativa na carga fúngica de ambas as linhagens de camundongos. Este achado sugere que o anticorpo anti-CD25 tenha bloqueado o efeito das células T reguladoras, deixando o sistema imune mais ativado e mais eficiente para o controle do fungo em ambos os períodos de infecção estudados.

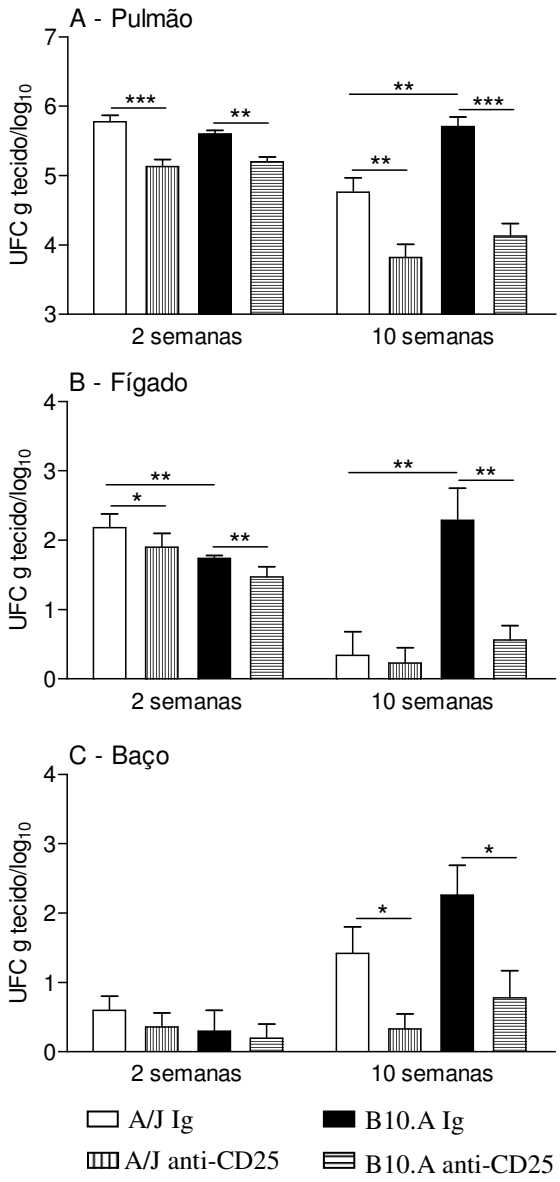

Figura 5: Análise do grau de infecção através de Unidades Formadoras de Colônias (UFC) obtido de camundongos A/J e B10.A depletados de células $CD25^+$ por tratamento com anticorpo monoclonal anti-CD25 ou Ig de rato, nas segunda e décima semanas após a infecção com $1x10^6$ células leveduriformes de *P. brasiliensis*. O grau da infecção foi determinado pela contagem de UFC nos órgãos (*P<0,05 **P<0,01 *** P<0,001).

4.6 Efeito da depleção de células $CD25^+$ nos níveis de óxido nítrico (NO) presentes nos pulmões de camundongos A/J e B10.A

Os níveis de NO foram quantificados nos homogenatos dos pulmões de camundongos A/J e B10.A na segunda e décima semana após a infecção pelo fungo.

Na figura 6 observamos que após 2 semanas de infecção os camundongos B10.A tratados com Ig apresentaram níveis maiores de NO quando comparado aos camundongos A/J do mesmo grupo e aos camundongos B10.A depletados com anti-CD25. Por outro lado, na décima semana após a infecção não observamos diferenças significantes entre os grupos.

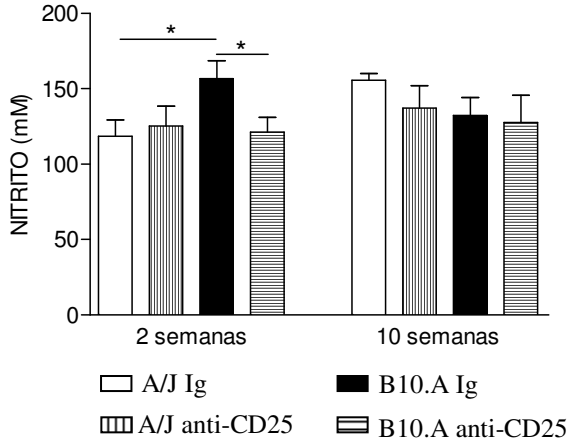

Figura 6: Quantificação de NO nos homogenatos de pulmão de camundongos A/J e B10.A tratados com anticorpo monoclonal anti-CD25 ou Ig normal de rato na segunda e décima semana após a infecção com $1x10^6$ leveduras do *P. brasiliensis*. Os dados apresentam a média +/- erro obtidos de grupos analisados nas segunda e décima semanas pós-infecção. O asterisco representa diferença estatisticamente significante (*P<0,05).

4.7 Efeito da depleção de células $CD25^+$ nos níveis de citocinas pulmonares do tipo 1 de camundongos A/J e B10.A

Na figura 7 estão mostrados os resultados obtidos para as citocinas do tipo 1 nos homogenatos de pulmão (Média +/- Erro Padrão) nos tempos de 2 e 10 semanas após a infecção com o fungo *P. brasiliensis* em camundongos B10.A e A/J tratados ou não com anticorpo anti-CD25. Os dados apresentados, são resultados de 2 experimentos independentes com resultados equivalentes (n=6).

No período de 2 semanas de infecção, não foram observadas diferenças significantes nas concentrações das citocinas TNF-α, IFN-γ, IL-2 e IL-12 presentes nos pulmões de camundongos B10.A e A/J. Já no período de 10 semanas de infecção, as citocinas dos padrões Th1, apresentaram alterações. Em ambas as linhagens observamos que a depleção com o anticorpo anti-CD25 ocasionou um aumento dos níveis das citocinas IL-2 e IL-12 quando comparado aos seus grupos controle.

É interessante verificar que os camundongos B10.A controle apresentaram maiores níveis de IL-2 e IL-12 que os camundongos A/J controle, tratados com Ig. Os níveis das citocinas IFN-γ e TNF-α não apresentaram diferenças entre os grupos (figura 7).

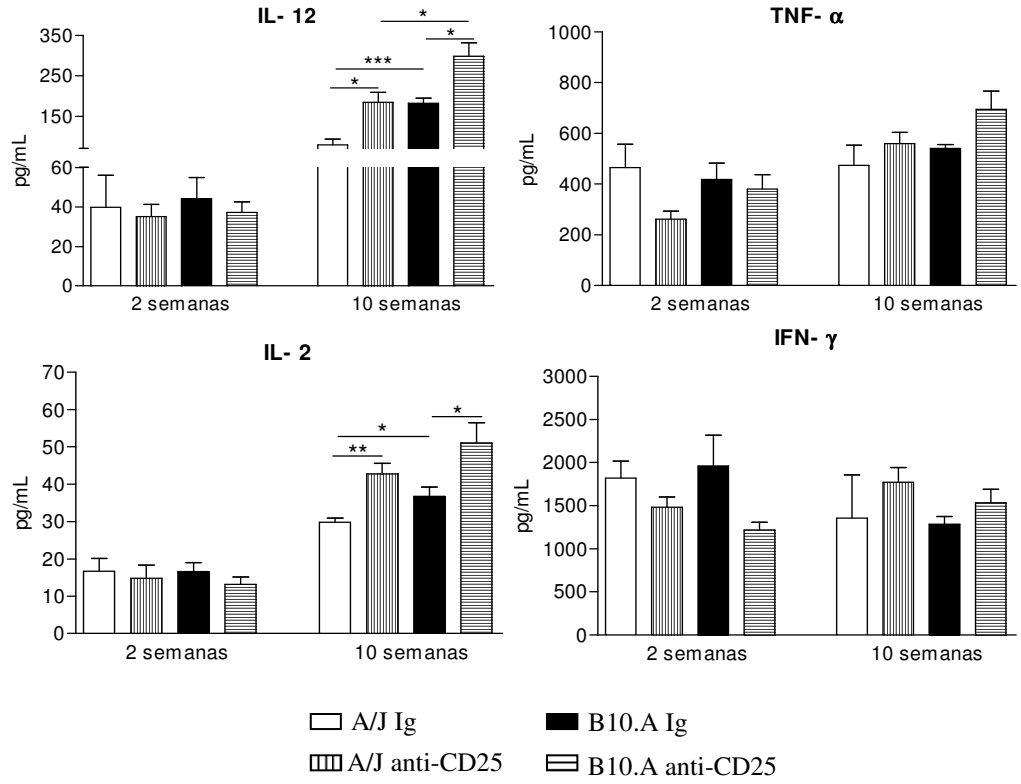

Figura 7: Quantificação dos níveis de citocinas tipo 1 (pg/mL) por ELISA em camundongos resistentes e susceptíveis após tratamento com anticorpo monoclonal anti-CD25 ou Ig de rato e subseqüente infecção com $1x10^6$ leveduras do *P. brasiliensis*. Os homogenatos pulmonares foram obtidos após 2 e 10 semanas de infecção (*P<0,05, **P<0,01, ***P<0,001).

4.8 Efeito da depleção de células $CD25^+$ nos níveis de citocinas pulmonares do tipo 2 de camundongos A/J e B10.A

As citocinas do tipo 2 (IL-4 e IL-10) e o fator de crescimento (GM-CSF) foram dosados em camundongos A/J e B10.A infectados com o fungo e depletados ou não com anticorpo anti-CD25.Os resultados estão apresentados na figura 8.

Na segunda semana após a infecção observamos que os camundongos A/J e B10.A depletados com anti-CD25 apresentaram diminuição nos níveis de IL-10 quando comparados aos seus controles.

Os camundongos A/J do grupo controle apresentaram maiores níveis de GM-CSF quando comparados aos camundongos A/J depletados com anti-CD25. Por outro lado, os níveis da citocina IL-4 não foram diferentes entre os grupos (figura 8).

Na décima semana após a infecção, tanto os camundongos A/J como os camundongos B10.A depletados com anti-CD25 apresentaram maiores níveis de IL-4, IL-10 e GM-CSF quando comparado aos seus grupos controle. Observamos ainda que a depleção promoveu um maior aumento da citocina IL-4 nos camundongos B10.A do que nos camundongos A/J que receberam o mesmo tratamento.

Em relação aos grupos controle, os camundongos B10.A apresentaram maiores níveis de GM-CSF do que camundongos A/J do mesmo grupo (figura 8).

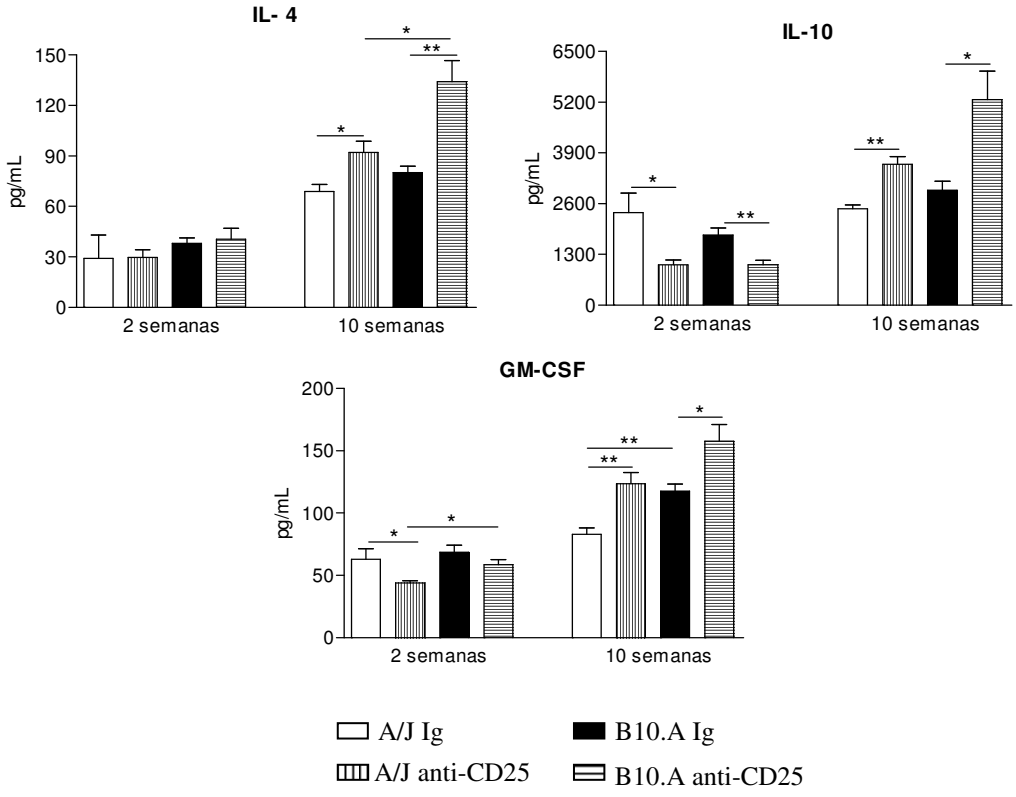

Figura 8: Quantificação dos níveis de citocinas tipo 2 (pg/mL) por ELISA em camundongos resistentes e susceptíveis após tratamento com anticorpo monoclonal anti-CD25 ou Ig de rato e subseqüente infecção com $1x10^6$ leveduras do *P. brasiliensis*. Os homogenatos pulmonares foram obtidos após 2 e 10 semanas de infecção (*P<0,05 **P<0,01).

4.9 Efeito da depleção de células $CD25^+$ nos níveis de citocinas pulmoanres do tipo Th17 de camundongos A/J e B10.A

A influência da depleção das células T reguladoras nos níveis das citocinas do tipo Th17 produzidos por camundongos suscetíveis e resistentes infectados pelo fungo e depletados ou não com anti CD25 também foi estudada.

Na segunda semana após a infecção, observamos na figura 9 que os níveis da citocina IL-6 foram menores nos camundongos A/J e B10.A depletados quando comparado aos controles. Observamos ainda, que em relação ao grupo dos depletados, os camundongos B10.A apresentaram níveis maiores de IL-6 que os camundongos A/J do mesmo grupo.

Já em relação aos níveis de TGF-β, observamos que a depleção ocasionou redução nos níveis desta citocina produzidos pelos camundongos A/J e B10.A quando comparados aos controles. Por outro lado, após a depleção, apenas os camundongos A/J apresentaram menor produção de IL-23 em comparação com camundongos A/J controle. Não observamos diferenças nos níveis de IL-17 entre os grupos estudados.

Os níveis das citocinas do tipo Th17 produzidas após 10 semanas de infecção também foram avaliadas. A produção de IL-6 e TGF-β esteve aumentada em ambas as linhagens depletadas quando comparadas aos grupos controle. Observamos ainda, que os camundongos B10.A controle e tratados apresentaram níveis maiores de IL-6 quando comparado aos camundongos A/J de grupos equivalentes. Nos camundongos B10.A depletados os níveis de TGF-β eram maiores do que os dos camundongos A/J de grupo equivalente.

Os níveis da citocina IL-23 foram maiores nos camundongos B10.A depletados quando comparados aos camundongos A/J do mesmo grupo. O mesmo pôde ser observado para a citocina IL-17. Além disso, observamos que os animais B10.A apresentavam diferenças significantes quanto aos níveis de IL-17, sendo que os animais depletados apresentaram aumento desta citocina quando comparado ao seu grupo controle

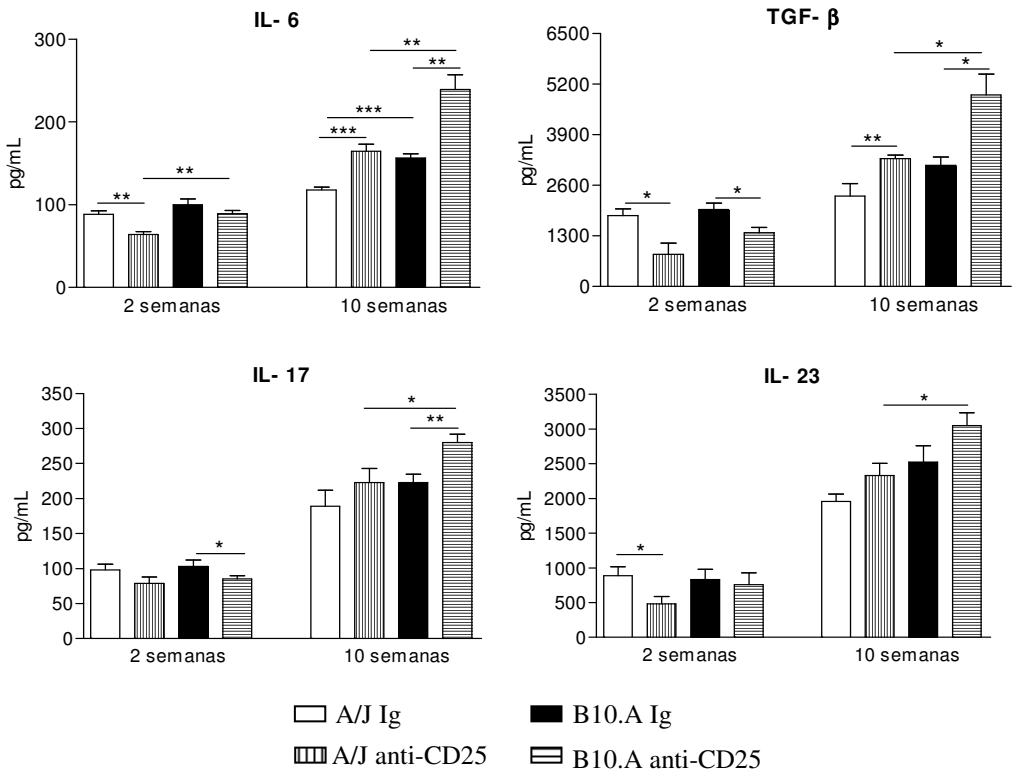

Figura 9: Quantificação dos níveis de citocinas tipo 17 (pg/mL) por ELISA em camundongos resistentes e susceptíveis após tratamento com anticorpo monoclonal anti-CD25 ou Ig de rato e subseqüente infecção com $1x10^6$ leveduras do *P. brasiliensis*. Os homogenatos pulmonares foram obtidos após 2 e 10 semanas de infecção (*P<0,05, **P<0,01, ***P<0,001).

4.10 Análise fenotípica de leucócitos infiltrantes de pulmão de camundongos B10.A e A/J na segunda e décima semanas de infecção

Há vários trabalhos que demonstram que a depleção de células T reguladoras pelo anticorpo monoclonal anti-CD25 tem grande influência nos vários mecanismos de regulação da resposta imunológica (revisado por BELKAID e TARBELL, 2009).

Como verificamos que a depleção alterava a carga fúngica e a produção de citocinas, resolvemos investigar o efeito deste tratamento no afluxo de células para o sítio de infecção. Assim, células infiltrantes dos pulmões foram obtidas na segunda e décima semanas após a infecção pelo fungo. A concentração foi ajustada para $1x10^6$ cels/mL e as mesmas foram marcadas com anticorpos específicos e analisadas por citometria de fluxo.

Estudamos o efeito do tratamento com anticorpo anti-CD25 no afluxo de linfócitos T totais, células B e granulócitos para os pulmões. Para isto utilizamos os marcadores CD3 para caracterizar a população de linfócitos T, CD19 para linfócitos B e GR1 para os granulócitos.

Na figura 10A, observamos que 2 semanas após a depleção com anticorpo anti-CD25, o número de células $CD3^+$, $CD19^+$ e $GR1^+$ apresentou-se aumentado nos camundongos A/J tratados quando comparados com os camundongos que receberam Ig. Em contraste, a depleção de células CD25 não alterou o número destas três populações celulares em camundongos B10.A. Observou-se também que camundongos A/J depletados apresentaram números maiores de células $CD3^+$, $CD19^+$ e $GR1^+$ que os camundongos B10.A que receberam o mesmo tratamento.

Na décima semana pós-infecção, os camundongos resistentes tratados com anti-CD25 apresentaram número reduzido das células $CD3^+$, $CD19^+$ e $GR1^+$ quando comparados ao grupo controle (figura 10B).

Por outro lado, a depleção de CD25 em camundongos B10.A diminuiu somente o número de células $GR1^+$ no pulmão.

É importante ressaltar que neste tempo pós-infecção os camundongos resistentes controle apresentam maior número de células T no pulmão do que os camundongos suscetíveis do grupo equivalente. A depleção com anti-CD25 aboliu esta diferença.

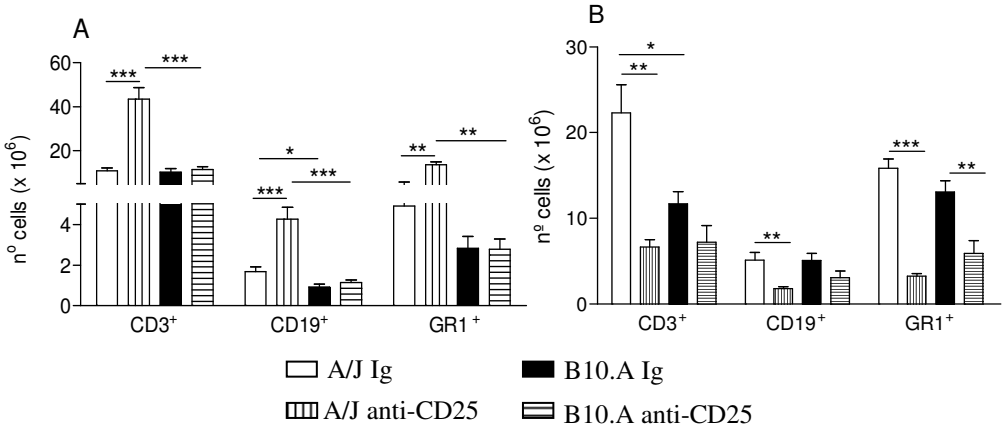

Figura 10: Quantificação de linfócitos T ($CD3^+$), linfócitos B ($CD19^+$) e de granulócitos ($GR1^+$) presente nos pulmões de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 2 e 10 semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (*P<0,05 **P<0,01 ***P<0,001).

Em camundongos A/J, a depleção de células $CD25^+$ causou na segunda semana pós-infecção o aumento do afluxo de células $TCD4^+$ e $TCD8^+$ naives e ativados (figura 11 A e B) para o pulmão. Em camundongos B10.A, entretanto, este tratamento não alterou o número destas populações celulares. É interessante notar que linfócitos $TCD4^+$ e $TCD8^+$ naives e ativados apareceram em maior número nos pulmões de camundongos resistentes A/J depletados em comparação com camundongos suscetíveis submetidos ao mesmo tratamento.

Na décima semana pós-infecção, observamos efeito contrário. O tratamento com anti-CD25 leva à diminuição no número de linfócitos $TCD4^+$ e $TCD8^+$ naives e ativados no pulmão de camundongos A/J. Em camundongos B10.A, entretanto, o tratamento diminuiu somente a presença de linfócitos $TCD4^+$ naives e ativados, porém não alterou as células $TCD8^+$ (figura 11 C e D).

É interessante notar que neste período pós-infecção há maior número de células $TCD4^+$ e $TCD8^+$ naives e ativadas no pulmão de camundongos resistentes controle quando comparado a camundongos suscetíveis controle.

Os resultados sugerem que na décima semana pós-infecção a depleção das células T reguladoras não ocasiona a expansão ou maior migração de células T para o foco inflamatório. Este fato pode estar relacionado com a diminuição da carga fúngica no pulmão dos camundongos A/J e B10.A depletados, onde possivelmente a diminuição de fungo ocasionou uma diminuição da estimulação imunológica.

Estes dados (figuras 11C e 11D) permitem também verificar que em camundongos depletados com anti-CD25 o número de linfócitos $TCD4^+$ e $TCD8^+$ pulmonares é equivalente nas linhagens B10.A e A/J, neste período pós infecção.

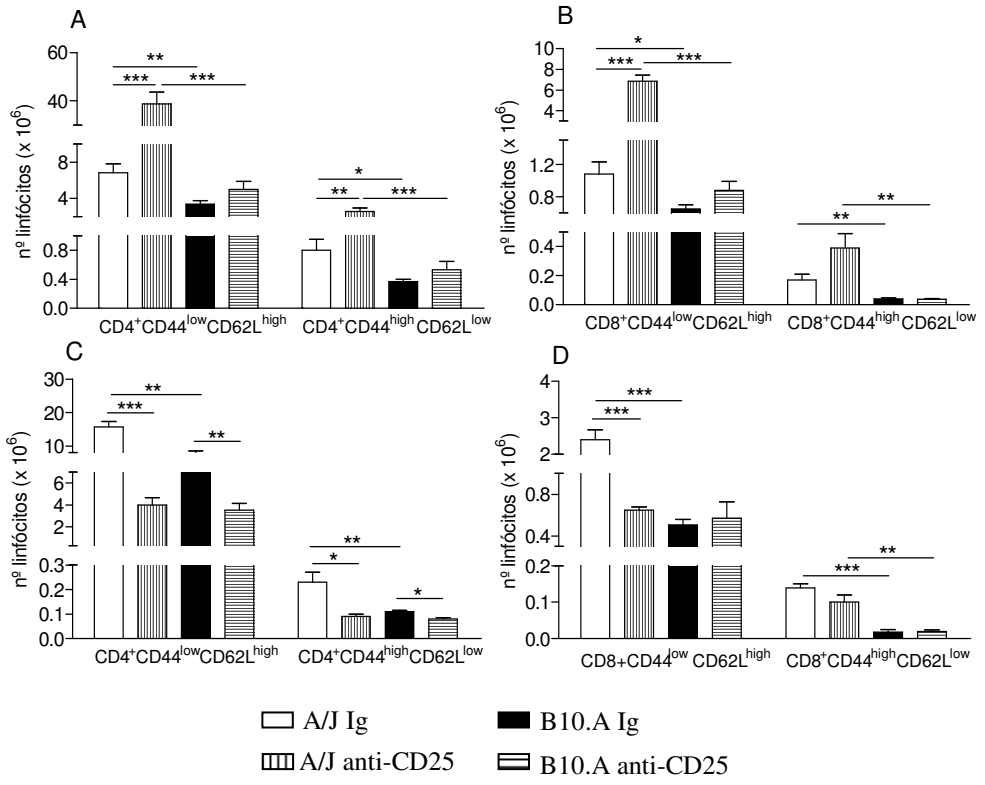

Figura 11: Quantificação de linfócitos TCD4$^+$ (A e C) e TCD8$^+$ (B e D) naives e ativados presentes nos pulmões de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 2 (A e B) e 10 (C e D) semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (**P<0,05 **P<0,01 ***P<0,001).

Ao estudarmos a expressão do marcador de ativação CD25$^+$ (cadeia α do receptor de IL-2) na segunda semana pós infecção, observamos na figura 12A que entre os grupos de camundongos tratados com Ig de rato, havia um maior número da população de linfócitos T CD4$^+$CD25$^+$ nos camundongos A/J quando comparados aos camundongos B10.A. Por outro lado, quando analisamos esta população celular nos camundongos depletados com anti-CD25 observamos uma diminuição desta população em ambas as linhagens, A/J e B10.A. Assim, comparando as linhagens entre si, constatamos que após a depleção ambos os grupos sofreram redução significativa quando comparados àqueles que somente receberam Ig de rato.

Sabemos que existem marcadores celulares que são específicos para cada fase de estimulação em que a célula se encontra. Por isso estudamos alguns outros marcadores que caracterizam a ativação celular; assim, resolvemos também caracterizar a expressão do marcador CTLA-4, que é uma molécula que aparece mais tardiamente na ativação e leva à desativação celular. Observamos que após 2 semanas de infecção os camundongos A/J

tratados com Ig apresentaram maior número de células T $CD4^+CTLA\text{-}4^+$ quando comparados aos camundongos B10.A do mesmo grupo. O marcador GITR está mais aumentado em células T ativadas e é especialmente expresso em células Treg. Tais células expressam em sua membrana celular marcadores como CD25, CTLA-4 e GITR, por exemplo. Sendo assim, resolvemos estudar o marcador GITR já que este também faz parte dos marcadores que caracterizam a população de células T reguladoras.

Observamos um aumento significativo de células $CD4^+GITR^+$ nos camundongos A/J depletados com anti-CD25 quando comparados aos camundongos A/J tratados com Ig de rato. O mesmo fenômeno pôde ser observado quando comparamos camundongos de ambos os grupos controle, onde pode-se verificar que os camundongos A/J apresentam maior número destas células quando comparado com os camundongos B10.A (figura 12A).

De maneira análoga, estudamos o efeito da depleção na presença de linfócitos duplo positivos ($CD25^+$, $CTLA\text{-}4^+$ e $GITR^+$) presentes nos pulmões na décima semana após a infecção.

A figura 12B mostra a análise das populações celulares $CD4^+CD25^+$, $CD4^+CTLA\text{-}4^+$ e $CD4^+GITR^+$. Podemos observar que o mesmo fenômeno ocorreu para as três populações celulares estudadas. Os camundongos A/J e B10.A depletados apresentam número de células significantemente menores quando comparados aos camundongos A/J e B10.A dos grupos controle. Além disso, entre os camundongos dos grupos controle, observamos maior número de células duplo positivas $CD4^+CD25^+$, $CD4^+CTLA\text{-}4^+$ e $CD4^+GITR^+$ nos camundongos A/J quando comparados aos camundongos B10.A.

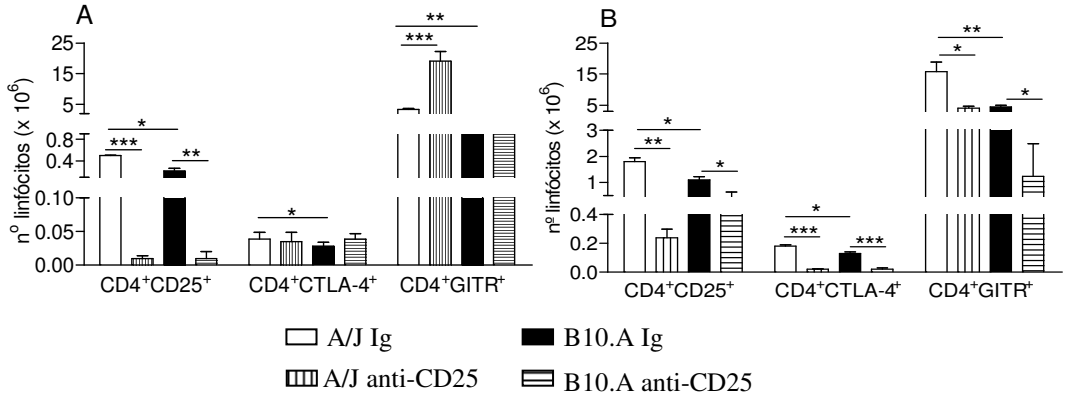

Figura 12: Quantificação de linfócitos $TCD4^+$ expressando $CD25^+$, $CTLA\text{-}4^+$ e $GITR^+$ presente nos pulmões de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 2 (A e B) e 10 (C e D) semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (*P<0,05 **P<0,01 ***P<0,001).

4.11 Análise fenotípica de macrófagos pulmonares de camundongos A/J e B10.A depletados ou não com anticorpo anti-CD25

Os marcadores de ativação dos macrófagos alveolares também foram estudados em animais A/J e B10.A depletados ou não com o anticorpo anti-CD25 após 2 e 10 semanas de infecção. A análise destes experimentos foi feita no FACS CANTO considerando o "gate" de tamanho (FSC) e granulosidade (SSC) para macrófagos.

A figura 13 mostra a análise das células $GR1^{+}F4/80^{+}$ (A – macrófagos), $F4/80^{+}IA^{k+}$ (B – macrófagos ativados) e $CD11c^{+}IA^{K+}$ (C – células dendríticas) após 2 semanas de infecção. Observamos que os camundongos A/J depletados apresentavam maior número destas três sub-populações quando comparados aos camundongos B10.A depletados. Além disto, os camundongos A/J depletados também apresentam número maior das três populações celulares quando comparados aos camundongos A/J tratado com Ig de rato. Em camundongos B10.A a depleção de células CD25+ não alterou o número destes fagócitos.

Já na décima semana após infecção, observamos que o número de células $GR1^{+}F4/80^{+}$ (A), $F4/80^{+}IA^{k+}$ (B) e $CD11c^{+}IA^{K+}$ (C) apresentaram-se diminuídos nos camundongos A/J depletados quando comparado aos camundongos A/J do grupo controle.

Por outro lado, verificamos que os camundongos B10.A depletados com anti-CD25 apresentaram redução no número de células $GR1^{+}F4/80^{+}$ quando comparados aos camundongos B10.A do grupo controle. O mesmo não pôde ser observado entre os camundongos B10.A para as outras populações celulares estudadas.

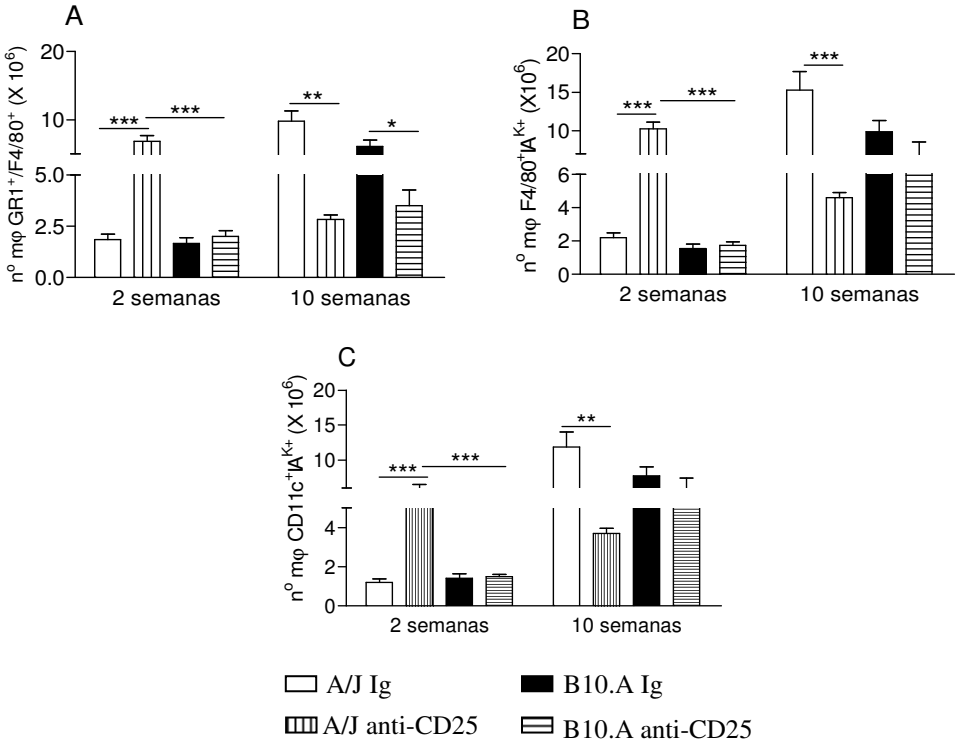

Figura 13: Quantificação de macrófagos infiltrantes do pulmão expressando os marcadores $GR1^+F4/80^+$ (A), $F4/80^+IA^{K+}$ (B) e $CD11c^+IA^{K+}$ (C) de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 2 e 10 semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (*P<0,05 **P<0,01 ***P<0,001).

Nossos dados mostram até o presente momento que após 2 semanas de infecção a depleção com anticorpo anti-CD25 promove em camundongos A/J um aumento de células T naives e ativadas e que ao contrário, após 10 semanas de infecção a depleção diminui o número destas células. Resolvemos também estudar o efeito da depleção de células CD25+ nas sub populações de células dendríticas presentes no pulmão, que são as mais eficientes apresentadoras de antígenos para o sistema imune.

Analisamos na figura 14 a presença de células dendríticas (DCs) mielóides ($CD11b^{high}CD11c^{high}$), linfóides ($CD11c^{high}CD8^+$) e plasmocitóides ($CD11c^{int}B220^+$) na segunda e décima semanas de infecção.

O tratamento na segunda semana pós-infecção causa um aumento das DCs mielóides e plasmocitóides de camundongos A/J e não altera a população linfóide. Por outro lado, este tratamento não altera o número de DCs nos pulmões de camundongos B10.A. Pôde-se também verificar que camundongos resistentes tratados com Ig apresentavam maior número de DCs mielóides que os camundongos B10.A do grupo equivalente. Após tratamento com

anti-CD25 os camundongos A/J apresentavam número maior de DCs mielóides e plasmocitóides que camundongos B10.A submetidos ao mesmo tratamento. Assim, a depleção de células CD25+ alterou somente a presença de DCs nos pulmões de camundongos resistentes (figura 14A).

Na décima semana pós-infecção novamente o fenômeno parece ser inverso. A depleção com anticorpo anti-CD25 diminui a presença de DCs mielóides e linfóides nos pulmões de camundongos A/J. Em camundongos B10.A somente a sub população mielóide diminuiu após o tratamento. Não foram observadas alterações quanto à presença de DCs plasmocitóides em ambas as linhagens (figura 14B).

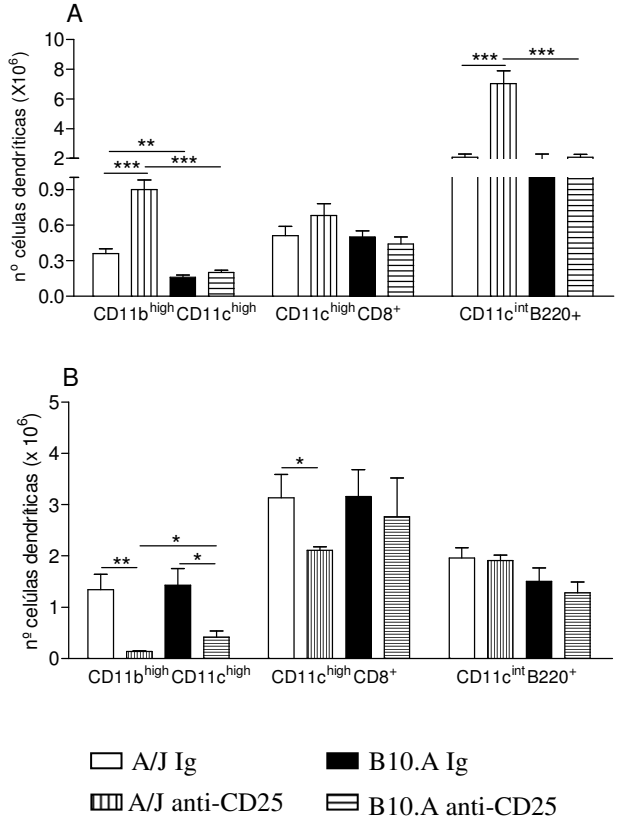

Figura 14: Quantificação de células dendríticas com perfil mielóide ($CD11b^{high}CD11c^{high}$), linfóide($CD11c^{high}CD8^{+}$) e plasmocitóide$CD11c^{high}B220^{+}$) infiltrantes do pulmão de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 2 (A) e 10 (B) semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP (*P<0,05 **P< 0,01 ***P<0,001).

4.12 Análise fenotípica de leucócitos infiltrantes de fígado de camundongos B10.A e A/J na décima semana de infecção

Nosso trabalho mostrou que a depleção com o anticorpo monoclonal anti-CD25 alterava a gravidade da infecção no fígado na fase crônica da doença. Independente do tratamento, os camundongos A/J apresentavam baixa carga fúngica no fígado; entretanto, os camundongos B10.A tratados com anti-CD25 apresentavam redução muito marcante na presença de fungos recuperados quando comparados ao grupo controle. Para compreendermos melhor este fato, resolvemos caracterizar os leucócitos infiltrantes do fígado no tempo de 10 semanas pós infecção.

Assim, células infiltrantes do fígado foram obtidas neste período pós infecção, a concentração foi ajustada para $1x10^6$ cels/mL e as mesmas foram marcadas com anticorpos específicos e analisadas por citometria de fluxo.

Primeiramente estudamos o efeito do tratamento com anticorpo anti-CD25 no afluxo de linfócitos T totais, células B e granulócitos para os pulmões. Para isto utilizamos os marcadores CD3 para caracterizar a população de linfócitos T, CD19 para linfócitos B e GR1 para os granulócitos.

Na figura 15, observamos o número de células $CD3^+$, $CD19^+$ e $GR1^+$ presentes no fígado, apresentou-se diminuído nos camundongos A/J tratados quando comparados com os camundongos controle. Em contraste, a depleção de células CD25 não alterou o número destas três populações celulares em camundongos B10.A. Observou-se também que camundongos A/J controle apresentavam números maiores de células $CD3^+$, $CD19^+$ e $GR1^+$ que os camundongos B10.A somente infectados.

É interessante também ressaltar que neste tempo pós-infecção, os camundongos resistentes controle apresentavam maior número de células T no fígado do que os camundongos suscetíveis do grupo equivalente, e que a depleção com anti-CD25 aboliu esta diferença.

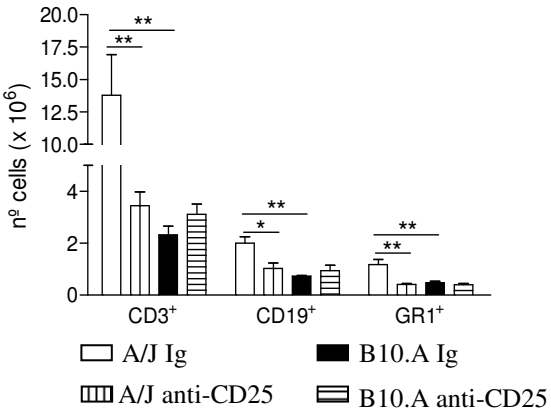

Figura 15: Quantificação de linfócitos T ($CD3^+$), linfócitos B ($CD19^+$) e de granulócitos ($GR1^+$) presentes no fígado de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 10 semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (*P<0,05 **P<0,01).

Na décima semana pós infecção, o fígado também apresentou alteração no afluxo de células $TCD4^+$ e $TCD8^+$ naives e ativadas. Em camundongos A/J controle observou-se maior número de células $TCD4^+$ e $TCD8^+$ naives e ativadas (figura 16 A e B) no fígado quando comparados aos camundongos B10.A do grupo equivalente. Além disso, os camundongos A/J depletados apresentavam redução no número de células $TCD4^+$ ativadas e $TCD8^+$ naives quando comparados aos camundongos A/J controle.

É interessante notar que após a depleção com anti-CD25 os linfócitos $TCD4^+$ naives e $TCD8^+$ naives e ativados apareceram em maior número no fígado de camundongos resistentes quando comparado aos camundongos suscetíveis submetidos ao mesmo tratamento. Apenas a população de células $TCD4^+$ ativadas apresentou-se em maior número nos camundongos B10.A depletados quando comparados aos camundongos A/J também tratados com anti-CD25.

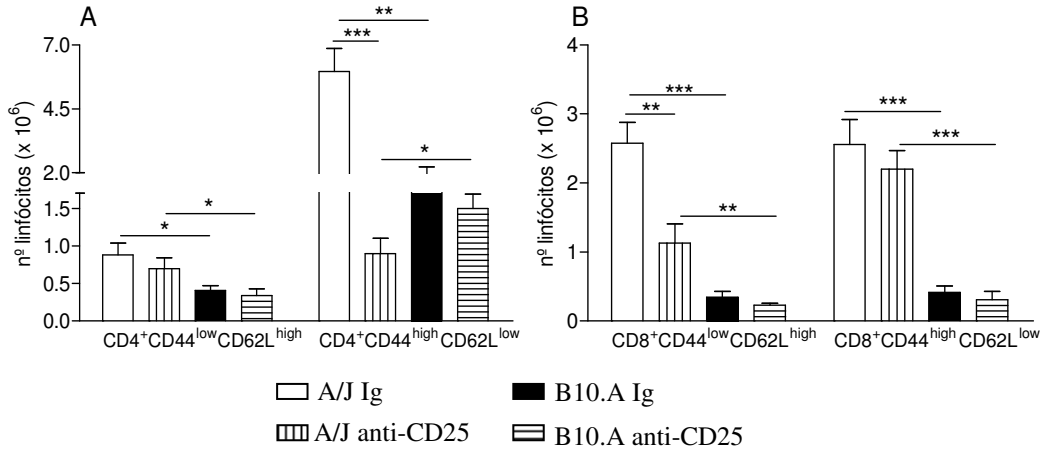

Figura 16: Quantificação de linfócitos $TCD4^+$ (A) e $TCD8^+$ (B) naives e ativados presentes no fígado de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 10 semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (**P<0,05 **P<0,01 ***P<0,001).

Também estudamos a expressão do marcador de ativação $CD25^+$ (cadeia α do receptor de IL-2) nas células do fígado e observamos que entre os grupos de camundongos tratados com Ig de rato, havia um maior número da população de linfócitos T $CD4^+CD25^+$ nos camundongos A/J quando comparados aos camundongos B10.A (figura 18). Além disso, os camundongos A/J controle apresentavam maior número desta população quando comparados aos camundongos A/J tratados.

Os marcadores CTLA-4 e GITR também foram estudados. Observamos que os camundongos A/J tratados com Ig apresentaram maior número de células T $CD4^+CTLA\text{-}4^+$ quando comparados aos camundongos B10.A do mesmo grupo.

Por outro lado, verificamos um maior número de células $CD4^+GITR^+$ nos camundongos A/J e B10.A controle quando comparados aos camundongos A/J e B10.A depletados. O mesmo fenômeno pôde ser observado quando comparamos camundongos A/J e B10.A de ambos os tratamentos, onde pode-se verificar que os camundongos B10.A apresentam menor número destas células quando comparados com os camundongos A/J (figura 17).

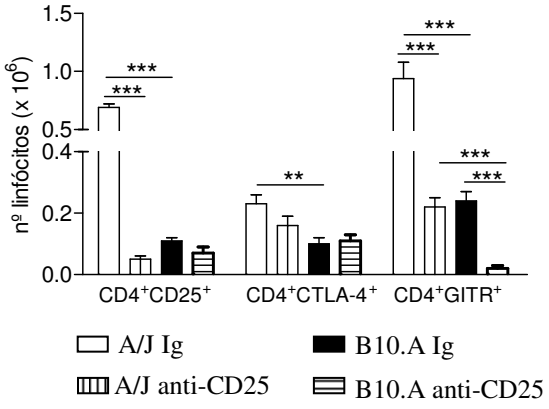

Figura 17: Quantificação de linfócitos $TCD4^+$ expressando $CD25^+$, $CTLA\text{-}4^+$ e $GITR^+$ presentes no fígado de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 10 semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (**P<0,01 ***P<0,001).

4.13 Análise fenotípica de macrófagos obtidos do fígado de camundongos A/J e B10.A depletados ou não com anticorpo anti-CD25 na décima semana de infecção

Os marcadores de ativação dos macrófagos presentes no fígado também foram estudados em camundongos A/J e B10.A depletados ou não com o anticorpo anti-CD25 após 10 semanas de infecção. A análise destes experimentos foi feita no FACS CANTO considerando o "gate" de tamanho (FSC) e granulosidade (SSC) para macrófagos.

A figura 18 mostra o resultado da análise das células $GR1^{+}F4/80^{+}$ (A – macrófagos), $F4/80^{+}IA^{K+}$ (B – macrófagos ativados) e $CD11c^{+}IA^{K+}$ (C – células dendríticas). Observamos que os camundongos B10.A controle apresentavam maior número de macrófagos ($GR1^{+}F4/80^{+}$) que os camundongos B10.A depletados e os camundongos A/J do grupo controle.

Já para a população de macrófagos ativados ($F4/80^{+}IA^{k+}$) observamos que os camundongos A/J apresentavam maior número desta população quando comparados aos camundongos B10.A, em ambos os tratamentos. Além disso, os camundongos B10.A tratados apresentavam redução no número de macrófagos ativados quando comparados aos camundongos B10.A controle. Não observamos diferenças quanto ao número de células dendríticas entre as linhagens e tratamentos.

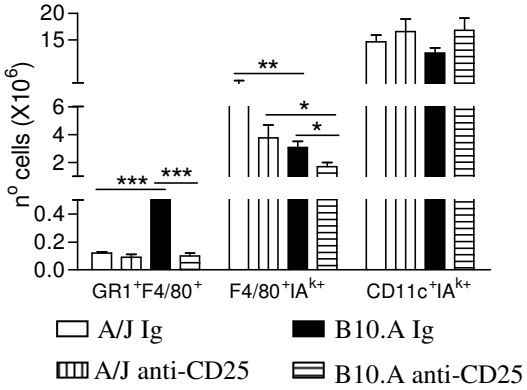

Figura 18: Quantificação de macrófagos infiltrantes do fígado expressando os marcadores $GR1^{+}F4/80^{+}$, $F4/80^{+}IA^{K+}$ e $CD11c^{+}IA^{K+}$ de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 10 semanas pós infecção com 1 x 10^{6} de *P. brasiliensis* por via i.t. A expressão destes marcadores foi determinada por citometria de fluxo. O valor absoluto de cada sub-população é apresentado como média ±EP. (*P<0,05 **P<0,01 ***P<0,001).

4.14 Efeito do tratamento com anticorpos anti-CD25 no número de células T reguladoras $CD4^+CD25^+FOXP3^+$ no pulmão e fígado de camundongos suscetíveis e resistentes

Após análise de linfócitos, macrófagos e células dendríticas no infiltrado celular do pulmão e fígado, fomos também caracterizar a presença de células T reguladoras $CD4^+CD25^+FoxP3^+$. O número destas células foi obtido pela quantificação de células triplamente positivas a partir de um gate de células T $CD4^+CD25^+$.

A figura 19A mostra que tanto na segunda como na décima semana de infecção, o número de células T reguladoras no pulmão de camundongos A/J tratados com Ig de rato foi maior que nos camundongos B10.A submetidos ao mesmo tratamento. Entretanto, após a depleção com anti-CD25 verificamos que ambas as linhagens A/J e B10.A apresentavam diminuição do número de células $CD4^+CD25^+Foxp3^+$quando comparadas aos seus grupos controles.

Vale ressaltar, que o número de células T reguladoras ($CD4^+CD25^+FoxP3^+$) foi diferente se observarmos os dois períodos de infecção. No período de 10 semanas, ambos os camundongos do grupo controle apresentaram número de células maior em relação ao período de 2 semanas. Entretanto, os camundongos resistentes parecem apresentar número de células T reguladoras sempre maiores que os camundongos suscetíveis.

No fígado observamos o mesmo fenômeno encontrado no pulmão, onde os camundongos A/J e B10.A controle apresentavam número de células T reguladoras maior quando comparados aos seus grupos tratados. Além disso, os camundongos A/J apresentavam número de células T reguladoras maior que os camundongos B10.A (figura 19B).

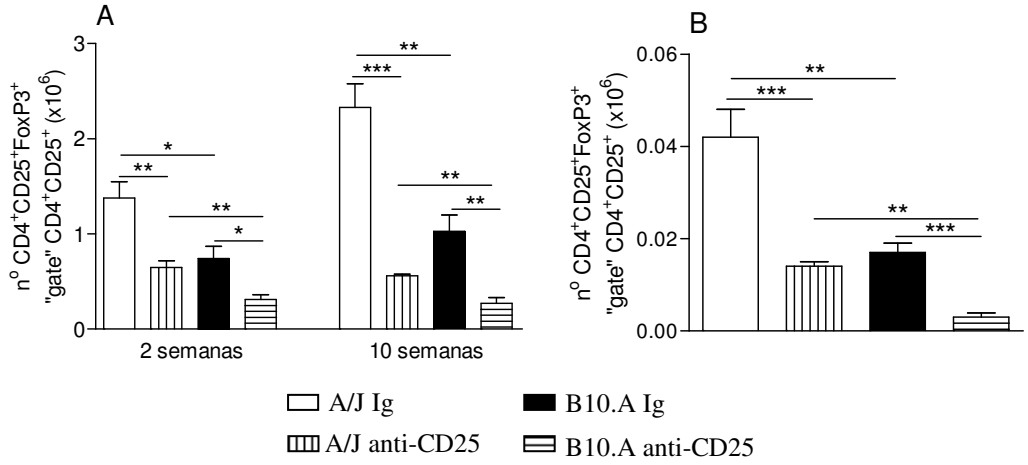

Figura 19: Quantificação de células T reguladoras infiltrantes do pulmão(A) e fígado (B) de camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e analisados 2 e 10 semanas pós infecção com 1 x 10^6 de *P. brasiliensis* por via i.t. O valor absoluto de cada sub-população é apresentado como média ±EP. (*P<0,05 **P<0,01 ***P<0,001).

4.15 Análise histopatológica dos pulmões

Um parâmetro adicional para avaliar a gravidade da doença foi a análise histopatológica dos pulmões dos camundongos A/J e B10.A tratados ou não com o anticorpo monoclonal anti-CD25 obtidos na segunda e décima semana pós-infecção i.t. pelo *P. brasiliensis.* Nestes períodos, o pulmão esquerdo de cada camundongo foi removido e fixado em 10% de formalina e embebido em parafina. Secções (5µm) foram coradas com hematoxilina e eosina (H&E) para análise das lesões e com a coloração Groccot, para visualizar os fungos.

Verificamos que na segunda semana de infecção, os camundongos A/J controle apresentam exuberante infiltrado linfohistiocitário pulmonar com grandes áreas de condensação e grande quantidade de fungos (figura 20A e 20E). Por outro lado, os camundongos B10.A do mesmo grupo apresentam infiltrado inflamatório linfohistiocitário presentes nos septos interlobulares com eventual esboço de granulomas e grande quantidade de fungos (figura 20B e 20F).

Já os camundongos A/J (figura 20C e 20G) e B10.A (figura 20D e 20H) tratados com anticorpo monoclonal anti-CD25 apresentam o mesmo perfil histológico. Observa-se a presença de um importante infiltrado inflamatório linfohistiocitário pulmonar com grandes áreas de condensação. Além disso, ambos os grupos apresentam moderada quantidade de fungos.

Neste período de infecção, análise do fígado não exibiu nenhuma anormalidade (dados não mostrados).

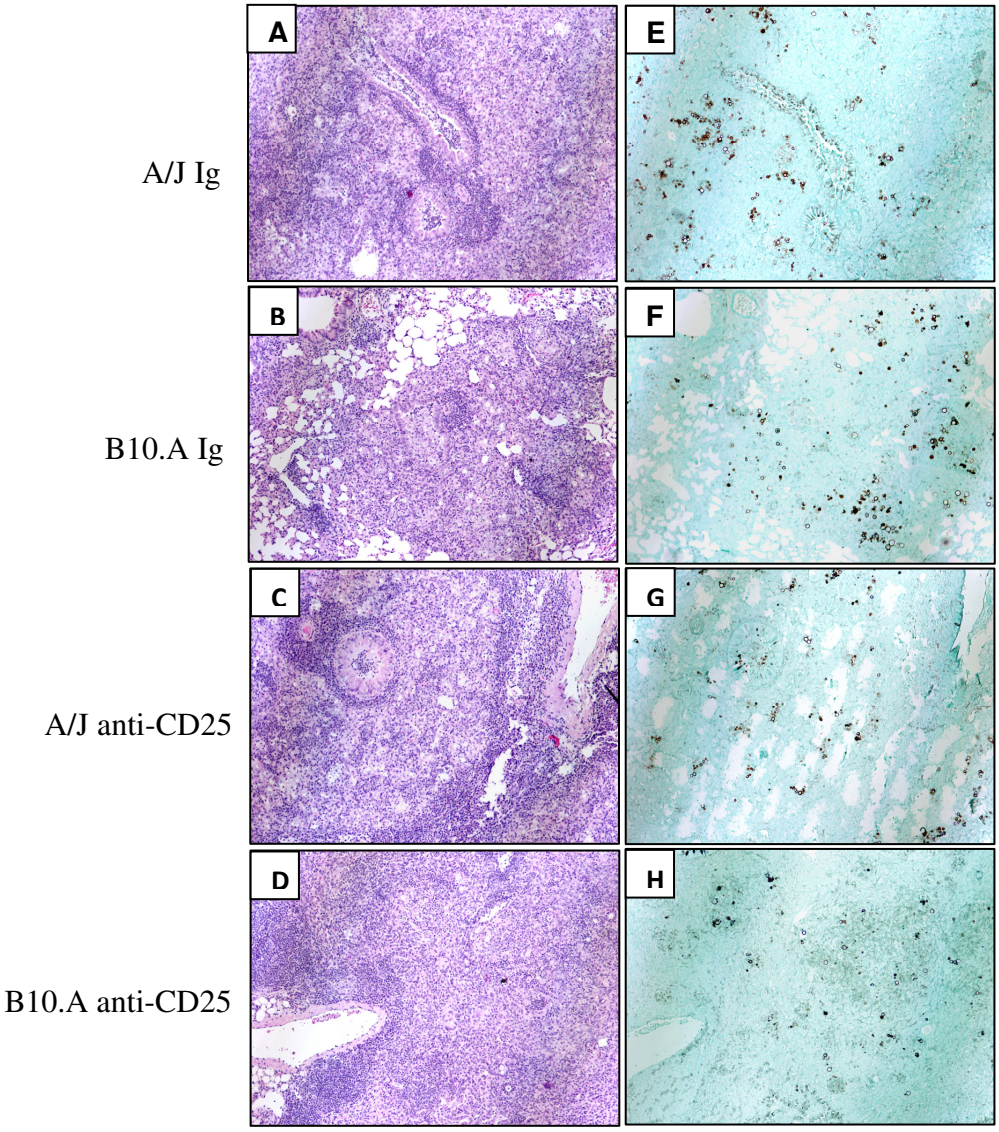

Figura 20: Fotomicrografia das lesões pulmonares de camundongos A/J e B10.A tratados ou não com anticorpo monoclonal anti-CD25 e infectados com $1x10^6$ leveduras de *Paracoccidioides brasiliensis* na segunda semana de infecção. Coloração H&E e Groccot (aumento 10x).

Por outro lado, observamos que na décima semana pós-infecção os camundongos A/J controle exibem no pulmão um infiltrado linfomononuclear e histiocitário, localizado preferencialmente nos septos interlobulares junto aos bronquíolos terminais. Existem acúmulos de histiócitos epitelióides que esboçam a formação de granulomas, entretanto, sem a presença de granulomas bem estruturados. O número de fungos é pequeno e esses estão por vezes ausentes (figura 21A e 21E). A análise do fígado não demonstra achados relevantes. Não há inflamação, presença de granulomas ou fungos (figura 22A e 22E).

Os camundongos B10.A controle exibem grandes granulomas envolvendo grandes áreas do parênquima pulmonar, contendo uma grande quantidade de fungos (figura 21B e 21F). No fígado, os camundongos apresentaram granulomas localizados nos lóbulos, contendo grande quantidade de fungos (figura 22B e 22F).

Já os camundongos A/J depletados com anticorpo monoclonal anti-CD25 mostram ausência de graunulomas, infiltrado inflamatório linfomononuclear e histiócitos nos septos interlobulares e peribronquiolares, semelhantes ao grupo dos camundongos A/J controle. Os fungos estão ausentes ou são poucos e dispersos no parênquima (figura 21C e 21G). No fígado não foram identificadas alterações histológicas relevantes (figura 22C e 22G).

Por outro lado, os camundongos B10.A depletados mostram granulomas pulmonares menores, isolados sem fungos ou com pequena quantidade desses (figura 21D e 21H). No fígado, nenhum animal mostrou presença de fungos ou qualquer outro comprometimento relevante (figura 22D e 22H).

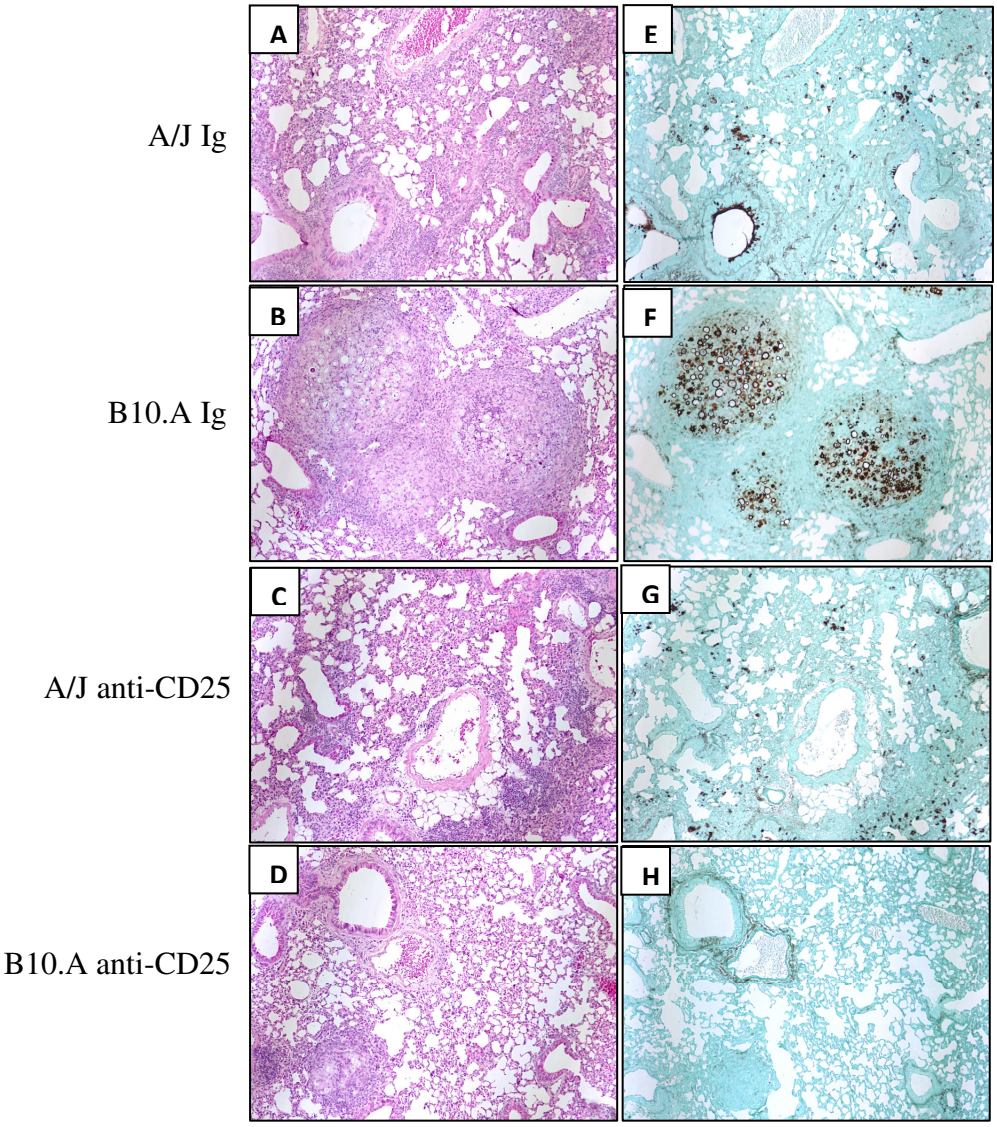

Figura 21: Fotomicrografia das lesões pulmonares de camundongos A/J e B10.A tratados ou não com anticorpo monoclonal anti-CD25 e infectados com $1x10^6$ leveduras de *Paracoccidioides brasiliensis* na décima semana de infecção. Coloração H&E e Groccot (aumento 10x).

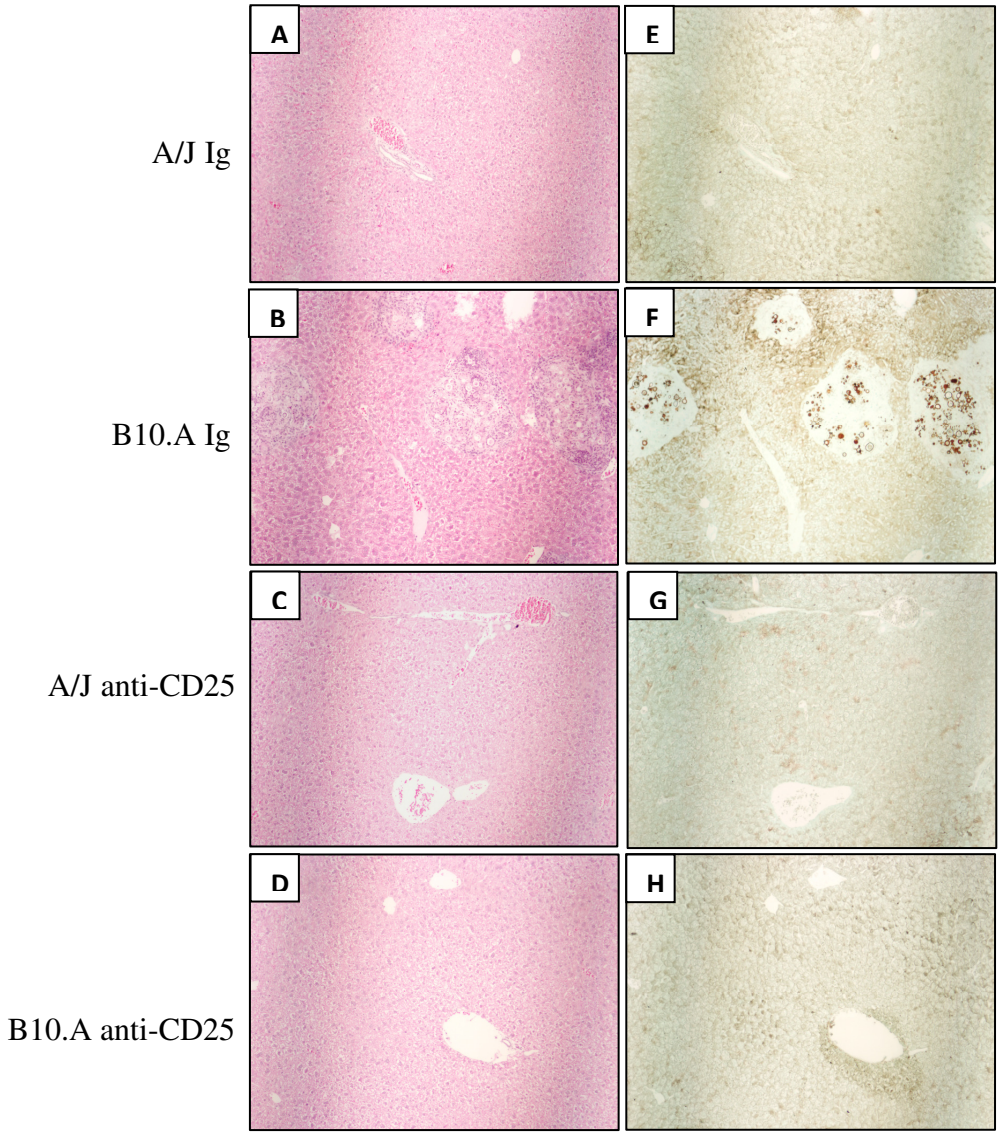

Figura 22: Fotomicrografia das lesões do fígado de camundongos A/J e B10.A tratados ou não com anticorpo monoclonal anti-CD25 e infectados com $1x10^6$ leveduras de *Paracoccidioides brasiliensis* na décima semana de infecção. Coloração H&E e Groccot (aumento 10x).

No período de 10 semanas de infecção a análise morfometríca das lesões pulmonares demonstrou que camundongos B10.A controle apresentavam maior área de lesão quando comparados aos camundongos A/J do mesmo grupo. Entretanto, após a depleção com anti-CD25 verificamos que ambas as linhagens A/J e B10.A apresentavam áreas menores de lesão menores quando comparadas aos seus grupos controle. Assim, o tratamento com anti-CD25 aboliu as diferenças histopatológicas dos pulmões entre as linhagens (figura 23).

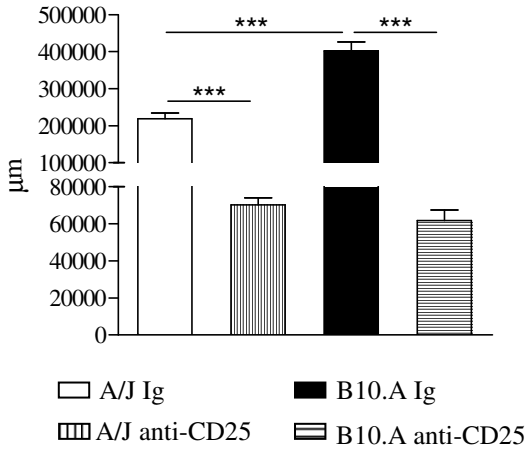

Figura 23: Análise da área total de lesões pulmonares em camundongos A/J e B10.A tratados ou não com anticorpo anti-CD25 e infectados com $1x10^6$ leveduras de *Paracoccidioides brasiliensis* na décima semana de infecção. A medida da área da lesão é apresentada como média±EP (***P<0,001).

4.16 Tempo de sobrevida

Após os dados obtidos mostrando que após a depleção de células CD25+ os camundongos A/J e B10.A apresentavam doença menos grave em ambas as fases da infecção, nos perguntamos se este tratamento influenciaria a mortalidade dos camundongos. Para isso, grupos de camundongos A/J e B10.A tratados ou não com anticorpo monoclonal anti-CD25 foram infectados com leveduras viáveis de *P. brasiliensis* e observados durante um período de 190 dias, sendo registrado o tempo de sobrevida (em dias) para cada animal.

Como observado na figura 24, a mortalidade dos camundongos B10.A controle inicia-se no dia 110 e após 190 dias apenas 1 camundongo continuava vivo; neste período, não foram registradas mortes dos camundongos A/J controle e A/J e B10.A tratados.

A análise estatística destes dados de sobrevida (método de comparação de curvas de sobrevida pelo teste de LogRank) demonstrou haver diferenças estatisticamente significantes entre os grupos A/J e B10.A controles e entre os grupos de camundongos B10.A controle e B10.A tratados.

Este experimento demonstrou que a depleção de células T reguladoras em período precoce da infecção aboliu as diferenças entre camundongos susceptíveis e resistentes ao *P. brasiliensis.* Ambas as linhagens tendem à resolução do processo infeccioso e sobrevivem ao mesmo.

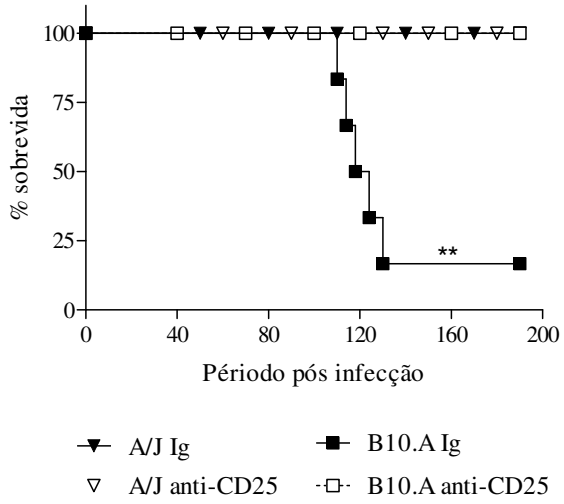

Figura 24: Tempo de sobrevida de camundongos A/J e B10.A tratados ou não com anticorpo monoclonal anti-CD25 infectados pela via i.t. com $1x10^6$ leveduras viáveis do *P. brasiliensis* (n=6). A sobrevida dos animais foi acompanhada por 190 dias. ** p<0,01.

4.17 Efeito da depleção de células Treg na carga fúngica dos camundongos sobreviventes da mortalidade após a infecção com *P. brasiliensis*

Aos 190 dias de infecção do experimento de mortalidade, sacrificamos os camundongos sobreviventes e analisamos os órgãos quanto ao número de fungos viáveis. Os resultados estão apresentados na figura 25.

Observamos que ambos os camundongos A/J controle e os camundongos A/J e B10.A tratados com o anticorpo monoclonal anti-CD25 apresentavam o mesmo número de fungos recuperados do pulmão (figura 25A).

Por outro lado, apesar de observarmos uma pequena disseminação, não observamos diferenças significativas quanto ao número de fungos recuperados no fígado de ambos os

grupos (figura 25B). No baço, nenhum grupo apresentou número de fungos viáveis recuperados.

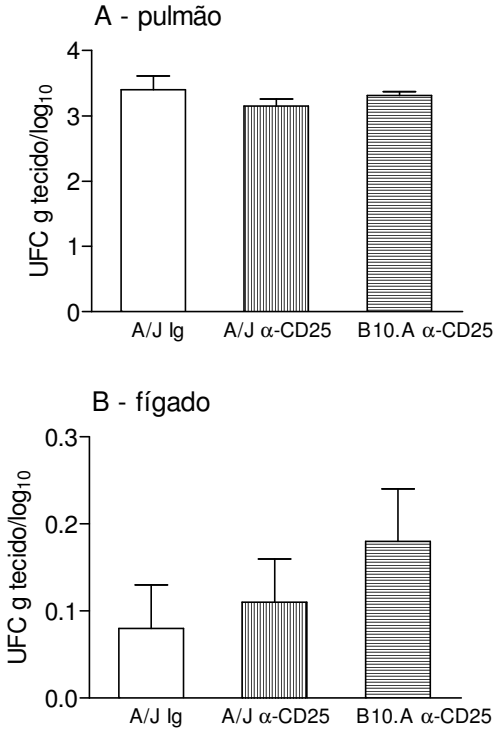

Figura 25: Análise do grau de infecção através de Unidades Formadoras de Colônias (UFC) obtido de camundongos A/J e B10.A depletados de células $CD25^+$ por tratamento com anticorpo monoclonal anti-CD25 ou Ig de rato, sobreviventes da mortalidade após a infecção com $1x10^6$ células leveduriformes de *P. brasiliensis*. O grau da infecção foi determinado pela contagem de UFC nos órgãos.

5 DISCUSSÃO

Muitos trabalhos têm descrito a função das células T reguladoras, especialmente o seu efeito controlador das doenças auto-imunes e das respostas imunológicas contra antígenos exógenos, principalmente patógenos (BELKAID e TARBELL, 2009). Na fase inicial de uma infecção pulmonar, os macrófagos alveolares e as células dendríticas interagem com o patógeno e as últimas migram para os linfonodos drenantes. Nos órgãos linfóides periféricos as células dendríticas têm a função de expandir a população de células T efetoras através da apresentação de antígenos no contexto de moléculas co-estimuladoras. Nesse processo são também expandidas as células T reguladoras que tanto podem reconhecer auto-antígenos como antígenos exógenos. O ambiente de citocinas e a maior expressão de moléculas co-estimuladoras é o que determina a eficiência dessa fase de ativação e o comprometimento das sub-populações linfocitárias para os padrões Th1, Th2 ou Th17, por exemplo. Já nesta fase as células T reguladoras podem controlar a expansão das células T efetoras e das células de imunidade inata. Uma vez estabelecida a resposta imune há a migração de várias sub-populações celulares para o sítio da infecção, uma vez que lá o processo inflamatório pôde alterar a expressão de adressinas do endotélio e induzir a produção de mediadores solúveis (citocinas, quimiocinas, mediadores lipídicos) que controlam a migração e ativação celular.

Ao início da infecção, as células dendríticas e os macrófagos podem (ou não) eficientemente controlar a carga fúngica. No caso da infecção de camundongos B10.A e A/J a resposta imune inata mais eficiente é a desenvolvida por camundongos suscetíveis, pois seus macrófagos e células dendríticas são facilmente ativáveis por IL-12 e IFN-γ e desenvolvem eficiente atividade fungicida. Por outro lado, macrófagos e células dendríticas de camundongos A/J secretam preferencialmente TGF-β que inibe a atividade fungicida, mas permite um posterior desenvolvimento de imunidade adaptativa provavelmente, pela presença concomitante de TNF-α e IL-1β (revisto por CALICH et al., 2008)

As células T reguladoras podem inibir a fase indutora da imunidade através da modulação negativa da atividade apresentadora de antígenos e secretora das células dendríticas. Na fase efetora, as T reguladoras podem inibir tanto as células da imunidade inata como adaptativa (TANG et al., 2006). Ainda em relação às células dendríticas, as células T reguladoras expressando CTLA-4 podem induzir a expressão da enzima indolamina 2,3-dioxigenase (IDO) que atua no catabolismo do aminoácido triptofano, metabolizando-o em

quinureninas, sendo estas essenciais para inibir a ativação de células T e promover a apoptose destas células (FALLARINO et al., 2003). Resultados do mesmo grupo apoiam a sugestão que a expressão de IDO pode induzir a expansão de células T regulatórias. Estes autores demonstraram o desenvolvimento de células regulatórias T $CD4^+$ expressando Foxp3 originárias de células T $CD4^+CD25^-$ naive depois de serem expostas à depleção de triptofano combinado com excesso de quinureninas (FALLARINO et al., 2006). Estas células parecem ter um controle muito importante sobre a resposta imune em um modelo murino contra colite. Após a ativação da resposta imune inata, as células T reguladoras se expandem e exercem efeito controlador desta patologia, minimizando o dano tecidual e prevenindo a resposta exacerbada contra a flora intestinal (POWRIE et al., 2003).

Neste trabalho investigamos o papel das células T reguladoras na gravidade da PCM desenvolvida por camundongos resistentes e suscetíveis ao *P.brasiliensis*, através da depleção de células $CD25^+$ com o anticorpo monoclonal anti-CD25. Estudamos o efeito na carga fúngica e caracterizamos também quais seriam as citocinas pulmonares modificadas pelo tratamento. Estudamos também, o efeito da depleção nas populações celulares (linfócitos e macrófagos) que compunham as lesões pulmonares de ambas as linhagens de camundongos na segunda e décima semanas pós-infecção. Além disso, estudamos o efeito supressor das células T reguladoras dos camundongos suscetíveis e resistentes ao fungo, bem como a presença dessas células em ambas as linhagens de camundongos após a infecção. Vale lembrar que o anticorpo monoclonal foi administrado nos dias -3 e +3 da infecção.

A primeira parte do nosso estudo demonstrou que as células T reguladoras estão presentes em camundongos sadios de ambas as linhagens, entretanto, o número destas células apresentou-se maior nos camundongos A/J. Após a infecção, verificamos um aumento significante da população de células $FoxP3^+$ tanto na fase inicial como na fase crônica da doença, demonstrando que tais células estão envolvidas no curso da resposta imune contra o *P. brasiliensis*. È importante ressaltar que tanto os camundongos A/J sadios e infectados apresentavam número maior destas células quando comparados aos camundongos B10.A. O mesmo fenômeno pôde ser observado para a intensidade de expressão do marcador $FoxP3^+$.

De maneira semelhante, o estudo dos marcadores de superfície e das citocinas intracelulares característicos desta sub população celular demonstrou que tanto na segunda como na décima semana de infecção os camundongos A/J apresentavam um maior número de células $TCD4^+CD25^+FoxP3^+GITR^+$, $TCD4^+CD25^+FoxP3^+CTLA\text{-}4^+$ e

TCD4+CD25$^+$FoxP3$^+$LAP$^+$ bem como células TCD4$^+$CD25$^+$FoxP3$^+$ produtoras de TGF-β e IL-10 quando comparados aos camundongos B10.A.

Relevante trabalho desenvolvido por Cavassani et al. (2006) demonstrou que pacientes com Paracoccidioidomicose apresentavam freqüência de células TCD4$^+$CD25$^+$ similar ao grupo de pacientes controles. Entretanto, a expressão dos marcadores CTLA-4, GITR, LAP e FoxP3 nas células TCD4$^+$CD25$^+$ dos pacientes foi maior do que o observado nas células do grupo controle. Em relação à atividade funcional da células TCD4$^+$CD25$^+$ os autores verificaram que as células dos pacientes apresentavam maior inibição da linfoproliferação quando comparado com as células do grupo controle. A análise da produção de citocinas intracelulares demonstrou que as células TCD4$^+$CD25$^+$ isoladas da lesão de pacientes com PCM apresentavam aumento de IL-10, TGF-β e IFN-γ quando comparado com as células TCD4$^+$CD25$^-$ obtidas da mesma lesão. Neste trabalho verificou-se que as células T reguladoras estão presentes no sangue e lesões dos pacientes com PCM, sugerindo que estas células poderiam controlar o desenvolvimento da resposta imune (CAVASSANI et al., 2006).

Nossos dados permitem afirmar que tanto nos camundongos A/J como nos camundongos B10.A há a expansão e ativação das células T reguladoras após a infecção e que estas células se fazem presentes tanto na fase inicial como na fase crônica da doença.

Em trabalho anterior desenvolvido pelo nosso grupo, demonstrou-se que os macrófagos alveolares de camundongos A/J produziam níveis maiores de TGF-β quando comparados aos camundongos B10.A (PINA; BERNARDINO; CALICH, 2008). Em nosso estudo pudemos demonstrar uma maior ativação de células T reguladoras em camundongos A/J e talvez este fato esteja correlacionado à grande produção de TGF-β produzida por macrófagos alveolares.

Os ensaios de supressão *in vitro* mostraram que as células TCD4$^+$CD25$^+$ isoladas dos pulmões de camundongos A/J e B10.A infectados pelo fungo foram capazes de suprimir a proliferação de células T CD4$^+$CD25$^-$ quando estimuladas com anti-CD3 e APC irradiadas de maneira dose-dependente. Entretanto, as células T reguladoras dos camundongos A/J foram mais eficientes em inibir a proliferação do que as células T reguladoras dos camundongos B10.A. Além disso, no curso da doença o número de células Treg em camundongos A/J é maior do que o observado em camundongos B10.A. Desta maneira a maior atividade reguladora negativa dos camundongos resistentes dá-se pelo maior número e maior atividade intrínseca das células Treg no decorrer da infecção.

Chen et al. (2005) também demonstraram que células $TCD4^{+}CD25^{+}$ de camundongos C57Bl/6 e BALB/c foram capazes de suprimir a proliferação de células $TCD4^{+}CD25^{-}$ estimuladas com anti-CD3 e APC irradiadas. Esta supressão foi maior com células de camundongos BALB/c que, além disso, apresentavam maior número de células Treg no timo e órgãos linfóides periféricos (CHEN; OPPENHEIN; HOWARD, 2005). Este dado foi correlacionado com a maior susceptibilidade dos camundongos BALB/c às infecções por patógenos intracelulares e à sua predisposição para o padrão Th2 de resposta imune.

Nosso estudo demonstrou que o tratamento com o anticorpo anti-CD25 levou à diminuição do crescimento fúngico no pulmão e fígado em ambas as linhagens na segunda semana pós infecção quando comparado ao dos camundongos somente tratados com Ig normal de rato. Vale ressaltar que neste período de infecção, as células T reguladoras dos camundongos A/J parecem ter exercido efeito negativo maior sobre a resposta imune do que as dos camundongos B10.A, pois nos camundongos A/J a depleção parece ter ocasionado uma diminuição maior na carga fúngica.

Na décima semana de infecção, a depleção com o anticorpo anti-CD25 na fase inicial da doença permitiu a manutenção da diminuição do crescimento fúngico em ambas as linhagens, ou seja, os camundongos continuaram apresentando redução da carga fúngica pulmonar quando comparados aos camundongos tratados com Ig. Observamos ainda que camundongos resistentes e suscetíveis tratados com Ig apresentaram disseminação fúngica para o fígado e baço e que após a depleção este fenômeno diminuiu de intensidade. Verificamos ainda que entre o grupo de camundongos tratados com Ig, os camundongos B10.A apresentavam maior carga fúngica pulmonar, hepática e esplênica quando comparados aos camundongos A/J. Entretanto, a depleção com anti-CD25 aboliu esta diferença.

Nossos dados demonstram que a presença de um número maior de células Treg nos camundongos A/J pode ser associado com o aumento da carga fúngica observado na segunda semana pós infecção. Verificamos que estas células são produtoras de TGF-β, IL-10 e expressam TGF-β latente de membrana (LAP). O TGF-β foi descrito como a citocina responsável pela diminuição da atividade fungicida exercida pelos macrófagos alveolares de camundongos A/J (PINA; BERNARDINO; CALICH, 2008). Assim, o aumento de células Treg produtoras de TGF-β parecem ter inibido o desenvolvimento da resposta imune dos

camundongos A/J permitindo o aumento do crescimento fúngico observado na fase inicial da doença.

Por outro lado, nossos dados obtidos com os camundongos B10.A demonstram que, apesar de aparecerem em menor número, as células Treg também devem ter contribuído com a redução de fungos observada nos camundongos tratados. Na segunda semana pós-infecção, os camundongos B10.A produziram níveis maiores de NO, e este mediador pode ter contribuído com a maior atividade fungicida dos macrófagos, resultando na diminuição do número de fungos recuperados dos pulmões.

Diferente dos nossos resultados, Kotner e colaboradores verificaram que após infecção com a cepa *Brazil* de *Trypanossoma cruzi* as células T reguladoras não desempenhavam papel importante no controle da infecção aguda. Os autores observaram que a resposta de fase aguda não se alterou após a administração do anticorpo anti-CD25. Além disto, observaram que a depleção de células T reguladoras antes do desafio com o parasita não melhorou a sobrevida dos camundongos, apesar de poderem identificar na fase crônica da infecção uma população de células T reguladoras em tecidos linfóides periféricos dos animais infectados (KOTNER et al., 2007).

Em relação ao mesmo parasito, outro grupo demonstrou que após a depleção com anticorpo anti-CD25 os camundongos apresentavam uma resistência maior à infecção com a cepa Colombiana de *T. cruzi,* caracterizada por uma redução da parasitemia e mortalidade. Entretanto, os autores relatam que para este parasita as células T reguladoras têm um papel limitado à fase aguda da resistência do hospedeiro (SALES JR et al., 2008).

Da mesma maneira, Mariano e colaboradores demonstraram que o tratamento com anticorpo anti-CD25, induzia em camundongos infectados com a cepa Y de *T. cruzi* uma maior parasitemia entre os dias 9 e 11 pós-infecção, sendo este perfil também observado nos camundongos tratados com anticorpo anti-GITR. Além disso, embora a sobrevida tenha sido de 60% no grupo controle, após a depleção houve redução significativa da sobrevida dos animais infectados com *T. cruzi*, demonstrando que as células T CD25+ exercem papel protetor na resistência à infecção (MARIANO et al., 2008*).*

Tais resultados demonstram que a ação das células T reguladoras está relacionada com o tipo da cepa de *T. cruzi* utilizado em cada trabalho. Além disso, devemos levar em

consideração que existem cepas que podem ser mais virulentas do que outras, o que talvez poderia estar relacionado com a importância ou não das células T reguladoras.

Por outro lado, Morampudi e colaboradores (2011) demonstraram que o tratamento com anti-CD25 em camundongos susceptíveis C57Bl/6 infectados com *Toxoplasma gondii* não acelerava a mortalidade destes camundongos quando comparado aos camundongos que receberam tratamento com placebo. Verificaram ainda que os camundongos resistentes BALB/c tratados com anti-CD25 apresentaram aumento da parasitemia na fase crônica quando comparado aos camundongos BALB/c não tratados. Este dado demonstra a contribuição das células T reguladoras na resistência à infecção por este protozoário (MORAMPUDI *et al.,* 2011).

Belkaid et al. mostraram em 2002 que a persistência da *Leishmania major* na pele é controlada por uma população endógena de células T reguladoras, e que após a remoção das células T reguladoras observa-se uma "cura estéril", um estado não compatível com a preservação da imunidade por longo tempo (BELKAID et al., 2002).

Em nosso modelo, podemos concluir que as células T reguladoras estão presentes nos dois períodos de infecção e que tanto na fase aguda como na crônica as células T reguladoras influenciam no controle do crescimento fúngico tanto em camundongos suscetíveis como resistentes ao fungo. Entretanto, os dados de carga fúngica sugerem que as células Treg são mais importantes para os camundongos A/J na fase aguda da doença enquanto que para os camundongos B10.A, o são na fase crônica.

Conforme descrito anteriormente, as citocinas produzidas em resposta à infecção pelo fungo apresentam importante papel no desenvolvimento da resposta imune inata e adaptativa. Sabemos que a citocina IFN-γ desempenha importante papel na resistência contra a infecção pelo *P. brasiliensis* (CANO et al., 1998; SOUTO et al., 2000). Sabemos também que a IL-4 pode ter efeito protetor ou exacerbador da doença, dependendo do padrão genético do hospedeiro (PINA et al., 2004; ARRUDA et al., 2004). Sendo assim, resolvemos caracterizar a presença de citocinas nos homogenatos pulmonares após 2 e 10 semanas de infecção, tanto em camundongos tratados com Ig como nos camundongos depletados.

Verificamos que na segunda semana pós-infecção o tratamento com anti-CD25 levou a uma diminuição das citocinas IL-10 e TGF-β nos camundongos A/J e B10.A quando comparados aos camundongos tratados com Ig. Sabe-se que estas citocinas estão envolvidas

com a expansão e função das células T reguladoras, sugerindo uma diminuição da sua atividade, uma vez que observamos diminuição no número de fungos recuperados após a depleção em ambas as linhagens.

Vale ressaltar que neste período de infecção, a depleção com anticorpo anti-CD25 ocasionou diminuição nos níveis das citocinas IL-6, IL-23 e do fator de crescimento GM-CSF nos camundongos A/J quando comparados aos camundongos do grupo controle. Observamos ainda que após a depleção, os camundongos B10.A apresentaram níveis maiores de IL-6 e GM-CSF quando comparados aos camundongos B10.A do grupo controle. Este dado é interessante, pois a IL-6 e a IL-23 estão envolvidas na indução e manutenção de células Th17 (LOCHNER et al., 2008; IVANOV et al., 2006). Assim, nesta fase da infecção, as células T reg poderiam desempenhar um efeito regulador positivo sobre as células Th17 em camundongos A/J e negativo em camundongos B10.A.

Por outro lado verificamos que na décima semana pós-infecção o tratamento levou a um aumento dos níveis das citocinas do tipo Th1, Th2 e Th17 em ambas as linhagens quando comparadas aos grupos somente tratados com Ig. Este fato pôde ser observado para os níveis de IL-12, IL-2, IL-4, IL-10, IL-6, IL-17, IL-23, TGF-β e para o fator de crescimento GM-CSF. Além disso, verificamos que os níveis das citocinas nos camundongos B10.A foi maior quando comparados aos dos camundongos A/J.

Com estes dados podemos sugerir que a depleção das células T reguladoras nos camundongos A/J e B10.A promoveu uma maior expansão e o desenvolvimento das células com perfil Th1, Th2 e Th17. Esta associação pode ser considerada levando em conta as citocinas necessárias para a geração e manutenção destas sub populações celulares. As citocinas IL-6 e IL-23 são necessárias para a geração e manutenção de células Th17 (HARRINGTON et al., 2005; LANGRISH et al., 2005). A IL-6 e o TGF-β estão relacionados com a diferenciação das células Th17. Estas citocinas induzem o fator de transcrição RORγt que é determinante para a diferenciação desta linhagem celular (YANG *et al*., 2008). Verificamos também o aumento de citocinas Th2 (IL-4 e IL-10) e Th1 (IL-2, IL-12) indicando a ativação de uma população mista Th1/Th2/Th17 na ausência de células Treg.

Dados de outros pesquisadores mostram que a presença de citocinas Th1 e Th2 inibem a polarização de células T reguladoras Foxp3$^+$. Descrevem ainda que a neutralização de IFN-γ e IL-4 pode aumentar a diferenciação de células T reguladoras Foxp3$^+$ antígeno-específica *in vivo* (WEI et al., 2007).

No nosso modelo observamos efeito semelhante, pois a inibição de células Treg induziu a maior expansão da imunidade Th1 e Th2 com grande resolução da carga fúngica dos órgãos. Além disso, o aumento dos níveis das citocinas com padrão Th1, Th2 e Th17 após a depleção com anticorpo anti-CD25 em camundongos A/J e B10.A não só permitiu uma resposta controlada de todas as subpopulações celulares bem como a diminuição da carga fúngica como também mostrou que a proteção contra o fungo não é somente dependente de uma resposta Th1 (CANO et al., 1998). Além disso, em trabalhos recentes temos verificado uma possível associação entre imunoproteção e expansão da subpopulação Th17 (LOURES et al., 2009, 2010, 2011).

Hadjur et al. (2009) descreveram que a diferenciação de células T estava relacionada com a produção de citocinas e fatores de transcrição. Após usarem anticorpos anti- IL-4 observaram indução de células $Foxp3^+$ em células T naive, e que tanto o fator de transcrição GATA 3 como PU.1 (fator de transcrição mielóide) inibiam a indução de $Foxp3^+$. Estes dados são importantes, pois sugerem que a diferenciação de células T reguladoras induzidas pode ser afetada pela polaridade das respostas imunes por sofrerem estímulo de citocinas.

Diferente dos nossos resultados, Mariano e colaboradores demonstraram que na fase aguda da infecção pelo *Trypanossoma cruzi* a depleção com anticorpo anti-CD25 levou a uma diminuição dos níveis das citocinas IL-10, TGF-β, IFN-γ e IL-12 quando comparado aos dados obtidos de camundongos tratados com Ig de rato. Entretanto, esta diminuição não pôde ser relacionada com diminuição da parasitemia (MARIANO et al., 2008).

Nossos dados demonstraram que após a infecção a supressão mediada pelas células Tregs inibia a eliminação do patógeno, pois verificamos que a depleção das células Tregs reduziu de maneira significante o número de fungos. Além disso, ambos os camundongos apresentaram o aumento do desenvolvimento de uma resposta Th1, Th2 e Th17. Sabemos que as células T reguladoras além de expressar o fator de transcrição FoxP3, podem perder as funções supressoras e manifestar algumas das funções efetoras das células T através da expressão dos fatores de transcrição específicos, como T-bet (células Th1) (KOCK et al., 2009) e IRF4 (células Th2) (ZHENG et al., 2009). Além disso, as células T podem expressar FoxP3+RORγT+ e que por diferentes estímulos podem diferenciar-se em células T reguladoras ou células Th17 (TARTAR et al., 2010).

Em elegante revisão, Campbell e Koch descreveram que defeitos na função da célula T reguladora podem resultar no desenvolvimento de uma doença inflamatória mediada por células com perfil Th1, Th2 e Th17, indicando que aquelas células são necessárias para a regulação adequada da resposta imune (CAMPBELL e KOCK, 2011). As principais causas da perda da expressão do FoxP3 incluem os ambientes inflamatórios com a presença de altos níveis de citocinas que geralmente estão envolvidos na indução de células T efetoras de vários sub-tipos (SHEVACH e DAVIDSON, 2010).

Em nosso modelo fica a sugestão de uma plasticidade na diferenciação das subpopulações Th na fase crônica da infecção. A depleção com o anticorpo monoclonal anti-CD25 permitiu o desenvolvimento não só de células Th1 e Th2, fundamentais para o controle do patógeno, mas principalmente o desenvolvimento de células com perfil Th17. Podemos sugerir que estas células estariam envolvidas no controle do crescimento fúngico, principalmente em camundongos B10.A onde a resposta imune adaptativa está suprimida desde o início da infecção. Entretanto, para se ter certeza da presença e função das células Th17 em nosso modelo, será necessário a caracterização fenotípica desta população através da caracterização da presença de IL-17 intracelular nas diversas subpopulações de leucócitos que afluem para o pulmão na fase crônica da doença.

A depleção das células T reguladoras por anticorpo anti-CD25 é alvo de questionamento metodológico uma vez que este anticorpo atinge outras populações celulares ativadas expressando o receptor de IL-2 de alta afinidade, além da célula T reguladora. Entretanto, acreditamos que esta seja uma escolha adequada para estudar tal população celular, pois as células T reguladoras expressam constitutivamente altos níveis de CD25 quando comparado às células T efetoras (revisado por BELKAID e TARBELL, 2009), e isso leva a uma depleção mais acentuada das primeiras, principalmente se realizado antes ou em fase muito precoce da infecção.

Nossos dados mostraram na segunda semana de infecção que após o tratamento com anticorpo anti-CD25 os números de células T naives e ativadas estavam elevados, o que significa que a depleção eliminou eficientemente as células supressoras, impedindo a sua expansão e migração.

Uma visão geral sobre o efeito da depleção de células $CD25^+$ ao início da infecção pelo *P. brasiliensis* sugere que as células T reguladoras controlam a carga fúngica pulmonar

de ambas as linhagens e controlam negativamente a inflamação tecidual mediada por células T e macrófagos principalmente na linhagem A/J. Assim, as células T reguladoras possivelmente atuam nas células de imunidades inata e adquirida de camundongos A/J, mas preferencialmente nos mecanismos efetores da imunidade inata de camundongos suscetíveis. A análise da presença de células T, linfócitos B e granulócitos demonstram claramente o maior efeito de células $CD25^+$ sobre estas populações celulares da linhagem A/J.

Os camundongos A/J depletados apresentavam aumento de todas as sub-populações de células estudadas, sendo elas $CD4^+$ naives ($CD4^+CD44^{low}CD62L^{high}$), $CD4^+$ ativadas ($CD4^+CD44^{high}CD62L^{low}$), $CD8^+$ naives ($CD8^+CD44^{low}CD62L^{high}$), $CD8^+$ ativadas ($CD8^+CD44^{high}CD62L^{low}$), $CD4^+$ ativadas/supressoras ($CD4^+GITR^+$), $CD4^+$ inibidoras ($CD4^+CTLA\text{-}4^+$), macrófagos ($F4/80^+GR1^+$), macrófagos ativados ($F4/80^+Ia^{K+}$), DCs ($CD11c^+Ia^{K+}$), DCs mielóides ($CD11b^{high}CD11c^{high}$), e DCs plasmocitóides ($CD11c^{int}B220^+$). Assim, a presença de células T reguladoras parece exercer uma maior influência na ativação de linfócitos e macrófagos dos camundongos A/J do que dos camundongos B10.A. Além disso, como a depleção com o anticorpo anti-CD25 ocorreu 3 dias antes da infecção com o fungo e 3 dias depois, podemos sugerir que seu efeito foi primariamente sobre células T reguladoras naturais que estariam presentes no sítio da infecção e aptas para suprimir a ativação celular nos camundongos A/J. Este efeito, entretanto, parece haver se mantido durante o curso da infecção, pois o número diminuído de células Treg (provavelmente Treg induzida) foi observado na fase crônica da doença.

Apesar de termos observado uma diminuição no número de fungos recuperados em camundongos B10.A após a depleção com anti-CD25, este fenômeno não pôde ser correlacionado com um aumento do infiltrado inflamatório nestes camundongos. Deve-se também lembrar que em camundongos B10.A a depleção de células Treg ocasionou, na segunda semana, uma diminuição dos níveis de NO dos pulmões, concomitante com redução de carga fúngica. Assim, a diminuição das células Treg levou à redução do NO, metabólito envolvido na imunossupressão de camundongos suscetíveis e poderia ter resgatado parcialmente mecanismos efetores T-dependentes, apesar de não muito evidentes.

Uma provável hipótese para explicar a diminuição do número de fungos recuperados em camundongos B10.A após a depleção com o anticorpo anti-CD25, sem aumento do afluxo celular, seria a grande eficiência da imunidade inata destes camundongos. Nos camundongos susceptíveis demonstrou-se previamente a ativação de uma resposta pró-inflamatória intensa

com a presença de macrófagos produtores de IL-12 e NO que exercem atividade fungicida bastante eficiente (PINA; BERNARDINO; CALICH, 2008). Na ausência de células T reguladoras os macrófagos e outras células de imunidade inata dos camundongos B10.A poderiam apresentar maior capacidade fagocítica, que associada com o aumento de citocinas levaria a diminuição do número de fungos tanto no pulmão como no fígado. É importante destacar que o tratamento com o anticorpo anti-CD25 levou ao aumento tardio da produção dos níveis de TGF-β comparado ao grupo controle e aos camundongos A/J. Em processos de excessiva ativação esta citocina é importante para controlar respostas inflamatórias excessivas e impedir danos teciduais.

Os membros moleculares da super família do receptor de TNF são capazes de afetar diretamente ou indiretamente o curso de uma resposta imune (WATTS, 2005). O marcador GITR, é membro desta família e têm sido implicado na regulação de ambas as respostas imunes, inata e adquirida (RONCHETTI et al., 2004; NOCENTINI et al., 2007). As células T reguladoras são conhecidas por expressar altos níveis de GITR, entretanto, este receptor também pode ser expresso em células T ativadas, células B, monócitos, macrófagos, células dendríticas e mastócitos (SHEVACH et al., 2006). Além disto, ele é pouco expresso em células T naive, ao contrário das células T ativadas (NOCENTINI et al., 2005). Em consonância com os resultados por nós obtidos, pode-se sugerir que a expressão de GITR não deva ser atribuído somente às células T reguladoras, pois ao observarmos a população celular $CD4^{+}GITR^{+}$ verificamos aumento deste marcador em células de camundongos A/J depletados quando comparados ao grupo controle. Isto significa que, apesar de ter ocorrido diminuição das células T reguladoras, o aumento das células T ativadas ao início da infecção poderia explicar o aumento do GITR observado nestes camundongos.

Por outro lado, os dados obtidos na décima semana pós-infecção demonstram que após a depleção os camundongos apresentavam diminuição da carga fúngica pulmonar associado a uma diminuição do influxo de células inflamatórias para o pulmão. Assim, podemos sugerir que na fase crônica da infecção a ausência das células T reguladoras permitiu o desenvolvimento de uma resposta imune mais eficiente contra o fungo e levou à diminuição do estímulo primário da resposta imune, isto é o antígeno. Este fato pode ser relacionado com o menor número de linfócitos T, células B e granulócitos nos pulmões após a depleção com

anti-CD25; mais uma vez a alteração do afluxo celular foi mais marcante em camundongos A/J.

Os camundongos A/J e B10.A depletados com anti-CD25 apresentaram diminuição no número de células $TCD4^{+}CD44^{low}CD62L^{high}$, $TCD4^{+}CD44^{high}CD62L^{low}$, $TCD4^{+}CD25^{+}$, $TCD4^{+}$CTLA-4^{+} $TCD4^{+}GITR^{+}$, $F4/80^{+}GR1^{+}$ e $CD11b^{high}CD11c^{high}$. Podemos sugerir que a presença das células T reguladoras na fase crônica da infecção pelo *Paracoccidioides brasiliensis* prejudica a eficiência da resposta imune por manter elevado o número de patógeno que induz inflamação excessiva que pode ser deletéria para os tecidos. Nossos dados demonstram também que os camundongos A/J do grupo controle (tratado com Ig) apresentam maior número de linfócitos T pulmonares quando comparados aos camundongos B10.A do mesmo grupo indicando um melhor desenvolvimento de imunidade celular pela linhagem resistente. Dados semelhantes da resposta imune desenvolvida contra o fungo por camundongos A/J foram mostrados por Calich et al. que descrevem uma eficiente resposta imune protetora em fases mais tardias da doença de camundongos resistentes (revisto em CALICH et al., 2008).

Um dado muito importante por nós obtido, foi a caracterização da presença de células T reguladoras $CD4^{+}CD25^{+}Foxp3^{+}$ no pulmão e fígado de camundongos A/J e B10.A tratados com Ig de rato e depletados com anticorpo anti-CD25 na segunda e décima semana pós-infecção. Verificamos que camundongos A/J controle apresentam maior número de células T reguladoras quando comparados aos camundongos B10.A. Por outro lado após a depleção, observamos diminuição significativa de células T reguladoras $Foxp3^{+}$ em ambas as linhagens. Este dado demonstra que o tratamento com o anticorpo monoclonal anti-CD25, afeta realmente a expansão das células T reguladoras, possivelmente as naturais e as induzidas.

Corroborando com os nossos resultados, Taylor e seus colaboradores mostraram que durante a infecção murina com *Litomosoides sigmodontis*, a morte do parasita adulto só acontece após a depleção de células T reguladoras bem como com o bloqueio de CTLA-4 em células T efetoras (TAYLOR et al., 2007).

Por outro lado, é cada vez mais claro que as células T reguladoras não são apenas células que suprimem a resposta imune a auto-antígenos ou impedem o desenvolvimento da auto-imunidade, mas também são importantes na regulação da imunidade a antígenos de patógenos, especialmente aqueles que estabelecem infecções persistentes como *Leishmania*

major, Helicobacter pylori, o vírus da Hepatite C, o HIV (BELKAID e ROUSE, 2005) e agora o *P. brasiliensis*.

Em um modelo de infecção pela *Leishmania major*, a presença de células Treg leva à persistência do parasita resultando em manutenção de memória imunológica e manutenção de imunidade protetora contra o parasita (SUVAS et al., 2004; BELKAID et al., 2002). Outra demonstração de que as células T reguladoras são importantes para manter o equilíbrio entre o hospedeiro e o patógeno é a de uma infecção ocular causada pelo vírus herpes simples (HSV) em camundongos. A partir de uma infecção com baixa dose do vírus, as células T reguladoras protegem os animais de patologia exacerbada mediada por células T, além de permitirem o estabelecimento da imunidade frente a uma re-infecção (SUVAS et al., 2004).

De maneira similar, na infecção de camundongos por *C. albicans*, a redução do número de células T reguladoras ocasionou em uma melhora no controle da infecção primária, porém reforçou a patologia tecidual devido à imunidade exacerbada associada com a perda de memória imunológica e proteção a uma re-infecção. Por outro lado, a imunidade contra uma re-infecção pôde ser readquirida mediante a transferência adotiva de células T reguladoras (MONTAGNOLI et al., 2002).

Assim, preconiza-se um papel importante para as células T reguladoras na manutenção da imunidade em infecções crônicas em que a resposta efetora não é tão eficiente e ocorre a persistência do patógeno. Assim, o sistema imune ficará constantemente ativado, necessitando de células T reguladoras para manter uma resposta protetora permanentemente ativada.

Em nosso modelo observamos que os camundongos B10.A controle apresentaram alta taxa de mortalidade quando comparados aos camundongos B10.A depletados com anticorpo anti-CD25. Podemos sugerir que após o tratamento os camundongos apresentaram o desenvolvimento de uma imunidade inata muito eficiente associada com o desenvolvimento de uma possível memória imunológica, ambas balanceadas que puderam controlar o crescimento fúngico. Além disso, podemos sugerir que o número de células T reguladoras observado após o tratamento foi suficiente para permitir a ativação do sistema imune, porém, sem levar a uma ativação exacerbada e patologia tecidual.

Um importante dado por nós obtido foi à caracterização do número de fungos recuperados dos camundongos sobreviventes da mortalidade. Observamos que tanto os camundongos A/J controle e tratados, como os camundongos B10.A tratados apresentavam o mesmo número de fungos recuperados, cerca de $3,5x\log_{10}$. Assim, podemos supor que a

depleção com o anticorpo anti-CD25 não resultou um cura estéril permitindo o desenvolvimento de memória imunológica capaz de controlar o crescimento fúngico nas lesões e em desafios posteriores.

Trabalho anterior desenvolvido em nosso laboratório (ARRUDA et al., 2007) demonstrou que se pode induzir imunidade protetora em camundongos susceptíveis à PCM através da pré-imunização de camundongos com fungos viáveis pela via sub-cutânea. Ao contrário, camundongos resistentes não desenvolvem proteção e talvez este fenômeno, não compreendido na época de sua publicação, esteja relacionado com o grande número de células Treg (provavelmente naturais) que os camundongos A/J demonstram ao início da infecção.

Na infecção por *P. brasiliensis* a disseminação do fungo para o fígado parece exercer papel preponderante na determinação da gravidade da doença (CANO et al., 1995; FELONATO et al., 2010). A análise da carga fúngica no fígado demonstrou maior número de fungos recuperados dos camundongos B10.A controle quando comparados aos tratados com anticorpo anti-CD25. Por outro lado, os camundongos A/J tratados ou não com anticorpo anti-CD25 apresentaram baixa carga fúngica no fígado. Sendo assim, fomos estudar o fenótipo do infiltrado celular presente no fígado para esclarecer um pouco melhor esta situação.

Os camundongos A/J apresentavam eficiente desenvolvimento de uma imunidade celular no pulmão e observamos também no fígado de camundongos A/J aumento no influxo de células $TCD4^{+}$e $TCD8^{+}$ naives e ativadas associado com o baixo número de fungos recuperados. Este afluxo aumentado de células não foi observado nos pulmões e fígado de camundongos B10.A.

Os camundongos B10.A controle por apresentarem no pulmão grande quantidade de fungo associada com uma deficiente ativação da imunidade celular parece permitir uma maior disseminação do fungo para o fígado

Analisamos ainda a presença de células T reguladoras presentes no fígado dos camundongos A/J e B10.A tratados ou não com o anticorpo anti-CD25 e verificamos que os camundongos A/J controle apresentavam maior número de células Treg quando comparados aos camundongos B10.A submetidos ao mesmo tratamento. Entretanto, após a depleção observamos que o número de células Treg diminuiu em ambos os camundongos, mas os camundongos A/J continuavam apresentando número maior desta população quando comparados aos camundongos B10.A.

Este dado permite compreender melhor o crescimento fúngico observado no fígado dos camundongos B10.A controle. O número expandido de células Treg observado nestes camundongos, associado com o baixo afluxo de linfócitos T para o pulmão, poderia ter permitido uma maior disseminação fúngica para o fígado que não foi observada nos camundongos B10.A depletados. A redução do número de células Treg nos camundongos tratados, apesar de não promover o maior afluxo de células para os órgãos, poderia ter permitido, entretanto, o desenvolvimento de uma imunidade inata mais eficiente do pulmão que poderia ter impedido a disseminação para o fígado.

Nossos dados demonstram que no desenvolvimento da resposta imunológica (fases aguda e crônica) os camundongos resistentes ao fungo apresentam número aumentado de células T reguladoras quando comparados aos camundongos suscetíveis. Vários fatores estão associados a esta resistência ao fungo (revisado por CALICH et al., 2008), mas agora sabemos também que o fato de existir grande número de células T reguladoras leva à anergia inicial do sistema imunológico em camundongos A/J, às custas de um menor controle da carga fúngica. Caso tal população não existisse, ou se expandisse em menor número como aqui induzido, pudesse ser adequadamente ativado levando a imunidade eficiente.

É interessante ressaltar, ainda, que os camundongos A/J e B10.A depletados de células Treg naturais nos dias -3 e +3 pós infecção, apresentaram ativação da resposta imune mais eficiente contra o fungo; entretanto, observamos que ambas as linhagens não apresentaram aumento do número de células FoxP3+ na fase tardia da doença. Estes dados sugerem que a diminuição de células Treg naturais na fase inicial da doença, leva a um decréscimo do estímulo pelo patógeno e uma consequente menor expansão de células Treg induzidas na fase tardia.

Em um recente trabalho desenvolvido em nosso laboratório, Pina e colaboradores demonstraram que células dendríticas (DCs) de animais A/J apresentam além de células mielóides, DCs com perfil plasmocitóide que produzem níveis altos de TGF-β e expansão de grande número de células T reguladoras *in vitro*. *In vivo,* há tolerância inicial ao crescimento fúngico e posterior desenvolvimento da resposta imune protetora caracterizada pela produção prevalente de IFN-γ. Por outro lado, camundongos B10.A desenvolvem células dendríticas com perfil mielóide com produção elevada de IL-12 e IL-23, porém com baixos níveis de

TGF-β e expansão diminuída de células T reguladoras. Nesta linhagem a presença de IDO e o aumento de NO também controlam a carga fúngica inicial, porém, o aumento de metabólitos do aminoácido triptofano decorrente da ação da enzima IDO e NO suprimem a ativação de linfócitos T, permitindo o desenvolvimento mais grave da doença nos camundongos suscetíveis. (PINA et al., 2011 submetido a publicação). No modelo *in vivo*, a inibição da enzima IDO pelo composto 1-metil 2,3-triptofano restaurou a imunidade celular $TCD4^{+}$ após 2 semanas de infecção pelo fungo em camundongos B10.A mas não em camundongos A/J (ARAÚJO, 2010).

Neste contexto, a regulação ou supressão da resposta imune de camundongos suscetíveis parece ser mediada pela produção de NO e IDO além de células Treg, como aqui demonstrado.

Em nosso modelo, o aumento de células dendríticas mielóides e plasmocitóides observado na segunda semana pós-infecção de camundongos A/J, poderia ser correlacionado com o aumento da imunidade adaptativa observada. Tal aumento foi observado em camundongos A/J depletados com anticorpo anti-CD25, onde também verificamos uma diminuição da presença de IL-10, TGF-β e da população de células T reguladoras. Pode-se então sugerir que nos camundongos A/J a presença de células Treg inibe a expansão e função de células dendríticas. Estas células de aparecimento precoce controlariam a resposta imune inata como a adquirida. Como o anticorpo anti-CD25 diminuiu o número de células T reguladoras, pode-se supor que a interação do fungo com as células dendríticas deixou de ser inibida e assim possibilitou uma maior expansão de células dendríticas mielóides de fenótipo $CD11b^{high}CD11c^{high}$ e de células dendríticas plasmocitóides de fenótipo $CD11c^{int}B220^{+}$.

Por outro lado, o aumento do número das células dendríticas observado na décima semana pós-infecção nos camundongos A/J e B10.A tratados com Ig pode estar relacionado com aumento do número de fungos observado nestes camundongos. A depleção de células Treg que leva à diminuição da carga fúngica, entretanto, leva à diminuição da sub população mielóide em ambas as linhagens, e isso poderia estar associado à falta de estímulo antigênico.

A análise histológica das lesões não revelou grande diferenças entre os grupos na segunda semana pós infecção. Neste período, os camundongos A/J e B10.A tratados ou não com o anticorpo anti-CD25 apresentavam semelhanças na análise histopatológica, porém diferenças na quantidade de fungos como observado na coloração pelo Groccot.

Por outro lado, a análise histológica na décima semana pós infecção mostrou que os camundongos A/J depletados e não depletados apresentavam o mesmo perfil inflamatório e pequeno número de fungos. Já os camundongos B10.A controle apresentavam grandes granulomas contendo grande quantidade de fungos. Após o tratamento com anti-CD25 os camundongos B10.A apresentavam redução significativa no número de granulomas e fungos.

A análise histológica do fígado na décima semana pós infecção adicionou informações importantes. Os camundongos B10.A controle apresentavam presença de granulomas hepáticos com grande quantidade de fungos, enquanto que os camundongos B10.A depletados apresentavam o mesmo perfil que os camundongos A/J: ausência de fungos e infiltrado inflamatório.

Neste período de infecção a análise morfométrica revelou que os camundongos B10.A controle apresentavam maior área de lesão quando comparados aos camundongos A/J controle. Da mesma maneira, ambos os camundongos A/J e B10.A controle apresentavam maior área de lesão quando comparado aos seus grupos tratados com anticorpo anti-CD25. Após a depleção observamos que camundongos B10.A e A/J apresentavam áreas de lesão equivalentes. Desta maneira, é tentador fazer algumas especulações para tentar esclarecer a diminuição do número de fungos nos camundongos B10.A tratados. Por um lado os camundongos A/J controle desenvolvem uma infecção regressiva, com pequeno número de fungos e controle da morfologia das lesões. Após a depleção não houve mudança nos achados histopatológicos. Há uma menor presença de patologia tecidual e inflamação residual associados com a quase ausência dos patógenos.

Por outro lado, os granulomas observados nos camundongos B10.A controle estariam apenas tentando conter o crescimento fúngico, mas não estariam matando o fungo nas lesões nem impedindo sua disseminação. Já nos camundongos B10.A tratados a presença de pequenos granulomas e um possível processo inflamatório seria responsável pela diminuição do fungo, devido a sua eficiente atividade efetora (macrófagos e leucócitos, por exemplo).

Estes resultados associados com o experimento de mortalidade são bastante motivadores, pois nos permitem sugerir que os camundongos susceptíveis na ausência de células T reguladoras conseguem promover o desenvolvimento de uma resposta imune suficiente para o controle do crescimento fúngico. Em outras palavras, os camundongos susceptíveis B10.A tendem a perder esta característica e "tornam-se resistentes" tanto quanto aos camundongos A/J, controlando o processo infeccioso.

Em conjunto, nossos dados demonstram que camundongos A/J apesar de desenvolverem precocemente grande número de células Treg conseguem desenvolver imunidade Th1/Th2/Th17 mediada por linfócitos T e macrófagos ativados, principalmente em fases mais tardias da doença. Entretanto, este tipo de ativação tem um preço a pagar, isto é o baixo controle da carga fúngica ao início da doença que ocasiona uma disseminação precoce do fungo para o fígado e baço. Este fato, entretanto, poderia levar a uma melhor resposta imune sistêmica que permitiria grande afluxo de células inflamatórias para o pulmão e controle relativo do patógeno.

Em camundongos B10.A, entretanto, os mecanismos de imunidade inata são muito eficientes no controle inicial da carga fúngica, mas por excessiva produção de NO e IDO ocorreria a imunossupressão, quase que irreversível, da resposta imune adaptativa. As células Treg contribuem com esta supressão, principalmente em fases mais tardias da doença.

Nosso modelo experimental de PCM é distinto dos modelos usuais de resistência e suscetibilidade à maioria dos patógenos. Ele demonstra que a convivência, a não excessiva reação inicial à colonização pelo patógeno, pode ser menos deletéria que tentativas exacerbadas de eliminação da infecção que podem resultar em supressão irreversível da resposta imune adaptativa.

É bastante interessante também verificar a existência de mecanismos reguladores da imunidade bem distintos, um que baseia-se principalmente na atividade de células Treg (A/J) e outro que decorre principalmente da produção excessiva de IDO e NO (B10.A).

Assim, nosso trabalho demonstrou que a resistência da linhagem A/J parece ser mediada por tolerância ao crescimento fúngico sem anergia total de imunidade adaptativa, enquanto a suscetibilidade de camundongos B10.A está relacionada com eficiente imunidade inata, menor ativação de células T reguladoras e excesso de mecanismos pró-inflamatórios que levam à anergia da resposta imune adaptativa. Além disso, nosso trabalho demonstrou pela primeira vez que frente à infecção pelo *P. brasiliensis* as células T reguladoras dos camundongos A/J agem acentuando a regulação do sistema imune adaptativo de maneira precoce, impedindo o controle fúngica na fase inicial da doença. Entretanto, a relação entre a função exercida pelas células T reguladoras e a função promovida pelo sistema imune na fase crônica da infecção, parece ser balanceada, pois os camundongos resistentes conseguem

conter a infecção sem, entretanto, apresentarem lesão tecidual provocado por um possível excesso de ativação não encontrado nestes camundongos.

Por outro lado, as células T reguladoras dos camundongos B10.A agem de maneira pouco eficiente na fase inicial da doença. Os camundongos apresentam uma eficiente resposta da imunidade inata, mas que é possivelmente bloqueada pela supressão das células T reguladoras na fase crônica da doença. Assim, a resposta inicial desenvolvida pelos camundongos não seria suficiente para controlar o crescimento fúngico e por ação das células Treg inibindo tal sistema, permitiria o crescimento e a disseminação para outros órgãos.

No conjunto, o tratamento com o anticorpo anti-CD25 promove uma melhora significativa da doença em ambos os camundongos: os camundongos A/J apresentaram aumento da ativação da resposta imunológica, enquanto que camundongos B10.A desenvolveram ativação menos evidente da resposta imune adaptativa, mas que possivelmente em conjunto com mecanismos de imunidade inata levou ao controle do crescimento fúngico. Em outras palavras, pode-se dizer que a depleção de Treg, resgatou a anergia inicial de células T nos camundongos A/J, mas não a de camundongos B10.A, que parece ao início da doença ser mediada por excessiva produção de NO e IDO (mediadores inflamatórios).

Esse é um mecanismo não usual de resistência e suscetibilidade a patógenos e abre novas perspectivas no entendimento da imunopatologia da PCM e talvez novas abordagens terapêuticas em casos graves da doença.

6 CONCLUSÕES

- ✓ O presente trabalho demonstrou que a expansão de um elevado número de células T reguladoras é prejudicial para os camundongos resistentes e susceptíveis.
- ✓ As células T reguladoras parecem exercer diferentes mecanismos supressores em camundongos A/J e B10.A.
- ✓ As células Treg estão envolvidas na tolerância fúngica desenvolvida por animais resistentes na fase inicial da doença (as células Treg exercem capacidade aumentada de supressão).
- ✓ As células Treg não estão envolvidas na anergia de células T de camundongos suscetíveis na fase inicial da doença (induzidas por mediadores inflamatórios, NO e IDO).
- ✓ A depleção de células Treg promove um novo equilíbrio entre os mecanismos efetores e reguladores da resposta imune que resulta em diminuição de carga fúngica e patologia tecidual menos grave no pulmão.

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ANEXOS

***Anexo* A – CD28 Exerts Protective and Detrimental Effects in a Pulmonary Model of Paracoccidioidomycosis**

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CD28 Exerts Protective and Detrimental Effects in a Pulmonary Model of Paracoccidioidomycosis▽

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T-cell immunity has been claimed as the main immunoprotective mechanism against *Paracoccidioides brasiliensis* infection, the most important fungal infection in Latin America. As the initial events that control T-cell activation in paracoccidioidomycosis (PCM) are not well established, we decided to investigate the role of CD28, an important costimulatory molecule for the activation of effector and regulatory T cells, in the immunity against this pulmonary pathogen. Using CD28-deficient ($CD28^{-/-}$) and normal wild-type (WT) C57BL/6 mice, we were able to demonstrate that CD28 costimulation determines in pulmonary paracoccidioidomycosis an early immunoprotection but a late deleterious effect associated with impaired immunity and uncontrolled fungal growth. Up to week 10 postinfection, $CD28^{-/-}$ mice presented increased pulmonary and hepatic fungal loads allied with diminished production of antibodies and pro- and anti-inflammatory cytokines besides impaired activation and migration of effector and regulatory T (Treg) cells to the lungs. Unexpectedly, CD28-sufficient mice progressively lost the control of fungal growth, resulting in an increased mortality associated with persistent presence of Treg cells, deactivation of inflammatory macrophages and T cells, prevalent presence of anti-inflammatory cytokines, elevated fungal burdens, and extensive hepatic lesions. As a whole, our findings suggest that CD28 is required for the early protective T-cell responses to *P. brasiliensis* infection, but it also induces the expansion of regulatory circuits that lately impair adaptive immunity, allowing uncontrolled fungal growth and overwhelming infection, which leads to precocious mortality of mice.

It has long been appreciated that cellular immunity is the most important resistance mechanism against fungal infections (14, 36, 64). $CD4^+$ and $CD8^+$ T-cell subpopulations have been described to have a fundamental role in the control of fungal growth, and disease severity is also controlled by regulatory T (Treg) cells, which prevent tissue pathology by controlling excessive inflammatory reactions (25, 45, 46, 65). Similar to other deep mycoses, the severity of paracoccidioidomycosis (PCM), the most severe pulmonary mycosis in Latin America, is controlled by cellular immunity and cytokine-activated phagocytes that are able to kill *Paracoccidioides brasiliensis*, the etiological agent of this infection (10, 20, 30, 60, 61). In humans and in murine models of PCM, resistance to the disease is associated with the secretion of gamma interferon (IFN-γ) and other type 1 cytokines, whereas impaired Th1 immunity and the prevalent secretion of Th2 cytokines correlate with a systemic and progressive disease (2, 6, 39, 59, 76). Studies with $CD4^+$ and $CD8^+$ T-cell-deficient mice revealed that both T-cell subsets are involved in the protective immunity against *P. brasiliensis* infection and indicated the prominent role of $CD8^+$ T cells (3, 21, 25). Besides the prevalent Th2 immunity, recent investigations have described alternative mechanisms underlying T-cell dysfunction in humans and experimental PCM. Increased apoptosis and overexpression of Fas and FasL in T cells suggest that activation-induced cell death (AICD) is a mechanism that controls T-cell expansion during the active disease (13, 19). In addition, the increased expression of CTLA-4 and the expansion of Treg cells were associated with severe patterns of the disease (24, 45, 46, 56). Thus, in addition to cytokine imbalance, other regulatory mechanisms can actively participate in the unresponsiveness of T cells in *P. brasiliensis*-infected hosts.

Optimal activation, proliferation, and cytokine production by antigen-specific T cells require two distinct signals from dendritic cells or other antigen-presenting cells (APCs). After T-cell receptor (TCR) occupancy by the antigen epitope/major histocompatibility (MHC) complex (first signal), a second signal is mediated by costimulatory molecules (43, 63), such as CD28 on T cells and their counter-receptors CD80 (B7-1) and CD86 (B7-2) expressed by APCs (1, 34). Soluble molecules, such as cytokines and chemokines, also participate in the activation process, which drives and controls T-cell numbers and fates (1). CD28 enhances the TCR-triggered activation of naïve T cells, promotes interleukin-2 (IL-2) secretion and prevents T-cell anergy (1, 37). Alternatively, CD28-independent T-cell activation can occur if a strong and sustained antigen-specific signal is available (40, 81). Like CD28, two other molecules, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and mouse inducible costimulatory molecule (ICOS), are selectively expressed by T cells, but the expression of these molecules depends on previous cell activation (50, 71). More recently, evidence has emerged that CD28 family members are also crucial regulators of natural and induced regulatory ($CD4^+CD25^+Foxp3^+$) T cells (9). These cells are induced in the thymus and in the periphery, respectively, and control self-tolerance and the activation of several components of innate and adaptive immunity (68). Treg cells can suppress im-

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mune responses through the production of immunosuppressive cytokines (mainly IL-10 and transforming growth factor β [TGF-β]), through the induction of the apoptosis of effector T cells and through the modification of the functional properties of antigen-presenting cells (70, 78).

Immunoprotection against microorganisms has been shown to be either CD28 dependent or independent. CD28-deficient ($CD28^{-/-}$) mice are highly susceptible to infection with *Salmonella enterica* serovar Typhimurium due to the poor ability of these mice to secrete IFN-γ (51). During some viral and parasitic infections, CD28 was shown to be required to mediate $CD8^+$ T-cell immunoprotection (8, 53). In contrast, $CD28^{-/-}$ mice infected with *Mycobacterium bovis* or *Listeria monocytogenes* control the bacterial burden and develop cell-mediated immunity (35, 52). In primary and opportunistic fungal infections, CD28 costimulation controls protective immunity, the expansion and function of regulatory T cells, and the intensity of inflammatory reactions (5, 54, 55, 66, 84).

Because CD28 is critical for T-cell activation in fungal infections, we investigated its role in a murine model of *P. brasiliensis* infection. We show that CD28 costimulation exerts contrasting roles in pulmonary PCM. Early in infection, CD28 expression results in efficient adaptive immunity that is able to control fungal growth. Late in infection, however, this costimulatory molecule induces significant expansion of regulatory T cells, diminished immunity, and uncontrolled fungal growth that eventually leads to the death of the mice. In contrast, the absence of CD28 costimulation results in impaired T-cell immunity, which appeared to be compensated by the absence of Treg cell expansion. This weak but persistent immunity was able to partially control fungal growth, organize granulomatous lesions, and guarantee the enhanced survival of the mice, suggesting the relative protection conferred by CD28-independent mechanisms.

MATERIALS AND METHODS

Mice. Breeding pairs of homozygous CD28-deficient ($CD28^{-/-}$) and wild-type (WT) control C57BL/6 mice (intermediate susceptibility to *P. brasiliensis*) were bred at the University of São Paulo animal facilities under specific-pathogen-free (SPF) conditions in closed-top cages. Clean food and water were given *ad libitum*. Mice were 8 to 11 weeks of age at the time of infection, and procedures involving animals and their care were approved by the Ethics Committee on Animal Experiments from Instituto de Ciências Biomédicas, Universidade de São Paulo.

Fungus and mice infection. *P. brasiliensis* 18 isolate (Pb18), which is highly virulent, was used throughout the study. To ensure the maintenance of its virulence, the isolate was used after three serial animal passages (38). Pb18 yeast cells were then maintained by weekly subcultivation in semisolid Fava Netto culture medium (29) at 35°C and used on the seventh day of culture. The fungal cells were washed in phosphate-buffered saline (PBS; pH 7.2) and counted in a hemocytometer, and the suspension was adjusted to 20×10^6 fungal cells/ml. The viability of fungal suspension, determined by Janus Green B vital dye (Merk, Darmstadt, Germany), was always higher than 80%. Mice were anesthetized and submitted to intratracheal (i.t.) *P. brasiliensis* infection as previously described (20). Briefly after intraperitoneal anesthesia, the animals were infected with 1×10^6 Pb18 yeast cells, contained in 50 μl of PBS, by surgical i.t. inoculation, which allowed dispensing of the fungal cells directly into the lungs. The skins of the animals were then sutured, and the mice were allowed to recover under a heat lamp. Mice were studied during an early period (2 and 10 weeks after infection) and a late period (16 and 26 weeks). Two or three experiments were performed separately.

Assay for organ CFU. The number of viable microorganisms in infected organs (lung, liver, and spleen) from experimental and control mice were determined by counting the number of CFU. Animals ($n = 6$ to 8) from each group were sacrificed, and the enumeration of viable organisms was done as previously described (74). Briefly, aliquots (100 μl) of the cellular suspensions and serial dilutions were plated on brain heart infusion agar (Difco, Detroit, MI) supplemented with 4% (vol/vol) horse serum (Instituto Butantan, São Paulo, Brazil), and 5% *P. brasiliensis* 192 culture filtrate, the last constituting a source of growth-promoting factor. The plates were incubated at 35°C, and colonies were counted daily until no increase in counts was observed. The number ($\log_{10}$) of viable *P. brasiliensis* colonies per gram of tissue were expressed as means ± standard errors (SE).

Mortality rates. Mortality studies were performed with $CD28^{-/-}$ and control WT mice inoculated i.t. with 1×10^6 yeast cells or PBS ($n = 9$ to 11). Deaths were registered daily for a 400-day period, and experiments were repeated twice.

NO production. Nitric oxide (NO) production was quantified by the accumulation of nitrite in the tissue homogenates by a standard Griess reaction. Briefly, 50 μl of supernatants was removed from 24-well plates and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0,1% naphthylene diamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. The conversion of absorbance to micromolar NO was deduced from a standard curve by using a known concentration of $NaNO_2$ diluted in RPMI medium. All determinations were performed in duplicate and expressed as micromolar NO.

Measurement of serum *P. brasiliensis*-specific isotypes. Specific isotype levels (total IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3) were measured by a previously described enzyme-linked immunosorbent assay (ELISA) (20) employing a cell-free antigen (18) prepared by using a pool of different *P. brasiliensis* isolates (Pb339, Pb265, and Pb18). The average of the optical densities obtained with sera from control mice (PBS inoculated) diluted 1:20 was considered the cutoff for each respective isotype. Optical densities for each dilution of experimental sera were compared to the control values. The titer for each sample was expressed as the reciprocal of the highest dilution that presented an absorbance higher than the cutoff.

Measurement of cytokines. Mice were infected i.t. with *P. brasiliensis* ($n = 6$ to 8), and their right lungs were aseptically removed and individually disrupted in 5.0 ml of PBS. Supernatants were separated from cell debris by centrifugation at $2{,}000 \times g$ for 15 min, passed through 0.22-μm-pore-size filters (Millipore, Bedford, MA), and stored at −70°C. The levels of IL-2, IL-12, IFN-γ, tumor necrosis factor alpha (TNF-α), IL-4, IL-5, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-23, IL-17, and TGF-β were measured by capture ELISA with antibody pairs purchased from Pharmingen. The ELISA procedure was performed according to the manufacturer's protocol. The concentrations of cytokines were determined with reference to a standard curve for several 2-fold dilutions of murine recombinant cytokines. As an additional control, lung homogenates were added to recombinant cytokines used to obtain standard curves; no interference was detected, indicating the absence of inhibitory substances (e.g., soluble cytokine receptors).

Lung and liver leukocyte isolation. Lungs from each mouse were excised, washed in PBS, minced, and digested enzymatically for 1 h in 15 ml/lung of digestion buffer (RPMI, 5% fetal calf serum, 1 mg/ml collagenase [Sigma Aldrich Inc.], and 30 μg/ml DNase). Livers from individual mice were obtained and submitted to organ perfusion using 10.0 ml of warm PBS via the portal vein, and organ fragments were pressed through a 70-μm cell strainer (Becton Dickson). After erythrocyte lysis using NH_4Cl buffer, cells were washed, resuspended in complete media, and centrifuged for 30 min at $1{,}200 \times g$ in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Total leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was always higher than 85%. The absolute number of a leukocyte subset was equal to the percentage of that cell subset multiplied by the total number of leukocytes recovered from the digested organ divided by 100.

Flow cytometry analysis. For surface staining alone, leukocytes were washed and resuspended at a concentration of 1×10^6 cells/ml in staining buffer (1× PBS, 2% serum calf bovine, and 0.5% NaN_3). Fc receptors were blocked by the addition of unlabeled anti-CD16/32 (Fc block; BD Pharmingen, San Diego, CA). The leukocytes were then stained for 20 min at 4°C with the optimal dilution of each antibody or fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated (BD Pharmingen). The following antibodies were used: $CD11b^+$, $CD11c^+$, Ia^{b+}, $CD80^+$, $CD86^+$, $CD40^+$, $CD4^+$, $CD8^+$, $CD44^+$, $CD25^+$, $CD69^+$, CTLA-4^+, $ICOS^+$, and $FasL^+$. Cells were washed twice with staining buffer resuspended in 100 μl, and an equal volume of 2% formalin was added to fix the cells. The stained cells were analyzed immediately on a FACSCalibur flow cytometer (BD Biosciences, CA) using the CellQuest software (BD Biosciences) gating on macrophages or lymphocytes as judged from forward and side light scatter. Ten thousand cells were counted, the data were expressed as the percentage or the absolute number of positive cells, which was calculated through

the percentage obtained by a fluorescence-activated cell sorter (FACS), and the number of cells was determined in Neubauer chambers. The intracellular detection of FoxP3, the X-linked forkhead/winged helix transcription factor, in leukocytes obtained from the lung lesions was performed in fixed and permeabilized cells using Cytofix/Cytoperm (BD Biosciences). Initially, the cells were labeled with antibodies for cell surface molecules, such as FITC-conjugated anti-CD4 and PE-conjugated anti-CD25. Next, the cells were fixed, permeabilized, and stained with Cy-conjugated anti-FoxP3 for 90 min at 4°C. Cells were then washed twice with staining buffer and resuspended in 100 μl, and an equal volume of 2% formalin was added to fix the cells. A minimum of 20,000 events were acquired on the FACSCalibur flow cytometer using the CellQuest software, as described above. Surface staining of CD25 and intracellular FoxP3 expression were backgated on the CD4 T-cell population. For flow cytometric analysis of apoptotic and necrotic lymphocytes, annexin V and propidium iodide labeling was used (82).

***In vivo* depletion of CD8$^+$ T cells.** The H-35 hybridoma (rat IgG1, anti-mouse CD8 monoclonal antibody [MAb]) was grown intraperitoneally (i.p.) in pristane (Sigma Chemical Co., St. Louis, MO)-primed BALB/c nu/nu male mice. The antibodies were purified from ascites as described elsewhere (49) and assessed for purity by SDS-PAGE electrophoresis. Groups of WT and CD28$^{-/-}$ C57BL/6 mice were given 200 μg of the H-35 MAbs or normal rat IgG (controls) by the i.p. route, 48 and 24 h before infection with *P. brasiliensis* cells, and 150 μg of the H-35 MAbs or rat IgG at days 6 and 12 after infection. Mice were sacrificed 48 h later, and lungs analyzed for CFU counts and the presence of lymphocytes and macrophages.

Histopathologic and morphometrical analyses. Groups of CD28$^{-/-}$ mice and their WT counterparts were killed at the second and tenth week postinfection. Lungs were collected, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were stained by hematoxylin-eosin (H&E) for an analysis of the lesions and silver stained for fungal evaluation. Pathological changes were analyzed based on the size, morphology, and cell composition of granulomatous lesions, presence of fungi, and intensity of the inflammatory infiltrates. Morphometrical analysis was performed using a Nikon DXM 1200c digital camera (magnification of ×10) and Nikon NIS Elements AR 2.30 software. The area of lesions was measured (in μm^2) in 10 microscopic fields per slide ($n = 4$ to 6). Results were expressed as the mean (± standard error of the mean [SEM]) total area of lesions for each animal.

Statistical analysis. All values are means ± SEM, unless otherwise indicated. Differences between two means were evaluated by Student's t test. Differences between survival times were determined with the log rank test using GraphPad Prism 5 for Windows (GraphPad Software). P values of ≤0.05 were considered significant.

RESULTS

CD28 expression induces an early protective but a late deleterious effect in the control of fungal growth. The evolution of the disease of i.t. infected CD28$^{-/-}$ mice and WT controls was monitored by counts of CFU in the lung, liver, and spleen at different times postinfection (2, 10, 16, and 26 weeks). At weeks 2 and 10 of infection, CD28-deficient mice showed higher counts of CFU in their lungs (Fig. 1A) than WT mice. At week 2, fungal dissemination to the liver was observed with CD28$^{-/-}$ mice, but the difference between the control and CD28$^{-/-}$ mice was significant only at week 10 (Fig. 1B). Unexpectedly, late in infection (weeks 16 and 26), no difference in the pulmonary fungal burden was observed between the two mouse strains. Importantly, by week 16, a reversion in the severity of the hepatic infection was detected: fungal loads of the WT mice were significantly higher than those of the CD28$^{-/-}$ mice, indicating a vigorous dissemination or uncontrolled fungal growth in the WT strain. This difference was even higher at week 26 postinfection, when the livers of the WT mice showed 6.12 ± 0.10 viable yeast cells, while 4.11 ± 0.52 fungal cells were detected in CD28$^{-/-}$ mice. No difference in fungal burdens was detected in the spleens (data not shown).

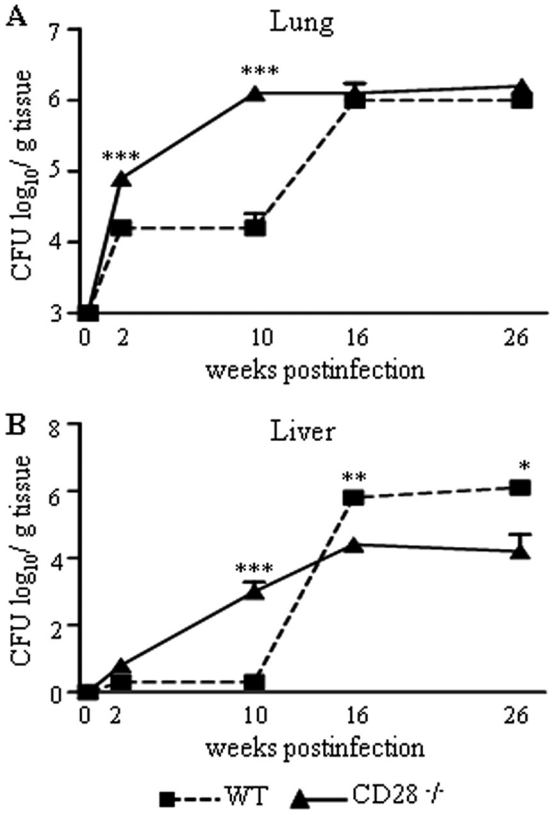

FIG. 1. Early in infection CD28 is protective, while at the chronic phase, CD28 signaling exerts a deleterious effect. CD28$^{-/-}$ and control WT C57BL/6 mice were i.t. infected with 1×10^6 fungal cells, and severity of infection was analyzed by CFU counts at four postinfection periods. (A) At weeks 2 and 10 after infection, increased fungal burdens in the lungs of CD28-deficient mice were observed. (B) By week 10, an increased dissemination to livers of CD28$^{-/-}$ mice was also seen. Unexpectedly, at week 16, a marked fungal growth was detected in the lungs, and the number of fungal cells in the livers of WT mice supplanted that observed with the deficient strain. At week 26 postinfection, no differences in the number of pulmonary CFU were noted, but the number of viable yeasts in the livers of WT mice remained higher than that of CD28-deficient mice. The points represent means ± SEM of the numbers of $\log_{10}$ CFU obtained from groups of six to eight mice. The results are representative of 3 experiments (except week 16). *, $P < 0.05$, and ***, $P < 0.001$, compared with WT controls.

CD28-deficient mice present diminished mortality rates. To better characterize the effect of CD28 signaling in the outcome of pulmonary paracoccidioidomycosis, mortality in *P. brasiliensis*-infected CD28-deficient and control mice was followed over a 57-week period (Fig. 2). Unexpectedly, WT mice showed increased mortality rates compared to CD28$^{-/-}$ mice. Thus, late in infection, CD28-dependent mechanisms appeared to result in deleterious effects to *P. brasiliensis*-infected hosts.

Absence of CD28 signaling leads to decreased synthesis of nitric oxide (NO) and an impaired humoral immune response. Synthesis of nitric oxide by cytokine-activated macrophages has been reported to be the most important mechanism in the control of *P. brasiliensis* growth in mice (11, 22). Accordingly, the better control of the fungal burden by the CD28-normal mice early in the infection was concomitant with increased

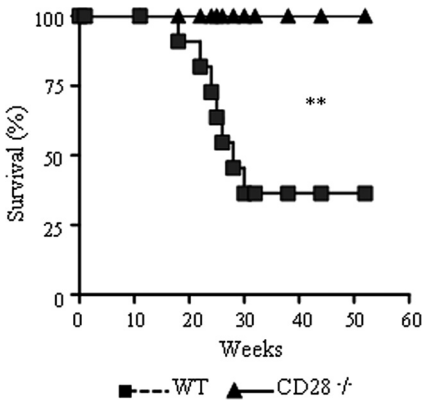

FIG. 2. CD28 costimulation results in increased mortality rates of *P. brasiliensis*-infected mice. Survival times of CD28$^{-/-}$ and WT control mice after i.t. infection with 1×10^6 *P. brasiliensis* yeast cells were determined for a period of 57 weeks. Significant differences in mortality rates were detected; the results are representative of two independent experiments; ($n = 6$). **, $P < 0.01$.

levels of nitric oxide in the lungs and liver homogenates of these mice (Fig. 3A).

Although specific antibodies may have a protective role in some fungal infections (23), antibody production in human and experimental PCM is a marker of disease severity (14, 17, 28). In addition, almost all *P. brasiliensis* components are T-dependent antigens and require Th cells to induce antibody production (12). In the severe forms of PCM, there is a prevalence of Th2 isotypes (IgG2b, IgG1, and IgA), while in the benign infection, IgG2a, a Th1 isotype, is preferentially produced (14). Because CD28 deficiency was shown to impair antibody production (73) and influenced the severity of *P. brasiliensis* infection, we decided to characterize humoral immunity at the second and tenth weeks postinfection. At week 2, the increased fungal loads of CD28$^{-/-}$ mice were associated with decreased levels of IgM compared with the control group. At week 10, the more severe and disseminated disease of CD28$^{-/-}$ mice paralleled a prominent impairment in the synthesis of all IgG isotypes except IgG3 (Fig. 3B). As a whole, the data on nitric oxide and antibody production demonstrate that CD28 signaling is necessary for the activation of the immune response against *P. brasiliensis*.

CD28-deficient mice produce decreased levels of pro- and anti-inflammatory cytokines. Similar to what has been observed with other intracellular infections, proinflammatory cytokines (IFN-γ, TNF-α, and IL-12) have been shown to be protective, while anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) are prevalent in the severe forms of PCM (6, 14, 15, 47). Cytokines associated with the Th17 pattern of immunity, however, have been shown to exert both protective and deleterious effects in pulmonary PCM: they increase the number and effector activity of neutrophils but also induce increased inflammatory reaction mixtures (46). Here, the levels of type 1 (IL-12, TNF-α, IL-2, and IFN-γ,), type 2 (IL-4, IL-5, and IL-10), and Th17-associated (IL-6, TGF-β, IL-23, and IL-17) cytokines were measured in the lungs and livers of CD28$^{-/-}$ mice and their normal counterparts at weeks 2 and 10 after *P. brasiliensis* infection. We could verify that all types of cytokines were present in the lungs and livers of CD28-deficient and WT mice (Fig. 4). The liver homogenates, however, exhibited the most important differences determined by CD28 deficiency. At week 2 postinfection, IL-2, IL-4, and GM-CSF appeared at

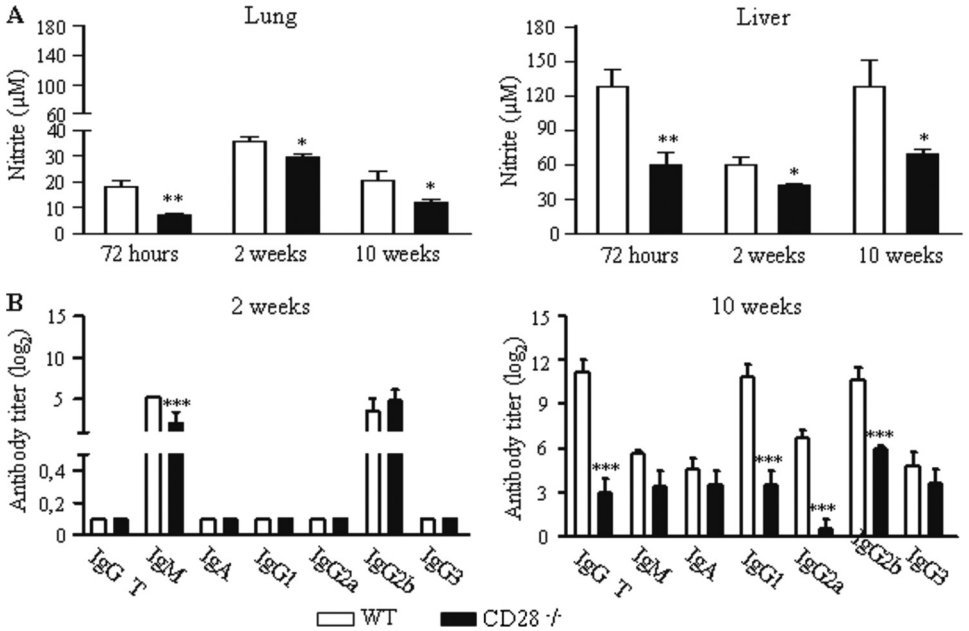

FIG. 3. CD28 deficiency determines impaired production of NO and decreased humoral immunity. Levels of nitric oxide (A) and *P. brasiliensis*-specific antibodies (B) were assayed, respectively, in organ homogenates and sera of CD28$^{-/-}$ and WT mice i.t. infected with 1×10^6 yeast cells. NO was measured by the Griess reaction, and sera were assayed for total IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 by using an isotype-specific ELISA as detailed in Materials and Methods. The bars depict means ± SE of NO levels or serum titers (6 to 8 mice per group). The results are representative of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT controls.

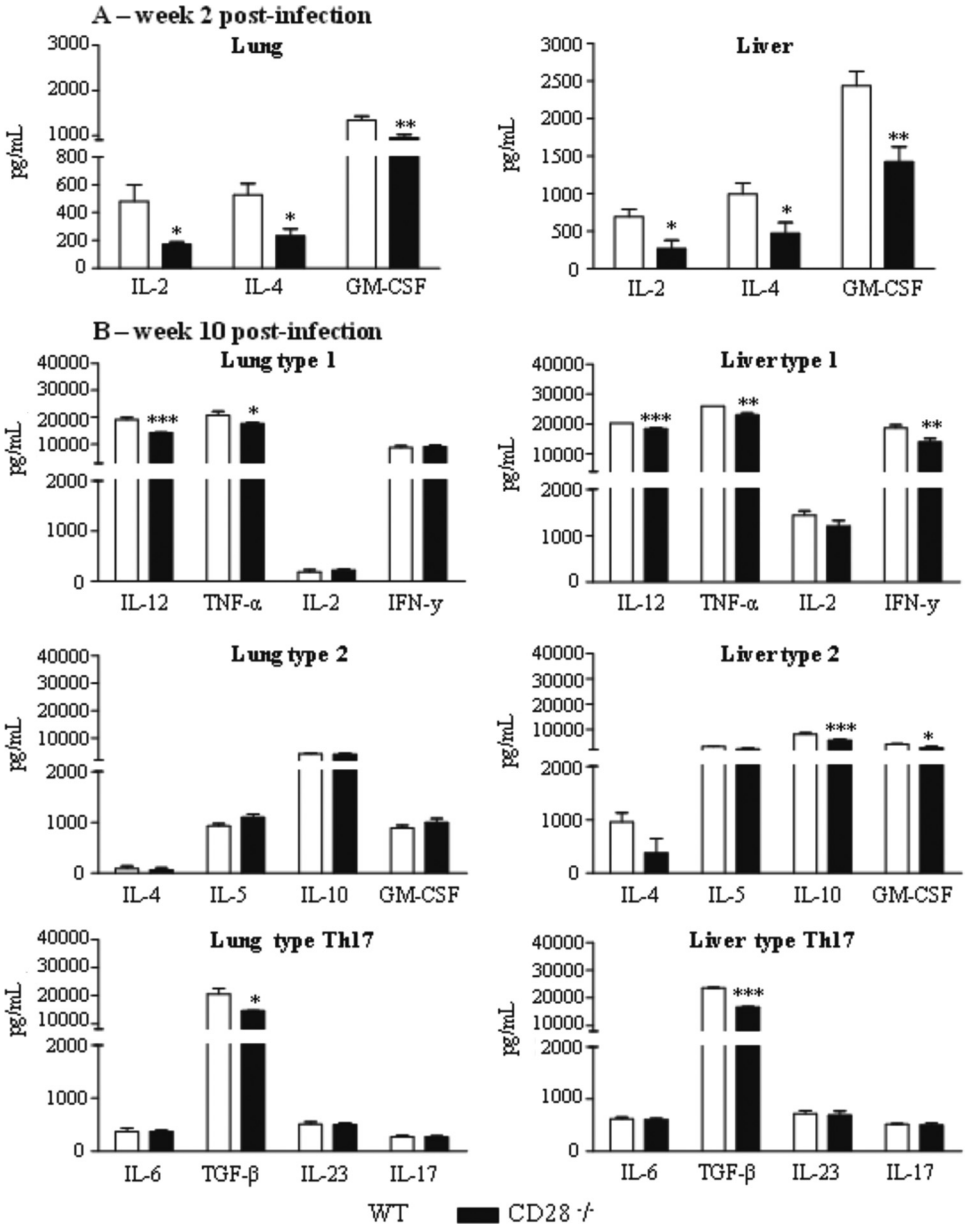

FIG. 4. CD28 deficiency causes a reduced production of pro- and anti-inflammatory cytokines in the lungs and livers of *P. brasiliensis*-infected mice. Levels of cytokines in organ homogenates of $CD28^{-/-}$ and WT mice were measured after i.t. infection with 1×10^6 yeast cells. Lungs and livers were collected at weeks 2 and 10 after infection and disrupted in 5.0 ml of PBS, and supernatants were analyzed for cytokine content by capture ELISA. (A) At week 2 after infection, CD28-deficient mice presented reduced levels of IL-2, IL-4, and GM-CSF in the lungs and livers. (B) Decreased levels of IL-12, TNF-α, and TGF-β in the lungs and IL-12, TNF-α, IFN-γ, IL-10, and TGF-β in the livers of deficient mice were seen at week 10 postinfection. The bars depict means ± SEM of cytokine levels (6 to 8 animals per group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT controls.

significantly lower levels in the lungs and livers of $CD28^{-/-}$ mice than in those of WT mice (Fig. 4A); the other cytokines assayed appeared at equivalent levels (data not shown). At week 10, diminished levels of IL-12 and TNF-α were present in the lungs, whereas lower levels of pro- and anti-inflammatory cytokines (IL-12, TNF-α, IFN-γ, IL-10 and GM-CSF) were observed in the liver homogenates of CD28-deficient mice (Fig. 4B). At this postinfection time point, no differences in IL-6, IL-23, or IL-17 were detected in organ homogenates, but TGF-β was detected at lower levels in the lung and liver homogenates of $CD28^{-/-}$ mice (Fig. 4B).

Early in infection, CD28 costimulation leads to the increased influx of activated macrophages, T cells, and regulatory T cells to the site of infection. To characterize the inflammatory infiltrates in the lung, the presence and activation status of monocytes/macrophages and T cells were studied at week 10 after *P. brasiliensis* infection. Figure 5A shows that increased numbers of $CD11b^+$-activated macrophages were

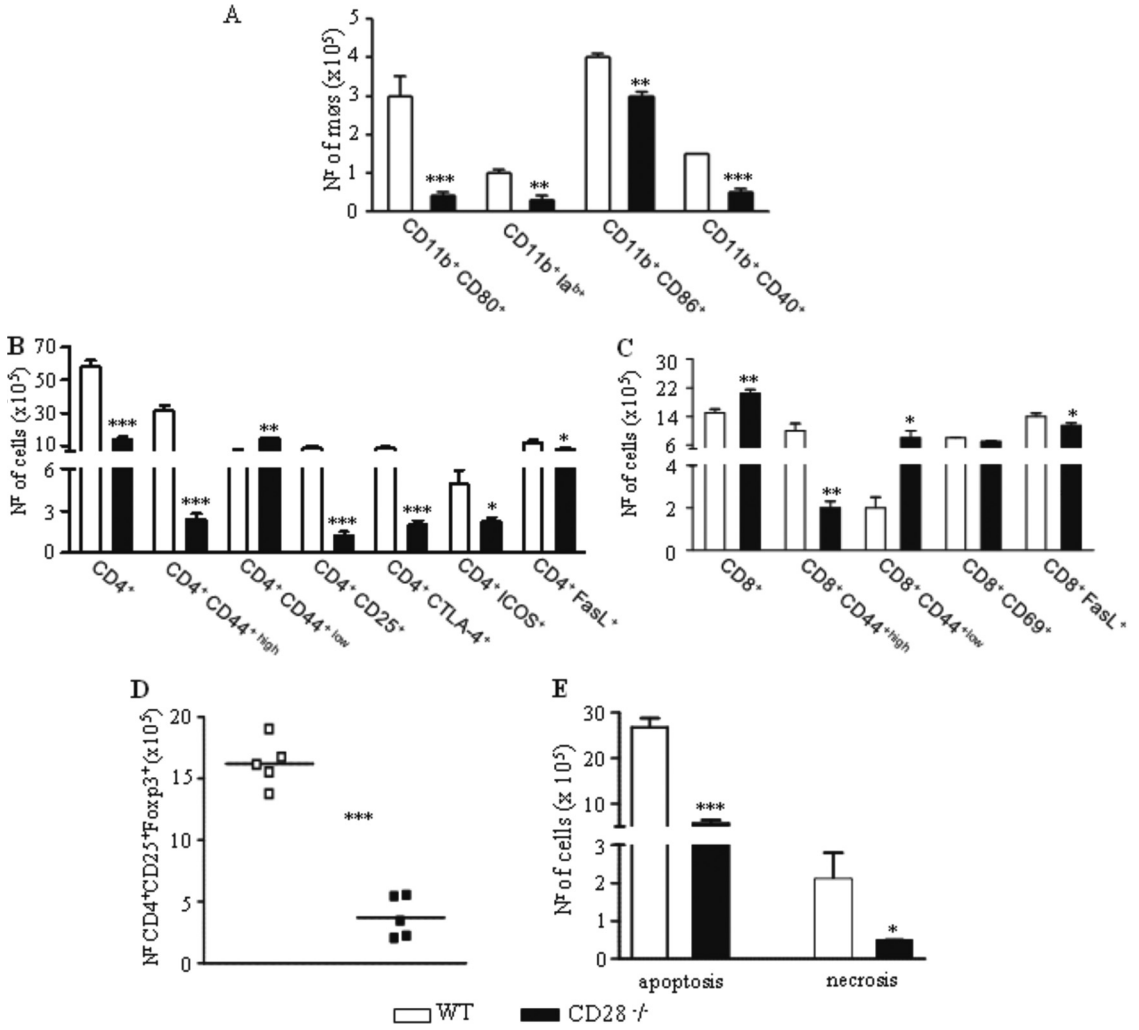

FIG. 5. WT mice presented an increased influx of activated macrophages, T cells, and regulatory $CD4^+CD25^+FoxP3^+$ T cells to the lungs, whereas CD28-deficient mice showed an increased presence of naïve $CD8^+$ T cells. Characterization of leukocyte subsets and the activation profile of cells by flow cytometry in the lung-infiltrating leukocytes (LIL) from $CD28^{-/-}$ and WT mice inoculated i.t. with 1×10^6 *P. brasiliensis* yeast cells. At week 10 after infection, lung cell suspensions were obtained and stained as described in Materials and Methods. The acquisition and analysis gates were restricted to macrophages or lymphocytes. (A) Macrophages (møs); (B) $CD4^+$ T cells; (C) $CD8^+$ T cells. (D) To characterize the expansion of regulatory T cells in LIL, surface staining of $CD25^+$ and intracellular FoxP3 expression were back-gated on the $CD4^+$ T-cell population. (E) The number of apoptotic and necrotic leukocytes was assessed by flow cytometry using FITC-labeled annexin V and propidium iodide. The data represent the mean ± SEM of the results from 6 to 8 mice per group and are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with WT controls. N^r, number.

present in the lungs of WT mice. $CD11b^+$ cells expressing costimulatory molecules were detected in diminished numbers in the lungs of CD28-deficient mice. The expression of CD69, CD25, CD44, ICOS, CTLA-4, and FasL by T cells freshly isolated from the lungs was used to determine the activation profile of $CD4^+$ and $CD8^+$ T cells that had migrated to the lungs of *P. brasiliensis*-infected mice. The marker CD69 is a very early activation antigen (86), as is CD25, the α-chain of the interleukin-2 receptor (69), and both markers are rapidly upregulated on activated T cells. CTLA-4 is a costimulatory molecule of which the main function is to attenuate the expansion and cytokine production of recently activated T cells (67). The expression of high levels of CD44 ($CD44^{high}$) has been observed with effector or memory T cells (80). Increased numbers of $CD4^+$ T cells expressing $CD44^{high}$, CD25, CTLA-4, and ICOS were observed with the lungs of CD28-sufficient mice (Fig. 5B), demonstrating that a high number of activated T cells migrated to the site of infection. Conversely, $CD8^+$ T cells migrated in higher numbers into the lungs of CD28-deficient mice. The increased number of $CD8^+CD44^{low}$ cells indicated, however, the prevalence of a naïve phenotype (Fig. 5C). No differences in CD69 expression were observed in $CD8^+$ T cells, but upregulated expression of FasL was detected in WT mice. Because the expression of CD28 is necessary for the expansion of Treg cells (33, 77), the presence of $CD4^+CD25^+Foxp3^+$ T cells in the lungs of both mouse strains was evaluated. Consistent with previous reports, increased expression of Foxp3 was observed in the $CD4^+CD25^+$ cells of CD28-normal mice (Fig. 5D). Because upregulated expression of FasL was detected in the lymphocytes of WT mice, we also assessed the number of

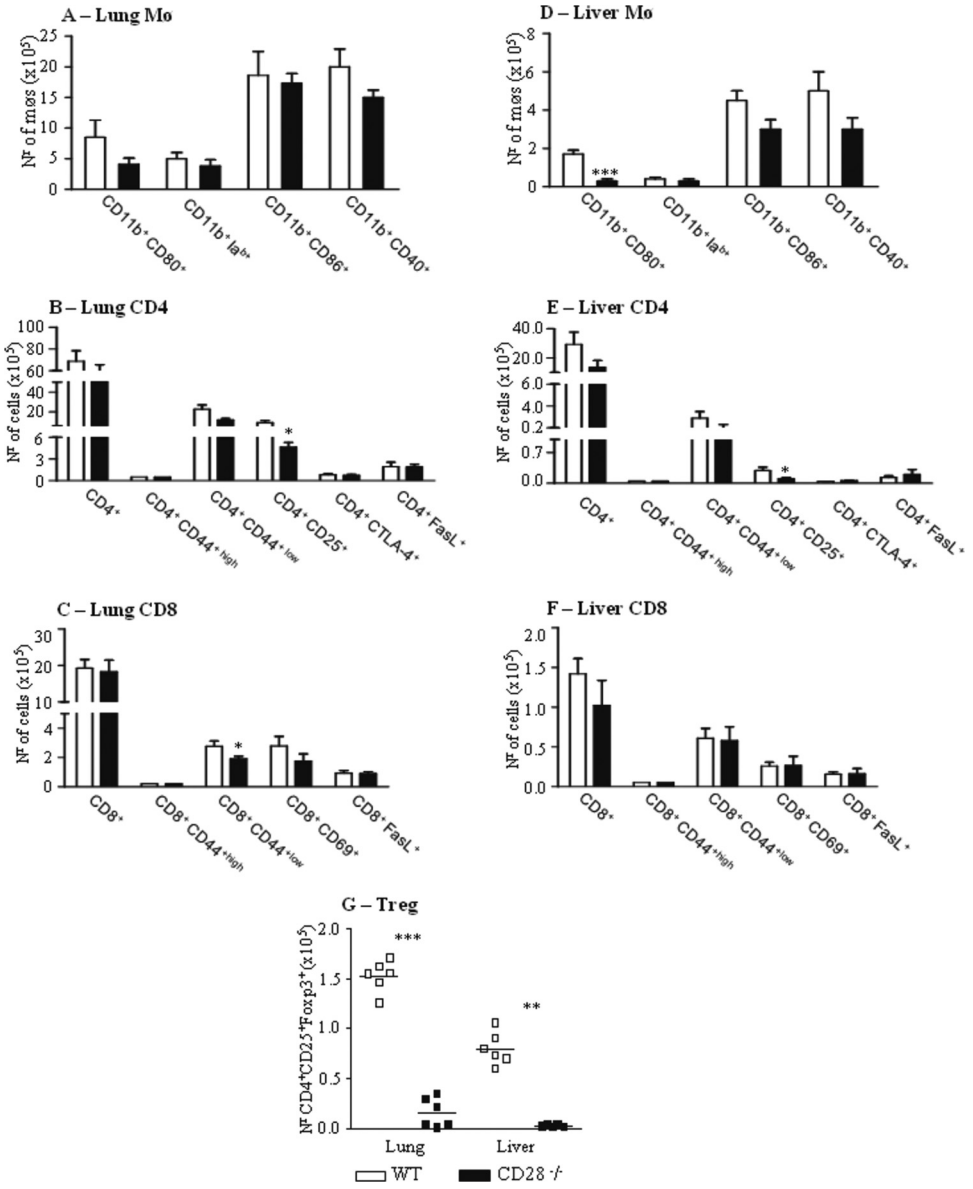

FIG. 6. At week 16, the numbers and levels of activation of inflammatory cells from lungs and livers of $CD28^{-/-}$ and WT mice were similar, but increased numbers of regulatory T cells were detected in the latter strain. Characterization of leukocyte subsets and the activation profile of cells by flow cytometry in the lung- and liver-infiltrating leukocytes (LIL) from $CD28^{-/-}$ and WT mice inoculated i.t. with 1×10^6 *P. brasiliensis* yeast cells. At week 16 after infection, lung and liver cell suspensions were obtained and stained as described in Materials and Methods. The acquisition and analysis gates were restricted to macrophages or lymphocytes. (A, B, and C) Lung macrophages and $CD4^+$ and $CD8^+$ T cells, respectively. (D, E, and F) Liver macrophages and $CD4^+$ and $CD8^+$ T cells, respectively. (G) To characterize the expansion of regulatory T cells in lungs and livers, surface staining of $CD25^+$ and intracellular FoxP3 expression were back-gated on the $CD4^+$ T-cell population. The data represent the mean ± SEM of the results from 6 to 8 mice per group and are representative of one experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT controls.

apoptotic and necrotic cells in the lungs of infected mice. Increased numbers of both apoptotic and necrotic cells were detected in WT mice (Fig. 5E).

In the chronic phase, WT mice showed a progressive deactivation of inflammatory cells associated with the persistent presence of regulatory T cells. Because the infection in WT mice was seen to be more severe than in $CD28^{-/-}$ mice from week 16 onward, we decided to characterize the inflammatory cell infiltrates of the lungs and livers at week 16 postinfection. Flow cytometric analysis of lung cells showed that almost all of the increased activation markers of macrophages and $CD4^+$ and $CD8^+$ T cells observed with WT mice at week 10 postinfection were lost during this later period (Fig. 6A, B, and C). The only significant difference that was maintained was the increased expression of CD25 (a marker of activated and regulatory T cells) on the $CD4^+$ T cells of WT mice. Indeed, the $CD4^+CD44^{high}$ and $CD8^+CD44^{high}$ phenotypes were observed in very low numbers in both mouse strains (Fig. 6B and

C). Interestingly, during this postinfection period, an increased number of macrophages was seen compared to week 10 (3- to 8-fold in WT and 5- to 10-fold in knockout [KO] mice), although no differences in the expression of activation markers were detected between the mouse strains (Fig. 6A). In addition, no marked differences in the number or activation profile of liver leukocytes were detected. WT mice, however, presented increased numbers of $CD11b^+CD80^+$ macrophages (Fig. 6D) and $CD4^+CD25^+$ lymphocytes (Fig. 6E), although no differences were detected in $CD8^+$ T cells (Fig. 6F). Importantly, augmented numbers of $CD4^+CD25^+Foxp3^+$ Treg cells were present in the cell exudates of the lungs and livers of WT mice (Fig. 6G). As a whole, these results demonstrated that the increased number and activation of macrophages and $CD4^+$ and $CD8^+$ T cells detected in WT mice at week 10 postinfection were lost during this later postinfection period. The same was true for the augmented number of naïve $CD8^+$ T cells from CD28KO mice. In contrast, the regulatory T cells were maintained in expanded numbers in the livers and lungs of the WT strain.

$CD8^+$ T lymphocytes are more protective in the WT than in CD28-deficient mice. In a study of the influence of CD28 costimulation in the pulmonary infection caused by *Pneumocystis carinii*, Rose et al. (66) observed an increased expansion of naïve $CD8^+$ T cells in the lungs of CD28-deficient mice. Because the same phenomenon was observed in our study, we asked whether $CD8^+$ T lymphocytes exerted a protective role in the CD28-deficient mouse strain. Thus, $CD28^{-/-}$ and WT mice were depleted of $CD8^+$ T cells *in vivo* by a monoclonal antibody and were infected with *P. brasiliensis*. CFU counts were determined 2 weeks later. As shown in Fig. 7A, a slight but significant increase in the number of fungal cells was seen in the lungs of both mouse strains. Depletion of $CD8^+$ T cells, however, abolished the differences in fungal loads observed between the mouse strains. Thus, $CD8^+$ T cells appear to exert a more efficient protective role in CD28-sufficient mice compared to CD28-deficient mice, and this agrees with the activated phenotype of the cells observed in WT mice. As expected, the anti-CD8 treatment selectively depleted $CD8^+$ T cells but had no influence on the numbers of $CD4^+$ T lymphocytes or $CD11b^+$ macrophages (Fig. 7B).

Early in infection, CD28 costimulation is protective and prevents fungal dissemination. Histopathological examination of the lungs was performed at weeks 2, 10, and 26 after fungal infection. As early as week 2 of infection, increased inflammatory reaction mixtures composed of macrophages, lymphocytes, and epithelioid cells were detected as organized granulomas in the lungs of CD28-normal mice. In CD28-deficient mice, the histological architecture of lungs was more preserved, and inflammatory cells did not organize into typical granulomas (data not shown). However, at week 10 postinfection, the lower fungal loads of WT mice were controlled by a reduced number of well-organized granulomas scattered through the lung tissue. These granulomas were composed of giant and epithelioid cells. A wide halo of mononuclear cells containing some plasmocytes was seen surrounding the epithelioid cells and xanthomatous macrophages (Fig. 8A and B). In contrast, CD28-deficient mice showed numerous well-organized epithelioid granulomas that generally presented central supurative necrosis surrounding a high number of budding yeasts. Hyperplasia of bronchoalveolar lymphoid tissue

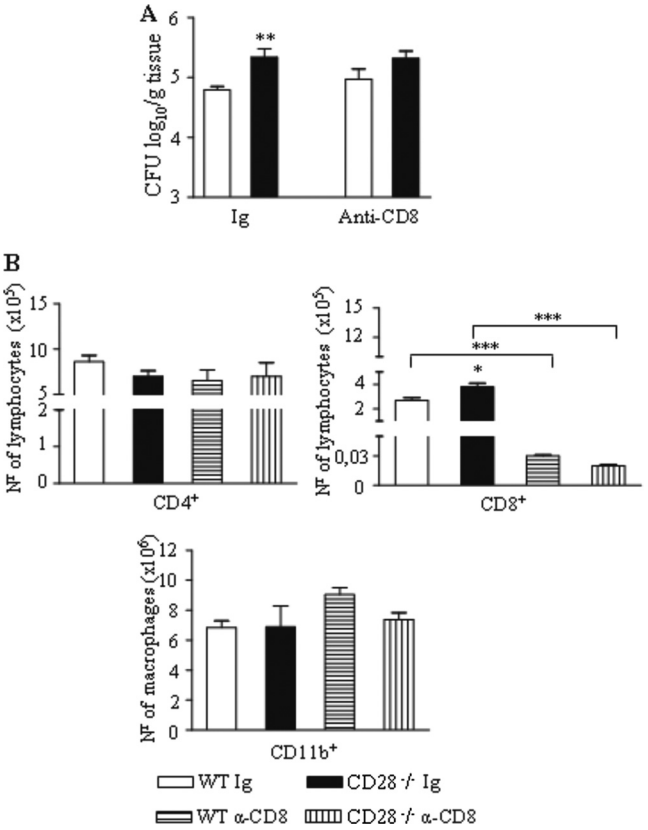

FIG. 7. *In vivo* depletion of $CD8^+$ T cells abrogated the differences in fungal loads between WT and CD28-deficient mice. Both mouse strains were treated *in vivo* with H35 anti-CD8 monoclonal antibodies or control immunoglobulin and i.t. infected with 1×10^6 *P. brasiliensis* yeasts. (A) The number of viable yeasts recovered from lungs was assessed at week 2 postinfection. (B) Lung-infiltrating $CD4^+$ and $CD8^+$ T cells and $CD11b^+$ macrophages were phenotyped at week 2 postinfection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with controls.

(BALT) was detected alongside the granulomas, which occupied an extensive area of lung parenchyma (Fig. 8C and D). No dissemination of yeast cells to the livers of WT mice was seen (Fig. 8E and F), but several granulomatous lesions were detected in CD28-deficient mice (Fig. 8G and H). Therefore, early in infection, WT mice appeared to develop an immune response able to control fungal growth in the lungs and dissemination to other organs.

Late in infection, CD28-sufficient mice develop a severe, disseminated infection. Histopathological analysis of the lungs at week 26 after infection did not show any differences in the pattern or intensity of the granulomatous lesions developed by CD28-deficient and WT control mice (Fig. 9A to D). In contrast, compared with those of $CD28^{-/-}$ mice, the livers of WT mice showed elevated numbers of fungal cells contained in wide lesions, which replaced large areas of the liver parenchyma (Fig. 9E to H). Morphometric analysis confirmed the significantly increased areas of hepatic lesions developed by WT mice compared with those of CD28-deficient mice (Fig. 9I).

At week 26 postinfection, almost no differences in pulmonary cytokines were observed (data not shown), and this fact

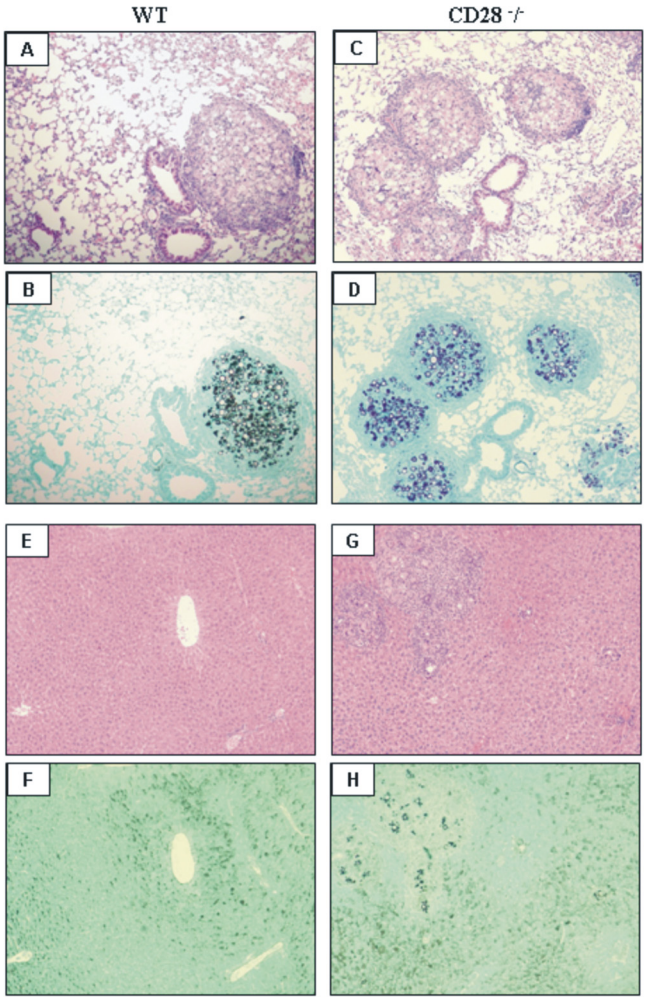

FIG. 8. Photomicrographs of pulmonary and hepatic lesions of WT and CD28$^{-/-}$ mice at week 10 postinfection with 1×10^6 *P. brasiliensis* yeasts. (A and B) WT mice showed well-organized granulomas containing an elevated number of yeasts surrounded by a halo of mononuclear cells containing a few plasmocytes; most of the lung tissue was preserved, with limited signs of inflammatory cell recruitment. (C and D) CD28-deficient mice presented an elevated number of well-defined epithelioid granulomas containing a large number of yeast budding cells; these lesions occupy a large area of lung tissue and were concomitant with an extensive expansion of the bronchoalveolar lymphoid tissue. No hepatic lesions were observed in WT mice (E and F), while CD28-deficient mice showed a large number of viable yeasts surrounded by granulomatous lesions scattered through the liver parenchyma (G and H). For panels A, C, E, and G: H&E, ×100; for panels B, D, F, and H: Groccot stain, ×100.

agrees with the similar numbers of fungal cells observed in the lungs. The intense growth of fungal cells in the livers of WT mice, however, was accompanied by increased levels of anti-inflammatory cytokines (IL-4, IL-10, TGF-β, and GM-CSF). Interestingly, IL-2, an important growth factor for Treg cells (31), was also present at elevated levels, but IL-12, IFN-γ, and TNF-α appeared in equivalent levels in both strains (Fig. 9J).

DISCUSSION

In this report, we examined the influence of CD28 costimulation on immunity to *P. brasiliensis* infection. Our results demonstrate that CD28 exerts both protective and deleterious effects in PCM and that *P. brasiliensis*-infected hosts can develop CD28-independent immunity.

The efficient immunological activation developed by WT mice was confirmed by the early control of fungal loads associated with the elevated production of antibody, NO, and cytokines. In addition, at week 10 postinfection, the inflammatory macrophages and T cells present in the lungs expressed high levels of activation/regulatory markers. In contrast, the absence of CD28 costimulation led to a severe pulmonary and hepatic infection and impaired the activation of the immune system, as revealed by the diminished levels of cytokines and NO in the lungs and the decreased production of all *P. brasiliensis*-specific IgG isotypes except IgG3. Interestingly, despite their more severe infection, CD28-deficient mice produced lower levels of *P. brasiliensis*-specific antibodies. This result is an unusual finding because in human and experimental PCM, the levels of antibodies have a direct correlation with disease severity (7, 16, 48). However, this result is in line with our previous demonstration that almost all *P. brasiliensis* antigens are T cell dependent (12). The lower levels of IgG1, IgG2a, and IgG2b isotypes regulated by IL-4, IFN-γ, and TGF-β, respectively (75), suggest that CD28 deficiency affected all of the T-helper subsets (Th1, Th2, and Th3) of CD4$^+$ T-cell immunity.

Analysis of the survival times of infected mice suggests an immunological paradox. Why does the presence of CD28 costimulation, which induces an early and efficient pattern of immunity that is able to control fungal growth and induces regulatory mechanisms to avoid excessive tissue pathology, result in increased mortality of hosts? The corollary is also contradictory: why does the inefficient immunity associated with inefficient control of pathogen replication and underdeveloped regulatory mechanisms not lead to overwhelming disease characterized by high fungal burdens and enhanced tissue damage? Our results lead us to suggest that the fatal outcome of infection developed by WT mice was due to imperfect fungal clearance during the first phase of immunity followed by the activation of vigorous CD28-dependent suppressive mechanisms mediated by Treg cells, suppressive cytokines, and other T-cell dysfunctions. This would allow a late uncontrolled fungal growth that would have deleterious effects on infected tissues. In contrast, the weak, CD28-independent immunity developed by CD28$^{-/-}$ mice was not impaired by regulatory mechanisms but was sufficient to partially restrain fungal growth without causing damage to important tissues.

The early immune response of the WT mice was composed of several cells and mediators previously shown to exert a protective effect in *P. brasiliensis*-infected hosts. Thus, the increased production of pulmonary IL-2, IL-4, and GM-CSF at week 2 postinfection indicates a balanced and concomitant activation of type 1 and type 2 immunity. The presence of high levels of IL-2 appears to guarantee the late expansion of Treg cells, which consistently appeared in the lungs and livers of WT mice. The increased synthesis of TNF-α and IL-12 in the lungs and the increased levels of IFN-γ in the livers indicated the existence of effector mechanisms able to kill *P. brasiliensis* yeasts and control fungal infection. Both in human and experimental PCM, cellular immunity and activated macrophages have been shown to be the most important protective mecha-

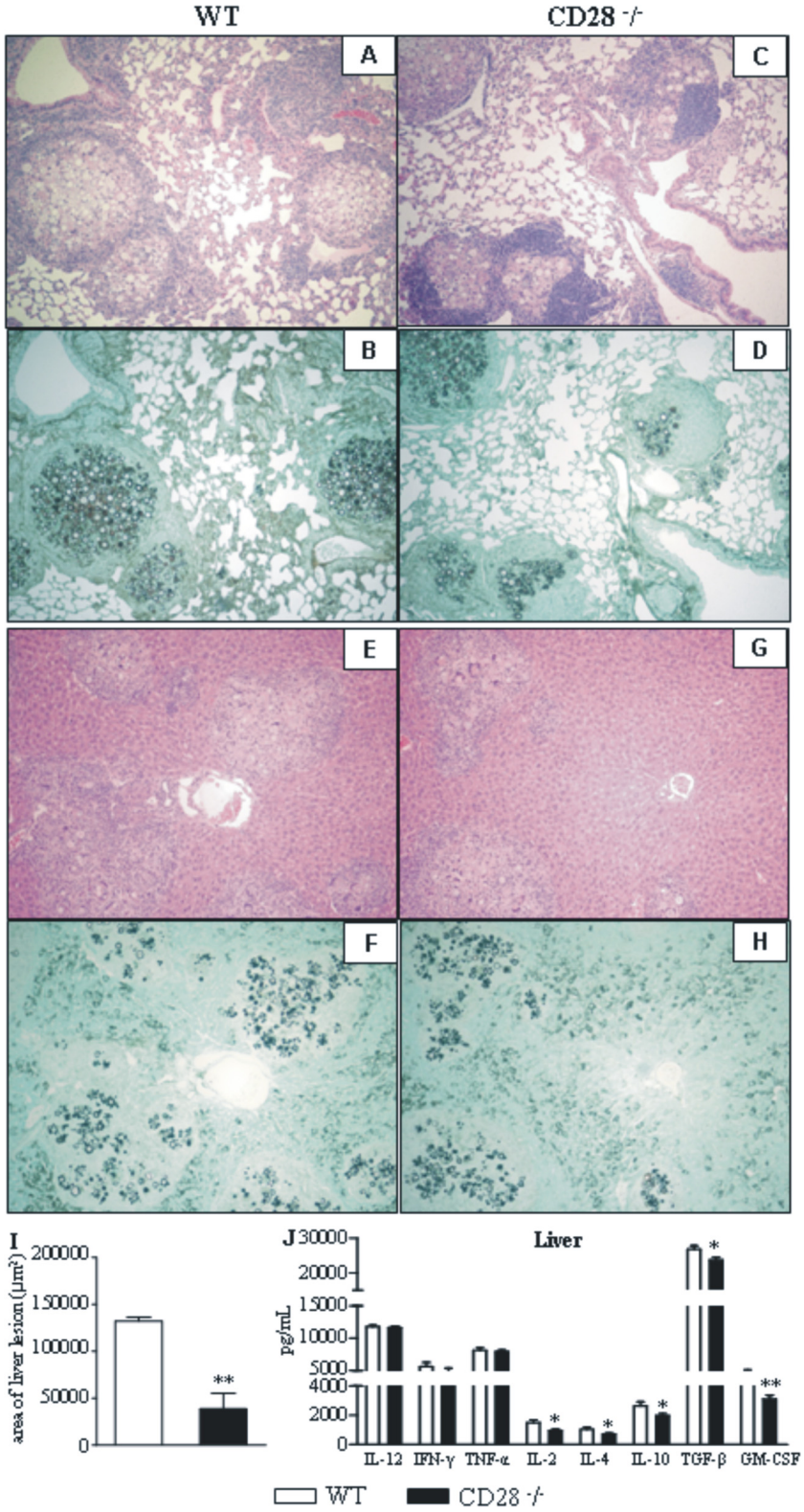

FIG. 9. At week 26 after infection, WT mice present large numbers of lesions and *P. brasiliensis* yeasts in their livers. $CD28^{-/-}$ and WT mice were i.t. infected with 1×10^6 fungal cells, and histopathological analyses of lungs and livers were performed at week 26 after infection. (A to D) No differences in pulmonary lesions were noted between WT and CD28-deficient mice. (E to H) Increased numbers of yeast cells and more-severe hepatic lesions were detected in WT mice. For panels A, B, E, and F: HE, ×100; for C, D, G, and H, Groccot stain, ×100. (I) Morphometrical analysis confirmed the more extensive areas occupied by the liver lesions of WT mice. (J) Levels of liver cytokines at week 26 postinfection. *, $P < 0.05$; **, $P < 0.01$, compared with WT controls.

nisms. In addition, NO, IL-12, TNF-α, and IFN-γ have been implicated in the fungicidal mechanisms developed by phagocytes following the prevalent activation of Th1 cells (3, 22, 47, 58). The efficiency of these mechanisms can be inferred from the diminished fungal loads observed with the lungs and livers of WT mice up to week 10 after infection. Moreover, lung macrophages appeared in elevated numbers and were positive for the expression of costimulatory molecules (CD80, CD86, CD40, and MHC class II), indicating efficient effector and antigen-presenting cell activities. The latter activity could be confirmed by the increased influx of activated $CD4^+$ and $CD8^+$ T cells to the lungs. These inflammatory lymphocytes showed upregulated expression of activation/regulatory markers ($CD44^{high}$, CD25, ICOS, CTLA4, and FasL), indicating that the significant activation of T cells was accompanied by the development of regulatory mechanisms to control excessive cell multiplication and inflammatory reactions (5, 86). Indeed, the high expression of CD25 and CTLA4 at week 10 postinfection was concomitant with an increased presence of Treg cells and cells undergoing apoptosis and necrosis (Fig. 5).

TGF-β, a cytokine involved in the induction of Th17 and Treg cells (42, 83), consistently appeared in elevated levels in the lungs and livers of WT mice. Because no Th17 polarization was observed in this strain, TGF-β appears to have preferentially driven Treg development. In CD28 KO mice, the production of TGF-β, probably by innate immune cells or Th3 lymphocytes, appears to have contributed to the control of inflammatory reactions but also to the constant and persistent fungal growth observed with this strain. As has been shown in murine candidiasis and aspergillosis (54, 55), our study demonstrates that in pulmonary PCM, CD28 signaling leads to Treg development, but in the absence of CD28 costimulation, production of IL-2 was impaired, and the commitment of T lymphocytes to the regulatory phenotype was practically abolished. Interestingly, with the opportunistic pathogen *Candida albicans*, CD28-dependent Treg cells were needed to restrain pathogen clearance, to control inflammatory reactions, and to ensure immunoprotection against a secondary challenge, but with *Blastomyces dermatitidis*, a primary pathogen, vaccine-induced memory was shown to be CD28 independent (54, 84). In our studies, WT mice developed a canonical pattern of immunity in which the activation process was tightly regulated by deactivation mechanisms that controlled excessive inflammatory reactions and consequent tissue damage. However, this was not observed in the chronic phase of infection developed by CD28-sufficient mice.

The data obtained at week 16 postinfection revealed the late deleterious effect mediated by CD28 costimulation in PCM. As can be seen in Fig. 1, at this time point, WT mice lost control of pulmonary fungal growth (CFU counts increased 65-fold), and fungal cells disseminated to the liver, reaching a value of 1 million fungal cells/g tissue. In addition, despite the equivalent number of fungal cells in the lungs of both mouse strains at week 26, the hepatic fungal loads of WT mice were 67-fold higher than those of CD28 KO mice. The inefficient late-T-cell immunity of WT mice was concomitant with Treg cell expansion, a fact not observed with CD28 KO mice, which maintained low-efficiency, but persistent, antifungal immunity. Furthermore, the inhibitory activity of Treg cells and anti-inflammatory cytokines was evidenced by the deactivated phenotype of the T cells and macrophages observed with the lungs and livers at week 16 postinfection (Fig. 6). Almost no $CD4^+CC44^{high}$ and $CD8^+CD44^{high}$ cells were detected in the lungs and livers of WT mice, and almost no significant differences in the number or activation status of macrophages were seen between the strains. The only activation/regulatory marker persistently expressed at elevated levels by WT mice was CD25, and this was consistent with the increased presence of Treg cells in the livers and lungs of WT mice.

As observed in chronic viral infections of the liver, the expression of programmed death-1 (PD-1, or CD279), a costimulatory receptor that inhibits T-cell receptor signaling, could have contributed to the inefficient late immunity and increased mortality of WT mice. Indeed, in cases of chronic viral infections, antigen-specific $CD8^+$ T cells express high levels of PD-1 and assume an exhausted phenotype characterized by low secretion of IL-2 and IFN-γ (26, 32, 62). Importantly, recent investigations have shown that $CD8^+$ T-cell exhaustion correlates with Treg expansion, and experimental ablation of these cells resulted in a significant increase of cytotoxic $CD8^+$ T-cell responses, decreased viral loads, and no functional exhaustion of $CD8^+$ T cells (57, 85). Because immune exhaustion has also been associated with increased expression of PD-1 for a fungal pathogen, *Histoplasma capsulatum* (41), it is tempting to suggest that the inefficient T-cell immunity developed by WT mice could be due to T-cell exhaustion controlled by increased expansion of Treg cells.

The immune response developed by $CD28^{-/-}$ mice was very different from that mounted by CD28-sufficient mice. The severe and disseminated infection in $CD28^{-/-}$ mice was only partially controlled by immunological mechanisms. However, these mechanisms were sufficient to organize granulomatous lesions (Fig. 8 and 9). $CD28^{-/-}$ mice secreted NO and produced pro- and anti-inflammatory cytokines in the lungs and livers, although at lower levels than WT mice. At week 10, the inflammatory exudates of the lungs were composed of diminished numbers of macrophages expressing low levels of costimulatory molecules and decreased numbers of activated $CD4^+$ and $CD8^+$ T cells compared to WT mice. The lymphocytes of $CD28^{-/-}$-deficient mice expressed low levels of activation/regulatory markers, which were associated with a small number of necrotic and apoptotic cells. Furthermore, the number of $CD4^+CD25^+Foxp3^+$ Treg cells was very low, which was likely due to the absence of CD28 costimulation (44, 79). Because Treg development involves a multistep process dependent on signals from the TCR, cytokines (IL-2 and IL- 15), and other receptors, the low levels of IL-2 produced by CD28-deficient cells appear to have contributed to the impaired expansion of Treg cells (44, 72, 79). Interestingly, as has been observed with *Pneumocystis carinii* infection (4, 66), the only cell population observed in increased numbers in the lungs of $CD28^{-/-}$ mice was of the $CD8^+$ T-cell phenotype. Depletion experiments, however, indicated that $CD8^+$ T cells were less protective in CD28-deficient mice than in WT mice, and this finding was consistent with the higher activation of $CD8^+$ T cells developed by WT mice. Despite the impaired $CD4^+$ T-cell immunity, T cells from CD28-deficient mice possibly used other members of the CD28 family of costimulatory molecules, such as ICOS, or other members of the tumor necrosis receptor superfamily, including CD40, OX40, 4-1BB, CD27, and

CD30, to deliver costimulatory signals (27) and activate T-cell immunity.

Analysis of hepatic cytokines gave some clues to interpret the late massive fungal growth in the livers of WT mice. At week 10, in addition to increased levels of anti-inflammatory cytokines (IL-10 and TGF-β), augmented levels of proinflammatory cytokines were also present in the livers of WT mice (Fig. 4, IL-12, TNF-α, and IFN-γ). At week 26 (Fig. 9J), all of the anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) remained at higher levels in WT mice, while the proinflammatory ones appeared in levels equivalent to those of KO mice. Thus, a prevalent anti-inflammatory pattern appears to progressively develop in WT mice, favoring *P. brasiliensis* growth. By week 26, WT mice showed, in addition to increased levels of IL-10 and TGF-β (effector cytokines of Treg cells), increased levels of IL-2, a fundamental growth factor for Treg cells (Fig. 9J), which were also found in elevated numbers at week 16 postinfection in the livers of this strain (Fig. 6G). Thus, the liver environment, particularly that of WT mice, appears to be heavily suppressive, leading us to suppose that the increased liver pathology observed (Fig. 9) was due much more to the uncontrolled fungal growth than to damage caused by inflammatory cells.

In summary, our work demonstrates for the first time the crucial importance of CD28 costimulation in adaptive immunity to *P. brasiliensis* infection. Early in infection, CD28 regulates the expansion of effector and regulatory T cells, restraining fungal growth without causing damage to infected tissues. Later in infection, however, suppressive mechanisms appear to prevail, allowing uncontrolled fungal growth and dissemination that induced increasing tissue damage and augmented the mortality of infected hosts. Our study also shows that *P. brasiliensis* is able to trigger CD28-independent immune responses that confer relative immunoprotection to the host.

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***Anexo B* ï MyD88 Signaling Is Required for Efficient Innate and Adaptive Immune Responses to *Paracoccidioides brasiliensis* Infection**

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MyD88 Signaling Is Required for Efficient Innate and Adaptive Immune Responses to *Paracoccidioides brasiliensis* Infection[▽]

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AQ: A **The mechanisms that govern the initial interaction between *Paracoccidioides brasiliensis*, a primary dimorphic fungal pathogen, and cells of the innate immunity need to be clarified. Our previous studies showed that Toll-like receptor 2 (TLR2) and TLR4 regulate the initial interaction of fungal cells with macrophages and the pattern of adaptive immunity that further develops. The aim of the present investigation was to assess the role of MyD88, an adaptor molecule used by TLRs to activate genes of the inflammatory response in pulmonary paracoccidioidomycosis. Studies were performed with normal and MyD88$^{-/-}$ C57BL/6 mice intratracheally infected with *P. brasiliensis* yeast cells. MyD88$^{-/-}$ macrophages displayed impaired interaction with fungal yeast cells and produced low levels of IL-12, MCP-1, and nitric oxide, thus allowing increased fungal growth. Compared with wild-type (WT) mice, MyD88$^{-/-}$ mice developed a more severe infection of the lungs and had marked dissemination of fungal cells to the liver and spleen. MyD88$^{-/-}$ mice presented low levels of Th1, Th2, and Th17 cytokines, suppressed lymphoproliferation, and impaired influx of inflammatory cells to the lungs,**
AQ: B **and this group of cells comprised lower numbers of neutrophils, activated macrophages, and T cells. Nonorganized, coalescent granulomas, which contained high numbers of fungal cells, characterized the severe lesions of MyD88$^{-/-}$ mice; the lesions replaced extensive areas of several organs. Therefore, MyD88$^{-/-}$ mice were unable to control fungal growth and showed a significantly decreased survival time. In conclusion, our findings demonstrate that MyD88 signaling is important in the activation of fungicidal mechanisms and the induction of protective innate and adaptive immune responses against *P. brasiliensis*.**

Fn1 Toll-like receptors (TLRs), one of the most important groups of innate immune receptors, have been shown to participate in the recognition of several pathogens (9, 19, 34). These receptors have been characterized as a family of evolutionarily conserved type I transmembrane proteins displaying leucine-rich extracellular domains. The cytoplasmic portions of TLRs are homologous to the intracellular signaling domain of the interleukin 1 (IL-1) receptor, and they are known as TIR domains. Intracellular pathways are activated by TLRs through the homophilic interactions of their TIR domains with those of adaptor proteins, the best characterized of which is myeloid differentiation factor 88 (MyD88). This adaptor protein is used by all TLRs except TLR3. MyD88 signaling leads to the activation of protein kinases and the expression of transcription factors that trigger the expression of genes involved in the inflammatory response (19, 27). In addition, the IL-1R family of cytokine receptors, including IL-18R and IL-33R, contains intracellular TIR domains and, like the majority of TLRs, uses MyD88 as an adaptor molecule to activate cells and induce the expression of specific genes such as the IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) genes (1, 11, 26).

IL-1β, IL-18, and IL-33 are members of the IL-1 family of inflammatory cytokines and are involved in the differentiation of the Th17, Th1, and Th2 immune responses, respectively (11, 26). Interestingly, MyD88-dependent TLR signaling has been implicated in the transcription of pro-IL-1β and pro-IL-18, the inactive precursors of IL-1β and IL-18, which are then cleaved to their active forms by different host- or pathogen-derived proteases (1, 26). Thus, deficient MyD88 signaling can affect multiple pathways of cell activation and cytokine production and can influence the development of innate and adaptive immune responses against pathogens.

Several studies have demonstrated the importance of MyD88 signaling in the immunoprotection against fungal infections caused by *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* (2, 5, 12). This adaptor molecule was shown to regulate the fungicidal mechanisms, the production of cytokines, and the activity of effector and Treg cells and to affect the efficiency of the immunological mechanisms induced by these pathogens.

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*, which constitutes the most prevalent deep mycosis in Latin America (16). The molecular mechanisms controlling the initial steps of *P. brasiliensis* and phagocyte interactions are not well understood. It is known, however, that unstimulated macrophages are permissive to *P. brasiliensis* growth while cytokine-activated macrophages are able to control *P. brasiliensis* proliferation (6, 8).

In previous work, our group demonstrated the role of TLR2 in pulmonary PCM. TLR2 deficiency leads to increased Th17 immunity associated with diminished expansion of Treg cells and increased lung pathology due to unrestrained inflamma-

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tory reactions (23). In addition, a more severe *P. brasiliensis* infection associated with increased production of Th17 cytokines, enhanced proinflammatory immunity, and impaired expansion of regulatory T cells was shown to be regulated by TLR4 expression (22). Moreover, TLR2, TLR4, and dectin-1 were suggested to be involved in the recognition and internalization of *P. brasiliensis* by human monocytes and neutrophils, indicating an important role for these pathogen receptors in the immune response against the fungus (3). Another group, however, reported that MyD88 is not essential for an effective defense against a systemic *P. brasiliensis* infection (17).

In the present work, we verified that the absence of MyD88 signaling by peritoneal macrophages resulted in decreased fungicidal ability associated with diminished synthesis of IL-12 and nitric oxide (NO). Accordingly, after pulmonary infection, MyD88$^{-/-}$ mice presented a severe infection, decreased levels of pulmonary Th1, Th2, and Th17 cytokines, impaired lymphoproliferative response, diminished expansion of Treg cells, and high mortality rates associated with extensive fungal lesions that affected several organs. Altogether, our findings demonstrate that the MyD88 adaptor molecule plays an important role not only in the fungicidal mechanisms of innate immune cells but also in the induction of the effector and regulatory cells of the adaptive immune response against this fungal pathogen.

MATERIALS AND METHODS

Fungus. *P. brasiliensis* Pb18, a highly virulent isolate, was used throughout this investigation (18). Pb18 yeast cells were maintained by weekly subcultivation in semisolid culture medium. Washed yeast cells were adjusted to 20×10^6 cells/ml (*in vivo* infection) and 4×10^4 cells/ml (*in vitro* infection) based on hemocytometer counts. Viability was determined with Janus Green B vital dye (Merck) and was always higher than 85%.

Mice and i.t. infection. MyD88$^{-/-}$ mice on a C57BL/6 background were kindly provided by S. Akira (Osaka University, Japan). C57BL/6 control (wild-type [WT]) mice were obtained from our Isogenic Breeding Unit (Departmento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil) and used at 8 to 12 weeks of age. Mice were anesthetized and subjected to intratracheal (i.t.) *P. brasiliensis* infection as previously described (10). Briefly, after intraperitoneal anesthesia, the animals were i.t. infected with 10^6 *P. brasiliensis* yeast cells, contained in 50 μl of phosphate-buffered saline (PBS). Mice were studied at 48 h and 8 weeks postinfection. The experiments were approved by the Ethics Committee on Animal Experiments of our institution.

PI labeling of *P. brasiliensis* yeast cells. *P. brasiliensis* yeast cells were washed in PBS and heat killed at 60°C for 1 h. Before the labeling, the yeast suspension was sonicated using 3 cycles of 10 s each (21% amplitude) with Sonics (Vibra Cell VCX 750; Sonics & Materials) to eliminate aggregates. The yeast cells were washed, adjusted to 1×10^6 cells/ml in PBS, and then incubated with propidium iodide (PI; 100 μg/ml; Sigma) for 30 min at 37°C. The yeast suspension was then washed three times with PBS and stored at 4°C.

Phagocytic and fungicidal assays. Thioglycolate-induced peritoneal macrophages were isolated by adherence (2 h at 37°C in 5% CO_2) to plastic-bottom tissue culture plates (1×10^6 cells/well in 24-well plates for fungicidal assays). Macrophages were washed to remove nonadherent cells and cultivated overnight with fresh complete medium (Dulbecco's modified Eagle's medium [DMEM]; Sigma) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence or absence of recombinant gamma interferon (IFN-γ; 20 ng/ml in culture medium; BD Pharmingen). For phagocytic assays, macrophage cultures were infected with *P. brasiliensis* yeast cells labeled with propidium iodide (PI) at a macrophage/yeast ratio of 1:1. The cells were cocultivated for 2 h at 37°C in 5% CO_2 to allow fungal adhesion and ingestion. Supernatants were removed and cells washed with PBS to remove any noningested or nonadhered yeast cells. Cells were then harvested and the macrophages were then labeled with anti-CD11b fluorescein isothiocyanate (FITC; eBioscience) for 20 min at 4°C. As *P. brasiliensis* yeast cells have high levels of variability in size and granularity (different sizes, numbers of buds, and numbers of nuclei), the granulocyte gates as defined by size (forward scatter [FSC]) and granularity (side scatter [SSC]) to determine the macrophage population were not used. The total cells present in the samples were analyzed by flow cytometry (FACSCalibur; BD Pharmingen). For fungicidal assays, IFN-γ-primed and unprimed macrophages were infected with *P. brasiliensis* yeast cells at a macrophage/yeast ratio of 25:1 and cocultivated for 2 h. The monolayers were then washed to remove nonadherent cells and incubated for an additional 48-h period. Plates were centrifuged ($400 \times g$, 10 min, 4°C) and supernatants obtained and stored at −70°C and further analyzed for the presence of nitrite and cytokines. The wells were washed with distilled water to lyse macrophages and suspensions collected in individual tubes. One hundred microliters of cell homogenates was assayed for the presence of viable yeast cells. All assays were done with five wells per condition in more than three independent experiments.

CFU assays, mortality rates, and histological analysis for determining severity of infections. The numbers of viable microorganisms in cell cultures and infected organs (lungs, liver, and spleen) from experimental and control mice were determined by counting the number of CFU. Animals from each group were sacrificed, and the enumeration of viable organisms was done as previously described (31). The numbers ($\log_{10}$) of viable *P. brasiliensis* per gram of tissue (*in vivo*) or per ml of cell homogenate (*in vitro*) are expressed as the means ± standard errors. Mortality studies were done with groups of 9 or 10 mice inoculated i.t. with 1×10^6 yeast cells or PBS. Deaths were registered daily, and experiments were repeated twice. For histological examinations, the left lung of the infected mouse was removed, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were stained by hematoxylin-eosin (H&E) for an analysis of the lesions and silver stained (Grocott stain) for fungal evaluation. Pathological changes were analyzed based on the size, morphology, and cell composition of granulomatous lesions, the presence of fungi, and the intensity of the inflammatory infiltrates. Morphometrical analysis was performed using a Nikon DXM 1200c digital camera (magnification of ×10) and Nikon NIS Elements AR 2.30 software. The area of lesions was measured (in μm^2) using 10 microscopic fields per slide for 6 animals per group. Results were expressed as the means ± standard errors for the total area of lesions for each animal.

Measurement of cytokines and NO. Supernatants from lung homogenates or cell cultures were separated and stored at −70°C. Cytokines (IL-1β, IL-33, transforming growth factor β [TGF-β], IL-4, IL-5, IL-23, IL-17, IL-12, IL-10, IL-6, TNF-α, and IFN-γ) levels were measured by a capture enzyme-linked immunosorbent assay (ELISA) with antibody pairs purchased from eBioscience or BD Pharmingen. The ELISA procedure was performed according to the manufacturer's protocol, and absorbances were measured with a Versa Max microplate reader (Molecular Devices). NO production was quantified by the accumulation of nitrite in the supernatants from *in vitro* and in *vivo* protocols by a standard Griess reaction (13). All determinations were performed in duplicate and expressed in μM NO.

Quantitative analysis of IL-18 mRNA expression. RNA was extracted from infected lungs using Trizol reagent (Invitrogen), and cDNA was synthesized from 2 μg RNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. IL-18 mRNA expression was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using assay-on-demand primers and probes, TaqMan universal master mix, and an ABI Prism 7000 apparatus (Applied Biosystems).

Assessment of leukocyte subpopulations in lung inflammatory exudates. After 48 h and 8 weeks of infection, lungs from each mouse were digested enzymatically for 30 min with collagenase (1 mg/ml) and DNase (30 μg/ml) in culture medium (Sigma). Lung cell suspensions were centrifuged in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris. Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was >85%. For differential leukocyte counts, samples of lung cell suspensions were cytospun (Shandon Cytospin) onto glass slides and stained by the Diff-Quik blood stain (Baxter Scientific). A total of 200 to 400 cells were counted from each sample. For flow cytofluorometry, lung leukocytes were resuspended at 10^6 cells/ml in staining buffer (PBS-0.1% NaN_3-1% fetal calf serum). Fc receptors were blocked by unlabeled anti-CD16/32 antibodies (BD Biosciences) and cells stained for 20 min at 4°C. Phycoerythrin (PE)-labeled anti-CD40, CD86, CD69, and dectin-1, fluorescein isothiocyanate (FITC)-labeled anti-IA^K, CD80, TLR-2, TLR-4, and CD4, and Alexa Fluor 488 anti-CD25, PE-Cy7 anti-CD4, and peridinin chlorophyll protein (PerCP) complex-Cy5.5 anti-CD11b and anti-CD8 monoclonal antibodies (MAbs; BD Biosciences) were used. Cells were fixed with 1% paraformaldehyde (Sigma) and stored in the dark at 4°C until they were analyzed in a flow cytometer. The acquisition and analysis gates were restricted to the lymphocytes or macrophages. Treg cells were characterized by intracellular staining for Foxp3, using a Treg staining kit from BD Biosciences. Surface staining of CD25$^+$ and intracellular FoxP3 (PE) expression were backgated on the CD4$^+$ T cell population. For intracellular cytokine (IL-4, IFN-γ, and IL-17) staining, cells were stimulated for 6 h in complete medium in the

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presence of 50 ng/ml phorbol 12-myristate 13-acetate, 500 ng/ml ionomycin (both from Sigma-Aldrich), and monensin (3 mM; eBioscience). After surface staining for CD4 (Pacific Blue anti-CD4) and CD8 (Alexa Fluor 488 anti-CD8), cells were fixed, permeabilized, and stained by PerCP-Cy5.5 anti-IFN-γ, PE-Cy7 anti-IL-4, and PE anti-IL-17 antibodies (eBioscience, San Diego, CA). The cell surface expression of leukocyte markers as well as intracellular expression of FoxP3, IL-4, IFN-γ, and IL-17 in lung-infiltrating leukocytes (LIL) were analyzed with a FACSCanto flow cytometer (BD Pharmingen) using the FlowJo software program (Tree Star, Inc., Ashland, OR).

Lymphoproliferation assay. Cells were assayed for proliferation using an *in vitro* fluorescence-based assay. Briefly, 1×10^6 cells from spleens were stained AQ: D with 1 μl (5 mM) of carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes) in PBS and 5% fetal calf serum for 15 min at room temperature. Stained cells were cultured for 3 days in the presence of anti-CD3 antibodies (0.3 μg/ml), anti-CD28 monoclonal antibodies (2.5 μg/ml) (BD Biosciences), or *P. brasiliensis* soluble antigen (100 μg/ml). A minimum of 100,000 events were acquired with a FACSCalibur flow cytometer using Cell-Quest software (BD Pharmingen). AQ: E The proliferation index (p) was calculated as the mean fluorescence intensity (MFI) of unstimulated cultures/MFI of stimulated cultures.

Statistical analysis. Data were analyzed by Student's t test or two-way analysis of variance depending on the number of experimental groups. Differences between survival times were determined with the log rank test using GraphPad Prism 5 for Windows (GraphPad Software). P values under 0.05 were considered significant.

RESULTS

MyD88 deletion leads to a less pronounced response of macrophages to *P. brasiliensis* infection. Macrophage cultures (1×10^6/well) performed in 24-well plates were preactivated or not with IFN-γ (20 ng/ml) and infected with 1×10^6 heat-killed PI-labeled yeast cells/well (1:1 fungus/macrophage ratio). After 2 h of incubation, supernatants were aspirated, the monolayer was gently washed with PBS, and the cells were harvested. To determine the number of ingested/adhered Pb yeast cells, macrophages were incubated with FITC-labeled anti-CD11b and analyzed by flow cytometry. Compared with the level for WT macrophages, a lower number of yeast cells were associated (ingested/adhered) with MyD88$^{-/-}$ macrophages F1 (Fig. 1A and C). The same was observed with IFN-γ-primed macrophages (Fig. 1B and C).

Macrophages were cultivated with *P. brasiliensis* yeast cells for an additional 48-h period. Supernatants were removed and assayed for the presence of nitric oxide and cytokines and cell homogenates plated for CFU determinations. As shown in Fig. 1D, the absence of MyD88 signaling led to increased recovery of viable yeast cells from untreated and IFN-γ-primed macrophages (20 ng/ml). In addition, higher levels of NO were produced by macrophages from WT mice than by those from MyD88$^{-/-}$ mice (Fig. 1E).

When cytokines in macrophage supernatants were measured, no differences in IL-10 and TNF-α were observed, but IL-12 was produced in decreased concentrations by MyD88$^{-/-}$ macrophages. The same occurred with the MCP-1 chemokine F2 (Fig. 2).

Absence of MyD88 signaling increases mortality rates associated with increased fungal loads and tissue pathology. To assess the influence of MyD88 deficiency in the disease outcome, the mortality of *P. brasiliensis*-infected MyD88$^{-/-}$ and WT ($n = 9$ or 10) mice was registered daily after infection with F3 1×10^6 *P. brasiliensis* yeast cells. As shown in Fig. 3A, at the 70th day of infection, all (10) MyD88$^{-/-}$ mice were dead. In the same period, 6 out of 9 WT mice were still alive.

After infection of groups ($n = 6$ to 8) of MyD88$^{-/-}$ and WT mice i.t. with 1×10^6 *P. brasiliensis* yeast cells, the severity of infection was assessed at early (48-h) and late (8-week) periods of the disease. As soon as 48 h after infection, MyD88$^{-/-}$ mice presented increased fungal loads in the lungs (Fig. 3B). At a later time (week 8), increased fungal burdens were recovered from lungs, livers, and spleens of MyD88$^{-/-}$ mice. The most striking differences in fungal loads were seen in the livers. No *P. brasiliensis* growth was detected in WT mice, whereas almost 1 million yeast cells were recovered from MyD88$^{-/-}$ mice (Fig. 3C). The augmented fungal burden of MyD88$^{-/-}$ mice observed in the lungs at 48 h of infection was accompanied by low levels of pulmonary NO (Fig. 3D). At week 8, however, elevated levels of NO were detected in the liver homogenates of MyD88$^{-/-}$ mice, which presented marked fungal loads (Fig. 3E). No measurable levels of NO were detected in the spleens of both mouse strains.

To better characterize the severity of *P. brasiliensis* infection, histopathological examination of lungs, livers, and spleens was done at week 8 of infection. As can be seen in Fig. 4, a more F4/AQ:F severe infection with intense destruction of tissue was observed in MyD88$^{-/-}$ mice. Pulmonary lesions in MyD88$^{-/-}$ mice replaced the largest part of normal tissue and were composed of confluent necrotic lesions of various sizes containing many budding yeast cells, surrounded by a small number of inflammatory mononuclear cells (Fig. 4G and H). The lesions in the lungs of WT mice (Fig. 4A) occupied a smaller area and were composed of sometimes confluent, organized granulomas of smaller sizes. Besides, the reduced numbers of fungal cells were surrounded by intense inflammatory reactions (Fig. 4A and B). The livers and spleens of WT mice presented a normal morphology (Fig. 4C to F), in contrast to those MyD88$^{-/-}$ mice which contained wide necrotic lesions with an elevated number of yeast cells accompanied by surrounding inflammatory exudates (Fig. 4I to L). The total areas of lesions in histological sections were quantified and are shown in panel M of Fig. 4. At week 8, the areas of lesions of MyD$^{-/-}$ mice were significantly larger than those of WT mice. Thus, the higher level of fungal growth observed in the organs of MyD88$^{-/-}$ mice was concomitant with increased tissue pathology.

MyD88-deficiency results in reduced levels of pro- and anti-inflammatory cytokines. Forty-eight hours after infection, at the innate phase of immunity, reduced levels of IL-12 and IL-1β were detected in the lungs of MyD88$^{-/-}$ mice (Fig. 5). F5 At week 8, reduced concentrations of IL-12, IL-4, IL-5, IL-10, TGF-β, IL-17, IL-23, IL-1β, and IL-33 were detected in the lungs of MyD88$^{-/-}$ mice (Fig. 6A). Besides these cytokines, F6 MyD88-deficient mice presented decreased levels of hepatic IL-6 (Fig. 6B). When splenic cytokines were measured, only reduced levels of TNF-α concomitant with elevated levels of IL-10 were detected in MyD88$^{-/-}$ mice (Fig. 6C). The message for IL-18 was also detected in the lungs, and impaired AQ: G expression was detected in the lungs at week 8 but not at the early postinfection period (48 h) of infection (Fig. 6D).

MyD88$^{-/-}$ mice exhibit decreased recruitment of PMN leukocytes to the lungs. To better characterize the inflammatory reaction at the site of infection, leukocyte recruitment to the lung tissues of *P. brasiliensis*-infected MyD88$^{-/-}$ and WT mice was studied at several postinfection periods. As can be seen in Fig. 7, lower frequencies (left panels) and numbers (right pan- F7 els) of polymorphonuclear (PMN) cells were observed in the lungs of MyD88$^{-/-}$ mice than in their normal controls. No

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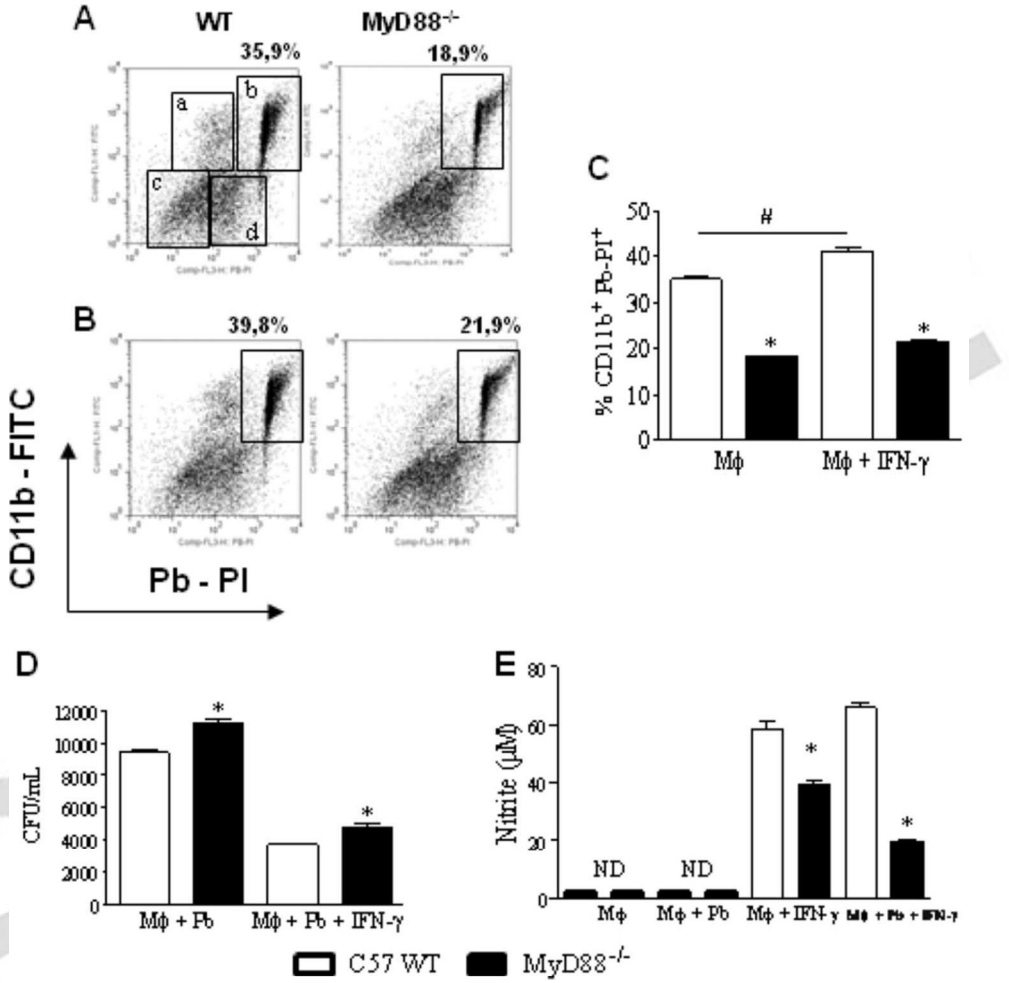

AQ: M FIG. 1. Macrophages from MyD88$^{-/-}$ mice have a decreased ability to interact with *P. brasiliensis* yeast cells. For phagocytic assays, unprimed (A, C) and IFN-γ-primed (20 ng/ml, overnight) (B, C) peritoneal macrophages from MyD88$^{-/-}$ and WT C57BL/6 mice were infected with 1×10^6/well heat-killed PI-labeled yeast cells (1:1 fungus/macrophage ratio). After 2 h, the supernatants were aspirated, the cells washed, and harvested macrophages labeled with FITC anti-CD11b antibodies and analyzed by flow cytometry. Four different cell populations were identified (regions a to d). (a) Single positive FITC-labeled cells (CD11b$^+$ cells that do not phagocytose or adhere to Pb-PI); (b) double-positive cells (PI-Pb–FITC macrophages); (c) dead cells; and (d) nonphagocytosed PI-Pb. For fungicidal assays, IFN-γ-primed and unprimed peritoneal macrophages were infected with *P. brasiliensis* yeast cells at a macrophage/yeast ratio of 25:1. After 48 h of cocultivation, the supernatants were obtained to characterize NO and cytokine production; monolayers were lysed and assayed for the presence of viable yeast cells by a CFU assay (D). Supernatants were used to determine the levels of nitrites using the Griess reagent (E). Data are means ± standard errors of the means (SEM) AQ: K of results from quintuplicate samples from one experiment representative of three independent determinations. # and *, $P < 0.05$.

important differences were noted in the total counts of lymphocytes and macrophages, except the significantly elevated numbers of lymphocytes in the lungs of WT mice at week 6 after infection (Fig. 7).

Absence of MyD88 signaling determines decreased numbers of activated mononuclear phagocytes and T cells. We have further analyzed the phenotype and activation status of lung infiltrating leukocytes at week 8 of *P. brasiliensis* infection (Fig. F8 8). To determine the activation profile of pulmonary mononuclear phagocytes (macrophages and dendritic cells), the expression of CD11b, major histocompatibility complex (MHC) class II (IA^K), CD80, CD86, CD40, dectin-1, TLR2, and TLR4 molecules was assessed by flow cytometry. As can be seen in Fig. 8A and B, besides diminished frequencies and numbers of CD11b$^+$ cells, MyD88$^{-/-}$ mice presented decreased presence of cells expressing IA^K and CD40 markers. No differences in AQ: H CD80, CD86, dectin-1, TLR2, and TLR4 expression by CD11b$^+$ phagocytes were detected. When lymphocytes were characterized, a significantly reduced recruitment of CD4$^+$ CD25$^+$ and CD8$^+$ CD69$^+$ T cells to the lungs of MyD88$^{-/-}$ mice was detected (Fig. 8C and D). The presence of CD4$^+$ CD25$^+$ FoxP3$^+$ T cells was characterized by flow cytometry in the CD4$^+$ subpopulation of lung-infiltrating lymphocytes. Surface staining of CD25$^+$ and intracellular FoxP3$^+$ expression were back-gated on the CD4$^+$ T cell population. As can be seen in Fig. 8C and D, diminished frequencies and numbers of CD4$^+$ CD25$^+$ FoxP3$^+$ T cells were observed in the lung inflammatory lymphocytes of MyD88$^{-/-}$ mice. Thus, in pulmonary PCM, MyD88 signaling appears to affect the induction of effector and regulatory T cells.

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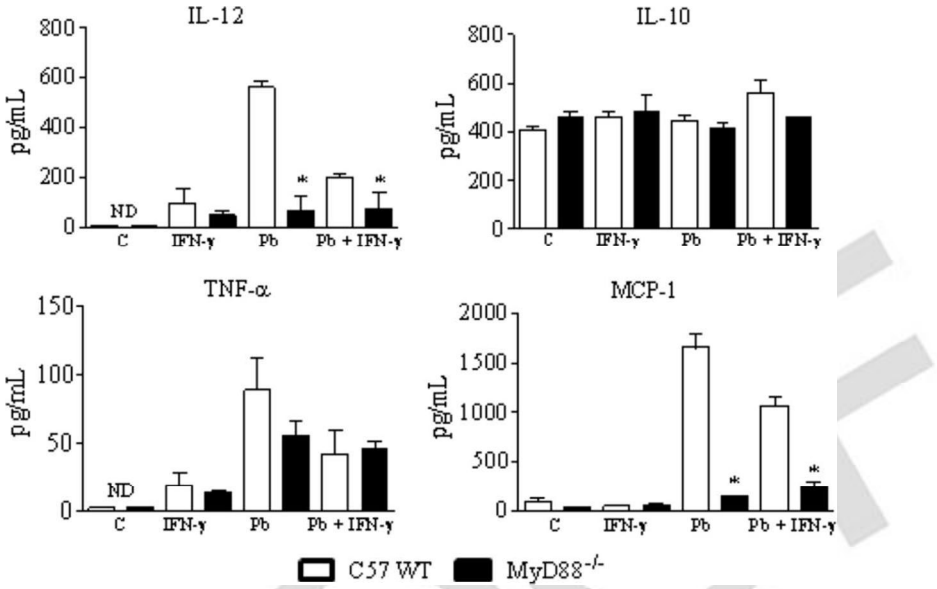

FIG. 2. Macrophages from MyD88$^{-/-}$ mice secrete diminished levels of IL-12 and MCP-1. IFN-γ-treated (20.00 ng/ml) or untreated macrophages of MyD-deficient and WT mice were challenged with viable *P. brasiliensis* yeast cells (1:25 fungus/macrophage ratio) and cultivated for 48 h at 37°C in 5% CO_2. Supernatants were then obtained and used for cytokine measurements using ELISA. Data are means ± SEM of results from triplicate samples from one experiment representative of 3 independent determinations. *, $P < 0.05$.

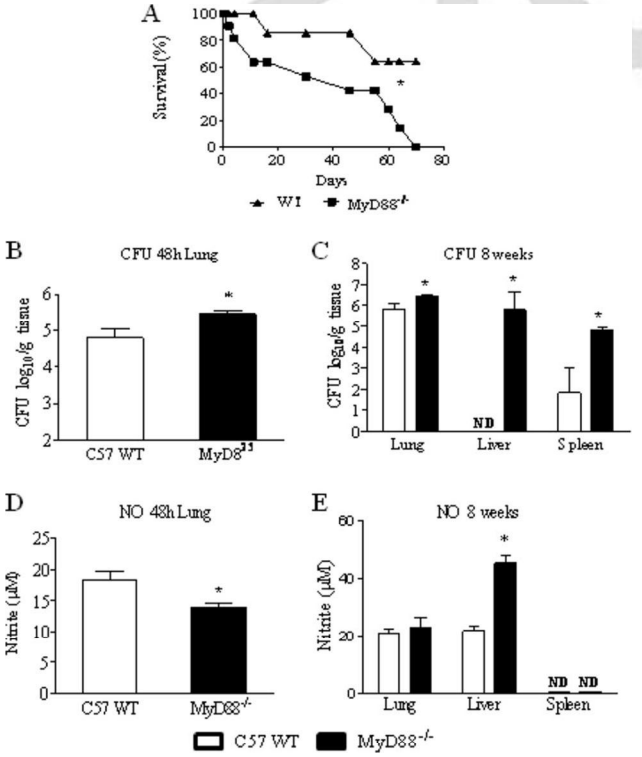

FIG. 3. Absence of MyD88 signaling increases mortality rates and tissue fungal burdens. (A) Survival times of MyD88$^{-/-}$ ($n = 9$) and WT ($n = 10$) mice after i.t. infection with 1×10^6 *P. brasiliensis* yeast cells was determined in a period of 70 days. The results are representative of two independent experiments. *, $P < 0.05$. (B and C) Recovery of fungal loads (CFU) from organs of MyD88$^{-/-}$ and WT mice. The bars represent mean ± SEM $\log_{10}$ numbers of CFU obtained from groups of 6 to 8 mice at 48 h (B) and 8 weeks (C) after infection. Levels of NO (μM) present in tissue homogenates obtained 48 h (D) and 8 weeks (E) after fungal infection. The results are representative of three experiments with equivalent results. *, $P < 0.05$.

MyD88$^{-/-}$ mice present an impaired lymphoproliferative response. To characterize the proliferative activity of lymphocytes, spleen cells were obtained from WT and MyD88$^{-/-}$ mice at week 8 of infection. CFSE-labeled lymphocytes were *in vitro* stimulated with *P. brasiliensis* antigen and anti-CD3 and anti-CD28 MAbs and cultivated for 72 h. As presented in Table 1, with all stimuli used, the lymphoproliferative response of MyD88$^{-/-}$ splenocytes was lower than that of WT mice. This experiment indicates that MyD88$^{-/-}$ mice mount a deficient T cell response as revealed by the diminished migration of T cells to the site of infection and the impaired lymphoproliferative activity detected.

MyD88$^{-/-}$ mice present decreased numbers of Th17 cells in the lungs. To better clarify the importance of MyD88 signaling in the polarization of T cell responses, the phenotypes of IL-17, IFN-γ, and IL-4-producing cells were defined in the inflammatory infiltrates of lungs at week 8 postinfection. These cytokines were assessed by intracellular staining in NK, Tγδ, CD4$^+$, and CD8$^+$ T cells. As shown in Fig. 9, significantly diminished frequencies and numbers of CD4$^+$ IL-17$^+$ T cells were detected in the lungs of MyD88$^{-/-}$ mice. Despite the increased percentages of CD4$^+$ IFN-γ$^+$, CD4$^+$ IL-4$^+$, and CD8$^+$IL-4$^+$ cells, no significant increases in the total numbers of these cells were detected in MyD88$^{-/-}$ mice. NK and Tγδ cells were negative for all intracellular cytokines assayed. These findings indicate that absence of MyD88 signaling induces a marked impairment of Th17 immunity of *P. brasiliensis*-infected hosts.

DISCUSSION

The ability to interact with distinct conserved molecules of pathogens makes TLRs key elements of innate immunity (27). Due to the broad activities of MyD88, infections in MyD88$^{-/-}$

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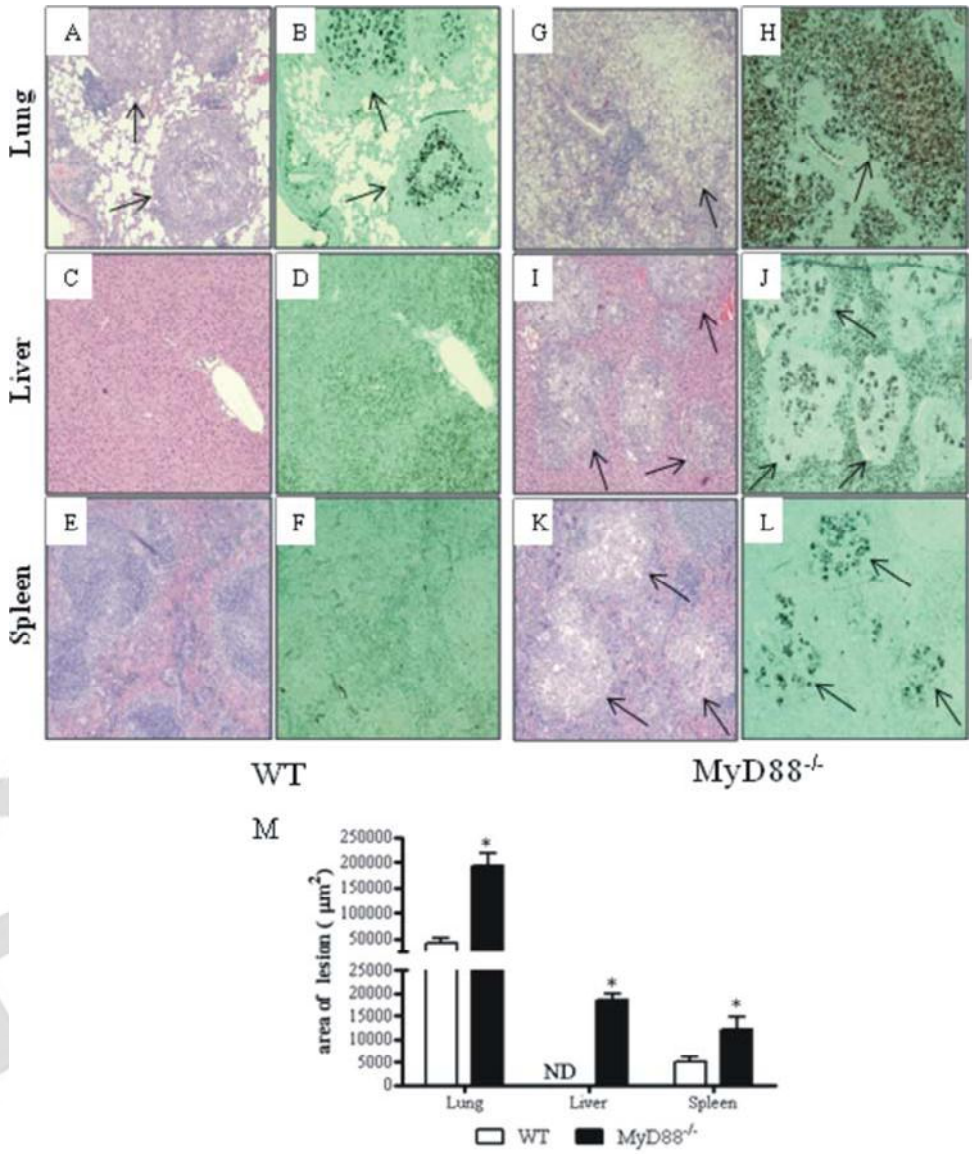

FIG. 4. Photomicrographs of lesions of WT (A to F) and MyD88$^{-/-}$ (G to L) mice at week 8 of infection with 1×10^6 *P. brasiliensis* yeast cells. Compared with those of MyD88$^{-/-}$ mice (G), the pulmonary lesions of WT mice (A) were smaller (arrows) and composed of organized granulomas containing lower numbers of yeast cells (B). The pulmonary lesions of MyD88$^{-/-}$ mice were composed of confluent, necrotic, unorganized granulomas of various sizes (G) containing an elevated number of fungal cells (arrow in H) and replaced almost all the normal tissue (G, H). The livers (C, D) and spleens (E, F) of WT mice presented a normal morphology; in contrast, the livers (I, J) and spleens (K, L) of MyD88$^{-/-}$ mice presented extensive necrotic lesions (arrows in I and K) containing an elevated number of yeast cells (arrows in panels J and L) surrounded by mononuclear inflammatory exudates. H&E (A, C, E, G, I, K)- and Groccot (B, D, F, H, J, L)-stained lesions (magnification, ×100). *, $P < 0.05$. (Lower panel) total area of lesions in the lungs, livers and spleen of mice ($n = 6$) at week 8 after infection. *, $P < 0.05$.

mice generally result in reduced survival, increased pathogen load, and diminished secretion of IL-12 (14, 24, 30, 35). Similar results were demonstrated in this study in a pulmonary model of infection caused by *P. brasiliensis*. In this report, we first verified that MyD88$^{-/-}$ macrophages have a decreased ability to interact with *P. brasiliensis* yeast cells, and this behavior was also observed when macrophages were primed with IFN-γ. The low phagocytic and fungicidal abilities of and the impaired NO and IL-12 production by these cells demonstrated that MyD88 signaling is important for macrophage activation and, consequently, for the control of *P. brasiliensis* growth. In addition, low IL-12 and MCP-1 production may negatively influence CD4$^+$ T cell activation and the migration of mononuclear cells to the site of infection, which have been observed in infected MyD88$^{-/-}$ mice. Although MyD88$^{-/-}$ macrophages were able to produce NO, the levels were lower than those produced by WT cells. In murine PCM and other fungal infections, the production of NO, an important fungicidal mediator (25), correlates with fungal loads and the synthesis of proinflammatory mediators, including cytokines, chemokines, and leukotrienes. Enhanced NO production and fungicidal function were detected in WT and MyD88$^{-/-}$ macrophages

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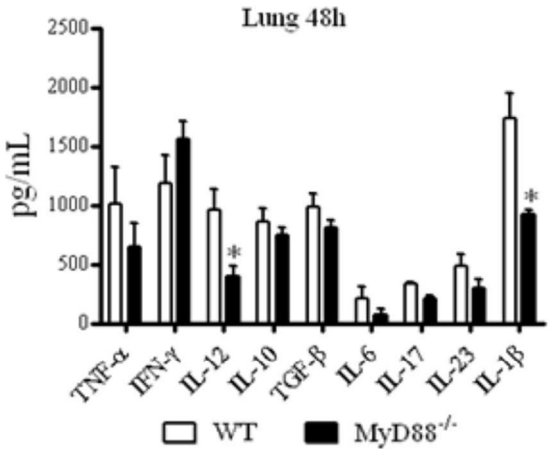

FIG. 5. Early after infection, lung homogenates of MyD88$^{-/-}$ mice presented decreased levels of IL-12 and IL-1β. MyD88$^{-/-}$ and WT mice were infected with 1×10^6 yeast cells of *P. brasiliensis*, and 48 h later, lungs were collected and disrupted in 5.0 ml of PBS, and supernatants were analyzed for cytokine content by capture ELISA. The bars depict means ± SEM of cytokine levels (6 to 8 animals per group). The results are representative of two independent experiments. *, $P < 0.05$.

activated by IFN-γ. Previous studies have indicated that IFN-γ R1 signaling is partially dependent on MyD88 (27, 32). Thus, it is possible that this low-level but evident NO secretion was induced by MyD88-independent IFN-γ R1 signaling or other inducible nitric oxide synthase (iNOS)-activating mediators induced by MyD88-independent pathogen recognition receptors (PRRs). Although no differences in TLR2, TLR4, or dectin-1 expression were detected in lung inflammatory mononuclear phagocytes, other pathogen receptors, such as C-type lectin receptors (e.g., mannose receptors [MRs] or dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin [DC-SIGN]), may be involved in the macrophage response in MyD88$^{-/-}$ mice.

The increased fungal burden observed in the early infection period (48 h) was consistent with data obtained using infected macrophages *in vitro*. A more severe infection associated with decreased IL-12 and IL-1β production was observed in MyD88$^{-/-}$ mice. These proinflammatory cytokines mediate phagocyte activation in the innate phase of immunity but are also involved in the induction of Th1 and Th17 adaptive immune responses, respectively (26). A more severe infection of the lungs, liver, and spleen was also detected at a later postinfection time point (8 weeks). Despite the high levels of NO produced, the dissemination and growth of fungal cells in the livers of MyD88$^{-/-}$ mice were extensive, suggesting that the activation of iNOS was induced by MyD88-independent mechanisms triggered by the elevated fungal burden.

Lower levels of Th1 (IL-12 and IL-18), Th2 (IL-33, IL-4, IL-5, and IL-10), and Th17 (TGF-β, IL-1β, IL-6, IL-23, and IL-17) cytokines were found in the lungs and/or livers of MyD88$^{-/-}$ mice after 8 weeks of infection. Moreover, the impaired cell migration to inflammatory sites and suppressed polyclonal and antigen-specific lymphoproliferative responses

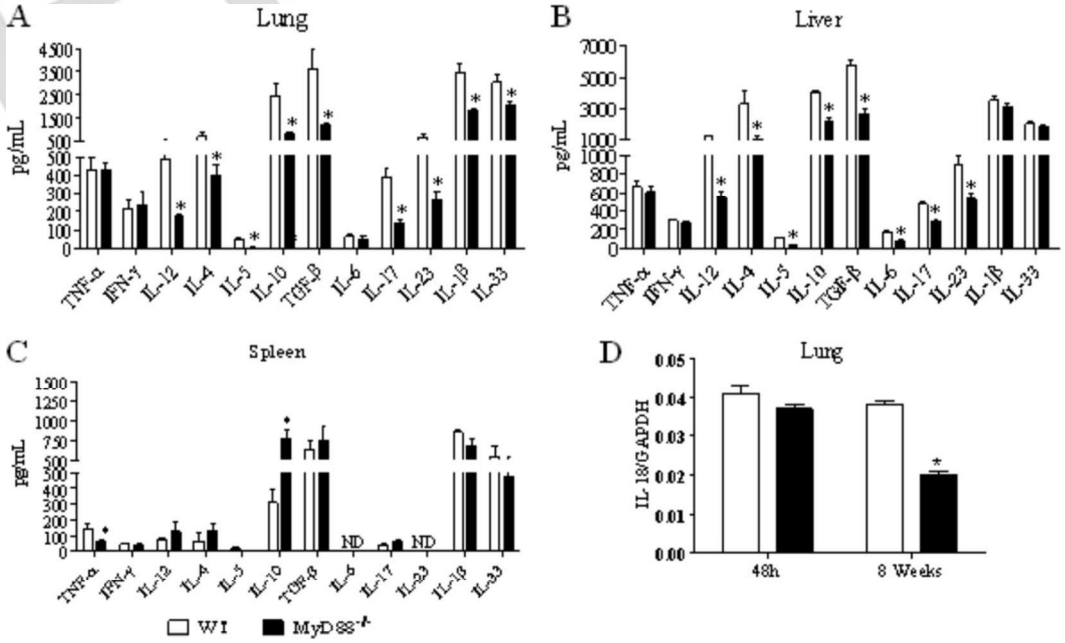

FIG. 6. At week 8 after infection, organs from MyD88$^{-/-}$ mice presented decreased levels of Th1, Th2, and Th17 cytokines. Lung (A), liver (B), and spleen (C) homogenates of MyD88$^{-/-}$ mice presented decreased levels of cytokines. At week 8 after i.t. infection with 1×10^6 yeast cells of *P. brasiliensis*, organs from MyD88$^{-/-}$ and WT mice were collected and disrupted in 5.0 ml of PBS and supernatants analyzed for cytokine content by capture ELISA. The bars depict means ± SEM of cytokine levels (6 to 8 animals per group). The results are representative of three independent experiments. *, $P < 0.05$. (D) Quantitative PCR analysis of IL-18 expression in the lungs of *P. brasiliensis*-infected WT and MyD88$^{-/-}$ mice. Total lung RNA was obtained, reverse transcribed, and cDNA amplified. Real-time PCR was performed using TaqMan universal master mix. Amplified products were normalized to the amount of GAPDH products. Data represent the means ± SEM of results from two independent experiments.

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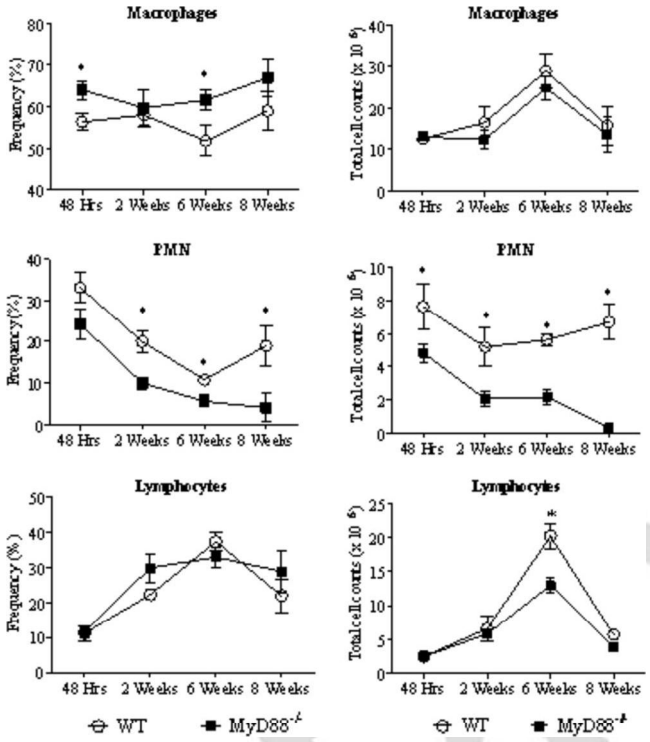

FIG. 7. MyD88 deficiency determines a decreased recruitment of PMN cells to the lungs. Frequency (left side panels) and absolute number (right panels) of leukocyte subsets (macrophages, PMN neutrophils, and lymphocytes) in the lung-infiltrating leukocytes (LIL) from MyD88$^{-/-}$ and WT mice inoculated i.t. with 1 million *P. brasiliensis* yeast cells. At different postinfection periods (48 h, 2, 6, and 8 weeks), lungs of both mouse strains ($n = 6$ to 8) were excised, washed in PBS, minced, and digested enzymatically. Lung cell suspensions were obtained and cytospun onto glass slides. Cells were stained by the Diff-Quik bloodstain. Data are expressed as means ± SEM. *, $P < 0.05$.

observed in MyD88$^{-/-}$ splenocytes suggest that absence of MyD88 signaling profoundly affects Th1, Th2, and Th17 immune responses in pulmonary PCM.

In addition to defective MyD88-dependent TLR signaling, deficient production of cytokines from the IL-1 family (IL-1β, IL-18, and IL-33) and cell signaling by their receptors (IL-1R, IL-18R, and IL-33R) appear to be involved in the deficient immunity observed in MyD88$^{-/-}$ mice. Indeed, IL-1β, IL-18, and IL-33 are involved in the differentiation of Th17, Th1, and Th2 cells of adaptive responses, respectively (11, 26). Interestingly, MyD88-dependent TLR signaling has been implicated in the transcription of pro-IL-1β and pro-IL-18 into their active forms by caspase 1 or other inflammatory or pathogen-derived proteases (1, 11, 26). Thus, the deficient cytokine production and impaired cell activation (likely due to defective IL-1R family signaling) observed here appear to have contributed to the suppressed T cell immunity developed by *P. brasiliensis*-infected MyD88$^{-/-}$ mice.

The reduced synthesis of IL-1β, TGF-β, and IL-23 was associated with defective Th17 responses in MyD88$^{-/-}$ mice. This deficiency was confirmed by the evaluation of intracellular cytokines, which demonstrated a lower number of CD4$^+$IL-17$^+$ T cells in the lungs of MyD88$^{-/-}$ mice. Interestingly, in murine candidiasis, dectin-1, dectin-2, and MRs, which are not involved in TIR domain-mediated signaling, were shown to be involved in fungal recognition and the induction of Th17 immunity (28, 29, 36). However, in pulmonary PCM, the two MyD88-dependent receptors, TLR2 and TLR4, have been shown to be antagonistically involved in Th17 development: TLR2 inhibited Th17 immunity but expanded Treg cell numbers, whereas TLR4 induced Th17 cells and inhibited Treg development (9, 22, 23).

In several models of infection, the control of neutrophil influx to the inflammatory sites is mediated by MyD88 (7, 37). Furthermore, Th17 immunity is generally associated with enhanced synthesis of CXC chemokines and the induction of neutrophils chemotaxis to inflammatory sites (20, 21, 38). The increased influx of neutrophils into the lungs of *P. brasiliensis*-infected WT mice paralleled the increase in production of Th17 cytokines. This finding is consistent with our previous report showing that Th17 polarization in pulmonary PCM was associated with PMN-rich inflammatory reactions. These cells appear to efficiently control fungal loads; however, they may also mediate deleterious effects due to their ability to cause tissue damage (23).

In addition to the reduced numbers of activated CD4$^+$ and CD8$^+$ T cells, MyD88 signaling affected the numbers and activation state of inflammatory myeloid cells. Reduced numbers of CD11b$^+$ mononuclear phagocytes expressing MHC class II (IA^K) and CD40 molecules were found in the lungs of MyD88$^{-/-}$ mice after 8 weeks of infection. In addition, a reduced influx of CD4$^+$ CD25$^+$ FoxP3$^+$ Treg cells was also observed. This result is consistent with the work by Sutmuller et al. (33), who previously showed that Treg cell proliferation is dependent on MyD88 signaling. Recently, the plasticity of Th/Treg cells has been demonstrated by studies showing that under certain *in vivo* or *in vitro* conditions, they can convert to other Th cell phenotypes. Thus, FoxP3$^+$ Treg cells (natural and induced Tregs) can acquire a Th17 phenotype in the presence of IL-6; however, these cells express transcription factors of both T cell subpopulations (39, 40). Our studies demonstrated that WT mice develop a high number of CD4$^+$IL-17$^+$ T cells in addition to elevated numbers of FoxP3$^+$ Treg cells. We have not assessed the concomitant presence of FoxP3 and RORγt in the IL-17-positive cells; however, this interconversion cannot be ruled out in our model.

The absence of MyD88 signaling resulted in increased mortality rates in infected mice. Interestingly, this phenomenon was not observed in TLR4- or TLR2-deficient mice (22, 23). Increased mortality was associated with uncontrolled fungal growth, impaired T cell immunity, and the absence of organized granulomatous lesions. We previously reported that impaired T cell immunity and excessive fungal growth in the livers of WT C57BL/6 mice were linked with the increased mortality observed in the late phase of infection (15). Thus, it is tempting to hypothesize that the marked growth of yeast cells in the livers of MyD88-deficient mice contributes to the augmented mortality that we observed. Working with *A. fumigatus*, Bretz et al. (5) also showed that MyD88 signaling is essential for controlling pulmonary fungal burden and organizing inflammatory reactions. A recent study, however, showed equivalent fungal growth and cytokine production in MyD88$^{-/-}$ and normal mice infected with *P. brasiliensis*, suggesting that MyD88

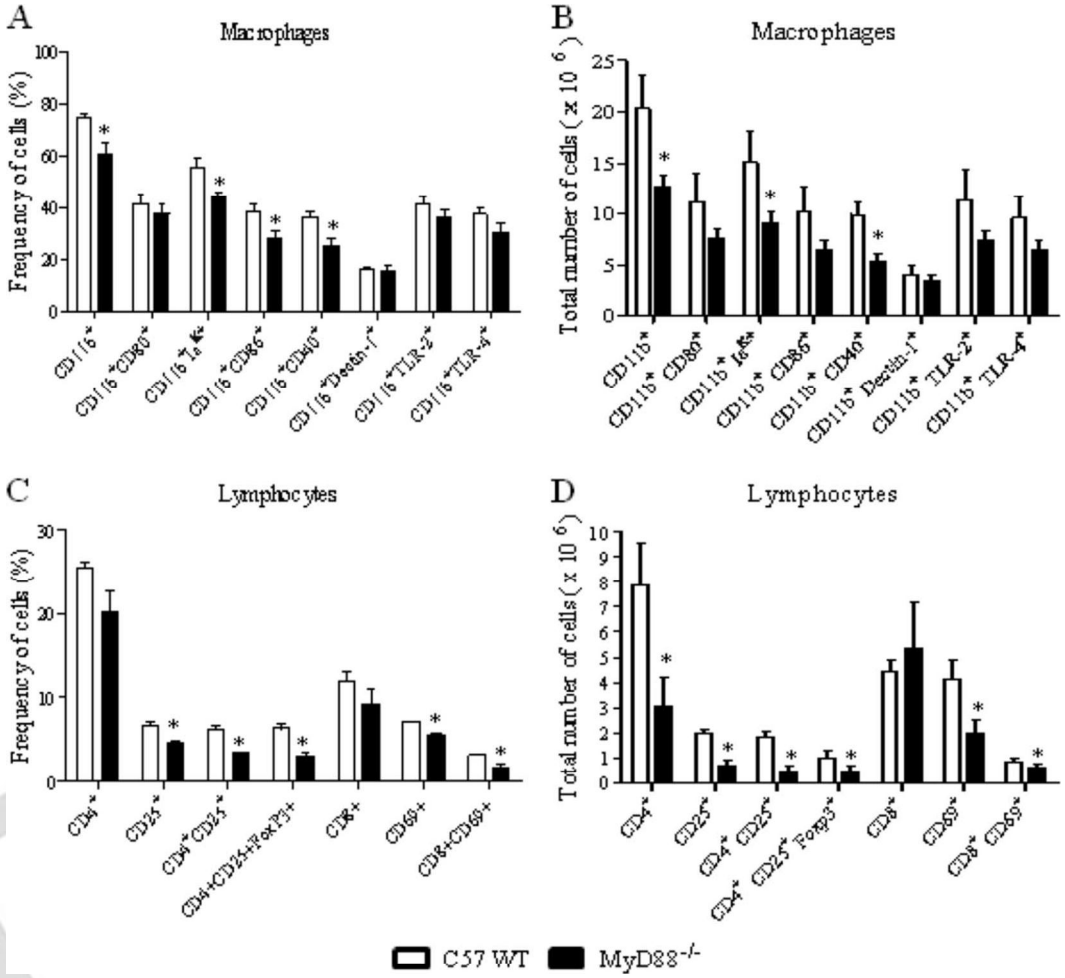

FIG. 8. Decreased numbers of activated macrophages, T lymphocytes, and regulatory T cells were detected in the lungs of MyD88$^{-/-}$ mice at week 8 of infection. Characterization of leukocyte subsets by flow cytometry in the lung-infiltrating leukocytes (LIL) from MyD88$^{-/-}$ and WT mice inoculated i.t. with 1×10^6 *P. brasiliensis* yeast cells. At week 8 after infection, lungs of both mouse strains ($n = 6$ to 8) were excised and digested enzymatically. Cell suspensions were obtained and stained as described in Materials and Methods. The stained cells were analyzed immediately with FACSCanto equipment gating on macrophages or lymphocytes, as judged from forward and side light scatters. Twenty thousand cells were counted, and the data are expressed as percentage and absolute number of positive cells. For characterization of Treg cells (CD4$^+$ CD25$^+$ FoxP3$^+$), surface staining of CD25$^+$ and intracellular FoxP3 expression were back-gated on the CD4$^+$ T cell population. Data are expressed as means ± SEM and are representative of two independent experiments. *, $P < 0.05$.

TABLE 1. Proliferation indexes of spleen lymphocytes obtained from *P. brasiliensis*-infected MyD88$^{-/-}$ and WT mice at week 6 of infection[a]

Treatment	*p*	
	MyD88$^{-/-}$	WT[b]
Lymphocytes-AgPb	0.86	1.87
Lymphocytes–anti-CD28	0.69	1.31
Lymphocytes–anti-CD28–AgPb	0.87	4.62

[a] Spleen cell suspensions were labeled with CFSE and cultured for 3 days in the presence of anti-CD3 (0.3 μg/ml) and anti-CD28 (2.5 μg/ml) antibodies and *P. brasiliensis* soluble antigen (100 μg/ml). The intensity of CFSE was assessed by flow cytometry.

[b] The proliferation index (*p*) was calculated as the mean fluorescence intensity (MFI) of unstimulated cultures/MFI of stimulated cultures. Data are representative of two independent experiments.

signaling is not essential for an effective defense against the fungus (17). This discrepancy may be due to the fact that those experiments were performed with a different strain and, perhaps more importantly, using a different infection route. It should be noted, however, that our previous studies (22, 23) showed that TLR2 and TLR4, which signal using MyD88 as an adapter molecule, influence the severity of PCM. Taken together, our results suggest that MyD88-dependent signaling pathways downstream from TLRs and IL-1Rs contribute to host defense against pulmonary paracoccidioidomycosis.

As demonstrated with another dimorphic fungal pathogen (4, 12), *P. brasiliensis* yeast cells appear to use MyD88-mediated signaling to mount protective Th responses that include prevalent Th17 expansion. This response pattern results in PMN-rich inflammatory reactions, which are able to restrain fungal growth and dissemination into other organs and tissues.

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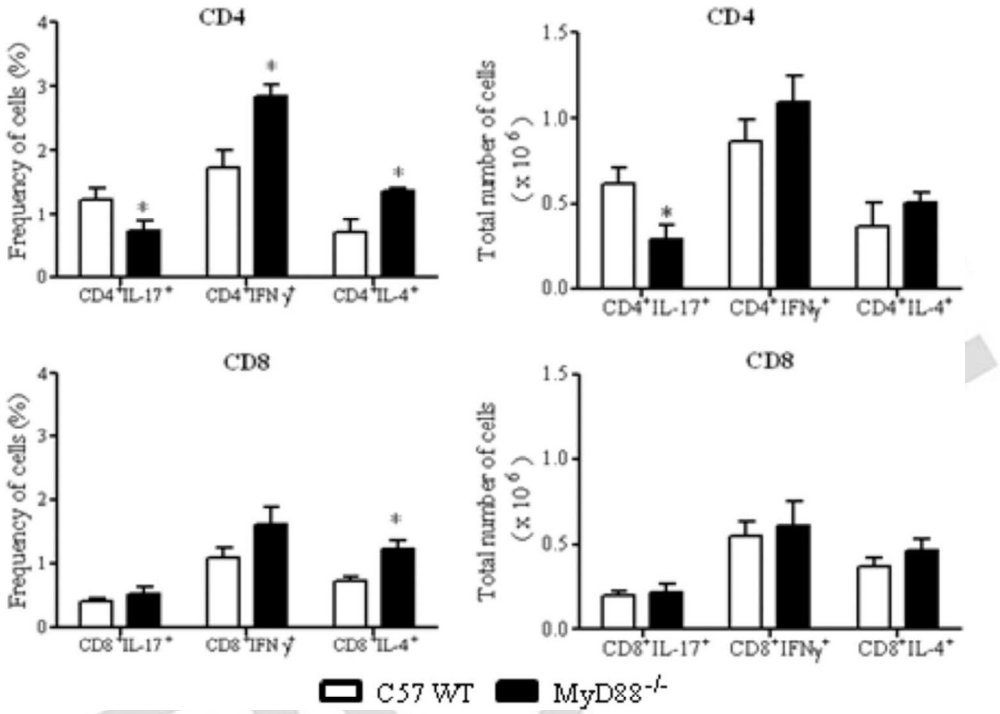

FIG. 9. Decreased numbers of $CD4^+IL\text{-}17^+$ cells were detected in the lungs of $MyD88^{-/-}$ mice. Groups ($n = 6$ or 7) of WT and $MyD88^{-/-}$ mice were infected with 1×10^6 *P. brasiliensis* yeast cells. The presence of IL-17^+, IFN-γ^+, and IL-4^+ $CD4^+$ and $CD8^+$ T cells in the lung-infiltrating leukocytes was assessed by intracellular cytokine staining by flow cytometry at week 8 after infection. Lung cells were restimulated *in vitro* with phorbol myristate acetate (PMA)-ionomycin for 6 h and subjected to intracellular staining for IL-17, IL-4, and IFN-γ. The lymphocyte population was gated by the forward/side scatters. The results are from one experiment and are representative of two independent experiments. *, $P < 0.05$.

Using a MyD88-influenced pathway of cell activation (12), WT mice developed $FoxP3^+$ Treg cells that were able to control adaptive immunity and excessive inflammation. This balanced immunity mediated by Th1/Th2/Th17 cells appeared to control fungal growth without significant tissue damage, which led to an extended survival time in WT mice. In contrast, the absence of MyD88 signaling appears to profoundly suppress the development of adaptive immunity, as shown by decreased levels of Th1/Th2 and Th17 cytokines, suppressed lymphoproliferative activity, and diminished activation and migration of mononuclear phagocytes and T cells ($CD4^+$ and $CD8^+$) to the site of infection. This defective innate immunity and impaired adaptive immunity, including deficient Treg expansion, resulted in uncontrolled fungal growth, which contributed significantly to the tissue pathology observed in $MyD88^{-/-}$ mice. In conclusion, the absence of MyD88 signaling in pulmonary PCM results in profound deleterious effects due to the combined deficiency of innate and adaptive immunity, which results in severe tissue pathology and precocious host mortality.

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***Anexo C* ï Toll-Like Receptor 4 Signaling Leads to Severe Fungal Infection Associated with Enhanced Proinflammatory Immunity and Impaired Expansion of Regulatory T Cells**

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Toll-Like Receptor 4 Signaling Leads to Severe Fungal Infection Associated with Enhanced Proinflammatory Immunity and Impaired Expansion of Regulatory T Cells▽

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Toll-like receptors (TLRs) present in innate immune cells recognize pathogen molecular patterns and influence immunity to control the host-parasite interaction. The objective of this study was to characterize the involvement of TLR4 in the innate and adaptive immunity to *Paracoccidioides brasiliensis*, the most important primary fungal pathogen of Latin America. We compared the responses of C3H/HeJ mice, which are naturally defective in TLR4 signaling, with those of C3H/HePas mice, which express functional receptors, after *in vitro* and *in vivo* infection with *P. brasiliensis*. Unexpectedly, we verified that TLR4-defective macrophages infected *in vitro* with *P. brasiliensis* presented decreased fungal loads associated with impaired synthesis of nitric oxide, interleukin-12 (IL-12), and macrophage chemotactic protein 1 (MCP-1). After intratracheal infection with 1 million yeasts, TLR4-defective mice developed reduced fungal burdens and decreased levels of pulmonary nitric oxide, proinflammatory cytokines, and antibodies. TLR4-competent mice produced elevated levels of IL-12 and tumor necrosis factor alpha (TNF-α), besides cytokines of the Th17 pattern, indicating a proinflammatory role for TLR4 signaling. The more severe infection of TLR4-normal mice resulted in increased influx of activated macrophages and T cells to the lungs and progressive control of fungal burdens but impaired expansion of regulatory T cells (Treg cells). In contrast, TLR4-defective mice were not able to clear their diminished fungal burdens totally, a defect associated with deficient activation of T-cell immunity and enhanced development of Treg cells. These divergent patterns of immunity, however, resulted in equivalent mortality rates, indicating that control of elevated fungal growth mediated by vigorous inflammatory reactions is as deleterious to the hosts as low fungal loads inefficiently controlled by limited inflammatory reactions.

Pathogen recognition receptors (PRRs) are a group of receptors present in the membrane and cytoplasm of innate immunity cells that recognize the presence of invading microbes by interacting with conserved pathogen structures, the so called "pathogen-associated molecular patterns" (PAMPs). This initial event of innate immunity is crucial for the control of pathogen growth and the subsequent activation of adaptive immunity. Toll like receptors (TLRs) constitute a major family of pattern recognition molecules and, like other PRRs, are able to respond to different structural homologies conserved in many microorganisms (2, 62). Activation of the TLRs is crucial for many aspects of microbe elimination, including microbial killing, recruitment of phagocytes to the site of infection, and activation of dendritic cells (DCs) (52). Early TLR activation results in the production of several inflammatory mediators, and the final balance among pro- and anti-inflammatory components regulates the type of adaptive immune response. Recent findings have shown that direct recognition of PAMPs by DCs is critical for priming appropriate T-cell responses, resulting in T helper 1 (Th1), Th2, or Th17 immunity (25, 31, 33, 60).

TLR4 is the key receptor that recognizes bacterial lipopolysaccharides (LPS), whereas TLR2 is involved in the interaction with bacterial peptidoglycans and lipoproteins (66). As reported for other microorganisms, TLRs have been shown to be involved in host defense against different fungal pathogens. *In vivo* and *in vitro* studies have demonstrated that *Cryptococcus neoformans* (7, 67), *Candida albicans* (43, 45), and *Aspergillus fumigatus* (24, 41) may signal through members of the TLR family, mainly TLR2 and TLR4. Different components of a certain pathogen can be used to stimulate the immune system. Thus, *C. albicans* phospholipomannan is sensed by TLR2 (34), while O-linked mannans are recognized by TLR4 (44). The contribution of individual TLRs to the immune response against pathogenic fungi depends on several factors, such as the fungal morphotype, fungal species, and route of infection. Activation signals mediated by innate immunity receptors, however, are not always beneficial to the host, and TLR activation can be used by pathogenic fungi to promote more-severe infections (6, 53).

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis* and constitutes the most prevalent deep mycosis in Latin America (28). The alveolar macrophages are the first host cells that interact with *P. brasiliensis* cells, and their activation is fundamental to the control of fungal growth. The molecular mechanisms controlling the initial steps of the in-

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teraction between *P. brasiliensis* and phagocytes are not well understood. It is known, however, that normal macrophages are permissive to *P. brasiliensis* growth, while cytokine-activated macrophages are able to restrain *P. brasiliensis* multiplication (12). Complement receptor 3 (CR3) and mannose receptor have been shown to play important roles in the initial interaction between *P. brasiliensis* cells and mouse peritoneal macrophages (14, 32, 50). Interestingly, recent work from our laboratory demonstrated that alveolar macrophages from susceptible mice (B10.A) are easily activated by *P. brasiliensis* infection and show efficient fungal killing associated with nitric oxide production, while pulmonary macrophages from resistant mice (A/Sn) are poorly activated and present inefficient killing activity associated with increased levels of transforming growth factor β (TGF-β) (49). Despite their inefficient innate immunity, A/Sn mice develop a balanced Th1/Th2 immune response that controls fungal growth without intense tissue pathology.

In previous work, our group demonstrated the influence of TLR2 on pulmonary PCM (15, 38). Using TLR2-normal and TLR2-deficient mice, we were able to show that the presence of TLR2 increases the severity of *in vitro* and *in vivo P. brasiliensis* infections. In addition, TLR2 deficiency results in increased Th17 immunity associated with diminished expansion of regulatory T cells (Treg cells) and increased lung pathology due to unrestrained inflammatory reactions (38). Characterizing the behavior of dendritic cells in murine PCM, Ferreira et al. observed increased expression of TLR2 by dendritic cells of susceptible, but not resistant, mice (26). Moreover, it has been suggested that TLR2, TLR-4, and dectin-1 are involved in the recognition and internalization of *P. brasiliensis* by human monocytes and neutrophils, indicating important roles for these pathogen receptors in the immune response against the fungus (10).

We decided to investigate the role of TLR4 in murine PCM by using *in vitro* and *in vivo* models of infection. Using TLR-competent C3H/HePas mice and C3H/HeJ mice, which possess a missense mutation in the TLR4 gene, we were able to demonstrate that both *in vitro* and *in vivo*, TLR4 signaling increases the severity of infection in association with increased activation of innate and adaptive immunity but decreased expansion of Treg cells. This pattern of immunity, however, was not beneficial to the hosts, due to the increased lung injury mediated by inefficient control of inflammatory reactions by Treg cells.

MATERIALS AND METHODS

Fungus. *P. brasiliensis* Pb18, a highly virulent isolate, was used throughout this investigation (36). Pb18 yeast cells were maintained by weekly subcultivation in semisolid culture medium. Washed yeast cells were adjusted to 20×10^6/ml (for *in vivo* infection) and 4×10^4/ml (for *in vitro* infection) based on hemocytometer counts. Viability was determined with Janus Green B vital dye (Merck) and was always higher than 85%.

Mice and i.t. infection. C3H/HeJ and C3H/HePas mice were obtained from our Isogenic Breeding Unit (Departmento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil) and were used at 8 to 12 weeks of age. The C3H/HeJ strain has a point mutation in the TLR4 gene, and the C3H/HePas strain has a functional TLR4 gene. In selected experiments, C57BL/6 mice that are genetically deficient in TLR4 (TLR4 knockout [KO]) and their normal C57BL/6 counterparts were used. Mice were anesthetized and subjected to intratracheal (i.t.) *P. brasiliensis* infection as previously described (18). Briefly, after intraperitoneal anesthesia, the animals were i.t. infected with 10^6 *P. brasiliensis* yeast cells, contained in 50 μl of phosphate-buffered saline (PBS). Mice were studied at 96 h, 2 weeks, and 11 weeks postinfection. The experiments were approved by the ethics committee on animal experiments of our institution.

Phagocytic and fungicidal assays. Thioglycolate-induced peritoneal macrophages were either isolated by adherence (2 h at 37°C under 5% CO_2) to plastic-bottom tissue culture plates (1×10^6 cells/well in 24-well plates for fungicidal assays) or plated onto 13-mm-diameter round glass coverslips (1×10^6 cells/well in 24-well plates) for phagocytosis. Macrophages were washed to remove nonadherent cells and were cultivated overnight with fresh complete medium (Dulbecco's modified Eagle's medium [DMEM; Sigma], containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) in the presence or absence of recombinant gamma interferon (IFN-γ; 20 ng/ml in culture medium; BD Pharmingen). Nonadherent cells were counted in order to evaluate the number of remaining adherent cells used in phagocytic and killing assays. For phagocytic assays, macrophage cultures were infected with *P. brasiliensis* yeasts at a macrophage/yeast ratio of 25:1. This ratio was previously determined and was shown not to be deleterious to macrophage cultures and to be adequate for phagocytosis and killing assays (18, 49). The cells were cocultivated for 4 h at 37°C under 5% CO_2 to allow adhesion and ingestion of fungi. Cells were washed twice with PBS to remove any noningested or nonadhered yeasts, and samples were processed for microscopy. Cells were fixed with methanol and stained with Giemsa stain (Sigma). Experimental conditions were performed in triplicate, and the number of phagocytosed or adhered yeasts per 1,000 macrophages was evaluated on at least three separate slides. For fungicidal assays, IFN-γ-primed and unprimed macrophage cultures were infected with *P. brasiliensis* yeasts as described above. After 48 h of culture at 37°C in a CO_2 incubator, plates were centrifuged ($400 \times g$, 10 min, 4°C), and supernatants were stored at −70°C and were further analyzed for the presence of nitrite and cytokines. The wells were washed with distilled water to lyse macrophages, and suspensions were collected in individual tubes. One hundred microliters of cell homogenates was assayed for the presence of viable yeasts. All assays were done with five wells per condition in more than three independent experiments.

CFU assay and histological and morphometric analyses. The numbers of viable microorganisms in cell cultures and infected organs (lungs, liver, and spleen) from experimental and control mice were determined by counting the number of CFU. Animals from each group were sacrificed, and the enumeration of viable organisms was done as previously described (59). The numbers ($\log_{10}$) of viable *P. brasiliensis* organisms per gram of tissue (*in vivo*) or per milliliter of cell homogenate (*in vitro*) are expressed as means ± standard errors. For histological examinations, the left lung of the infected mouse was removed, fixed in 10% formalin, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) for analysis of the lesions and were silver stained for fungal evaluation. Pathological changes were analyzed based on the size, morphology, and cell composition of granulomatous lesions, the presence of fungi, and the intensity of the inflammatory infiltrates. Morphometric analysis was performed using a Nikon DXM 1200c digital camera (magnification, ×10), and Nikon NIS Elements AR 2.30 software. The areas of lesions were measured (in square micrometers) in 10 microscopic fields per slide for 10 animals per group. Results were expressed as the mean total area of lesions for each animal ± the standard error.

Measurement of cytokines and NO. Supernatants from lung homogenates or cell cultures were separated and stored at −70°C. The levels of interleukin-2 (IL-2), IL-4, IL-5, IL-23, IL-17, IL-12, IL-10, tumor necrosis factor alpha (TNF-α), IFN-γ, and the chemokine MCP-1 (monocyte chemoattractant protein 1) were measured by a capture enzyme-linked immunosorbent assay (ELISA) with antibody pairs purchased from BD Pharmingen. The ELISA procedure was performed according to the manufacturer's protocol, and absorbances were measured with a Versa Max microplate reader (Molecular Devices). NO production was quantified by the accumulation of nitrite in the supernatants from *in vitro* and in *vivo* protocols by a standard Griess reaction. All determinations were performed in duplicate, and results were expressed as micromolar concentrations of NO.

Measurement of serum *P. brasiliensis*-specific isotypes. Levels of specific isotypes (total IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3) were measured by a previously described ELISA (18) employing a cell-free antigen prepared by using a pool of different *P. brasiliensis* isolates (Pb339, Pb265, and Pb18). The average of the optical densities obtained with sera from control mice (PBS inoculated), diluted 1:20, was considered the cutoff for each respective isotype. Optical densities for each dilution of experimental sera were compared to control values. The titer for each sample was expressed as the reciprocal of the highest dilution that presented an absorbance higher than the cutoff.

Assessment of leukocyte subpopulations in lung inflammatory exudates. After 2 and 11 weeks of infection, lungs from each mouse were digested enzymat-

ically for 30 min with collagenase (1 mg/ml) and DNase (30 μg/ml) in culture medium (Sigma). Lung cell suspensions were centrifuged in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris. Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was >85%. For differential leukocyte counts, samples of lung cell suspensions were cytospun (Shandon Cytospin) onto glass slides and stained with the Diff-Quik blood stain (Baxter Scientific). A total of 200 to 400 cells were counted from each sample. For flow cytofluorometry, lung leukocytes were resuspended at 10^6/ml in staining buffer (PBS with 0.1% NaN_3 and 1% fetal calf serum). Fc receptors were blocked by unlabeled anti-CD16/32 antibodies (BD Biosciences), and cells were stained for 20 min at 4°C. Phycoerythrin (PE)-labeled monoclonal antibodies against CD40, CD86, CD11b, CD25, and CD69 and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against IA^k, CD80, CD4, and CD8 (BD Biosciences) were used. Treg cells were characterized by intracellular staining for Foxp3, using the Treg staining kit of BD Bioscience. Surface staining of $CD25^+$ and intracellular FoxP3 expression were backgated on the $CD4^+$ T-cell population. Cells were fixed with 1% paraformaldehyde (Sigma) and were stored in the dark at 4°C until analysis in a flow cytometer. The acquisition and analysis gates were restricted to the lymphocytes or macrophages. The expression of leukocyte markers on the cell surface and the intracellular expression of FoxP3 in lung-infiltrating leukocytes were analyzed in a FACScalibur flow cytometer (BD Pharmingen) using FlowJo software (Tristar).

***Limulus* amoebocyte lysate activity assay.** Solutions used for the preparation of yeast cell suspensions and the cultivation of macrophages were tested for the presence of LPS using the *Limulus* amoebocyte lysate chromogenic assay (E-TOXATE; Sigma) and always showed LPS levels lower than 0.015 endotoxin unit (EU)/ml.

Statistical analyses. Data were analyzed by Student's *t* test or two-way analysis of variance depending on the number of experimental groups. *P* values under 0.05 were considered significant.

RESULTS

TLR4 deficiency leads to less-severe fungal infection of macrophages, associated with decreased synthesis of NO, IL-12, and MCP-1. Before performing fungicidal studies, we asked whether the initial interactions between *P. brasiliensis* yeasts and peritoneal macrophages from C3H/HeJ and C3H/HePas mice were equivalent. Macrophage cultures (1×10^6 cells/well), performed in round glass coverslips, were preactivated with IFN-γ (20 ng/ml) or not and were infected with 4×10^4 viable yeasts (fungus/macrophage ratio, 1:25). After 4 h of incubation, supernatants were aspirated, the monolayers were gently washed with PBS, and the cells were stained with Giemsa stain. An average of 1,000 macrophages were counted, and the number of ingested and/or adherent yeasts was determined. As shown in Fig. 1A, TLR4-deficient macrophages presented a lower number of associated (ingested/adhered) yeasts than normal macrophages.

Macrophages were cultivated with *P. brasiliensis* yeasts for an additional 48 h. Supernatants were removed and assayed for the presence of nitric oxide and cytokines, and cell homogenates were plated for CFU determinations. As can be seen in Fig. 1B, TLR4 signaling resulted in increased recovery of viable yeasts from untreated and IFN-γ-primed macrophages. In addition, higher levels of NO were produced by IFN-γ-activated macrophages from TLR4-normal mice than by those of TLR4-defective mice (Fig. 1C). *P. brasiliensis* infection of unprimed macrophages did not induce NO production, which was detected only in IFN-γ-treated macrophages.

We further asked if TLR4 expression or signaling was involved in the decreased fungal loads observed with TLR4-defective macrophages. Since C3H/HeJ mice have a defect in the intracellular signaling domain but a normal extracellular moiety, we comparatively assessed the behavior of macrophages genetically deficient for TLR4 expression. Thus, $TLR4^{-/-}$ macrophages from C57BL/6 mice were infected and compared with their $TLR4^{+/+}$ counterparts. TLR4-deleted macrophages showed decreased phagocytic ability (Fig. 1D), and decreased numbers of viable yeasts were recovered from killing assays (Fig. 1E). As with C3H/HeJ macrophages, $TLR4^{-/-}$ cells produced diminished levels of NO. This result suggested that TLR4 signaling influenced the endocytic and killing ability of *P. brasiliensis*-infected macrophages.

To further characterize the role of TLR4 in *P. brasiliensis* infection of C3H macrophages, culture supernatants obtained from killing assays were tested for the presence of some macrophage-activating cytokines (IL-12 and TNF-α), a deactivating cytokine (IL-10), and a chemokine involved in mononuclear cell chemotaxis, MCP-1. As depicted in Fig. 2, IFN-γ-treated and untreated macrophages from TLR4-defective mice secreted lower levels of IL-12 and MCP-1 than those of TLR4-normal mice. IL-10 and TNF-α, however, appeared at similar levels.

***In vivo*, the absence of TLR4 signaling induces lower fungal loads and diminished NO production.** To study the *in vivo* role of TLR4, groups of C3H/HeJ and C3H/HePas mice (six to eight animals per group) were infected i.t. with 1 million *P. brasiliensis* yeast cells and were evaluated in the course of infection. Diminished fungal burdens were detected in the lung tissues of mice lacking functional TLR4 at all postinfection times (96 h and 2 and 11 weeks) assayed, as can be seen in Fig. 3A. In both strains, no fungal growth was observed in liver and spleen tissues (data not shown). Decreased NO levels were detected at 96 h and at week 11 after infection, although by week 2 similar levels were observed (Fig. 3B).

Defective TLR4 signaling determines decreased inflammatory reactions characterized by lower numbers of activated macrophages and T cells. We further analyzed the phenotype and activation of lung inflammatory cells at weeks 2 and 11 of *P. brasiliensis* infection (Fig. 4). To determine the activation profile of pulmonary macrophages, the expression of CD11b, major histocompatibility complex (MHC) class II (IA^K), CD80, CD86, and CD40 antigens was assessed by flow cytometry. As can be seen in Fig. 4A, all activation markers were expressed at lower levels by deficient macrophages, although significant differences were noticed with CD11b, the MHC class II antigen, and CD86. To determine the lymphocyte influx and the activation profile of $CD4^+$ and $CD8^+$ T cells in the lungs of *P. brasiliensis*-infected mice, we determined the expression of CD69 and CD25 by T cells freshly isolated from the lungs. The marker CD69 is a very early activation antigen (70), as well as CD25, the α-chain of the interleukin-2 receptor (56), which is rapidly upregulated on activated T cells. Compared with the control group, at week 2 of infection, TLR4-deficient mice presented significantly reduced recruitment of $CD4^+$ and $CD8^+$ T cells to the lungs, and the latter subpopulation also showed decreased expression of CD69 (Fig. 4B). Studies at week 11 postinfection confirmed those of week 2. TLR4-normal mice presented increased numbers of $CD11b^+$, $CD11c^+$, and $CD40^+$ macrophages (Fig. 4C), besides augmented numbers of $CD4^+$, $CD8^+$, and $CD8^+$ $CD69^+$ T lymphocytes, in the inflammatory exudates of lungs (Fig. 4D).

The limited inflammatory reaction of TLR4-deficient mice was associated with increased numbers of Treg cells. Because

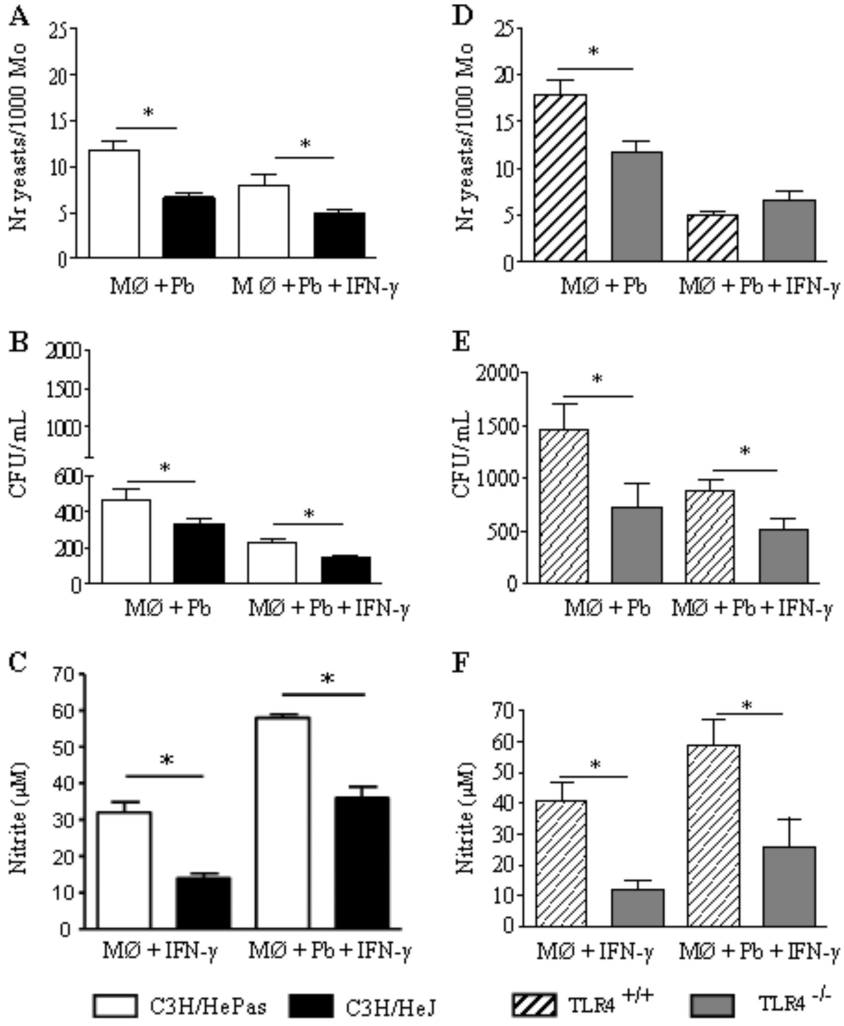

FIG. 1. TLR4 deficiency leads to less-severe fungal infection of macrophages (Mϕ) associated with decreased synthesis of NO. The phagocytic and fungicidal abilities of macrophages from mice with defective TLR4 signaling (C3H/HeJ) or defective TLR4 expression (C57BL/6 TLR4$^{-/-}$) were compared with those of their TLR4-normal controls (C3H/HePas and C57BL/6 TLR4$^{+/+}$, respectively). (A and D) For phagocytic assays, IFN-γ-primed (20 ng/ml, overnight) and unprimed macrophage cultures were infected with *P. brasiliensis* yeasts at a macrophage/yeast ratio of 25:1. The cells were cocultivated for 4 h at 37°C under 5% CO_2 to allow adhesion and ingestion of fungi. Cells were washed, fixed, and stained with Giemsa stain; an average of 1,000 macrophages were analyzed, and the number of macrophages with adhered or ingested yeasts was determined. (B and E) For fungicidal assays, IFN-γ-primed and unprimed macrophages were infected with yeast cells as described for panel A. After 48 h at 37°C under 5% CO_2, plates were centrifuged, and supernatants were used to determine levels of nitrite and cytokines. The monolayers were washed with distilled water to lyse macrophages, and 100 μl of cell homogenates was assayed for the presence of viable yeasts by a CFU assay. (C and F) Supernatants from fungicidal assays were used to determine the levels of nitrites using the Griess reagent. Data are means ± standard errors of the means for quintuplicate samples from one experiment representative of three independent determinations. *, $P < 0.05$.

Treg cells control the expansion of effector T cells, and because the number and function of these cells have been shown to be influenced by TLR4 activation (33), we investigated the presence of CD4$^+$ CD25$^+$ FoxP3$^+$ T cells in the lung cell infiltrates of TLR4-defective and normal mice (Fig. 5). At both postinfection periods studied, TLR4-defective mice showed increased numbers of CD4$^+$ CD25$^+$ FoxP3$^+$ Treg cells in their lungs (Fig. 5).

TLR4 dysfunction leads to diminished production of proinflammatory and Th17 cytokines. Levels of cytokines associated with Th1, Th2, and Th17 cells were assessed in lung homogenates obtained at different periods of infection. The production of type 1 (IL-12, TNF-α, and IFN-γ) and type 2 (IL-4, IL-5, and IL-10) cytokines, as well as that of the Th17-associated (IL-17, IL-6, TGF-β, and IL-23) cytokines, was studied 96 h, 2 weeks, and 11 weeks after infection. Mice lacking the ability to signal through TLR4 showed early (96 h) deficient production of IL-12, TNF-α, IL-17, and IL-6 (Fig. 6A). By week 2, IL-17 and IL-23 appeared at lower levels in the lungs of TLR4-defective mice (Fig. 6B). This decreased production of cytokines was confirmed at week 11, when these mice presented decreased amounts of IL-12, IL-17, and TGF-β (Fig. 6C). Interestingly, IL-17 and MCP-1 were constantly produced at higher levels by TLR4-normal mice (Fig. 6).

TLR4-defective mice produced lower levels of *P. brasiliensis*-specific antibodies. Although in some fungal infections specific

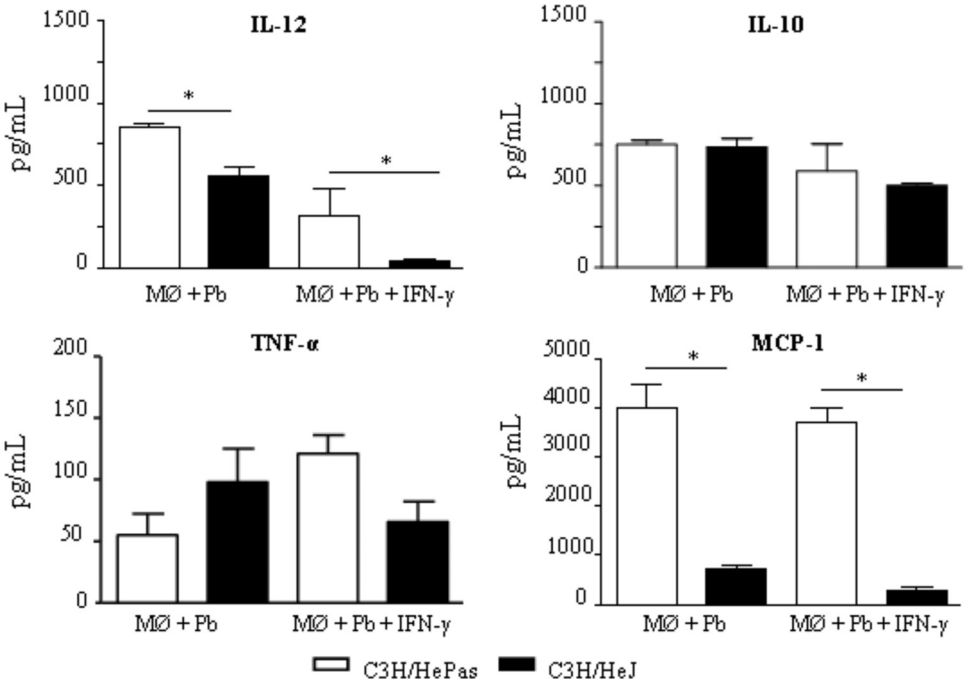

FIG. 2. Macrophages (Mϕ) from TLR4-defective mice secrete diminished levels of IL-12 and MCP-1. IFN-γ-treated (20.00 ng/ml) or untreated macrophages of TLR4-competent (C3H/HePas) and TLR4-defective (C3H/HeJ) mice were challenged with viable *P. brasiliensis* yeasts (fungus/macrophage ratio, 1:25) for 4 h, washed, and further cultivated for 48 h at 37°C under 5% CO_2. Plates were then centrifuged, and supernatants were used for cytokine measurements by ELISA. Data are means ± standard errors of the means for triplicate samples from one experiment representative of three independent determinations. ∗, $P < 0.05$.

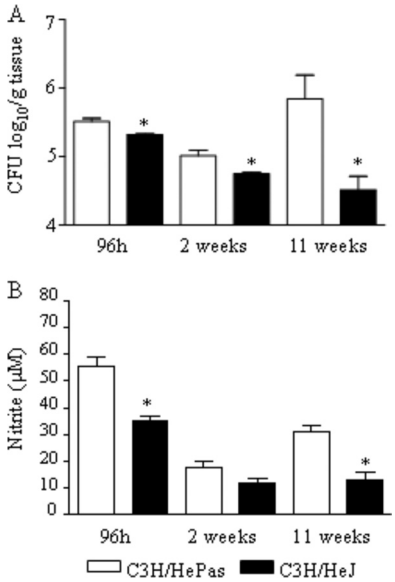

FIG. 3. *In vivo*, TLR4 dysfunction leads to less-severe fungal infection. (A) Recovery of CFU from the lungs of TLR4-defective and TLR4-normal control mice infected i.t. with 1×10^6 yeasts. (B) Lung homogenates were used to determine the levels of nitrites using the Griess reagent. Data are means ± standard errors of the means for groups of six to eight mice at 96 h, 2 weeks, and 11 weeks after infection. The results are representative of three independent experiments. ∗, $P < 0.05$.

antibodies may have a protective role (19), in human and experimental PCM antibody production is a marker of disease severity (13, 17). Since expression of TLRs has been shown to influence B-cell activation (30, 40), we decided to characterize the humoral immunity of TLR4-deficient and normal mice at week 11 postinfection. The less-severe infection of TLR4-deficient mice was associated with decreased production of IgG1-, IgG2b-, and IgM-specific isotypes (Fig. 7).

The absence of TLR4 signaling does not increase mortality rates but results in less-severe inflammatory reactions in the lungs. To verify the influence of TLR4 deficiency in the disease outcome, the mortality of *P. brasiliensis*-infected C3H/HeJ and C3H/HePas mice was registered daily for a 250-day period, and the median survival time was calculated for each strain. Surprisingly, despite the significant differences in fungal burdens, no differences between mortality data ($P = 0.9$) were detected (Fig. 8A). The mean survival times of C3H/HeJ and C3H/HePas mice were 221 and 203 days, respectively. At day 250 (35 weeks postinfection), the remaining survivors were sacrificed, and the numbers of CFU in organs were determined. Compared with those at other postinfection periods assayed, lower fungal burdens were detected in both mouse strains, but TLR4-normal mice still had higher pulmonary fungal loads than TLR4-defective mice (Fig. 8B). To further characterize the severity of *P. brasiliensis* infection, histopathological examination of lungs was conducted at week 11 of infection. As can be seen in Fig. 8C and E, similar patterns of inflammatory reactions were detected in the lungs of the two mouse strains, but better-preserved lung tissue was seen in TLR4-defective

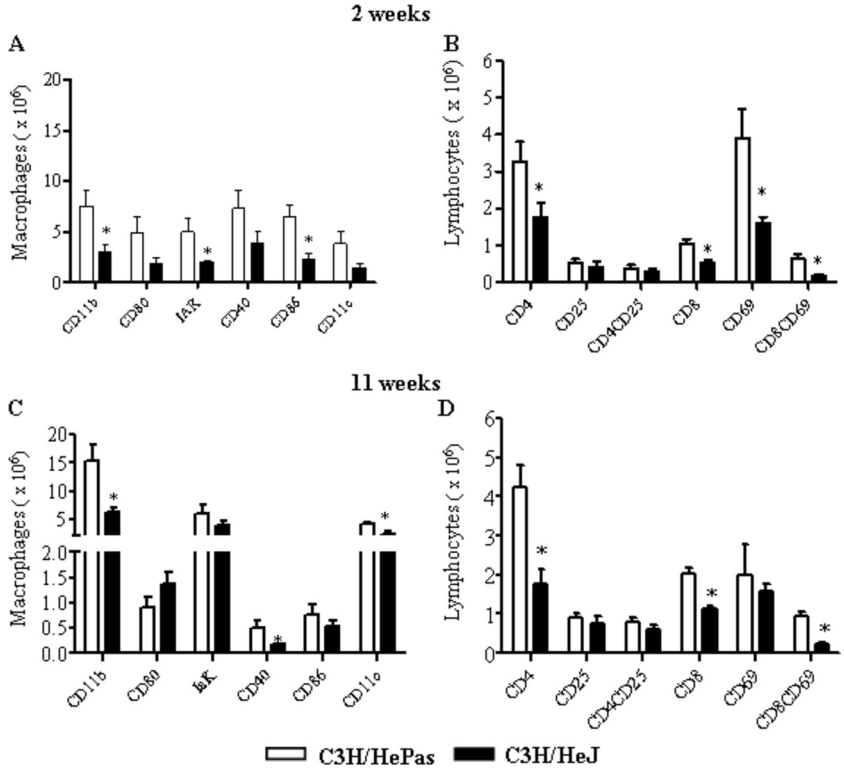

FIG. 4. Increased numbers of activated macrophages, $CD4^+$ T lymphocytes, and $CD8^+$ T lymphocytes were detected in the lungs of TLR4-competent mice at weeks 2 and 11 of infection. Leukocyte subsets in the lung-infiltrating leukocytes (LIL) from TLR4-defective and TLR4-normal mice inoculated i.t. with 1 million *P. brasiliensis* yeast cells were characterized by flow cytometry. Lungs of C3H/HePas and C3H/HeJ mice (six to eight mice per group) were excised, washed in PBS, minced, and digested enzymatically. At weeks 2 and 11 after infection, lung cell suspensions were obtained and stained as described in Materials and Methods. The acquisition and analysis gates were restricted to lymphocytes or macrophages. The data are mean results from six to eight mice per group ± standard errors of the means and are representative of two independent experiments. *, $P < 0.05$.

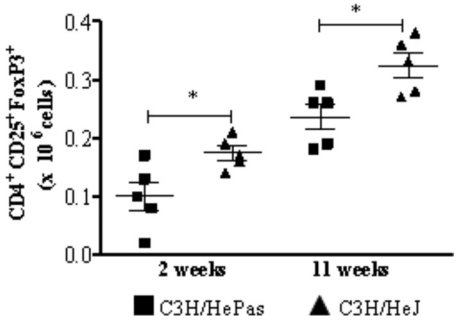

FIG. 5. TLR4-defective mice presented increased numbers of Treg cells in the lungs. FoxP3 expression by lung lymphocytes from TLR4-defective (C3H/HeJ) and normal (C3H/HePas) mice inoculated i.t. with 1 million *P. brasiliensis* yeast cells was determined by flow cytometric analysis. Lungs of six to eight mice per group were excised, washed in PBS, minced, and digested enzymatically; at 2 and 11 weeks after infection, cell suspensions were obtained and stained as described in Materials and Methods. The expression of leukocyte markers on the cell surface, as well as intracellular FoxP3 expression in lung-infiltrating leukocytes, was analyzed by flow cytometry. Surface staining of $CD25^+$ cells and intracellular FoxP3 expression were backgated on the $CD4^+$ T-cell population. The data are numbers of $CD4^+$ $CD25^+$ $FoxP3^+$ cells for individual mice (five or six per group) and are representative of two independent experiments.

mice. The pulmonary tissue presented several confluent or isolated granulomas of various sizes containing yeast cells with preserved morphology (Fig. 8D and F). Large aggregates of macrophages, rare epithelioid cells, and a poor mantle of lymphocytes made up the granulomas, which were usually in the interlobular septa. Plasma cells, eosinophils, and multinucleated cells were scarcely seen. The total areas of lesions were quantified in histological sections, and the results are shown in Fig. 8G. At week 11, the areas of lesions of TLR4-normal mice were significantly larger than those presented by TLR4-deficient mice. Thus, the higher influx of inflammatory cells observed in the lungs of TLR4-normal mice was concomitant with increased pathology of lung tissue.

DISCUSSION

The innate immune mechanisms of hosts infected with *P. brasiliensis* are poorly defined, but macrophages and their pathogen recognition receptors are thought to play a crucial role in the initial interaction of this fungus with the immune system (16, 26, 29, 49, 50). Despite several studies with diverse fungal pathogens (16, 43), the role played by TLRs in paracoccidioidomycosis is still unclear. In a previous report we were able to show the dual role played by TLR2 in the immunity to *P. brasiliensis* infection. TLR2 activation prevented un-

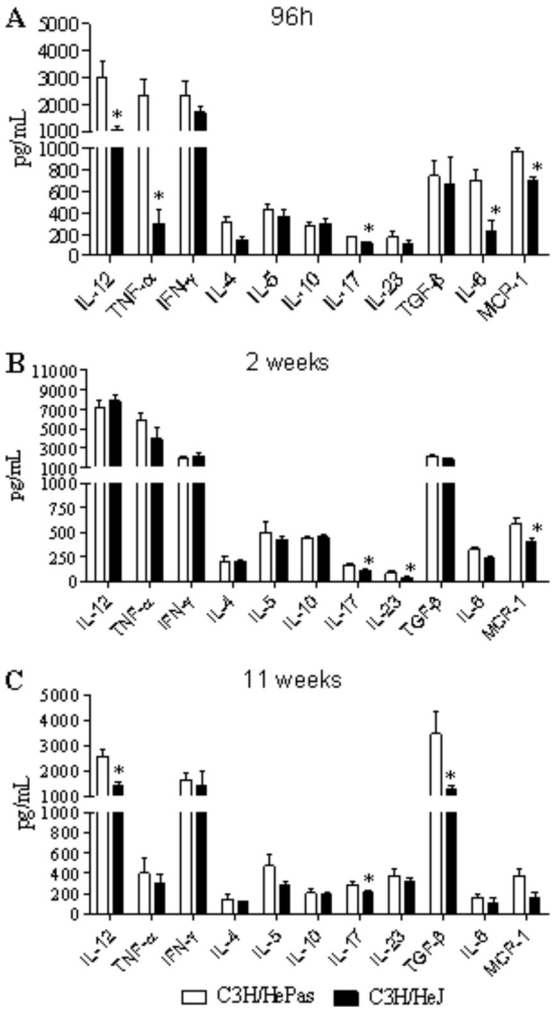

FIG. 6. Lung homogenates of TLR4-competent mice presented increased levels of proinflammatory cytokines. At 96 h, 2 weeks, and 11 weeks after i.t. infection with 10^6 *P. brasiliensis* yeast cells, lungs from TLR4-defective and TLR4-competent mice were collected and disrupted in 5.0 ml of PBS, and supernatants were analyzed for cytokine contents by capture ELISAs. Data are mean cytokine levels ± standard errors of the means (six to eight animals per group). The results are representative of three independent experiments. *, $P < 0.05$.

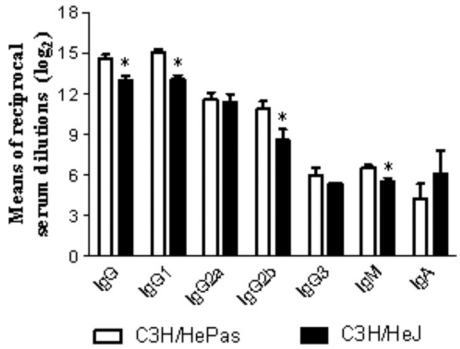

FIG. 7. TLR4 deficiency determines impaired humoral immunity. Levels of *P. brasiliensis*-specific antibodies in the sera of TLR4-defective (C3H/HeJ) and normal (C3H/HePas) mice at week 11 after i.t. infection with 1×10^6 yeast cells are shown. Sera were assayed for total IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 by using an isotype-specific ELISA as detailed in Materials and Methods. Data are mean serum titers (log_2) ± standard errors (six to eight mice per group). *, $P < 0.05$ for comparison with controls.

controlled inflammatory reactions in pulmonary paracoccidioidomycosis associated with increased expansion of Th17 cells and diminished function of Treg cells (38).

Initially we characterized the influence of TLR4 on the phagocytic and fungicidal abilities of macrophages. Both the absence of TLR4 expression by $TLR4^{-/-}$ C57BL/6 mice and defective TLR4 signaling (C3H/HeJ mice) resulted in deficient *P. brasiliensis* ingestion/adherence and lower fungal loads recovered 48 h after cocultivation. In both deficient mouse strains, lower levels of nitric oxide (and cytokines with C3H/HeJ cells) were detected, indicating that the lower CFU counts recovered were not due to increased activation of phagocytes and enhanced fungal killing but probably were due to decreased endocytosis of *P. brasiliensis* yeasts. TLRs usually do not act as phagocytic receptors, but their engagement by pathogen components results in strong activation of inflammatory responses (8, 9). There are, however, several examples demonstrating that cell signaling can influence endocytosis and vice versa (20, 23). Indeed, TLR4 was shown to actively participate in bacterial phagocytosis (4) and to be rapidly internalized by human monocytes after *in vitro* interaction with *P. brasiliensis* yeasts or *A. fumigatus* conidia (10, 21). In addition, a recent paper has clearly demonstrated that TLR4 and TLR2 synergize with class A scavenger receptor to mediate phagocytosis of Gram-negative and Gram-positive bacteria, respectively (3). Thus, we can suppose that TLR signaling facilitated the endocytosis of *P. brasiliensis* and further induced the activation of proinflammatory pathways, which, however, were not sufficient to control the early increased fungal loads. Since equivalent results were obtained with macrophages lacking TLR4 expression, we believe that TLR4 signaling could have influenced phagocytosis mediated by another pathogen receptor. Although our experiments have not identified the main PRR involved in initial *P. brasiliensis* recognition (particularly due to the number and complexity of components that comprise fungal cell walls), we have clearly demonstrated that TLR4 participates in the activation of innate immune cells required for the initial interaction with *P. brasiliensis* yeasts. Our *in vitro* findings were validated by *in vivo* experiments, which demonstrated that early in infection, TLR4-normal mice presented higher fungal loads than their TLR4-defective counterparts and that this was accompanied by increased activation of the immune system. Additional experiments with PRR agonists and antagonists using TLR4-normal and -deficient cells are needed, however, to further clarify the role of TLRs in pulmonary PCM.

Our *in vivo* data showed that mice expressing defective TLR4 developed a less-severe infection associated with lower production of nitric oxide and cytokines and less migration of inflammatory cells to the site of infection. The decreased presence of activated macrophages expressing CD11b, CD86, CD40, and MHC class II molecules was concomitant with reduced synthesis of MCP-1. In addition, the diminished presence of $CD4^+$ T cells and recently activated $CD69^+$ $CD8^+$ T cells in the lungs of TLR4-defective mice demonstrates that

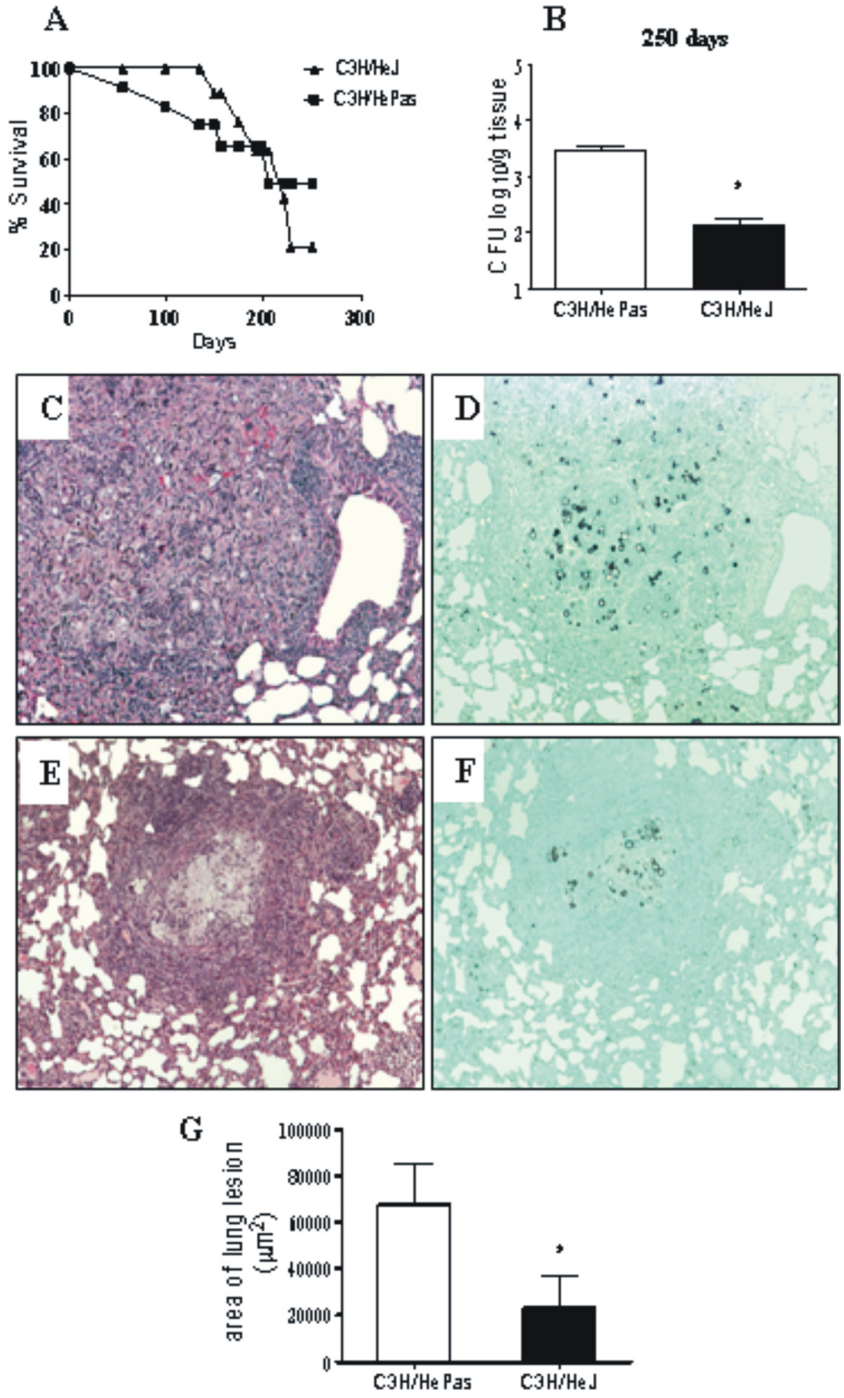

FIG. 8. Compared with TLR4-normal mice, TLR4-defective mice present decreased fungal loads and lung pathology but equivalent survival times. (A) Survival times of TLR4-defective and control mice after i.t. infection with 1×10^6 *P. brasiliensis* yeast cells were determined for a period of 250 days. No significant differences were seen between the median survival times of the two mouse strains. The results are representative of two independent experiments. (B) By 250 days after infection, survivor mice (three to six per group) were sacrificed, and CFU counts in tissues were determined. No viable yeasts were recovered from livers and spleens. (C to F) Photomicrographs of pulmonary lesions of TLR4-competent C3H/HePas mice (C and D) and TLR4-defective C3H/HeJ mice (E and F) at week 11 of infection with 1 million *P. brasiliensis* yeasts. At this period, the morphology of lesions was equivalent in the two mouse strains; fungal cells were surrounded by confluent or isolated granulomas scattered through the lung tissue. Lesions were stained with H&E (C and E) or with Grocott stain (D and F); magnification, ×100. (G) Total areas of lesions in the lungs of mice ($n = 10$) at week 11 after infection. *, $P < 0.05$.

TLR4 signaling is necessary to the proper activation of adaptive immunity to *P. brasiliensis* and enhanced migration of inflammatory cells into the lungs. Consistent with these observations, several reports have demonstrated that TLR4 signaling is needed for the activation and maturation of dendritic cells, which acquire the competent phenotype to preferentially differentiate naïve T cells to the Th1 or Th17 pattern (57, 64, 65). No differences in Th1 and Th2 cytokines, however, were detected in lung homogenates. The increased production of IL-12 and TNF-α concomitant with unaltered synthesis of Th2 cytokines (IL-4, IL-5, and IL-10) indicated, however, that TLR4 signaling promoted a cytokine milieu biased toward a proinflammatory balance. This cytokine response could have protected C3H/HePas mice from high fungal burdens due to the enhanced fungicidal mechanisms of activated phagocytes. Indeed, in experimental and human PCM, cytokine-activated phagocytes (activated mainly by IFN-γ, IL-12, and TNF-α) were shown to be the most important effector cells against *P. brasiliensis* infection (36, 39, 46, 55). Our data on cytokine production showed an additional fact not previously reported in PCM. The expression of TLR4 facilitated the expansion of IL-17-producing cells, since IL-17 and other Th17-associated cytokines (IL-6 and IL-23) appeared at higher levels in the lungs of TLR4-competent mice. In our previous report, we could verify that the absence of TLR2 signaling induced enhanced expansion of Th17 cells and that both $CD4^+$ and $CD8^+$ T cells displayed intracellular IL-17 (38). Further studies of the TLR4-deficient model will help us to characterize the phenotype of cells involved in IL-17 production.

TLR4 ligation is important for the activation of Th1 or Th17 responses (65), while TLR4-deficiency can lead to increased expansion of $CD4^+$ $CD25^+$ regulatory T cells (47, 48). When the presence of Treg cells in the lungs of Toll-deficient and control mice at weeks 2 and 11 of infection was assessed, increased numbers of $CD4^+$ $CD25^+$ $FoxP3^+$ cells were found in the lungs of TLR4-defective mice. This finding was associated with decreased fungal loads and diminished influx of inflammatory cells to the site of infection. Since Treg cells have been shown to control the inductive and effector phases of immunity against pathogens (5), we can suppose that Treg cells could have negatively controlled the expansion and migration of *P. brasiliensis*-specific T cells to the lungs. Thus, the advantage of low fungal loads conferred by TLR4 deficiency appeared to be negatively compensated for by deficient T-cell immunity and increased numbers of Treg cells, which appear to hamper the total clearance of fungal cells from the lungs.

At week 11 of infection, decreased levels of IL-12 were detected, probably due to the decreased migration of macrophages to the lungs. Interestingly, in C3H/HeJ mice, decreased levels of IL-17 were concomitant with diminished levels of TGF-β, indicating that another cytokine or costimulatory signal could have participated in the increased expansion of Treg cells (27).

Since the expression of TLRs has been shown to influence B-cell activation (40), we decided to analyze the levels of anti-*P. brasiliensis* isotypes in our model and observed an impaired humoral immune response in TLR4-defective mice. At week 11 of infection (Fig. 7), TLR4-deficient mice produced lower levels of IgG1-, IgG2b-, and IgM-specific antibodies. This could be due to the diminished fungal loads or the decreased production of cytokines observed in this mouse strain. Alternatively, since almost all TLR ligands were recently shown to induce the expansion and differentiation of B cells (30), we can suppose that TLR4 agonists present in *P. brasiliensis* yeasts could have exerted a stimulatory effect on B cells of TLR4-normal mice, resulting in increased humoral immunity. Independently of the mechanisms used, this is the first demonstration on the stimulatory role of TLR4 in the humoral immunity of *P. brasiliensis*-infected hosts.

TLR4 recognizes LPS of Gram-negative bacteria and favors Th1immunity due to the increased ability of LPS-stimulated DCs to produce IL-12 and TNF-α (51). In some fungal infections, however, cell wall polysaccharides have been reported to function as TLR agonists (42, 63). To our knowledge, no studies of paracoccidioidomycosis have addressed the characterization of TLR agonists. Although LPS or LPS-like components have not been characterized in *P. brasiliensis*, a few investigations have described the presence of polysaccharides, lipids, and glycolipids in *P. brasiliensis* cell walls (35, 61). The alkali-soluble fraction of *P. brasiliensis* cell walls has been shown to contain a high proportion of galactomannan (35), and it is tempting to suppose that this component could play a role in TLR4 activation.

Dectin and TLR4 signaling by microbial agonists has been reported to induce prevalent expansion of Th17 cells (1, 22, 34, 37). In our model, mice that possessed functional TLR4 were shown to have increased levels of IL-17 and other Th17-associated cytokines in their lungs. Although not investigated in the present work, IL-17-mediated immunity has been shown to exert deleterious or protective effects in infectious processes (54, 58). Actually, Th17 immunity can protect hosts due to its proinflammatory and chemotactic effect on polymorphonuclear (PMN) cells. Conversely, the enhanced oxidative metabolism and increased synthesis of metalloproteinases can result in tissue pathology and a detrimental effect on the hosts (11, 68, 69). In our previous work on the role of TLR2 in pulmonary PCM, we could demonstrate the dual role of Th17 immunity. The increased presence of inflammatory neutrophils conferred immune protection by reducing fungal loads but also resulted in tissue pathology equivalent to that induced by higher fungal burdens (38).

Mortality studies, unexpectedly, demonstrated that TLR4 signaling does not influence disease outcome, since TLR4-competent and -deficient mice presented equivalent survival times. In the course of the disease, both mouse strains were able to control fungal growth and to develop granulomatous reactions. However, the higher fungal loads, the enhanced Tcell immunity, and the lower expansion of Treg cells resulted in more-extensive inflammatory lesions, which exerted a deleterious effect on the lungs of TLR4-normal mice. On the other hand, the inefficient T-cell immunity of TLR4-deficient mice, tightly controlled by Treg cells, was not sufficient to totally clear the diminished fungal loads of TLR4-defective mice, abolishing the initial advantage conferred by their defective phagocytic ability. In sum, our findings indicate that high fungal loads accompanied by enhanced inflammatory responses mediated by uncontrolled T-cell immunity are equivalent to low fungal loads poorly controlled by a deficient T-cell response. Both mechanisms of immunity result in the chronic evolution of infection and equivalent mortality rates.

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***Anexo D* ï TLR2 Is a Negative Regulatory of Th17 Cells and Tissue Pathology in a Pulmonary Model of Fungal Infection**

Loures, F.V.; Pina, A.; Felonato, M.; Calich, V.L.G.

TLR2 Is a Negative Regulator of Th17 Cells and Tissue Pathology in a Pulmonary Model of Fungal Infection[1]

Flávio V. Loures, Adriana Pina, Maíra Felonato, and Vera L. G. Calich[2]

To study the role of TLR2 in a experimental model of chronic pulmonary infection, TLR2-deficient and wild-type mice were intratracheally infected with *Paracoccidioides brasiliensis*, a primary fungal pathogen. Compared with control, TLR2$^{-/-}$ mice developed a less severe pulmonary infection and decreased NO synthesis. Equivalent results were detected with in vitro-infected macrophages. Unexpectedly, despite the differences in fungal loads both mouse strains showed equivalent survival times and severe pulmonary inflammatory reactions. Studies on lung-infiltrating leukocytes of TLR2$^{-/-}$ mice demonstrated an increased presence of polymorphonuclear neutrophils that control fungal loads but were associated with diminished numbers of activated CD4$^+$ and CD8$^+$ T lymphocytes. TLR2 deficiency leads to minor differences in the levels of pulmonary type 1 and type 2 cytokines, but results in increased production of KC, a CXC chemokine involved in neutrophils chemotaxis, as well as TGF-β, IL-6, IL-23, and IL-17 skewing T cell immunity to a Th17 pattern. In addition, the preferential Th17 immunity of TLR2$^{-/-}$ mice was associated with impaired expansion of regulatory CD4$^+$CD25$^+$FoxP3$^+$ T cells. This is the first study to show that TLR2 activation controls innate and adaptive immunity to *P. brasiliensis* infection. TLR2 deficiency results in increased Th17 immunity associated with diminished expansion of regulatory T cells and increased lung pathology due to unrestrained inflammatory reactions. *The Journal of Immunology,* 2009, 183: 1279–1290.

The initial interaction between immune cells and microorganisms is mediated by several types of receptors that recognize molecular patterns of pathogens and are collectively called pathogen recognition receptors. The TLRs constitute a molecular family that recognizes a wide range of microbes and their products known as pathogen-associated molecular patterns. TLRs are expressed in diverse innate immune cells such as polymorphonuclear neutrophils (PMN),[3] macrophages, dendritic cells, and lymphocytes. Their activation triggers a signaling cascade that results in an inflammatory response through production of proinflammatory cytokines and up-regulation of costimulatory molecules expression leading to initiation of antigenic-specific adaptive immune response (1–3).

Importantly, the TLR expression was also shown to induce anti-inflammatory mediators and to discriminate the functional states of distinct T cell subsets (4, 5). Naive CD4$^+$ T cells do not express significant levels of TLR2 and TLR4 mRNA and intracellular proteins, although activated and memory T cells express high levels of membrane-bound TLR2 and TLR4 (5). Besides its influence in the activation of innate immunity cells, recent evidences suggest that TLR2 signaling may regulate the expansion and function of CD4$^+$CD25$^+$ regulatory T cells (Tregs) (5). Indeed, the administration of TLR2 ligands to wild-type (WT) mice results in increased number CD4$^+$CD25$^+$ Tregs and TLR2$^{-/-}$ mice were shown to contain significantly fewer CD4$^+$CD25$^+$ Tregs than control mice (5–8).

As described for other microorganisms, TLRs were shown to be involved in host defense against different fungal pathogens. In vivo and in vitro studies demonstrated that *Cryptococcus neoformans* (9, 10), *Candida albicans* (11, 12), and *Aspergillus fumigatus* (13, 14) may signal through members of the TLR family, mainly TLR2 and TLR4. The contribution of individual TLRs to the immune response against pathogenic fungi depends on several factors such as the route of infection, the fungal morphotype, or the fungal species. Activation signals mediated by innate immunity receptors, however, are not always beneficial to the host and TLR activation can be used by pathogenic fungi to promote more severe infections (15–17).

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis* and constitutes the most prevalent deep mycosis in Latin America (18). Individuals from endemic areas who have inhaled mycelial fragments or fungal spores usually develop PCM infection which is characterized by positive delayed-type hypersensitive reactions but absence of specific Abs. The acute or severe form of the disease is associated with deficient cell immunity, high levels of Abs, and preferential secretion of type 2 cytokines, whereas the benign localized forms demonstrate preserved cell-mediated immunity, prevalent production of type 1 cytokines, and low levels of Abs (19, 20).

Our studies on the genetic susceptibility of hosts to *P. brasiliensis* infection characterized B10.A as a susceptible mouse strain due to its progressive and disseminated disease associated with impaired macrophage activation and the presence of high fungal loads in nonorganized lesions. On the other hand, A/J mice showed a regressive pattern of disease with well-organized lesions containing low numbers of yeast cells, positive cellular immunity, and

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; KO, knockout; Treg, regulatory T cell; PCM, paracoccidioidomycosis; PI, proliferation index; WT, wild type; i.t., intratracheal(ly); MFI, mean fluorescence intensity.

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macrophage activation. These features are similar to the severe and benign forms of human PCM (20, 21).

The alveolar macrophages are the first host cells that interact with *P. brasiliensis* cells and their activation is fundamental to control pathogen growth. The molecular mechanisms controlling the initial steps of *P. brasiliensis* and phagocytes interaction are not well understood. It is known, however, that normal macrophages are permissive to *P. brasiliensis* growth while cytokine-activated macrophages are able to restrain *P. brasiliensis* multiplication (22). It was previously demonstrated that C3b, mannose receptor, and gp43, the immunodominant Ag of *P. brasiliensis*, play an important role in the initial interaction between *P. brasiliensis* cells and mouse peritoneal macrophages (23–25). Interestingly, a recent work of our laboratory demonstrated that alveolar macrophages from susceptible mice are easily activated by IL-12 and IFN-γ and display an efficient fungal killing associated with increased secretion of NO and proinflammatory cytokines. In contrast, pulmonary macrophages from resistant mice are poorly activated by both cytokines, present inefficient killing activity and NO secretion, and this behavior was associated with the increased activity of endogenous TGF-β (26). Despite their inefficient innate immunity, A/Sn mice develop a balanced Th1/Th2 immunity which controls fungal growth without intense tissue pathology.

Since the contribution of TLR in *P. brasiliensis* infection was never studied before, we decided to investigate the role of TLR2 in murine PCM using in vitro and in vivo models of infection. Using TLR2-normal and TLR2-deficient C57BL/6 mice, we were able to demonstrate that, both in vitro and in vivo, the presence of TLR2 causes a more severe infection. Both approaches demonstrated that TLR2 are used by *P. brasiliensis* yeast to infect host cells inducing enhanced secretion of NO and cytokines. Unexpectedly, TLR2-deficient and WT mice presented similar survival times and equivalent severe lesions in the lungs. The lower fungal loads of TLR2-deficient mice were, however, associated with prevalent activation of Th17 immunity and exacerbated pulmonary inflammation containing high numbers of PMN leukocytes but diminished presence of Tregs. Altogether, our data indicate that expression of TLR2 has a beneficial effect on fungal pulmonary infection due to its negative control on Th17 immunity and tissue pathology. Furthermore, the present findings demonstrate that uncontrolled inflammatory response of hosts to *P. brasiliensis* infection is as deleterious as uncontrolled fungal growth by absence or inadequate activation of immunity.

Materials and Methods

Fungus

P. brasiliensis Pb18, a highly virulent isolate, was used throughout this investigation (27). Pb18 yeast cells were maintained by weekly subcultivation in semisolid culture medium. Washed yeast cells were adjusted to 20×10^6 cells/ml (in vivo infection) and 4×10^4 cells/ml (in vitro infection) based on hemocytometer counts. Viability was determined with Janus Green B vital dye (Merck) and was always higher than 85%.

Mice and intratracheal infection

TLR2$^{-/-}$ mice on a C57BL/6 background were provided by S. Akira (Osaka University, Osaka, Japan). C57BL/6 control (WT) mice were obtained from our Isogenic Breeding Unit (Departmento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo) and used at 8–12 wk of age. Mice were anesthetized and submitted to intratracheal (i.t.) *P. brasiliensis* infection as previously described (28). Briefly, after i.p. anesthesia, the animals were i.t. infected with 10^6 *P. brasiliensis* yeast cells contained in 50 μl of PBS. Mice were studied at 48 h, 2 wk, and 11 wk postinfection. The experiments were approved by the ethics committee on animal experiments of our institution.

Phagocytic and fungicidal assays

Thioglycolate-induced peritoneal macrophages were isolated by adherence (2 h at 37ºC in 5% CO_2) to plastic-bottom tissue culture plates (1×10^6 cells/well in 24-well plates for fungicidal assays) or plated onto 13-mm round glass coverslips (1×10^6 cells/well in 24-well plates) for phagocytosis. Macrophages were washed to remove nonadherent cells and cultivated overnight with fresh complete medium (DMEM (Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin) in the presence or absence of recombinant IFN-γ (20 ng/ml in culture medium; BD Pharmingen). Equivalent procedures were performed with alveolar macrophages obtained from *P. brasiliensis*-infected mice at week 2 of infection by bronchoalveolar lavage with 1.5 ml of warm PBS (26). For phagocytic assays, macrophage cultures were infected with *P. brasiliensis* yeast in a macrophage:yeast ratio of 50:1. The cells were cocultivated for 4 h at 37ºC in 5% CO_2 to allow fungi adhesion and ingestion. Cells were washed twice with PBS to remove any noningested or nonadhered yeast cells and samples were processed for microscopy. Cells were fixed with methanol and stained with Giemsa (Sigma-Aldrich). Experimental conditions were performed in triplicate, and the number of phagocytosed or adhered yeast cells per 1000 macrophages was evaluated on at least five separate slides. For fungicidal assays, IFN-γ-primed and unprimed macrophage cultures were infected with *P. brasiliensis* yeast as above described. After 48 h of culture at 37ºC in a CO_2 incubator, plates were centrifuged ($400 \times g$, 10 min, 4ºC), supernatants were stored at −70ºC, and further analyzed for the presence of nitrite and cytokines. The wells were washed with distilled water to lyse macrophages and suspensions were collected in individual tubes. One hundred microliters of cell homogenates were assayed for the presence of viable yeast cells. All assays were done with five wells per condition in more than three independent experiments.

CFU assays, mortality rates, and histologic analysis to determine severity of infections

The numbers of viable microorganisms in cell cultures and infected organs (lungs, liver, and spleen) from experimental and control mice were determined by counting the number of CFU. Animals from each group were sacrificed and the enumeration of viable organisms was done as previously described (29). The numbers ($\log_{10}$) of viable *P. brasiliensis* per g of tissue (in vivo) or per ml of cell homogenates (in vitro) are expressed as the means ± SEs. Mortality studies were done with groups of 9–11 mice inoculated i.t. with 1×10^6 yeast cells or PBS. Deaths were registered daily for a 250-day period and experiments were repeated twice. For histology examinations, the left lung of infected mice was removed, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were stained by H&E for an analysis of the lesions and silver stained (Grocott stain) for fungal evaluation. Pathologic changes were analyzed based on the size, morphology, and cell composition of granulomatous lesions, presence of fungi, and intensity of the inflammatory infiltrates.

Measurement of cytokines and NO

Supernatants from lung homogenates or cell cultures were separated and stored at −70°C. Cytokine (IL-2, IL-4, IL-5, IL-23, IL-17, IL-12, IL-10, IL-6, TNF-α, and IFN-γ) and chemokine (MCP-1 and KC) levels were measured by capture ELISA with Ab pairs purchased from BD Pharmingen. Active and acid-activatable latent TGF-β forms were also measured (kits from R&D Systems) in our biological fluids. The ELISA procedure was performed according to the manufacturer's protocol and absorbances were measured with a Versa Max Microplate Reader (Molecular Devices). NO production was quantified by the accumulation of nitrite in the supernatants from in vitro and in vivo protocols by a standard Griess reaction. All determinations were performed in duplicate and expressed as μM NO.

Assessment of leukocyte subpopulations in lung inflammatory exudates

After 2 and 11 wk of infection, lungs from each mouse were digested enzymatically for 30 min with collagenase (1 mg/ml) and DNase (30 μg/ml) in culture medium (Sigma-Aldrich). Lung cell suspensions were centrifuged in the presence of 20% Percoll (Sigma-Aldrich) to separate leukocytes from cell debris. Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was >85%. For differential leukocyte counts, samples of lung cell suspensions were cytospun (Shandon Cytospin) onto glass slides and stained by the Diff-Quik blood stain (Baxter Scientific). A total of 200–400 cells was counted from each sample. For flow cytofluorometry, lung leukocytes were resuspended at 10^6 cells/ml in staining buffer (PBS plus 0.1% NaN_3 plus and 1%

FCS). FcRs were blocked by unlabeled anti-CD16/32 Abs (BD Biosciences) and cells were stained for 20 min at 4°C. PE- labeled anti-CD40, anti-CD86, anti-CD11b, anti-CD25, and anti-CD69 and FITC-labeled anti-IA^K, anti-CD80, anti-CD4, and anti-CD8 mAbs (BD Biosciences) were used. Cells were fixed with 1% paraformaldehyde (Sigma-Aldrich) and stored in the dark at 4°C until analyzed in a flow cytometer. The acquisition and analysis gates were restricted to the lymphocytes or macrophages. Tregs were characterized by intracellular staining for Foxp3 using a Treg staining kit from BD Biosciences. Surface staining of $CD25^+$ and intracellular FoxP3 expression were back-gated on the $CD4^+$ T cell population. For intracellular cytokine staining, cells were stimulated for 6 h in complete medium in the presence of 50 ng/ml PMA, 500 ng/ml ionomycin (both from Sigma-Aldrich), and monensin (3 mM; eBioscience). After surface staining for CD4 and CD8, cells were fixed, permeabilized, and stained by PerCP-Cy5 anti-IL-17 Abs (eBioscience). The cell surface expression of leukocyte markers as well as intracellular expression of FoxP3 and IL-17 in lung-infiltrating leukocytes were analyzed in a FACSCalibur flow cytometer (BD Pharmingen) using the FlowJo software (Tree Star).

Fungicidal ability of neutrophils

To study the role of neutrophils in the control of fungal growth, PMN leukocytes from WT and TLR2 KO mice were in vivo depleted by i.p. administration of 0.5 ml of saline-diluted (1/5) rabbit anti-mouse PMN polyclonal Ab (Accurate Chemical & Scientific) at days −1, +3, and +5 of i.t. infection (0 time) with 1 million *P. brasiliensis* yeast cells. Control mice received equivalent amounts of normal rat IgG. The number of blood PMN cells was evaluated before treatment (at day −2) and 24 h after the mAb inoculation. Groups of IgG-treated and PMN-depleted mice (n = 4–6) were sacrificed 2 and 6 days after *P. brasiliensis* infection and severity of disease evaluated by CFU counts. Mortality studies were done with IgG-treated and PMN-depleted groups of eight to nine mice inoculated i.t. with 1×10^6 yeast cells. Deaths were registered daily for a 10-day period and experiments were repeated twice. Alveolar neutrophils were obtained by bronchoalveolar lavage of mice infected 2 wk earlier with 1 million yeast cells. The number of PMN leukocytes in the nonadherent fraction of lung cells was adjusted to 2×10^5 cells, plated in 96-well plates, challenged with 1×10^5 yeast cells, and cocultivated for 1 h at 37°C. One hundred microliters of cell homogenates was assayed for the presence of viable yeast cells. All assays were done with three wells per condition in more than two independent experiments.

Lymphoproliferation assay

Cells were assayed for proliferation using an in vitro fluorescence-based assay. Briefly, 1×10^6 cells from spleens were stained with 1 μl (5 mM) of CFSE (Molecular Probes) in PBS and 5% FCS for 15 min at room temperature. Stained cells were cultured for 3 days in the presence of anti-CD3 Abs (0.3 μg/ml), anti-CD28 (2.5 μg/ml) mAbs (BD Biosciences), *P. brasiliensis*-soluble Ag (100 μg/ml), or Con A (1 μg/ml; Sigma-Aldrich). A minimum of 20,000 events was acquired on a FACSCalibur flow cytometer using CellQuest software (BD Pharmingen). The proliferation index (PI) was calculated as the mean fluorescence intensity (MFI) of unstimulated cultures/MFI of stimulated cultures.

Statistical analysis

Data were analyzed by Student's t test or two-way ANOVA depending on the number of experimental groups. Values of $p < 0.05$ were considered significant.

Results

TLR2 deficiency leads to less severe fungal infection of macrophages associated with decreased synthesis of NO, IL-10, and MCP-1

Before performing fungicidal studies, we asked whether the initial interaction between *P. brasiliensis* yeast cells and peritoneal macrophages from $TLR2^{-/-}$ and WT mice was equivalent. Macrophage cultures (1×10^6/well), performed in round glass coverslips, were preactivated or not with IFN-γ (20 ng/ml) and infected with 2×10^4 viable yeast cells (1:50 fungus:macrophage ratio). After a 4-h incubation, supernatants were aspirated, the monolayers were gently washed with PBS, and the cells were stained with Giemsa. An average of 1000 macrophages was counted and the number of ingested and/or adherent yeast cells was determined. Compared with WT macrophages, a lower number of yeast cells associated (ingested/adhered) with $TLR2^{-/-}$ macrophages was observed (Fig. 1*A*). Peritoneal macrophages were cultivated with *P. brasiliensis* yeast cells for an additional 48-h period. Supernatants were removed and assayed for the presence of NO and cytokines and cell homogenates were plated for CFU determinations. As shown in Fig. 1*B*, the presence of functional TLR-2 led to increased recovery of viable yeast cells from untreated and IFN-γ-primed macrophages (20 ng/ml). In addition, higher levels of NO were produced by macrophages from WT mice than those of $TLR2^{-/-}$ mice (Fig. 1*B*). Additional experiments were performed with alveolar macrophages obtained from bronchoalveolar lavage fluids of normal, uninfected, WT, and TLR2-deficient mice (Fig. 1*C*). Lower CFU counts were recovered from untreated TLR2-deficient cells, although no significant differences were seen with IFN-γ-activated macrophages. As observed with peritoneal cells, TLR2-deficient alveolar macrophages synthesized lower levels of NO.

To better characterize the role of TLR2 in *P. brasiliensis* infection, culture supernatants of peritoneal macrophages were evaluated for the presence of some macrophage-activating cytokines (IL-12 and TNF-α), a deactivating cytokine (IL-10), and for a chemokine involved in mononuclear cell chemotaxis, MCP-1. As depicted in Fig. 2, IFN-γ-treated and untreated macrophages from $TLR2^{-/-}$ mice secreted decreased levels of IL-10 and MCP-1 than those from WT mice. IL-12 and TNF-α, however, appeared in similar levels.

In vivo, absence of TLR2 induces lower fungal loads but increased lung pathology

We infected groups (n = 6–8) of $TLR2^{-/-}$ and WT mice i.t. with 1 million *P. brasiliensis* yeast cells and evaluated parameters of infection and local inflammatory pathology over the course of infection. Diminished fungal burdens were detected in the lung tissue of $TLR2^{-/-}$ mice at all postinfection periods (48 h and 2 and 11 wk) assayed (Fig. 3*A*). These lower fungal burdens were accompanied by lower NO levels in lung homogenates (Fig. 3*B*). Equivalent fungal counts were detected in the liver and spleen tissue (our unpublished data).

To assess the influence of TLR-2 deficiency in disease outcome, the mortality of *P. brasiliensis*-infected $TLR\text{-}2^{-/-}$ and WT mice was registered daily for a 250-day period and the median survival time was calculated for each strain. Unexpectedly, despite the significant differences in fungal burdens, no differences between mortality data ($p = 0.104$) were detected (Fig. 4*A*). The mean survival times of $TLR\text{-}2^{-/-}$ and WT mice were 221 and 190 days, respectively. To better understand this result, histopathological examination of lungs was done at weeks 2 and 11 of infection. As can be seen in Fig. 4, *B–E*, an equivalent and severe pattern of inflammatory reactions was detected in the lungs of both mouse strains. The pulmonary lesions replaced almost all of the normal parenchyma and were composed of confluent granulomas of various sizes containing many fungal cells surrounded by a small ring of mononuclear cells. Some lesions were also surrounded by an evident fibrotic layer. Multinucleated cells were scarcely seen. Lesions of TLR2-deficient mice showed a more prominent presence of inflammatory polymorphonuclear cells and decreased numbers of *P. brasiliensis* yeast cells (Fig. 4, *D* and *E*).

$TLR2^{-/-}$ deficiency determines a sustained recruitment of PMN cells to the lungs

To better characterize the inflammatory reaction at the site of infection, leukocyte recruitment to the lung tissue of *P. brasiliensis*-infected $TLR2^{-/-}$ and WT mice was studied at weeks 2 and 11 of infection. As can be seen in Fig. 5, *A* and *B*, at both postinfection

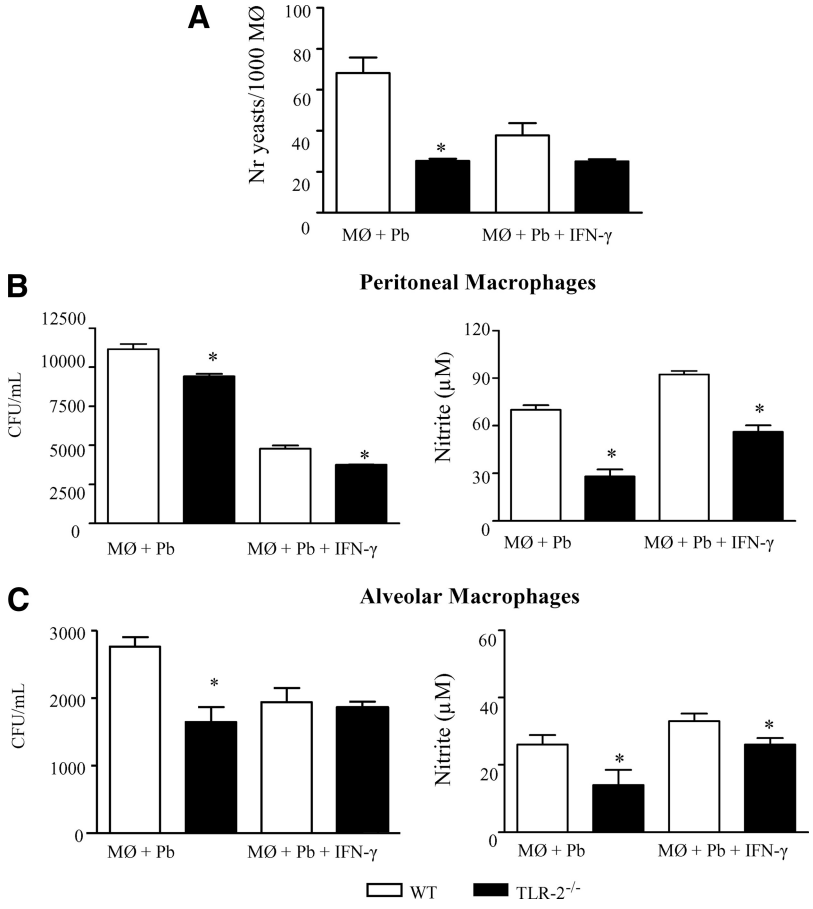

FIGURE 1. Macrophages (Mϕ) from TLR2-deficient mice have a decreased ability to interact with fungal yeast cells. *A*, For phagocytic assays, IFN-γ-primed (20 ng/ml, overnight) and unprimed peritoneal macrophage cultures were infected with *P. brasiliensis* yeast cells in a macrophage:yeast ratio of 50:1. The cells were cocultivated for 4 h at 37ºC in 5% CO_2 to allow fungi adhesion and ingestion. Cells were washed, fixed. and stained with Giemsa; an average of 1000 macrophages was analyzed and the number of macrophages with adhered or ingested yeast cells was determined. *B*, For fungicidal assays, IFN-γ-primed and unprimed peritoneal macrophages were infected with yeast cells as above described. After 48 h at 37ºC in 5% CO_2, plates were centrifuged and supernatants were used to determine the levels of nitrite and cytokines. The monolayers were washed with distilled water to lyse macrophages and 100 μl of cell homogenates was assayed for the presence of viable yeast cells by a CFU assay. Supernatants from fungicidal assays were used to determine the levels of nitrites using the Griess reagent. *C*, Alveolar macrophages were collected from bronchoalveolar lavage fluids of normal mice and studied as in *B* for fungicidal activity and NO production. Data are the mean ± SEM of quintuplicate samples from one experiment representative of three independent determinations. *, $p < 0.05$ between strains.

periods, increased numbers of polymorphonuclear cells (PMN) were observed in the lungs of TLR2$^{-/-}$ mice than in their normal controls. Consistent with these findings, 72 h after infection, significantly increased influx of PMN cells were detected in the bronchoalveolar lavage fluids of TLR2$^{-/-}$ mice (our unpublished data). At both postinfection periods, lower numbers of macrophages were recovered from lungs of TLR2-deficient mice, but only at week 2 was this difference significant. We further characterized the presence of neutrophil- and macrophage-mobilizing chemokines in the lung homogenates of WT and TLR2$^{-/-}$ mice. In good agreement with cell phenotypes detected at weeks 2 and 11 of infection, increased levels of KC were detected in

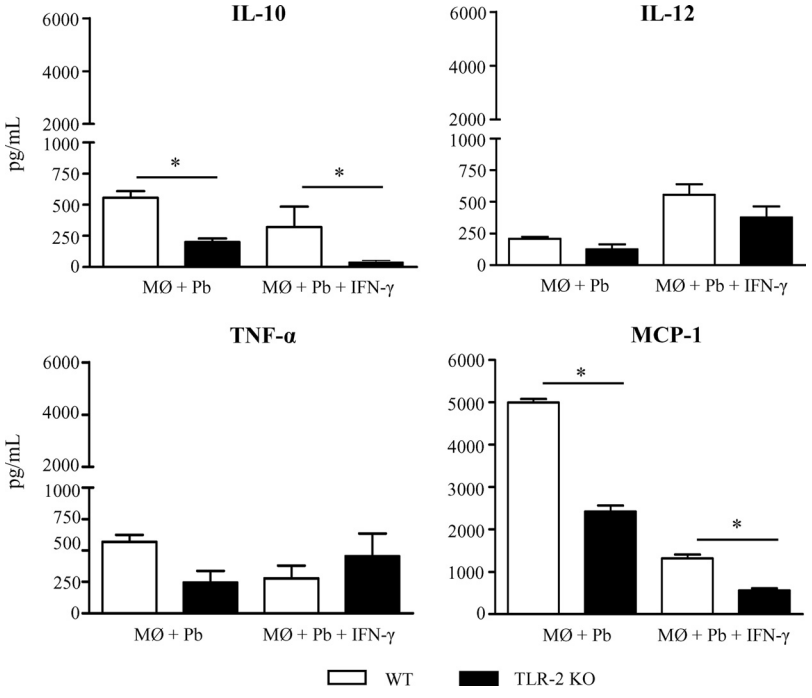

FIGURE 2. Macrophages (Mϕ) from TLR2$^{-/-}$ mice secrete diminished levels of IL-10 and MCP-1. IFN-γ treated (20.00 ng/ml) or untreated macrophages of TLR2$^{-/-}$ and TLR2$^{+/+}$ C57BL/6 mice were challenged with viable *P. brasiliensis* yeast cells (1:50, fungus:macrophage ratio) during 4 h, washed, and further cultivated for 48 h at 37°C in 5% CO_2. Plates were then centrifuged and supernatants used for cytokine measurements using ELISA. Data are the means ± SEM of triplicate samples from one experiment representative of three independent determinations. *, $p < 0.05$ between strains.

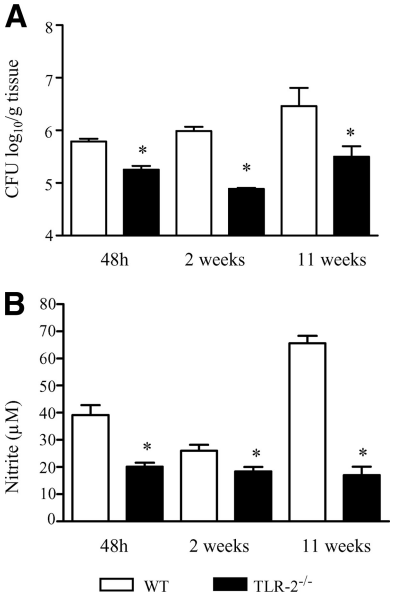

FIGURE 3. In vivo, absence of TLR2 induces lower fungal loads and NO synthesis. Depicted is the recovery of CFU (*A*) and NO (*B*) from the lungs of TLR2$^{-/-}$ and WT control mice infected i.t. with 1×10^6 yeast cells. The bars represent means ± SEM of $\log_{10}$ CFU obtained from groups of six to eight mice at 48 h and 2 and 11 wk after infection. The results are representative of three experiments. *, $p < 0.05$ between strains.

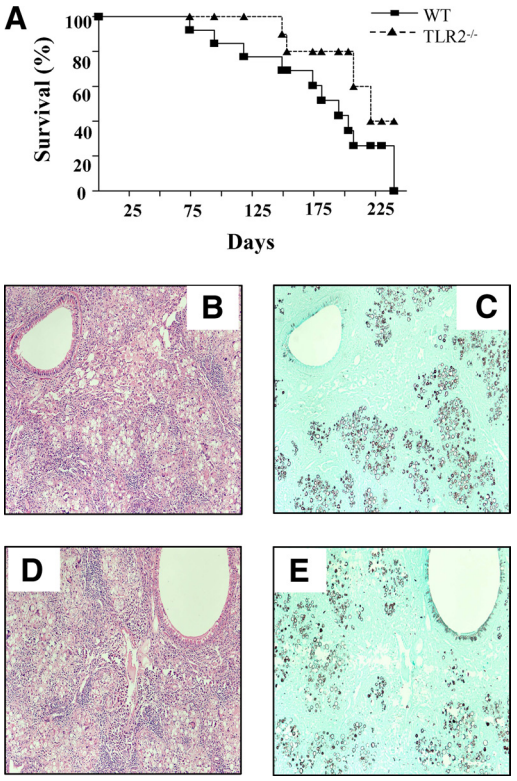

FIGURE 4. Despite the different fungal burdens, TLR2$^{-/-}$ and TLR2$^{+/+}$ mice present equivalent survival times and lung histopathology. *A*, Survival times of TLR2$^{-/-}$ and WT control mice after i.t. infection with 1×10^6 *P. brasiliensis* yeast cells was determined in a period of 250 days. No significant differences were seen in the median survival times of both mouse strains; the results are representative of two independent experiments. Photomicrographs of pulmonary lesions of WT (*B* and *C*) and TLR2$^{-/-}$ (*D* and *E*) mice at week 11 of infection with 1 million *P. brasiliensis* yeast cells. At this period, despite the higher fungal loads detected in WT mice, no differences in the severity of lesions between TLR2-deficient and control mice were noted. Both mouse strains presented extensive and confluent lesions occupying almost all lung parenchyma which presented an elevated number of yeast cells. H&E (*B* and *D*; ×100) and Grocott-stained lesions (*C* and *E*; ×100).

the lungs of deficient mice, whereas augmented levels of MCP-1 were found in WT supernatants (Fig. 5, *C* and *D*). Thus, TLR2 deficiency resulted in increased KC production and PMN recruitment while in TLR2-normal mice a prevalent MCP-1 synthesis led to enhanced macrophage chemotaxis to the site of infection.

Depletion of PMN leukocytes increases fungal loads and mortality rates of TL2-deficient but not normal mice

The finding that PMN cells were abundantly recruited to the lungs of TLR2$^{-/-}$ mice in the presence of diminished fungal growth led us to verify whether these cells had a significant antifungal effector function in our model of pulmonary PCM. Thus, TLR2$^{-/-}$ and TLR2$^{+/+}$ mice were in vivo depleted of PMN cells by a polyclonal Ab and infected i.t. with the fungus. By days 2 and 6 after infection, PMN-depleted TLR2$^{-/-}$ but not normal mice presented increased fungal burdens in the lungs (Fig. 6, *A* and *B*). The protective effect of PMN cells to deficient mice was confirmed by the precocious mortality developed by PMN-depleted mice. By day 5 of infection, 50% of PMN-depleted TLR2$^{-/-}$ mice died, although no deaths were observed in the PMN-depleted WT group (Fig. 6*C*). We have also evaluated the killing ability of alveolar PMN cells obtained from *P. brasiliensis*-infected mice. At week 2 of infection, airway PMN cells were obtained from TLR2$^{-/-}$ and normal mice and in vitro challenged with yeast cells (2:1, PMN:yeast ratio) for 1 h. As can be seen in Fig. 6*C*, equivalent numbers of viable yeast cells were recovered from both mouse strains, indicating that PMN cells from TLR2-deficient mice did not have an increased killing ability when compared with those from normal mice. Hence, the higher CFU counts detected in PMN-depleted TLR2$^{-/-}$ mice could be attributed to the higher influx of PMN leukocytes to the lungs but not to an intrinsically enhanced killing ability of these cells.

TLR deficiency determines diminished T cell reactivity and influx to the lungs

We have further analyzed the phenotype and activity of lung inflammatory cells of TLR2$^{-/-}$ and WT mice infected i.t. with 1 million *P. brasiliensis* yeast cells. To determine the activation profile of pulmonary macrophages, the expression of CD11b, MHC class II (I-A^k), CD80, CD86, and CD40 Ags was assayed by flow cytometry. As can be seen in Fig. 7, no differences in lung macrophages were detected at weeks 2 and 11 of infection. The phenotypic analysis of lymphocyte subsets was also performed. Thus, the expression of CD4, CD25, CD8, and CD69 molecules was studied in lung-infiltrating lymphocytes. As depicted in Fig. 7*A*, at week 2 of infection, no differences in the number and activation of T cells were observed. However, a decreased frequency of activated CD4$^+$ T lymphocytes expressing CD25 (CD4$^+$CD25$^+$) was detected in TLR2$^{-/-}$ animals at this period of infection (data not shown). By week 11, however, significantly decreased numbers of CD4$^+$, CD8$^+$, and CD8$^+$CD69$^+$ T cells were detected in TLR2$^{-/-}$ animals (Fig. 7*B*).

To characterize the lymphoproliferative activity of spleen cells obtained from WT and TLR2$^{-/-}$ mice at week 4 of infection, CFSE-labeled lymphocytes were in vitro stimulated with *P. brasiliensis* Ag,

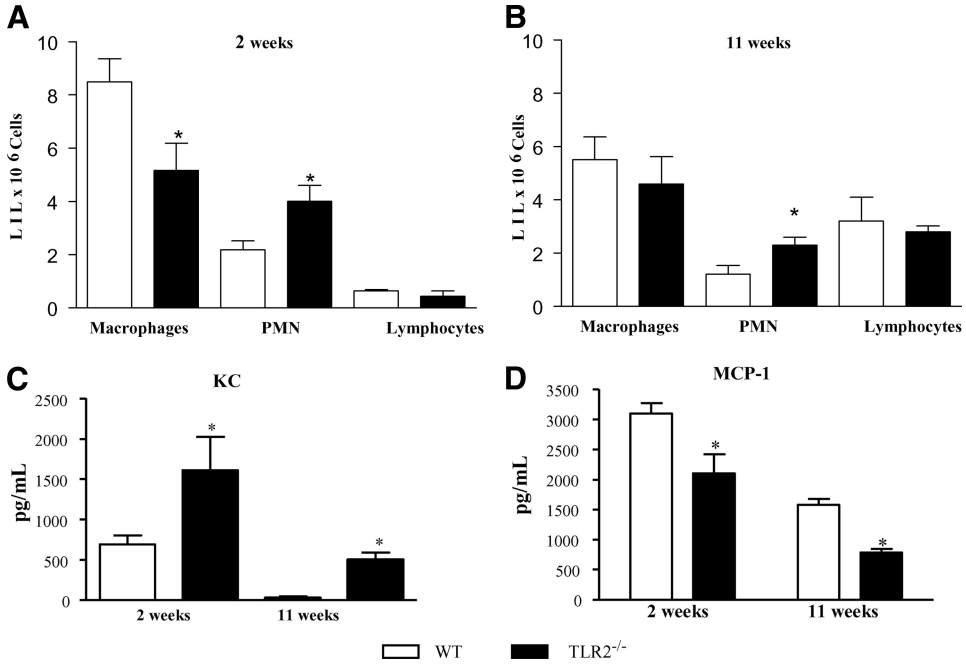

FIGURE 5. TLR2$^{-/-}$ deficiency determines a sustained increased recruitment of PMN cells to the lungs. Number of leukocyte subsets (macrophages, PMN neutrophils, and lymphocytes) in the lung-infiltrating leukocytes (LIL) from TLR2$^{-/-}$ and WT mice inoculated i.t. with 1 million *P. brasiliensis* yeast cells. By 2 (*A*) and 11 (*B*) wk after infection, lungs of TLR2$^{-/-}$ and WT mice ($n = 6–8$) were excised, washed in PBS, minced, and digested enzymatically. Lung cells suspensions were centrifuged in the presence of 20% Percoll (Sigma-Aldrich) to separate leukocytes from cell debris. Cell suspensions were cytospun onto glass slides and stained by the Diff-Quik blood stain. Lung cell homogenates were also obtained after 2 and 11 wk of infection and analyzed by ELISA for the content of KC (*C*) and MCP-1 (*D*) chemokines. Data are expressed as means ± SEM. ∗, $p < 0.05$ between strains.

anti-CD3 plus anti-CD28 mAbs, or Con A and cultivated for 72 h. As depicted in Table I, compared with TLR2-deficient mice, control mice developed higher lymphoproliferative activity with all stimuli used, including the polyclonal T cell activator Con A. A similar result was detected with unstimulated control lymphocytes, probably due to the presence of yeast cells in the spleens of infected mice at this postinfection period. Together, these experiments demonstrated that TLR2-deficient mice mount a deficient T cell response as revealed by the diminished migration of T cells to the site of infection and the impaired lymphoproliferative activity.

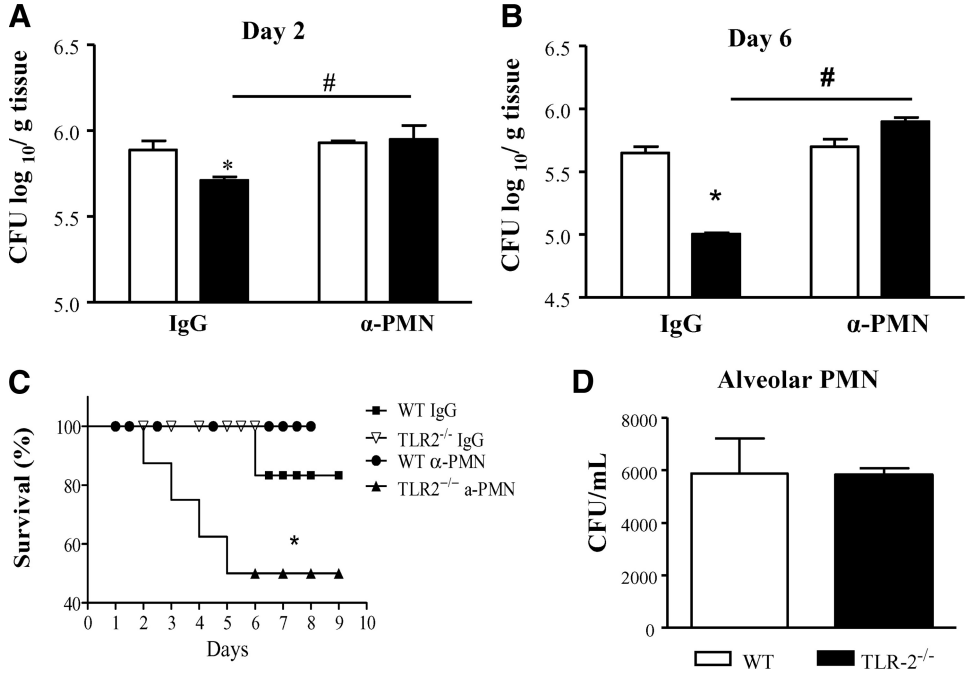

FIGURE 6. Fungicidal activity of PMN neutrophils. *A* and *B*, PMN leukocytes were in vivo depleted from WT and TLR2$^{-/-}$ mice by i.p. administration of 0.5 ml of a 1/5 dilution of a rabbit anti-mouse PMN polyclonal Ab at days −1, +3, and + 5 of i.t. infection (0 time) with 1 million *P. brasiliensis* yeast cells. Control mice received equivalent amounts of normal rat IgG. This treatment resulted in 75 and 87% of PMN depletion at days 0 and 6 of infection, respectively. IgG-treated and PMN-depleted mice ($n = 4–6$) were sacrificed at days 2 and 6 days after fungal infection and severity of disease evaluated by CFU counts in lung homogenates. *C*, Mortality data of PMN-depleted mice. Groups of mice ($n = 8–9$) were treated as above described and increased mortality was observed only in the PMN-depleted TLR2$^{-/-}$ group. Data were obtained from two independent experiments. *D*, Alveolar neutrophils were obtained by bronchoalveolar lavage of mice infected 2 wk earlier with 1 million yeast cells. The number of PMN leukocytes in the nonadherent fraction was adjusted to 2×10^5 cells, challenged with 1×10^5 yeast cells, and cocultivated for 1 h at 37°C. One hundred microliters of cell homogenates were assayed for the presence of viable yeast cells. All assays were done with three wells per condition in over two independent experiments. ∗, $p < 0.05$ compared with WT mice; #, $p < 0.05$ compared with IgG controls.

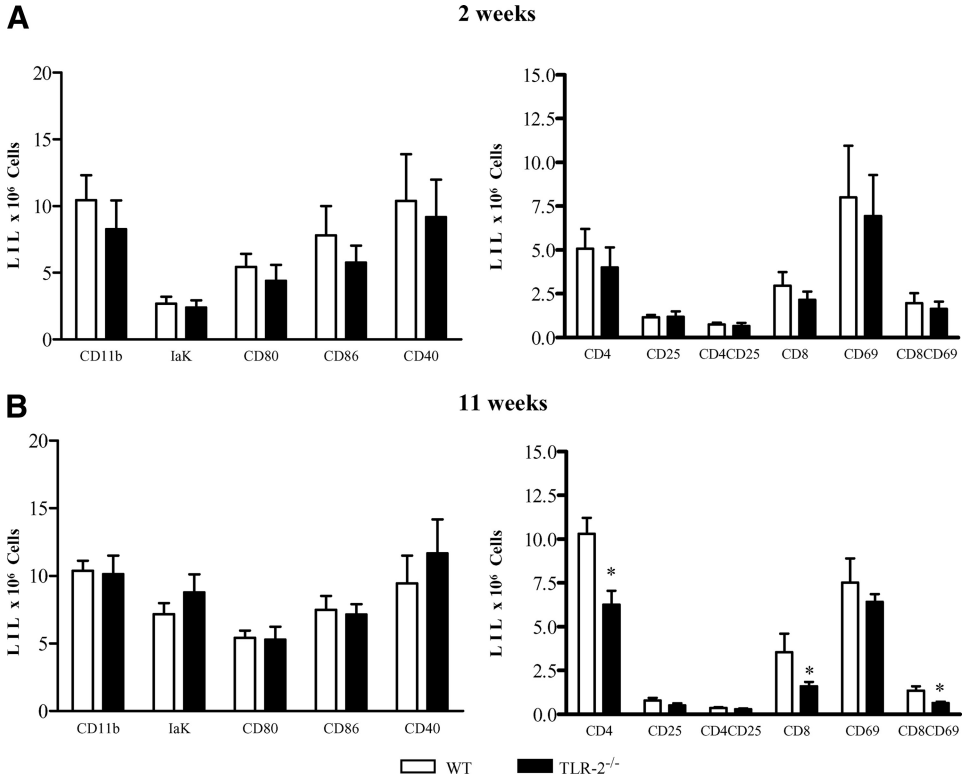

FIGURE 7. Characterization of macrophages and T lymphocytes in the pulmonary lesions of TLR2$^{-/-}$ mice. Equivalent numbers of macrophages were detected in the lungs of TLR2$^{-/-}$ and WT mice but the latter presented increased numbers of CD4$^+$ and CD8$^+$ T lymphocytes. Characterization of leukocyte subsets by flow cytometry in the lung-infiltrating leukocytes (LIL) from TLR2$^{-/-}$ and WT mice inoculated i.t. with 1 million *P. brasiliensis* yeast cells. Lungs of TLR2$^{-/-}$ and WT mice ($n = 6-8$) were excised, washed in PBS, minced, and digested enzymatically. At weeks 2 (*A*) and 11 (*B*) after infection lung cell suspensions were obtained and stained as described in *Materials and Methods*. The acquisition and analysis gates were restricted to lymphocytes or macrophages. The data represent the mean ± SEM of the results from six to eight mice per group and are representative of two independent experiments. *, $p < 0.05$ compared with WT mice.

TLR2-deficient mice develop a Th17-skewed T cell response

Because the equilibrium between pro- and anti-inflammatory cytokines determines the efficiency and cellular composition of inflammatory reactions (30) and because recent investigations have demonstrated an increased presence of PMN cells associated with prevalent IL-17 secretion (31), cytokines associated with Th1 (IL-12, TNF-α, IL-2, and IFN-γ), Th2 (IL-4, IL-5, and IL-10), and Th17 (TGF-β, IL-6, IL-17, and IL-23) immunity were assessed in lung homogenates obtained at different periods of infection (Figs. 8 and 9*A*). However, 48 h after infection, at the innate phase of immunity, higher levels of TGF-β and IL-17 were detected in the lungs of TLR2-deficient mice. At week 2, this strain produced higher amounts of IL-6, TGF-β, IL-17, and IL-23. By week 11, TLR2$^{-/-}$ mice showed a sustained increase of IL-23 associated with lowered concentrations of IL-10 and IL-12. We have further defined the phenotype of IL-17-producing cells. As shown in Fig. 9*B*, after 72 h and 2 wk of *P brasiliensis* infection, significantly increased numbers of IL-17$^+$CD4$^+$ T cells were detected in the lungs of TLR2$^{-/-}$ mice. No differences were observed in the total numbers of CD8$^+$ T cells, although the frequency of this T cell subset was significantly augmented in TLR2-deficient mice. These findings demonstrate that TLR2 deficiency induces, since the early phase of pulmonary infection, a Th17-skewed immune response, without severely impairing Th1 and Th2 immunity.

TLR2 deficiency results in lower expansion of CD4$^+$CD25$^+$FoxP3$^+$ Tregs

In murine candidiasis, TLR2 deficiency was associated with decreased IL-10 production and deficient expansion of CD4$^+$ Tregs (4, 32). In addition, differentiation of Tregs has an inhibitory effect on Th17 expansion (33, 34). Although Th17 cells and Tregs require TGF-β to their differentiation, the concomitant presence of some proinflammatory cytokines such as IL-1 or IL-6 results in preferential development of Th17 cells and impaired Treg expansion (34). These findings led us to ask whether the prevalent Th17 immunity developed by TLR2-defficient mice was associated with decreased expansion and recruitment of Tregs to the lungs. Thus, TLR2$^{-/-}$ and WT mice were sacrificed at weeks 2 and 11 after infection and the presence of CD4$^+$CD25$^+$FoxP3$^+$ T cells was characterized by flow cytometry in the CD4$^+$ subpopulation of lung-infiltrating lymphocytes. Surface staining of CD25$^+$ and intracellular FoxP3$^+$ expression was back-gated on the CD4$^+$ T cell population. As can be seen in Fig. 10, no differences were detected at week 2 but, at week 11 a lower number of CD4$^+$CD25$^+$FoxP3$^+$ T cells was observed in the lung inflammatory lymphocytes of TLR2-deficient than in WT mice.

Discussion

Recent studies have established the central role of TLRs in the innate immune recognition of a wide variety of microorganisms, including fungal pathogens (12, 15, 17). Since the contribution of TLR in *P. brasiliensis* infection was never investigated, the present report assessed the role of TLR2 in the innate and acquired phases of immunity to this pulmonary fungal pathogen. Data here presented demonstrate that TLR2 promotes fungal infection, but it has a concomitant beneficial effect to the host due to its inhibitory effect on the development of inflammatory reactions associated with prevalent Th17 immunity. Furthermore, it was also shown that TLR2 activation positively controls the expansion of Tregs, which modulate innate and adaptive immune cells and restrain lung inflammatory pathology.

Although TLR usually do not mediate fungal uptake (35), our in vitro studies suggested that *P. brasiliensis* yeast cells use TLR2 to interact with macrophages and to activate innate immunity cells. *P. brasiliensis* yeast cells appear to be recognized by TLR2 molecules, resulting in increased adherence/ingestion and augmented production of NO and cytokines (IL-10 and MCP-1). Despite this increased activation, 48 h after infection an increased number of viable fungi were recovered from TLR2-normal cells. This finding

Table I. *PI of spleen lymphocytes obtained from* P. brasilienis*-infected TLR2$^{-/-}$ and WT mice at week 4 of infection*[a]

Treatment	TLR2$^{-/-}$ (PI)	WT (PI[b])
Control lymphocytes	0.78	2.82
Lymphocytes + AgPb	0.99	2.43
Lymphocytes + anti-CD28	0.66	2.66
Lymphocytes + Con A	1.3	6

[a] Spleen cell suspensions were labeled with CFSE and cultured for 3 days in the presence of anti-CD3 (0.3) Ab, anti-CD28 (2.5 μg/ml) Ab, *P. brasiliensis*-soluble Ag (100 μg/ml), or Con A (1 μg/ml). The intensity of CFSE was assessed by flow cytometry.

[b] The PI was calculated as the MFI of unstimulated cultures/MFI of stimulated cultures. Data are representative of two independent experiments.

appears to be paradoxical, since the killing ability of murine macrophages was previously reported to be associated with NO production by cytokine-stimulated phagocytes (36). Although the killing mechanisms were not here addressed, it appears that the increased NO secretion by TLR2-normal macrophages was not sufficient to abolish the increased fungal interaction mediated by TLR2 expression. Furthermore, the concomitant increase in IL-10 production could have inhibited the fungicidal ability of TLR2-normal macrophages. Along the same line, we could hypothesize that the lower IL-10 synthesis by TLR2$^{-/-}$ macrophages would enhance IDO activity which would control *P .brasiliensis* growth and inhibit inducible NO synthase activity. This possibility is under investigation in our laboratory and would accommodate the lower fungal loads and NO production observed with TLR2$^{-/-}$ macrophages (16, 30). Similarly to our results, TLR2-deficient macrophages exhibited an increased ability to contain another fungal pathogen, *C. albicans*, but this fact was not associated with different patterns of cytokine production (37).

The in vivo infection of TLR2-deficient mice resulted in decreased fungal burdens in all postinfection periods assayed. The less severe infection was always associated with low NO production. Again, the differences in fungal burdens could be explained by the different fungal interaction with alveolar macrophages at early phases of infection and not by differences in NO secretion. Forty-eight hours after infection, production of pulmonary cytokines was similar in both mouse strains, except for the augmented synthesis of TGF-β and IL-17 by TLR2-deficient mice. Interestingly, IL-17 is a cytokine involved with proinflammatory activity and increased PMN chemotaxis due to its ability to promote the synthesis of CXC chemokines (38–40). Consistent with the augmented IL-17 production, 72 h after infection increased PMN influx was detected in the alveolar spaces of TLR2$^{-/-}$ mice (our unpublished data) and these cells could have efficiently controlled fungal growth but also sustained the synthesis of IL-23 and IL-17

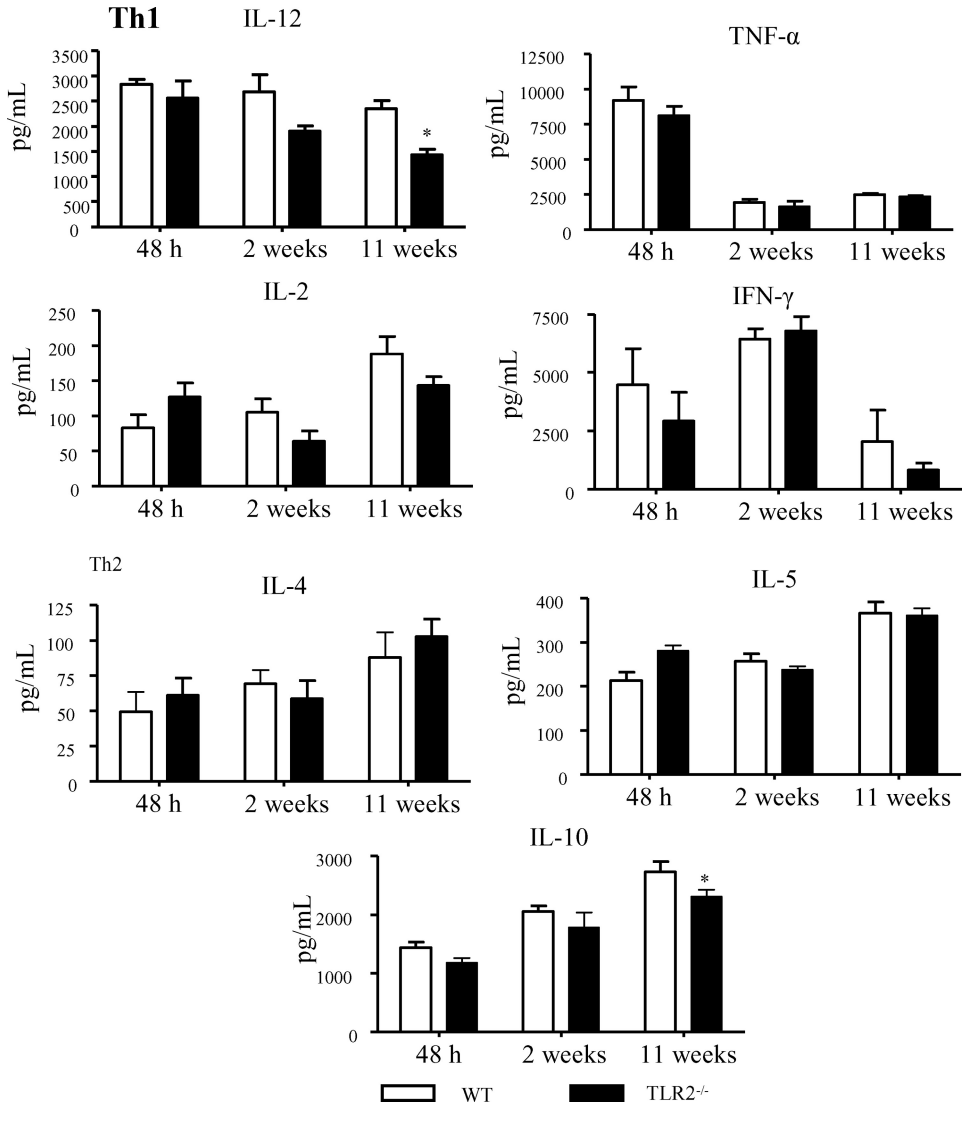

FIGURE 8. Type 1 and type 2 cytokines in lung homogenates of TLR2$^{-/-}$ and WT mice. At 48 h and 2 and 11 wk after i.t. infection with 10^6 yeast cells of *P. brasiliensis*, lungs from TLR2$^{-/-}$ and WT control mice were collected and disrupted in 5.0 ml of PBS and supernatants were analyzed for cytokines content by capture ELISA. The bars depict means ± SEM of cytokine levels (6–8 animals per group). *, $p < 0.05$ between strains.

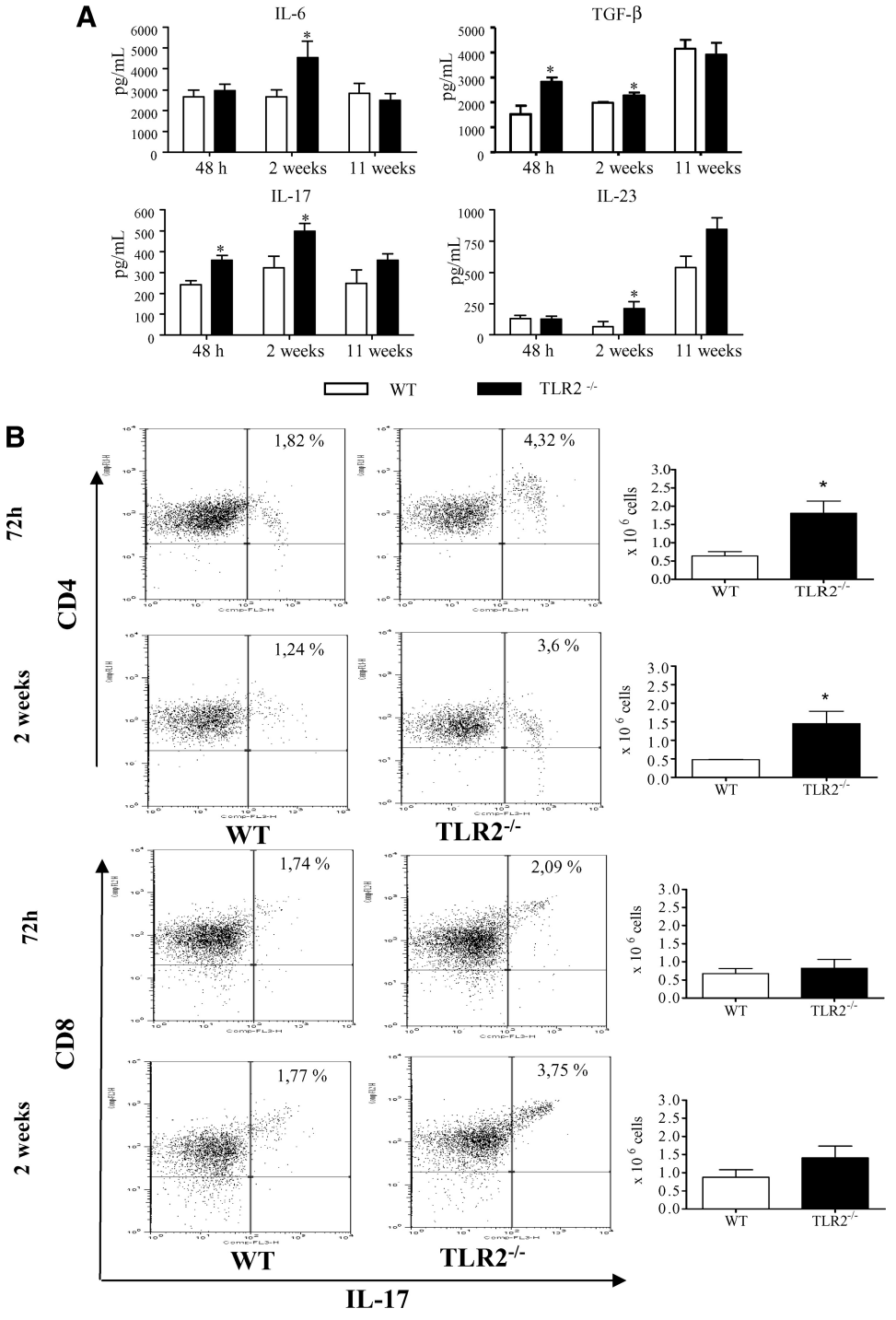

FIGURE 9. Lung homogenates of TLR2$^{-/-}$ mice presented increased levels of Th17 cytokines and IL-17$^+$ CD4$^+$ T cells. *A*, At 48 h and 2 and 11 wk after i.t. infection with 10^6 yeast cells of *P. brasiliensis*, lungs from TLR2$^{-/-}$ and WT control mice were collected and disrupted in 5.0 ml of PBS and supernatants were analyzed for cytokines content by capture ELISA. Compared with WT controls, TLR2$^{-/-}$ mice showed increased levels of pulmonary IL-6, TGF-β, IL-23, and IL-17. The bars depict means $\pm$ SEM of cytokine levels (6–8 animals/group). *B*, Flow cytometry analysis of IL-17-producing cells. Lung cells were restimulated in vitro with PMA/ionomycin for 6 h and subjected to intracellular staining for IL-17. The lymphocyte population was gated by forward/side scatters. Dot plots show a representative analysis from one of four to six mice. Results are from one experiment and are representative of two independent experiments. $*$, $p < 0.05$ between strains.

(41). Consistent with this possibility, lung homogenates of PMN-depleted TLR2$^{-/-}$ mice showed diminished levels of IL-23 (data not shown), suggesting that neutrophils could have a positive influence in the differentiation and expansion of Th17 cells. Although IL-17 production is usually associated with the Th17 subset of CD4$^+$ T cells, this cytokine was also described to be produced by innate immune cells such as $\gamma\delta$ T cells, dendritic cells, PMN cells, or NKT lymphocytes (41–45). In our model, CD4$^+$ T cells were shown to be the main source of IL-17 because increased numbers of IL-17$^+$CD4$^+$ T cells were present in the lung inflammatory infiltrates of TLR2$^{-/-}$ mice since the early phase of infection. This fact led us to suppose that TLR2 signaling could impair interactions of macrophages with fungal agonists that preferentially induce cytokines associated with the Th17 pathway of T cell differentiation, a fact not previously described in PCM.

At week 2 of infection, TLR2-deficient mice produced low levels of MCP-1 associated with augmented synthesis of pulmonary TGF-β, IL-6, IL-17, and IL-23. This pattern of cytokine secretion points to a preferential activation of Th17 cells without affecting other Th1/Th2 cytokines. Indeed, Th17 cell development occurs in the presence of TGF-β and IL-6 and is maintained in the presence of IL-23, an IL-12-related cytokine (46, 47). Interestingly, all of those mediators involved in the development and maintenance of Th17 immunity were found in lung homogenates of TLR2-deficient mice and increased numbers of

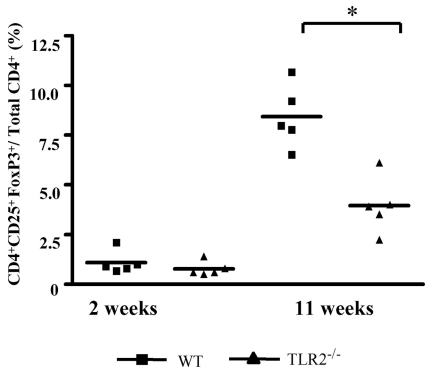

FIGURE 10. TLR2-deficient mice do not expand the number of Tregs. FoxP3 expression by lung lymphocytes from TLR2$^{-/-}$ and WT mice inoculated i.t. with 1 million *P. brasiliensis* yeast cells was determined by flow cytometric analysis. Lungs of TLR2$^{-/-}$ and WT mice ($n = 6-8$) were excised, washed in PBS, minced, and digested enzymatically; 2 and 11 wk after infection cell suspensions were obtained and stained as described in *Materials and Methods*. The cell surface expression of leukocyte markers as well as intracellular FoxP3$^+$ expression in lung-infiltrating leukocytes were analyzed by flow cytometry. Surface staining of CD25$^+$ and intracellular FoxP3 expression were back-gated on the CD4$^+$ T cell population. The data represent the mean ± SEM of the results from six to eight mice per group and are representative of two independent experiments. *, $p < 0.05$ compared with WT mice.

IL-17$^+$CD4$^+$ T cells were found in the lungs of these mice after 72 h and 2 wk of *P. brasiliensis* infection. This Th17-biased immunity resulted in altered cellular influx to the site of infection. At weeks 2 and 11 of infection, increased production of KC, a CXC chemokine involved in PMN chemotaxis, and augmented influx of neutrophils to the lungs of TLR2-KO mice were detected; these findings are in agreement with previous reports showing increased participation of PMN leukocytes in inflammatory exudates governed by Th17 immunity (38–40, 48, 49). Consistent with our results, association of IL-17 synthesis and increased PMN influx to the site of infection was seen in bacterial pneumonia (50), inflammatory lung disorders (51), and other fungal infections (31, 52). In addition, the diminished presence of MCP-1, a chemokine chemotactic for macrophages, was associated with decreased numbers of these cells in the lung cell infiltrates of TLR2-deficient animals.

The Th17-induced PMN accumulation in inflammatory reactions can play antagonistic effects to the hosts, being protective due to its microbicidal ability or deleterious due to the release of tissue-damaging components (41). In our model, the higher number of lung-infiltrating PMN associated with Th17 immunity was responsible, at least partially, by the better fungal clearance observed in TLR2$^{-/-}$ mice. This was shown by in vivo depletion of PMN cells that led to increased fungal burdens only in TLR2$^{-/-}$ mice. The immunoprotective effect of neutrophils was also verified by the early mortality of PMN-depleted TLR2$^{-/-}$ mice. These data are consistent with previous findings in murine candidiasis describing a more severe systemic infection associated with deficient IL-17 production and impaired influx of neutrophils to infected organs (53) However, as described in other models of prevalent Th17 immunity, we believe that PMN cells exerted a dual effect in murine PCM. The protection granted by the increased influx and efficient fungicidal ability was probably accompanied by tissue damage caused by the release of protein-degrading enzymes such as neutrophil elastase, metalloproteinases, and other cytotoxic compounds as reactive oxygen species (31, 41, 50–54). Since equivalent fungicidal ability was detected in airway neutrophils from TLR2$^{-/-}$ and control mice, we believe that the increased number of PMN cells that migrate to the lungs and not the intrinsic activation of neutrophils was responsible for the control of fungal growth.

Despite the control of fungal growth, our data also suggest that Th17 immunity and PMN cells have also exerted a deleterious effect in TLR2 $^{-/-}$ mice. Indeed, the diminished fungal burdens of TLR2$^{-/-}$ mice were concomitant with pulmonary lesions as severe as those displayed by WT mice. In both mouse strains nonorganized inflammatory reactions replaced almost all lung parenchyma, suggesting that high fungal loads (developed by WT animals) or prevalent Th17 immunity (mounted by TLR2$^{-/-}$ mice) have similar deleterious effects to *P. brasiliensis*-infected mice.

The phenotypic analysis of lymphocyte subsets performed after 2 and 11 wk of infection showed a decreased frequency of CD4$^+$ and CD8$^+$ T cells in TLR2$^{-/-}$ mice. This fact could be attributed to the lower fungal burden detected in this mouse strain or to the Th17-skewed immunity developed by the deficient animals. The early secretion of TGF-β and IL-6 by TLR2$^{-/-}$ mice appeared to have induced a precocious and prevalent Th17 immunity which resulted in diminished macrophage and T cell migration to the lungs. These cells were, however, partially replaced by PMN neutrophils which characterize Th17-mediated inflammatory exudates.

By week 11 of infection, TLR2$^{-/-}$ mice appear to maintain their prevalent Th17 immunity, whereas WT mice presented increased fungal burdens associated with augmented production of IL-10, IL-12, and significant expansion of Tregs. It can be supposed that those Tregs exerted an enhancing effect on fungal growth but an inhibitory activity on inflammatory reactions by down-modulating innate and adaptive immunity. Consistent with this interpretation, a recent work elegantly demonstrated the association of Tregs and increased fungal burdens in an experimental model of PCM using CXCR5 KO mice (55). The absence of TLR2 associated with decreased differentiation of Tregs here observed is also consistent with the increased secretion of TGF-β, which, associated with IL-6 (or other proinflammatory cytokine), could have inhibited FoxP3 production and expansion of Tregs (30).

In murine candidiasis, TLR2-deficient mice were shown to develop a more severe infection (56), a fact not subsequently confirmed (4, 16). The increased resistance of TLR2-deficient mice was associated with diminished IL-10 production and a decreased expansion of Tregs, which led to a more efficient Th1 immunity (4, 16). Similarly to the candidiasis model, PCM in TLR2$^{-/-}$ mice results in lower fungal loads and decreased expansion of Tregs. However, expression of TLR2 appears to be protective to pulmonary PCM because of its inhibitory activity on the development of Th17 immunity and tissue pathology. In addition, the decreased expansion of Tregs could have contributed to the maintenance of pathogenic inflammatory reactions.

The prevalent Th17 immunity developed by TLR2-deficient mice could be explained by the use of different pattern recognition receptors at the initial phase of infection. Other experimental models have implicated the use of Dectin-1 or TLR4 in the induction of Th17 immunity (57–59). Despite the initial pattern recognition receptor used, our results add a new mechanistic pathway which can lead to deleterious Th17 immunity in fungal infections in the absence of TLR2 signaling.

In summary, we have demonstrated for the first time that TLR2 are important innate receptors for *P. brasiliensis* which appears to use TLR2 as a virulence mechanism, which facilitates the access of fungal cells into murine macrophages. Most important, this is the

first study to demonstrate that TLR2 has a protective effect in pulmonary fungal infections due to its ability to deviate T cell responses from pathogenic Th17 immunity to a balanced Th1/Th2 response modulated by Tregs.

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Disclosures

The authors have no financial conflict of interest.

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***Anexo E* ï Toll-like receptors and fungal infections: the role of TLR2, TLR4 and MyD88 in paracoccidioidomycosis**

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MINIREVIEW

Toll-like receptors and fungal infections: the role of TLR2, TLR4 and MyD88 in paracoccidioidomycosis

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Abstract

The aim of this minireview is to present a concise view of the most important pattern recognition receptors used by the innate immune system to sense and control pathogen growth into host tissues. A brief review of the role of Toll-like receptors (TLRs) in fungal infections followed by some recent results on the function of TLR4, TLR2 and the MyD88 adaptor molecule in the pathogenesis of paracoccidioidomycosis are presented.

Innate immunity and pattern recognition receptors

Cells of the innate immune system constantly sense the presence of invading microorganisms using several kind of conserved, transmembrane or intracytoplasmatic receptors called pattern recognition receptors (PRRs). These receptors recognize conserved molecular structures shared by groups of microorganisms and known as pathogen-associated molecular patterns (PAMPs). Because PAMPs are produced by pathogens but not by the host cells, their recognition by PRRs allows for self–nonself discrimination (Janeway & Medzhitov, 2002). The most important types of PRR are the Toll-like receptors (TLRs), the non-TLRs such as intracellular nucleotide-binding oligomerization domain (NOD)-like proteins and the C-type lectin receptors (CLRs) (Gordon, 2002; Brown & Gordon, 2003; Inohara & Nunez, 2003; Akira *et al*., 2006). The interaction between PAMPs and PRRs leads to the activation of cells of the innate immune system and subsequent production of mediators that are used to eliminate the invading pathogen and to control the adaptative immune responses. TLRs are crucial for many aspects of microbial elimination, including recruitment of phagocytes to the site of infection, microbial killing and activation of dendritic cells (DCs), which become immunogenic and endowed with a unique ability to induce full activation of T cells (Reis e Sousa, 2004). Interestingly, recent findings indicate that direct recognition of PAMPs by DCsis critical for priming appropriate T-cell responses. TLR signaling resulted in activated DCs that primed an effective T helper 1 (Th1) or Th2 response, whereas indirect activation by inflammatory mediators alone (proinflammatory cytokines) induced DCs that, although supporting expansion of $CD4^+$ T-cell clones, did not promote Th1 or Th2 effector differentiation (Spörri & Reis e Sousa, 2005).

Thus far, 13 TLRs have been described that recognize a wide variety of pathogen structures including triacyl lipopeptides (TLR1 in association with TLR2), lipoteichoic acid and lipoproteins of Gram-positive bacteria (TLR2), double-stranded RNA (TLR3), lipopolysaccharide of Gram-negative bacteria (TLR4), bacterial flagellin (TLR5), diacyl lipopeptides (TLR6 in association with TLR2), single-stranded RNA (TLR7) and nonmethylated CpG of bacterial DNA (TLR9) (Takeda & Akira, 2005). Intracellular NOD proteins sense the presence of intracellular muropeptides (Inohara & Nunez, 2003). Upon ligand binding, innate

immune receptors engage intracellular signaling pathways that result in the activation of conserved transcription factors for cell activation. One key transcription factor used by TLR-mediated innate immunity is NFkB. Almost all TLRs signal via MyD88, an adaptor protein, for NFkB activation, subsequent inflammatory cytokine production and control of adaptative immunity (Takeda & Akira, 2005; Akira *et al.*, 2006). Besides their well-described role in the immunorecognition of conserved pathogen motifs, TLRs can be used to enhance microbial pathogenicity. Thus, *Yersinia pestis* was shown to evade the host's immune system by TLR2 activation and subsequent production of IL-10, one important macrophage deactivating cytokine (Sing *et al.*, 2002). Furthermore, studies with whole pathogens or purified components have shown distinct patterns of TLR usage: TLR2 and TLR4 control *in vivo Brucella abortus* infection whereas only TLR4 is activated by the purified bacterial lipopolysaccharide (Campos *et al.*, 2004).

TLR-activated DCs can induce the differentiation of distinct T-cell-mediated effector mechanisms. Lipopolysaccharide-stimulated DCs produce low levels of IL-10 but high levels of IL-12 and tumor necrosis factor-α (TNF-α), favoring Th1 immunity. In contrast, peptidoglycan-activated DCs secrete low levels of IL-12 associated with prevalent production of IL-10, resulting in prevalent Th2 immunity (Qi *et al.*, 2003). Interestingly, the recognition of microbial products by TLRs was found to block the suppressive effect of T regulatory (Treg) cells on pathogen-specific adaptative immune response and this effect was partially due to the synthesis of IL-6 by TLR-stimulated DCs (Pasare & Medzhitov, 2003).

Dendritic cell-specific intercelullar adhesion molecule-3-grabbing nonintegrin (DC-SIGN), mannose receptor (MR) and dectin-1 are C-type lectins that recognize glycoproteins and carbohydrates in pathogen cells. This interaction controls phagocytosis, microbicidal activity and signaling processes that direct cell adhesion and migration. As fungal cell walls are carbohydrate-rich structures, these PRRs are directly involved in the host's innate immunity to these pathogens. DC-SIGN as well as MRs are primarily activated by IL-4 and associated with the Th2 pattern of the immune response (Brown & Gordon, 2003; Cambi *et al.*, 2005; Koppel *et al.*, 2005). Dectin-1 activation by *Candida albicans* or curdlan, a dectin-1-specific β-glucan, was recently shown to induce the preferential secretion of tumor growth factor (TGF)-β and IL-6 and the subsequent activation of Th17 lymphocytes. This T-cell subset secretes IL-17, induces chemokine secretion at sites of infection, causes recruitment of neutrophils and is important in defense against extracellular pathogens, including *Candida albicans* (Leibundgut-Landmann *et al.*, 2007; Palm & Medzhitov, 2007).

Upon ligand binding, innate immunoreceptors engage intracellular signaling pathways that converge to the activation of conserved transcription factors and subsequent cell activation. Almost all TLRs signal via MyD88 for NFkB activation, resulting in Th1 immunity associated with the prevalent IL-12 secretion. TLR2 activation, however, induces high levels of IL-10, and expansion of regulatory T cells (Treg) which further control T effector lymphocytes (Gordon, 2002; Akira *et al.*, 2006; Sutmuller *et al.*, 2006). Interestingly, recent work has shown that the preferential activation of dectin-1 by the selective agonist curdlan, and possibly dectin-2 by *Candida albicans* hyphae, is mediated by the caspase recruitment domain (CARD9) adaptor protein, resulting in increased IL-23 secretion and preferential induction of Th17 cells (Gross *et al.*, 2006; Leibundgut-Landmann *et al.*, 2007; Palm & Medzhitov, 2007). The fungal morphotype is also recognized by different PRRs and this fact was suggested to be exploited as an escape mechanism by fungal cells. For instance, *Candida* hyphae are recognized only by TLR2, inducing prevalent secretion of anti-inflammatory cytokines, whereas *Candida* blastoconidia interact with TLR4, Dectin-1 and TLR2, resulting in a complex pattern of cell activation (Romani, 2004; Netea *et al.*, 2006).

In summary, PRR activation orchestrates the development of innate and adaptative immune responses, which are necessary for protection against infection, reinfection or containment of chronic infections. However, if activation of innate immune receptors is excessive, high levels of proinflammatory mediators [IFN-γ, TNF-α, nitric oxide (NO)] are secreted and can exert a deleterious effect to the host. Septic shock induced by lipopolysaccharide and TLR4 activation by Gram-negative bacteria is a good example of inadequate activation of immunity, which results in severe host pathology.

Recognition of fungi by TLR

Netea *et al.* (2002) were the first to describe the use of TLRs by a fungal pathogen, *Candida albicans*. C3H/HeJ mice, which express a defective TLR4 gene, present an increased susceptibility to disseminated candidiasis and impaired recruitment of neutrophils to the site of infection when compared with normal, C3H/HeN mice. In addition, the chemokines keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP-2) were shown to be released in lower amounts by TLR4-defective macrophages. Following this pioneer work, other groups reported their studies on the role of TLRs and the MyD88 adaptor protein in *Candida albicans* infections. The main biological effects observed in diverse experimental approaches are summarized in Table 1. As can be seen, some discrepant findings were obtained with TLR2- and TLR4-deficient hosts (Netea *et al.*, 2002, 2004; Villamón *et al.*, 2004b; Murciano *et al.*, 2006), although MyD88 deficiency appears consistently to lead to impaired protection or phagocyte–fungus interaction. This adaptor protein was shown to be

Table 1. The role of TLRs and MyD88 adaptor protein in some experimental models of *Candida albicans* infection

PRR deficiency	Biological effect	Reference
TLR4	High susceptibility; normal PMN and macrophage killing activity; normal TNF-α, low KC, MIP-2 and impaired PMN influx	Netea *et al*. (2002)
TLR4	No increased susceptibility to disseminated infection. TLR4-deficient mice mount Th1 immunity	Murciano *et al*. (2006)
TLR2	High resistance; TLR2 induces IL-10, Treg cells and suppressed immunity	Netea *et al*. (2004)
TLR2	High susceptibility; low TNF-α, MIP-2; decreased PMN influx; no effect phagocytosis and NO production	Villamón *et al*. (2004a)
MyD88	Hyphae: impaired phagocytosis, killing and cytokine secretion	Marr *et al*. (2003)
MyD88	High susceptibility, impaired production of cytokine, low type-1 $CD4^+$ and $CD8^+$ T cells	Villamón *et al*. (2004b)
MyD88, TLR4, TLR2, TLR9	MyD88: high susceptibility. TLR signaling: depends on morphotypes, route of infection. Th1 response: DC-MyD-dependent	Bellocchio *et al*. (2004a)

Table 2. The role of TLRs and MyD88 adaptor protein in some experimental models of *Cryptococcus neoformans* infection

PRR deficiency	Biological effect	Reference
TLR2, TLR4, CD14	Glucoronoxylomannan stimulates cells via CD14 and TLR4; no TNF-α synthesis	Shoham *et al*. (2001)
TLR-2, TLR4, MyD88, CD14	MyD88 has a major role in protection; CD14 and TLR2, minor roles; TLR4, no effect	Yauch *et al*. (2004)
TLR-2, TLR4, MyD88	MyD88 and TLR2 have a major role in protection; TLR4 not important	Biondo *et al*. (2005)
TLR2 and TLR4	Not important to protection	Nakamura *et al*. (2006)

involved in the induction of protective immune responses by DCs (Bellocchio *et al*., 2004a), as well as in the phagocytosis, killing and synthesis of cytokines by *Candida*-infected cells (Marr *et al*., 2003; Villamón *et al*., 2004a).

TLR2 usage was shown to have protective or detrimental effects in models of *Candida albicans* infection; the conflicting results, however, could be attributed to the use of different experimental protocols (Netea *et al*., 2004; Villamón *et al*., 2004a, b), but brought important contributions to the understanding of immunopathology of infectious processes. The deleterious effect of TLR2 signaling during infection was associated with increased synthesis of IL-10 and an enhanced survival of $CD4^+CD25^+$ Treg cells, resulting in deficient T-cell immunity and impaired fungal clearance (Netea *et al*., 2004).

As observed with *Candida albicans*, the role of TLR in *Cryptococcus neoformans* infection needs to be further explored (Table 2). Glucuronoxylomannan, the major component of the polysaccharide capsule of *Cryptococcus neoformans*, is shed from the fungus and circulates in blood and cerebrospinal fluid of infected hosts. This polysaccharide was reported to activate cells transfected with CD14 and TLR4 but this interaction results in incomplete activation of cells, and no secretion of TNF-α (Shoham *et al*., 2001). *In vivo*, MyD88 and TLR2 but not TLR4 were shown to be required to induce protection against *Cryptococcus neoformans* infection (Yauch *et al*., 2004; Biondo *et al*., 2005). A more recent report, however, suggests that TLR2 and TLR4 do not or only marginally contribute to the host response to this pathogen (Nakamura *et al*., 2006).

TLR2-, TLR4- and MyD88-dependent activation of host cells were shown to play a role in cytokine secretion, polymorphonuclear neutrophil (PMN) activation and susceptibility to infection by another opportunistic fungal pathogen, *Aspergillus fumigatus* (Wang *et al*., 2001; Marr *et al*., 2003; Meier *et al*., 2003; Netea *et al*., 2003; Bellocchio *et al*., 2004a, b; Braedel *et al*., 2004; Dubordeau *et al*., 2006). As described for *Candida albicans*, the germination from conidia to hyphae was proposed as an escape mechanism of *A. fumigatus* as conidia cells are recognized by TLR4 and TLR2, resulting in the production of proinflammatory cytokines, while hyphae stimulate production of IL-10 using a TLR2-dependent mechanism (Netea *et al*., 2003). Although some experimental approaches have revealed the important role of MyD88 adaptor protein in cell signaling and protective responses (Mambula *et al*., 2002; Bellocchio *et al*., 2004a), other reports claimed that MyD88 signaling and activation of NFκB are not important for fungal clearance (Marr *et al*., 2003; Dubordeau *et al*., 2006) (Table 3).

As a whole, several reports have illustrated the use of different TLRs by a single fungal species, resulting in diverse biological activities. Studies with purified components of fungal cell walls revealed the major PRR and signaling pathways used by host cells to recognize fungal PAMPs; however, this picture is less clear when whole pathogens are used to infect normal or PRR-deficient hosts. The final activation, although influenced by the missing receptor, is mediated by the remaining PRRs, which can compensate or not the deficient receptor.

PRR and *Paracoccidioides brasiliensis* infection

Paracoccidioides brasiliensis, the causative agent of human paracoccidioidomycosis, is primarily a respiratory pathogen, infecting the host through inhalation of airborne spores.

Table 3. The role of TLRs and MyD88 adaptor protein in some experimental models of *Aspergillus fumigatus* infection

PRR deficiency	Biological effect	Reference
TLR4, CD14, TLR2	TLR4 but not TLR2 recognizes hyphae	Wang *et al*. (2001)
TLR4, TLR2	TLR4 and TLR2 recognize conidia and hyphae, and induce TNF-α and MIP-2; impaired PMN influx	Meier *et al*. (2003)
TLR4, TLR2	Fungal antigens recognized by TLR4 and TLR2; enhanced phagocytosis and cytokine synthesis; activation and maturation of DCs	Braedel *et al*. (2004)
TLR4, TLR2, TLR3, etc	Individual TLRs activate human PMNs for specialized antifungal effector functions	Bellocchio *et al*. (2004b)
TLR4, TLR2	Conidia use TLR4 and TLR2 to induce proinflammatory cytokines; hyphae use only TLR2 to produce IL-10	Netea *et al*. (2003)
MyD88, TLR2, TLR4	MyD88 and TLR2 required for optimal signaling responses of cells	Mambula *et al*. (2002)
MyD88, TLR2, TLR4	TLR2, TLR4 and MyD88 signaling dispensable for fungal clearance	Dubordeau *et al*. (2006)
MyD88	Normal phagocytosis and killing of conidia; normal cytokine secretion	Marr *et al*. (2003)
MyD88 TLR4, TLR2, TLR9	MyD88: high susceptibility. TLR signaling: depends on morphotypes, route of infection. Th1 response: DC-MyD-dependent	Bellocchio *et al*. (2004a)

The great majority of infected subjects develop an asymptomatic pulmonary infection, although some individuals present clinical manifestations, which give rise to the localized (benign) or disseminated (severe) forms of the disease. Clinical and experimental evidence indicates that cell-mediated immunity plays a significant role in host defense against *P. brasiliensis* infection, whereas high levels of specific antibodies are associated with the most severe forms of the disease (Borges-Walmsley *et al*., 2002; Calich *et al*., 2008). Our laboratory developed a murine pulmonary model of infection in which A/Sn mice developed a chronic benign, pulmonary-restricted paracoccidioidomycosis whereas B10.A mice developed a progressive disseminated disease. The main immunological characteristics of this model are described elsewhere (Calich & Blotta, 2005).

Although the importance of innate immunity in resistance to fungal infection is well recognized (Roeder *et al*., 2004; Romani, 2004), the molecular mechanisms underlying recognition of *P. brasiliensis* by innate immune cells are not well known (Calich & Blotta, 2005; Calich *et al*., 2008). C3b receptors (CR3, CD11b/CD18) are membrane integrins that recognize iC3b of the complement system, several β-glucans and other cell-wall components expressing high mannose content (Brown & Gordon, 2003). We were the first to demonstrate that *P. brasiliensis* interaction with peritoneal macrophages was enhanced by iC3b opsonization of yeast cells (Calich *et al*., 1979). Studying murine macrophages, Jimenez *et al*. (2006) verified that CR3 and MR were involved in the phagocytosis of *P. brasiliensis* spores (conidia). In addition, gp43, the immunodominant antigen of *P. brasiliensis*, was shown to bind to MR and to inhibit the phagocytic and fungicidal ability of peritoneal macrophages from resistant and susceptible mice (Popi *et al*., 2002). This finding led the authors to postulate the expression or secretion of gp43 as an escape mechanism of fungal cells.

TLR4 and *P. brasiliensis* infection

Comparative studies of *in vivo* susceptibility of different mouse strains to *P. brasiliensis* intraperitoneal infection led us to verify that TLR4-deficient (C3H/HeJ) mice were more resistant than TLR4 normal (C3H/HePas) animals (Calich *et al*., 1985). Recent findings from our laboratory (F.V. Loures, unpublished data) demonstrated that, compared with the normal strain, macrophages from TLR4-deficient mice had a lower phagocytic ability, which appears to influence the decreased number of viable *P. brasiliensis* yeasts recovered after cocultivation for a 72 h period. Deficient macrophages secrete lower levels of NO, IL-12 and monocyte chemoattractant protein-1 (MCP-1) but produced equivalent amounts of TNF-α. In contrast, IL-10 was synthesized in higher amounts by TLR4-deficient macrophages. Consistent with *in vitro* results, 96 h after *in vivo* pulmonary infection, TLR4-deficient mice presented decreased fungal loads in the lungs associated with lower levels of NO and proinflammatory cytokines [IL-12, and granulocyte macrophage colony-stimulating factor (GM-CSF)]. Similar results were observed at week 11 after infection of TLR4-mutant mice: decreased CFU counts associated with low IL-12 levels but high IFN-γ secretion. Paralleling its mild infection, the deficient strain secreted low levels of IgG1, IgG2b and IgM *P. brasiliensis*-specific isotypes (F.V. Loures, unpublished data).

Cytospin preparations of lung-infiltrating leukocytes at week 2 of infection showed an increased number of PMN neutrophils associated with decreased numbers of lymphocytes and monocytes. Diminished expression of $CD25^{+}$, $CD86^{+}$ and $CD86^{+}IA^{k+}$ cells were also observed by fluorescence-activated cell sorter (FACS) analysis of lung lymphocytes. In addition, by week 2 of infection no differences in the lymphoproliferative activity of TLR4-normal and TLR4-deficient spleen cells were detected. Although

differences in CFU counts and synthesis of some inflammatory mediators occur in TLR4-deficient mice, such differences were not sufficient to alter their mean survival times (Loures; F.V. Loures & V.L.G. Calich, unpublished data).

TLR2 and *P. brasiliensis* infection

We have also comparatively analyzed *P. brasiliensis* infection of TLR2-normal (WT) and TLR2-gene knockout (KO) mice in a C57BL/6 background (F.V. Loures & V.L.G. Calich, unpublished data). *In vitro* infection of KO macrophages resulted in lower phagocytic indexes, decreased recovery of viable yeasts after 72 h of cocultivation and low levels of NO and MCP-1 in culture supernatants. Compared with WT mice, 48 h after infection KO mice presented diminished pulmonary fungal loads and NO, but these findings were not associated with differences in the levels of pro- and anti-inflammatory cytokines. Analysis of bronchoalveolar lavage fluids obtained 72 h after i.t. infection showed an increased influx of neutrophils to the airspaces of TLR2 KO mice in comparison with WT animals. Cytospin preparations of lung-infiltrating leukocytes at weeks 2 and 4 of infection showed a decreased proportion of macrophages but an increased number of PMN neutrophils and lymphocytes. In addition, a decreased number of IA^{k+} macrophages and $CD4^+$ $CD25^+$ T cells was detected in TLR2 KO mice. Further studies are needed, however, to characterize this T-cell subset more completely, which can exert effector or regulatory functions. Indeed, in a previous report Netea *et al*. (2004) showed that TLR2 KO mice are less susceptible to *Candida albicans* infection due to the decreased presence of regulatory T cells and a more efficient fungal-specific immunity. At week 11 of infection TLR2-deficient and normal mice presented similar humoral immunity but the former strain presented increased fungal burden in the lungs. Despite this difference, both mouse strains exhibited equivalent mortality rates.

MyD88 adaptor molecule and *P. brasiliensis* infection

When macrophages were *in vitro* infected with *P. brasiliensis* yeasts for 72 h, an increased number of viable fungi was recovered from MyD88 KO macrophages in comparison with normal cells. This diminished fungicidal ability paralleled a decreased synthesis of NO and IL-12. The early *in vivo* infection reproduced the *in vitro* findings: higher fungal loads were found in MyD88 KO mice associated with lower levels of pulmonary NO and IL-12. This appears to indicate that absence of MyD88 molecule causes profound effects in cell activation, resulting in more severe infection. Mortality studies confirmed the higher susceptibility of MyD88 KO mice to *P. brasiliensis* infection, as their mean survival time was significantly lower than that of WT controls (F.V. Loures & V.L.G. Calich, unpublished data).

Concluding remarks

An increasing number of reports document the primary importance of innate immunity not only by providing the first line of defense against invading pathogens but also by controlling essential mechanisms that induce and regulate adaptative immunity. Our results on the role of TLRs in paracoccidioidomycosis suggest *P. brasiliensis* yeasts use TLR2 and TLR4 to gain entry into macrophages and infect mammalian hosts. Indeed, *P. brasiliensis* yeasts appear to be recognized by TLR2 and TLR4, resulting in increased phagocytic ability, NO secretion and fungal infection of macrophages. These data appear to be paradoxical, but the killing activity usually associated with NO secretion was not able to reduce the fungal growth provided by the presence of TLRs. Thus, interaction with TLRs could be considered a pathogenicity mechanism of *P. brasiliensis*, which would use host receptors of innate immunity (TLR2 and TLR4) to infect cells and to guarantee its own multiplication.

The *in vivo* infection of TLR-deficient mice resulted in decreased fungal burdens, again suggesting that TLRs are used by *P. brasiliensis* yeasts to infect hosts. The opposite result, however, was seen with MyD88-deficient macrophages and mice as more severe infections were observed probably due to the intact fungal recognition mediated by the expression of normal PRR but impaired ability of cell activation resulting in diminished fungicidal ability.

As a whole, TLR deficiency caused less severe infections associated with altered secretion of NO, cytokines and chemokines, resulting in altered cellular influx to the site of infection. In both PRR-deficient strains, the lung inflammatory infiltrate was composed of a diminished number of macrophages associated with an increased presence of PMN neutrophils. The phagocytic and killing abilities of the latter cells perhaps contribute to the decreased fungal inoculum at the site of infection. Furthermore, the low level of MCP-1 was parallel to the decreased number of lung-infiltrating monocytes.

An interesting observation was the decreased number of $CD4^+CD25^+$ T cells in the lungs of TLR2 KO mice, although an equivalent situation was not observed in TLR4-deficient animals.

Mortality studies have shown that TLR deficiency was not able to change the late course of infection as no differences were observed between TLR-deficient and TLR-normal mice. Compensatory mechanisms appear to abolish the immunological differences caused by PRR deficiencies. The same was not true for MyD88 deficiency, which causes higher mortality of infected mice.

In summary, our studies suggest that MyD88 deficiency is more important than TLR2 or TLR4 deficiency and *P. brasiliensis* yeasts appear to use TLRs as a virulence mechanism, which facilitates the access of fungal cells into murine macrophages. Despite their TLR-mediated activation, macrophages are not able to control fungal growth, both *in vitro* and *in vivo*. However, the final balance between fungal growth and activation of the immune system appears to control disease outcome as the low fungal loads and impaired immunity of TLR-deficient mice and the high fungal burdens and enhanced immunity of normal-TLR mice result in equivalent survival times. Furthermore, our studies have also suggested that, besides TLR, other PRRs play a role in the host immune response against by *P. brasiliensis* infection.

In conclusion, studies aimed to characterize the role of TLRs in fungal infections are firmly demonstrating their important participation in the effector and regulatory mechanisms of innate and adaptative immunity against these pathogens. TLR2, TLR4 and the adaptor molecule MyD88 appear to control the phagocytic rates, cell migration and activation, cytokine and chemokine secretion as well as the expression of costimulatory molecules that affect dendritic cell activation and their competence as antigen-presenting cell (APC) to naïve T cells. The interaction between TLR and other PRRs, in a synergistic or antagonistic way with fungal agonists, can result in different effector (Th1, Th2 and Th17) and regulatory responses (Treg), which ultimately determine disease outcome. Despite the important information in the current literature, additional investigation is needed to characterize further the influence of TLRs in the immunopathology of fungal infections.

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***Anexo F* ï Innate immunity to *Paracoccidioides brasiliensis* infection**

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Innate immunity to *Paracoccidioides brasiliensis* infection

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Abstract Innate immunity is based in pre-existing elements of the immune system that directly interact with all types of microbes leading to their destruction or growth inhibition. Several elements of this early defense mechanism act in concert to control initial pathogen growth and have profound effect on the adaptative immune response that further develops. Although most studies in paracoccidioidomycosis have been dedicated to understand cellular and humoral immune responses, innate immunity remains poorly defined. Hence, the main purpose of this review is to present and discuss some mechanisms of innate immunity developed by resistant and susceptible mice to *Paracoccidioides brasiliensis* infection, trying to understand how this initial host-pathogen interface interferes with the protective or deleterious adaptative immune response that will dictate disease outcome. An analysis of some mechanisms and mediators of innate immunity such as the activation of complement proteins, the microbicidal activity of natural killer cells and phagocytes, the production of inflammatory eicosanoids, cytokines, and chemokines among others, is presented trying to show the important role played by innate immunity in the host response to *P. brasiliensis* infection.

Keywords Cytokines · Dendritic cells · Innate immunity · Macrophages · NK and PMN Cells · *Paracoccidioides brasiliensis* infection

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Introduction

Innate immunity has been defined as the first phase of immune response and is based in pre-existing elements of the immune system that directly interact with all types of microbes leading to their destruction or growth inhibition. Innate immunity, which is not clonally specific for a particular pathogen and does not generate specific memory, is mediated by physical barriers, chemical elements, and cell components of the immune system. The adaptative immunity, involving more slowly developing, long-lived, and highly antigen-specific responses are mediated by cell-mediated immunity and antibody production. Several elements of innate immunity act in concert to control initial pathogen growth and have profound effect on the adaptative immune response that further develops. Furthermore, most effector mechanisms of innate immunity are identical to those of adaptative immunity that are activated at later phases of immune response.

Several mechanisms of innate immunity such as the activation of complement proteins, the microbicidal activity of natural killer (NK) cells and phagocytes, the production of inflammatory cytokines and chemokines among others, have been shown to play an important role in the early host response to pathogens [1]. Besides their intrinsic complexities, innate immunity mechanisms present important peculiarities which depend on the site they take place [2]. The innate immune response against *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Blastomyces dermatitides*, and *Histoplasma capsulatum*, primary fungal pathogens which infect hosts through the respiratory tract, occurs in the lungs. The lung response to infection is initiated by the secretion of several antimicrobial proteins by the pulmonary epithelium and the phagocytic activity of resident alveolar macrophages. The cell-wall-degrading enzyme lyzozyme, the iron-chelating protein lactoferrin and the membrane-permeabilizing members of the defensin, cathelicidin, and pentraxin families are the initial antimicrobial proteins secreted in the alveolar lining layer of the pulmonary epithelium. Innate immunity recognition of microorganism is mediated by germ-line encoded receptors ("pattern recognition receptors, PRR") which interact with conserved pathogen structures, the so-called "pathogen associated molecular patterns" or "PAMP" [3–5]. The initial macrophage-pathogen interaction results in internalization by the activated cell which can kill the organism through the action of reactive oxygen species and lytic enzymes or extracellular microbial containment. In addition, the secretion of chemokines and cytokines orchestrates the expression of cell adhesion and chemotactic molecules which further control the influx and activation of inflammatory cells to the site of infection [1–5].

Although most studies in human PCM have been dedicated to understand cellular and humoral immune responses, innate immunity remains poorly defined. This is easily understood when one reminds that PCM infection and disease in human beings are recognized at a later and undefined period after initial infection, making difficult to evoke the early events which resulted in controlled infection or overt disease. In this aspect, experimental models are powerful tools to study the initial events that govern hosts-*P. brasiliensis* interactions. Thus, the main purpose of this review is to present and discuss some mechanisms of innate immunity to *P. brasiliensis* infection, trying to understand how this initial host-pathogen interface interferes with the protective or deleterious adaptative immune response that will dictate disease outcome. This review does not intend to be a comprehensive revision of the PCM literature that has been reported elsewhere [6–10], but to present a personal view, mainly based in the murine model of genetic resistance and susceptibility to *P. brasiliensis*, of how innate immunity can influence PCM severity and the adaptative immune response to this pathogen.

The isogenic murine model of resistance/susceptibility of paracoccidioidomycosis mimics the human disease

Our laboratory established a genetically controlled murine model of paracoccidioidomycosis (PCM), which allowed us to investigate several parameters of host-parasite interactions. Most of these studies were recently reviewed [10–13] and clearly showed the diverging immune responses mounted by genetically susceptible (B10.A) and resistant (A/Sn or A/J) mice to *P. brasiliensis* infection. One important characteristic of our model is the similarity with the human disease, B10.A mice mimicking the progressive, severe forms of the disease and A/Sn mice showing similar features of the regressive or localized forms of the infection (Fig. 1). As in the human disease, our experimental model demonstrated that resistance is associated with immune responses that favor cellular immunity and activation of phagocytes, whereas susceptibility is associated with impairment of cellular immune responses and preferential activation of B cells [10–12].

After an intra-tracheal (i.t.) infection, the susceptibility and resistance patterns observed following i.p. infection were maintained, as reflected by the high mortality rates of B10.A mice and the regressive disease developed by the A/J strain. The susceptible mice were not able to restrain the infection to the lungs and, 2 months after infection, dissemination to liver and spleen was seen, characterizing a chronic, progressive and disseminated form of the disease; in the resistant mice, on the other hand, no organ

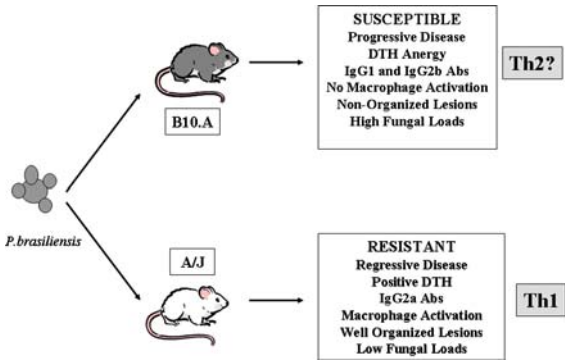

Fig. 1 Main features of the isogenic murine model of resistance and susceptibility to *Paracoccidioides brasiliensis* infection

dissemination occurred and a pulmonary-restricted chronic disease was observed. Unexpectedly, early in the i.t. infection (2nd and 4th weeks), A/J mice presented higher pulmonary CFU counts than B10.A mice suggesting that susceptible mice developed a more efficient innate immunity than resistant animals. The adaptative immunity of resistant mice appears to compensate their ineffective innate immunity (Fig. 2). Accordingly, from week 8 of infection onward, positive DTH responses, marked control of fungal burdens, secretion of pulmonary type 1 and type 2 cytokines and preferential production of IgG2a antibodies were seen, leading to a regressive pattern of disease. On the contrary, the anergy of DTH reactions, the preferential synthesis of IgG1 and IgG2b antibodies and the progressively increased fungal burdens of susceptible mice resulted in severe disseminated disease leading to decreased survival times [14, 15].

Innate immunity

Genetic control of susceptibility

Clinical studies suggested that susceptibility to *P. brasiliensis* is dependent on several factors, including genetic background, and host's hormonal function [4–7]. A fungal receptor for estrogen was identified and appears to block the conversion of conidia or mycelium to the infecting yeast form [16]. This finding was further explored in an animal model of infection demonstrating the enhanced resistance of female animals [17, 18] and may explain the unusual susceptibility of male individuals of endemic areas [6, 8, 19].

Genetic studies performed by our group have shown the existence of an autosomal dominant gene (*Pbr* gene), which control *P. brasiliensis* resistance [20] and appears to be similar to the *Nramp* gene, that control resistance to *Mycobacterium sp, Leishmania sp*, and *Salmonella sp* infection [21]. Further studies with *Nramp1* congenic macrophages (B10R and B10S expressing or not the Nramp1 protein, respectively) showed that B10R macrophages, in comparison with B10S cells, expressed higher levels of mannose receptors, presented higher phagocytic ability and increased inhibitory effect on the conidia to yeast conversion [22].

Complement system and chemokines

P. brasiliensis cells are able to activate the alternative pathway of complement and yeast cells-adherent C3b

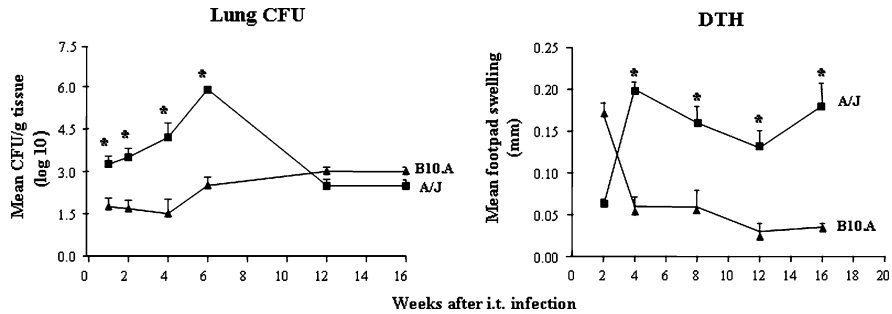

Fig. 2 At the onset of infection, susceptible (B10.A) mice show a better control of pulmonary fungal loads than resistant (A/J) mice. A/J and B10.A mice were i.t. infected with one million *P. brasiliensis* yeast cells. The graph on the left shows the recovery of viable fungal cell from lungs (colony forming unit counts, CFU), and the graph on the right the delayed hypersensitivity (DTH) responses measured during 16 weeks of infection

molecules can contribute to fungi phagocytosis by macrophages [23–25]. On the other hand, chemokines which play a major role in regulating the migration of specific leukocytes subsets in both the acute and chronic inflammatory processes [26], were shown to control mononuclear cell recruitment to the lungs of *P. brasiliensis*-infected C57BL/6 mice [27]. Unpublished results from our laboratory suggest that increased and sustained expression of IP-10, RANTES and the chemokine receptor CXCR3 is associated with the resistant behavior of A/Sn mice (C. Arruda and V. L. G. Calich, unpublished observations). This is in accordance with the sustained T cell response mounted by resistant mice at the acquired phase of the immune response [14, 15].

Lipid mediators (Eicosanoids) and *P. brasiliensis* lipids

During an inflammatory reaction, the enzymatic oxidation of aracdonic acid (AA) by cyclooxigenase produces prostaglandins, thromboxanes, and prostacyclins, whereas the 5-LO is an enzyme that catalyzes the oxidation of AA for the synthesis of leukotrienes (LT). The importance of LT as cellular activators and chemotactic factors for neutrophils and eosinophils is very well established, however, little is known about the function of these lipid mediators in the host defense against infectious agents [28, 29].

As the role of LT in pulmonary PCM was never investigated, we asked whether they would have a regulatory function in the severity of PCM of resistant (A/J) and susceptible (B10.A) mice and in the fungicidal and secretory ability of their macrophages. Our results showed that in vivo and in vitro *P. brasiliensis* infection induces LT synthesis. Compared with A/J mice, levels of pulmonary LT were higher in B10.A animals and increases in the course of infection. To evaluate the importance of LT in PCM, an inhibitor of LT synthesis (MK-0591) and an antagonist of LT receptor (montelukast) were studied in *P. brasiliensis* infection. In vitro, LT inhibitors significantly reduced the recovery of *P. brasiliensis* yeasts from normal and IFN-γ primed macrophages. At 48 h of in vivo infection, montelukast treatment of B10.A mice induced diminished fungal loads, impaired influx of PMN leukocytes, and increased number of monocytes in the lungs of *P. brasiliensis*-infected mice. Furthermore, in susceptible mice montelukast treatment led to increased levels of pulmonary IL-10 concomitant with diminished amounts of IL-12, TNF-α, and GM-CSF. In contrast, at the chronic phase of the disease, LT inhibition did not alter the fungal loads of B10.A and A/J mice. In conclusion, our results showed for the first time that LT are important mediators of the acute inflammatory reaction induced by *P. brasiliensis* infection affecting fungal recovery, cellular influx, and cytokines synthesis by susceptible mice [30, L. R. R. Ribeiro and V. L. G. Calich, unpublished observations]. Importantly, our findings with LT inhibition appear to demonstrate that the activation of innate immunity can result in increased ingestion and survival of *P. brasiliensis* yeasts which can evolve to a more severe disease.

Several lines of evidence suggest that prostaglandins production has a deleterious role for *P. brasiliensis*-infected hosts. In murine PCM, at early steps of infection, secretion of PGE_2 was shown to have an immunossupressive activity by inhibiting IL-12 production and up-regulating IL-4 and IL-10 synthesis [31]. In addition, studies with normal and IFN-γ activated human macrophages demonstrated that prostaglandins secretion inhibited their fungicidal ability which depends on the levels of hydrogen peroxide produced [32, 33]. Interestingly, recent studies showed that virulent and low virulence strains of *P. brasiliensis* are able to synthesize prostaglandins by a cyclooxigenase-dependent pathway and that these lipid mediators are required for *P. brasiliensis* survival [33].

Besides the importance of hosts lipid mediators such as the eicosanoids in innate immunity, other lipid components of pathogen membranes or walls have also been shown to play a role in the host-parasite interaction [34, 35]. Studies on the influence of *P. brasiliensis* lipid fractions in the fungicidal and secretory activities of B10.A macrophages were developed in our laboratories. Although all *P. brasiliensis* lipid fractions are potent inducers of NO synthesis, they can inhibit or enhance the fungicidal ability of macrophages. The previous in vitro treatment of macrophages by F1 (phospholipids + neutral lipids) and F2 (short chain glycolipids) fractions resulted in increased phagocytic activity of cells, and recovery of higher numbers of viable yeasts from infected macrophages, despite the presence of high

NO levels. On the other hand, fractions 3a (glycosylphosphatidylinositol-anchored glycoproteins) and F3b (long chain glycolipids) caused an opposite behavior; they inhibited the phagocytic ability of macrophages leading to decreased recovery of viable yeasts. As a whole, secretion of IL-10, IL-12, MCP-1, and GM-CSF induced by *P. brasiliensis* infection was inhibited by the previous pre-incubation with all lipid fractions [36, F. V. Loures, I. Almeida and V. L. G. Calich, unpublished data]. The different behavior of the studied lipid fractions could be attributed to the different physicochemical structures of these components which would interact with macrophages membranes through different PRR, and the subsequent balance of pro- and anti-inflammatory cytokines and chemokines secreted. Indeed, a further characterization of these lipid fractions will permit us to better understand the innate host response to *P. brasiliensis* infection.

Toll Like and other macrophage receptors

In mammalian cells, the Toll-Like Receptors (TLR) are transmembrane proteins, which interact with invariant molecular structures from pathogens (PAMP) and are involved in the activation of the innate immune system. Several typical pathogen components such as lipopolysaccharides, flagelin, peptidoglycans, DNA motifs, among others, are recognized by different TLR [37–42]. Early TLR activation results in the production of several inflammatory mediators and the final balance among pro- and anti-inflammatory components will regulate the type of adaptative immune response [37–42]. The TLR 4 is the key receptor that recognizes bacterial lipopolysaccharides, whereas TLR 2 is involved in the interaction with bacterial peptidoglycans and lipoproteins [38, 39]. TLR have been implicated in the resistance of mammalian hosts to several microorganisms [42–44] including fungal pathogens such as *Candida albicans, Aspergillus fumigatus*, and *Cryptococcus neoformans* [5]. Interestingly, our previous studies with *P. brasiliensis* infection showed that the LPS-resistant, TLR 4 deficient, C3H/HeJ strain is more resistant to i.p. infection than the congenic LPS-susceptible, TLR 4 normal, C3HeB/FeJ strain [45]. Our recent in vitro studies with TLR 4 normal (C3HeB/FeJ) and deficient (C3H/HeJ) macrophages have demonstrated that this receptor interacts with *P. brasiliensis* cells resulting in macrophage activation as shown by increased synthesis of nitric oxide, IL-12, MCP-1, and enhanced phagocytic activity; this activation, however, was associated with augmented recovery of viable yeast cells from infected macrophages. In the acute phase of pulmonary infection, the presence of TLR 4 induces a more severe disease, with increased numbers of viable yeasts in the lungs associated with elevated synthesis of NO and IL-12. Moreover, even in the chronic phase, higher fungal burdens were seen in the lungs of TLR-4-normal mice, associated with increased levels of pulmonary IL-12 and serum antibodies (IgM and IgG). Thus, the early macrophage activation induced by TLR 4 usage is not able to control *P. brasiliensis* infection [36, F. V. Loures and V. L. G. Calich, unpublished observations]. As LPS unresponsiveness of C3H/HeJ mice was linked to a point mutation in the *TLR 4* gene, it is tempting to suggest that recognition of *P. brasiliensis* components (LPS like?) by TLR 4 has a not yet described contribution to the control of PCM. We have also preliminary in vitro and in vivo studies with TLR 2 knockout mice in a C57Bl/6 background demonstrating a more severe infection in TLR-normal hosts or cells. Altogether, our findings with TLR-deficient animals are unusual since PRR are most commonly used by phagocytes to recognize molecular patterns of pathogens, and their interaction usually results in cell activation, enhanced secretion of pro-inflammatory cytokines and chemokines, and increased microbicidal activity. In our model, the increase production of nitric oxide and IL-12 by TLR-normal macrophages was not sufficient to control fungal growth and subsequent disease severity (F. V. Loures and V. L. G. Calich, unpublished results). These receptors appear to be used by *P. brasiliensis* yeast cells to gain access into macrophages and to escape from other fungicidal or fungistatic mechanisms of innate immunity.

Some reports have described the importance of mannose receptors in *P. brasiliensis* ingestion by phagocytic cells. Phagocytosis of yeasts by adherent peritoneal macrophages of susceptible and resistant mice was inhibited by gp-43, a *P. brasiliensis* glycoprotein most recognized by patients antibodies, as well as by *Saccharomyces cerevisiae* derived α-mannan. Gp 43 was also shown to inhibit NO

production and killing ability of cytokine-stimulated macrophages [46]. Immature dendritic cells of resistant mice appear to use mannose receptors to internalize *P. brasiliensis* yeasts [47]. In addition, comparative studies with *Nramp1* gene congenic macrophages (B10R and B10S) have demonstrated that B10R cells were better inhibitors of conidia to yeast conversion and expressed more mannose receptors than B10S macrophages, whereas both cell lines expressed similar levels of complement receptor 3 (C3R) [20]. As described below, CR3 was also shown to play an important function in *P. brasiliensis* adherence and ingestion by phagocytic cells [23, 24].

Polymorphonuclear leukocytes and NK cells

Differently from macrophages, murine PMN leukocytes are able to kill *P. brasiliensis* yeasts through the oxidative metabolism [48, 49]. In an air-pouch model of infection and compared with PMN leukocytes from susceptible mice, cells from A/J mice presented superior fungicidal ability associated with their enhanced oxidative burst [50]. The antifungal activity of murine and human PMN leukocytes was shown to be enhanced by IFN-γ, GM-CSF, or IL-1β, but not by TNF-α or IL-8 [51]. In contrast, TNF-α was shown to better enhance *P. brasiliensis* killing by human macrophages than IFN-γ [52].

Comparing the early influx of inflammatory cells to the lungs of susceptible and resistant mice, Cano [53] demonstrated an equivalent mononuclear cell influx, but a more prominent migration of neutrophil and eosinophil PMN cells into the lung of susceptible mice. This early PMN influx was also seen early in the infection of BALB/c mice [54]. Furthermore, only in susceptible mice this early (24 h after infection) PMN influx affects disease outcome and acquired immunity further established. Interestingly, the more severe disease of PMN-depleted susceptible mice was associated with the increased presence of pulmonary IL-12 and IFN-γ suggesting that the production of pro-inflammatory mediators not always leads to immunoprotection. Differently from primary infection, neutrophil depletion did not alter immunoprotection in secondary paracoccidioidomycosis. As a whole, our data showed that the genetic pattern of hosts exerts an important influence on the immunoprotective and immunoregulatory functions of neutrophils which appear to be essential in situations devoid of cell-mediated immunity [55].

The role of NK cell has not been well studied in *P. brasiliensis* infection, but the few available investigations in this area suggest that this lymphocyte subpopulation has a complex function in PCM that varies according to the type of host or site where these cells were obtained. In the peripheral blood of PCM patients, NK cells were found in elevated number but they displayed low cytotoxic activity [56]. In vitro studies showed a direct inhibitory effect of murine NK cells on *P. brasiliensis* growth [57] and in a hamster model of infection, NK cells were shown to be activated at the first weeks of infection followed by an impairment of its activity associated with depressed cell-mediated immunity [58].

Our findings of illness exacerbation after in vivo depletion of IL-12 or IFN-γ in euthymic and athymic BALB/c mice [59, 60] suggested that NK cells would have a protective role in pulmonary PCM. In vivo depletion of NK cells by anti-Asialo GM1 polyclonal antibody resulted in a more severe disease of both mouse strains, but the depletion effect was more pronounced in the NK-depleted athymic than euthymic mice. Anti-NK cell treatment led to increased antibody production by the former strain but did not modify the humoral immunity of euthymic animals, indicating that the isotype class switch in T cell deficient mice is influenced by NK cells cytokines. In addition, NK cells were shown to control PMN leukocytes influx to the lungs of infected mice. Hence, NK cells seem to have a protective effect in pulmonary PCM and their function appears to be more prominent in T-cell deficient than in T-cell sufficient mice [61, R. C. Valente-Ferreira and VLG Calich, unpublished data].

Macrophages and nitric oxide

The crucial role of the mononuclear phagocytic system in the resistance to *P. brasiliensis* infection was demonstrated by the fact that reticuloendothelial system blockade, induced by colloidal carbon inoculation previous to *P. brasiliensis* infection (i.p. route), increased the severity of the disease in both resistant and susceptible animals [62].

The infection by *P. brasiliensis* occurs by inhalation of airborne propagules of the mycelial phase of the fungus, which reach the lungs, eventually evade the host defenses and disseminate via the bloodstream and/or lymphatics to virtually all parts of the body [4, 8, 10, 19]. Alveolar macrophages (AM) are believed to be important in the initial containment of the microorganisms through nonspecific or innate immune mechanisms. AM or dendritic cells (DC) also phagocytose particles and microbial organisms and carry them via lymphatics to regional hilar lymph nodes, where specific immune responses are believed to be generated.

P. brasiliensis proliferates ex vivo in a variety of mouse macrophages, including resident peritoneal alveolar and peripheral blood derived monocytes until the cells are lysed and killed by a yet unknown mechanism. However, the immunological activation of these cells efficiently inhibits fungal growth [48]. When alveolar macrophages were analyzed after pulmonary infection, absence of hydrogen peroxide production was observed with cells obtained from susceptible mice, whereas macrophages from resistant mice produced increased levels of this metabolite in the course of disease [14]. These different activities parallel the DTH anergy and the evident DTH reactivity developed by susceptible and resistant mice, respectively.

Brummer et al. [48, 63] have demonstrated that activation of mouse peritoneal macrophages by IFN-γ enhances the fungicidal activity of these cells but fungal killing is independent of the respiratory burst. Further investigations showed the fundamental role of nitric oxide in the fungicidal ability of activated macrophages, which appear to use an iron-restriction mechanism to inhibit the transformation of ingested conidia to yeast cells [64, 65]. We have confirmed the fundamental role of NO in the murine PCM [64]. In the course of infection, peritoneal macrophages from resistant mice secrete low levels of NO associated with high amounts of TNF-α; the opposite was seen with glass adherent cells from susceptible mice. Interestingly, in vitro inhibition of NO production by aminoguanidine treatment of B10.A macrophages led to increased production of TNF-α indicating the inhibitory role of NO on cytokine secretion. More importantly, the disease of i.p. infected C57BL/6 mice genetically deficient for inducible nitric oxide-synthase (iNOS KO) and in resistant and susceptible mice in vivo treated with aminoguanidine and incapable of secreting NO, is more severe [66].

The dual role of NO in murine PCM was further confirmed in the pulmonary model of infection. Compared with wild type mice, a lower fungal load was observed at week 2, although at week 10, increased number of fungi was detected in the lungs of mice genetically deficient of inducible NO-synthase (iNOS KO). The better control of fungal loads by iNOS KO mice at week 2 of infection appeared to be TNF-α mediated, since its in vivo neutralization abolished this difference [67, S. Bernardino and V. L. G. Calich, unpublished results). In agreement, Gonzales et al. [68] showed that TNF-α-activated peritoneal macrophages, although not producing NO, were able to inhibit the transition of *P. brasiliensis* conidia to yeast cells. Interestingly, our studies also demonstrated that iNOS KO mice, despite the more intense fungal infection by week 10 of infection, developed better organized granulomas. Thus, the increased secretion of TNF-α, the increased influx of activated T cells to the lungs, and the better organized lesions appear to compensate the genetic deficiency of NO. This was further confirmed by the equivalent survival times showed by iNOS KO and WT mice, despite the higher fungal loads in the former strain [67, S. Bernardino and V. L. G. Calich, unpublished data).

Recent studies were also performed aimed to understand the interaction between alveolar macrophages from resistant and susceptible mice and *P. brasiliensis*. Normal alveolar macrophages of B10.A mice, in vitro infected with *P. brasiliensis* yeasts, can be activated by small doses of exogenously added IFN-γ, secrete high levels of IL-12, nitric oxide and display a very efficient fungal killing activity. In contrast, macrophages from A/J mice were poorly activated by low doses of IFN-γ, secrete low amounts of IL-12, NO and present a poor fungicidal ability concomitant with the production of high levels of active TGF-β. The fungicidal ability of B10.A macrophages was modulated by aminoguanidine, whereas TGF-β was the main negative regulator of A/J macrophages. Thus, alveolar macrophages of susceptible mice seem to be more efficient than those of resistant mice and interaction of *P. brasiliensis* with these cells probably occurs through different macrophage receptors [69 A. Pina and V. L. G. Calich, unpublished observations]. These findings

appear to explain the apparently discrepant result we had observed when the pulmonary model of infection was first described: at the beginning of infection higher number of viable yeast cells were recovered from lungs of resistant mice as compared with susceptible ones [14]. Furthermore, IFN-γ, the most efficient macrophage activator was found in higher levels in the lung homogenates of susceptible mice [59]. Thus, the innate immunity appears to be much more efficient in the susceptible strain than in the resistant one. This hyperactivity is concomitant with high levels of NO production that is able to restrain fungal growth but also interferes with acquired immune responses leading to a subsequent immunosuppression of T-cell mediated immunity [66, A. Pina and V. L. G. Calich, unpublished data].

Dendritic cells and other APCs

T cells have clonal receptors (TCR) educated to see antigen epitopes presented by major histocompatibility complex (MHC) molecules of antigen presenting cells (APC). Several cell types can exert the APC function such as macrophages, B cells and endothelial cells, but the dendritic cells (DC) are considered the "professional APC" due to their special ability to activate T cells. DC are derived from hematopoietic stem cells in the bone marrow and form a network of a heterogeneous cell populations. Many DC reside and traffic through nonlymphoid peripheral tissues, continuously surveying the environment for invading microorganisms [70]. During infection, DCs in the periphery are activated by interaction with microorganisms or inflammatory mediators to increase their expression of MHC and co-stimulatory molecules such as CD80, CD86, and CD40. They also modify their expression of chemokines receptors and adhesion molecules, causing migration from the periphery to the T cell zone of draining lymph nodes. Activated DC then display pathogen encoded antigens to naïve antigen-specific T cells which initiate primary T cell responses [71, 72]. In the course of maturation, DC are subject to profound changes. The endocytic capacity is downmodulated, while there is a marked up-regulation of MHC class II expression, from an already high constitutive level [72].

As with other infectious pathologies, some studies on the importance of different antigen presenting cells (DC, macrophages, and B cells) in the resistance to *P. brasiliensis* infection were reported. It was shown that gp43, the immunodominant antigen for humoral immunity in PCM [73], was mainly presented by macrophages and stimulated a preferential Th1 cytokine production in resistant mice. In contrast, in susceptible mice gp43 was predominantly presented by B lymphocytes and led to preferential secretion of Th2 cytokines. In addition, no differences in T cell reactivity of resistant and susceptible mice were detected. [74]. Another report from the same group showed that the s.c. injection of mature DC, macrophages and B cells primed naïve susceptible and resistant mice and induced T cell proliferation. In this study, however, macrophages and B cells from both mouse strains displayed equivalent stimulatory activity inducing a preferential secretion of IL-10 and IL-4; DC from resistant animals, however, when compared with B10.A DC, stimulated a higher production of IFN-γ, equivalent levels of IL-12 and higher expression of MHC class II and CD80 molecules. B10.A macrophages were also shown to secrete high levels of IL-6 while IL-12 was secreted in similar levels by DC of both strains. Hence, it was suggested that DC of resistant mice preferentially drive Th1 development while B cells and macrophages from both mouse strains appeared to induce the differentiation of a Th0 or Th2 phenotype [75]. Further studies with resistant [76] and susceptible mice derived DC [77] demonstrated an equivalent behavior of gp-43 stimulated DC. Thus, gp43 treatment as well as *P. brasiliensis* infection down-regulated MHC class II, CD80, CD86, CD54, and CD40 expression as well as IL-12 and TNF-α secretion by LPS-treated DC. So, no major differences were reported in the activities of DC obtained from resistant and susceptible mice, unless they were previously activated by LPS. The i.v. infusion of DC previously treated with gp-43 plus LPS, but not with each of these components individually, increased pulmonary CFU counts and altered granulomas morphology of *P. brasiliensis*-infected mice [77].

We have also preliminary results comparing the behavior of DC from resistant and susceptible mice. Bone marrow derived DC were obtained and activated with LPS, *P. brasiliensis* yeast cells or a soluble whole yeast cells antigen. DC from both mouse strains exhibited MHC class II and co-stimulatory molecules (CD80, CD86, CD11c,

CD40) when activated by LPS or fungal yeast cells although A/J DC presented higher CD11c and CD86 expression. Compared with A/J cells, B10.A DC secreted higher levels of IL-12, IL-10, and NO, whereas the former are more able to produce active TGF-β. DC of susceptible mice induced a high proliferative activity of A/J T cells but not of B10.A lymphocytes while A/J DC stimulated T cell proliferation of both mouse strains. Thus, T cell anergy was only detected when B10.A DC were co-cultivated with homologous lymphocytes indicating that B10.A DC does not lack the ability to properly present *P. brasiliensis* antigens and that B10.A lymphocytes are appropriately activated when *P. brasiliensis* antigens were presented by A/J DCs (A. Pina and V. L. G. Calich, unpublished results).

Cytokines

As a more detailed review on the role of cytokines in PCM was reported elsewhere [10, 13], only a brief analysis of those studies will be presented here. IFN-γ is the most important protective cytokine to susceptible, intermediate, and resistant mice to *P. brasiliensis* infection [59, 78]. TNF-α and IL-12 are also very important protective cytokines [60, 78, 79]. IL-4 has a dual role (protective or disease promoting) in pulmonary PCM depending on the genetic pattern of the host [80, 81]. Despite the less severe disease induced by administration of rIL-12 [79], the strong inflammatory reaction in the lungs demonstrated the harmful effect of this cytokine. IL-10 appears to be one important macrophage-deactivating cytokine in pulmonary PCM, and its genetic absence appears to result in the aseptic cure of infected mice (Fig. 3) (T. A. Costa and V. L. G. Calich, unpublished results). Altogether, studies with cytokine-deficient mice showed that the Th1/Th2 paradigm can be applied to explain fungal growth (or dissemination) in liver and spleen: IL-4 and IL-10 are disease-promoting cytokines while IL-12 and IFN-γ are protective ones. However, the control of fungal growth in the lungs is more complex and both, Th1 (e.g., IL-12) and Th2 cytokines (e.g., IL-4) can have antagonistic effects. IL-10 is a disease-promoting cytokine and appears to have a more prominent role in the control of pulmonary PCM than IL-4.

Summarizing, our studies on innate immunity to *P. brasiliensis* infection suggest that a highly efficient innate immunity can lead to severe paracoccidioidomycosis. The following findings appear to support such inference: at the onset of infection, susceptible mice display a better control of lung fungal loads; IL-4 protects susceptible mice from severe infection; exogenous IL-12 leads to increased lung pathology; TLR usage leads to increased macrophage activation associated with increased fungal loads; susceptible mice secrete higher levels of LT and its inhibition results in milder pathology; PMN depletion causes more severe PCM associated with increased secretion of pro-inflammatory cytokines; early NO secretion can induce more severe infection. As a whole, it appears that "the more reactive the host innate immunity the more severe is the initial *P. brasiliensis* infection."

The influence of innate immunity in the resistance to *P. brasiliensis* infection

Protective immunity in paracoccidioidomycosis (PCM) is believed to be mainly mediated by cellular immunity [82]. In the human disease the Th1/Th2 dichotomy of $CD4^+$ T cells appears to partially explain the behavior of PCM patients and healthy infected individuals. So, the most evident Th1 immunity is observed when lymphocytes from healthy infected subjects or cured patients are in vitro activated by gp 43 and a clear production of IL-2 and IFN-γ is concomitant with a vigorous lymphoproliferative response [83, 84]. The acute form of the disease appears to be the Th2 pole of reactivity, where IL-4, IL-5, and IL-10 are produced and associated with low T cell proliferation which, however, can be reverted by in vitro treatment with rIL-12 and anti-IL-10 antibodies [85]. The severe form of the chronic disease also appears to present a Th2 pattern of reactivity. Most individuals of the chronic form of PCM, however, do not display polarized Th1/Th2 immune responses and their hyporesponsiveness appears to be not linked to imbalanced cytokine synthesis and may be due to other immunoregulatory mechanisms such as T cell anergy, T cell deletion by apoptosis or suppressive activity of natural regulatory T cells [10, 86–88]. Indeed, a recent paper showed a direct correlation

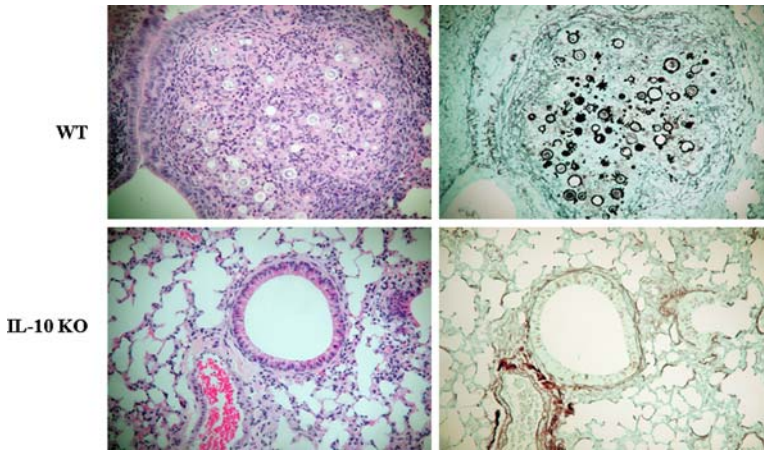

Fig. 3 Photomicrographs of pulmonary lesions developed by wild type (WT, upper micrographs) and IL-10-deficient (IL-10 KO, lower micrographs) C57BL/6 mice at week 8 after intratracheal infection with one million fungal cells. WT mice presented extensive, fungi rich, confluent lesions, occupying almost all lung parenchyma, whereas in IL-10 KO mice a diffuse inflammation, with no evident fungal cells, affected smaller areas of lungs. Left, H&E; right, Groccot stained lesions (100×)

between the number of natural regulatory T cells in the lesions and peripheral blood and the severity of PCM [88]. In human PCM the function of $CD8^+$ T cells, however, was poorly investigated.

We have some studies that characterized the function of $CD4^+$ and $CD8^+$(T cells in the immunity developed by susceptible (B10.A), intermediate (C57Bl/6) and resistant (A/J) mice after pulmonary infection with *P. brasiliensis* yeasts. In susceptible mice, anti-CD4 treatment did not alter disease severity and cellular immunity. However, anti-CD8 treatment led to increased fungal loads and DTH reactivity indicating the antagonistic effects of $CD8\alpha^+$ cells. In resistant mice, besides a protective type 1 immunity mediated by $CD8\alpha^+$ T cells, neutralization studies revealed the concomitant presence of Th1 and Th2 cells. In addition, deficiency of whole T and $CD8\alpha^+$ T cells but not of $CD4^+$ T or B cells in the C57Bl/6 background led to more severe PCM and increased mortality rates. In conclusion, our studies demonstrated that in pulmonary PCM: (a) fungal loads are mainly controlled by $CD8\alpha^+$ T cells; (b) genetic susceptibility of hosts appears to be associated with deletion or anergy of $CD4^+$ T cells, and finally, (c) a balanced type1/type2 immunity is associated with genetic resistance to *P. brasiliensis* infection [10].

The concomitant analysis of innate and adaptative immunity in murine PCM lead us to propose a model on the immunopathogenesis of pulmonary paracoccidioidomycosis. Alveolar macrophages of susceptible mice are very reactive to *P. brasiliensis* components and pro-inflammatory mediators are secreted by cells involved in the innate immunity of lungs. The high production of IL-12 stimulates NK cells to secrete elevated amounts of IFN-γ that induces the secretion of high levels of nitric oxide and other pro-inflammatory mediators by macrophages which develop a very efficient fungicidal ability. Leukotrienes and the TLR expression appear to activate macrophages and to contribute with *P. brasiliensis* endocytosis. Anti-inflammatory cytokines such as IL-10 and/or TGF-β are secreted in low levels. Although not extensively studied, equivalent activities were found with B10.A dendritic cells. This behavior results in a very effective innate immunity and precocious control of fungal growth and would result in preferential activation of Th1 $CD4^+$ cells. The excessive and continuous production of NO, however, inhibits the initial development of $CD4^+$ T-cell-immunity by active induction of T cell anergy or deletion. The elevated expression of co-stimulatory molecules (MHC class I, CD40, CD80, for example) by macrophages or DC could directly activate $CD8^+$ T cells without the help of $CD4^+$ T lymphocytes [89, 90]. This pattern of immunity could explain the very efficient mechanism of innate immunity resulting, however, in

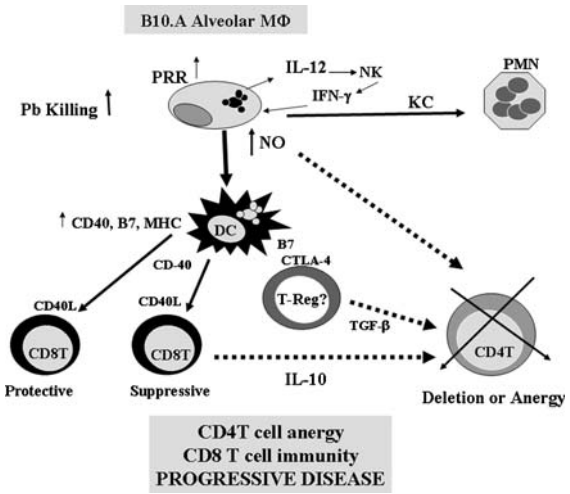

Fig. 4 Hypothesis on the innate and acquired immunological mechanisms leading to susceptibility to *P. brasiliensis* infection. At the initial stage of infection, macrophages from susceptible mice secrete high amounts of IL-12 and nitric oxide (NO) resulting in efficient fungal clearance. The same secretory pattern was seen with B10.A dendritic cells (DC). The excessive NO secretion, however, induces anergy or deletion of $CD4^+$ T cells. The expression of co-stimulatory molecules such as CD40 by antigen presenting cells induces a preferential activation of $CD8^+$ T cells that is not sufficient to efficiently activate macrophages and to control disease progression. (PRR, pattern recognition receptors; co-stimulatory molecules: CD40, B7, CTLA-4; MHC, major histocompatibility complex; KC, chemokine chemotactic for PMN cells; NK, natural killer cells; TGF-β, tissue growth factor beta; Treg, natural regulatory $CD4^+$ T cells)

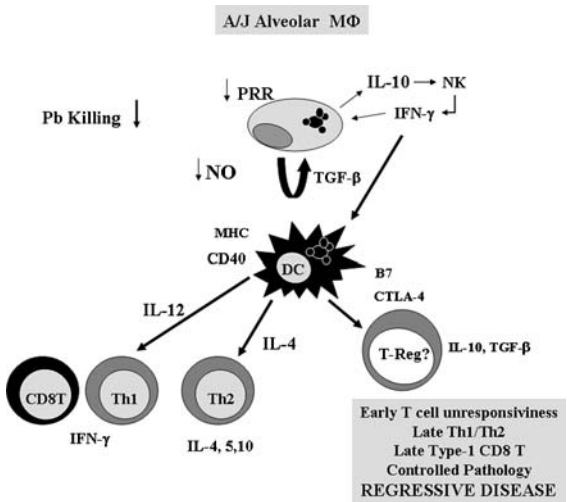

Fig. 5 Hypothesis on the innate and acquired immunological mechanisms leading to resistance to *P. brasiliensis* infection. At the innate phase of immunity, macrophages, and dendritic cells (DC) from resistant mice secrete low amounts of IL-12 associated with high levels of TGF-β resulting in impaired nitric oxide (NO) secretion and inefficient fungal clearance. This mild pattern of activation, however, results in slow activation of Th1 and Th2 $CD4^+$ T cells and type1 $CD8^+$ T lymphocytes which are able to induce efficient macrophage activation, controlled inflammation, and regressive disease. (PRR, pattern recognition receptors; co-stimulatory molecules: CD40, B7, CTLA-4; MHC, major histocompatibility complex; NK, natural killer cells; TGF-β, tissue growth factor beta; Treg, natural regulatory $CD4^+$ T cells)

poor T-cell mediated immunity (Fig. 4). It would also explain the DTH anergy, the non-organized lesions, the high levels of antibodies, and the progressive and severe disease developed by susceptible mice.

Alveolar macrophages and DC from resistant mice respond to *P. brasiliensis* infection by secreting low amounts of IL-12, but high levels of TGF-β and TNF-α. This results in poor NK cell activation, IFN-γ production, NO secretion, and initial inefficient fungal killing. This activity characterizes the low efficient natural immunity of resistant mice. However, the production of cytokines and NO in low levels do not impair T-cell immunity. So, resistant animals slowly develop *P. brasiliensis* specific $CD4^+$ and $CD8^+$ T lymphocytes, which control fungal growth and organize lesion morphology (Fig. 5). This model does not exclude the previously proposed Th1/Th2 model of *P. brasiliensis* control. It tries, however, to put together many results obtained with studies on innate and adaptative immunity in the murine model of pulmonary infection, which eventually may contribute to enhance our knowledge on the immunopathogenesis of human paracoccidioidomycosis.

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