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PAPEL DO RECEPTOR P2X7 EM MODELO MURINO DE INFECÇÃO POR *Mycobacterium tuberculosis*.

PORTO ALEGRE
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Dissertação apresentada como requisito parcial para obtenção do Título de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul.

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PORTO ALEGRE

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RESUMO

A tuberculose (TB) continua sendo uma das principais causas de morte no mundo, devido à grande capacidade de adaptação do bacilo que pode sobreviver em diferentes condições dentro e fora do hospedeiro humano. Estudos prévios mostraram evidências de que polimorfismos no receptor purinérgico P2X7 estão associados ao aumento da suscetibilidade à TB. O presente estudo objetivou analisar o papel do receptor purinérgico P2X7, na infecção por *M. tuberculosis* em camundongos, e os possíveis mecanismos de interação do receptor P2X7 com o hospedeiro, em modelos *in vivo* e *in vitro*. Nos experimentos para avaliação da viabilidade da micobacteria intracelular *in vitro* foi utilizada a linhagem de macrófagos murinos, RAW 264.7. Nossos resultados demonstraram que o tratamento das células RAW 264.7 com ATP (3 e 5 mM) resultou em uma redução estatisticamente significativa da contagem de unidades formadoras de colônias (CFUs). Nos experimentos *in vivo* foram utilizados camundongos machos C57BL/6 (tipo selvagem) e camundongos *knockout* para o receptor P2X7 ($P2X7R^{-/-}$) (25-30 g). Os resultados com imuno-histoquímica mostraram que a expressão do receptor purinérgico P2X7 foi encontrada significativamente aumentada nos pulmões de camundongos infectados com *M. tuberculosis*. Além disso, camundongos $P2X7R^{-/-}$ infectados com *M. tuberculosis* mostraram um aumento da carga da micobacteria no tecido pulmonar, quando comparado com camundongos do tipo selvagem infectados. Camundongos infectados mostraram um aumento marcante no peso do baço quando comparado com animais não infectados, indicando a ocorrência de esplenomegalia. Em baços de camundongos $P2X7R^{-/-}$, observou-se uma diminuição significativa nas populações de Treg (CD4 + Foxp3 +), células T (CD4 +, CD8 + CD25 + e CD4 + CD25 +), células dendríticas (CD11c +) e células + B220. No entanto, houve um aumento significativo de células CD11b + em camundongos $P2X7R^{-/-}$, quando comparados aos animais do tipo selvagem. Nos pulmões, camundongos $P2X7R^{-/-}$ infectados com *M. tuberculosis* apresentaram infiltrado pulmonar contendo um aumento de células Treg (CD4 + Foxp3 +), células T (CD4 + e CD8 +) e uma diminuição nas células + B220, quando comparado com camundongos do tipo selvagem infectados *com M. tuberculosis*. Portanto, os resultados observados no presente estudo fornecem novas evidências sobre o papel dos receptores P2X7 na patogênese e controle da tuberculose. O uso de agonistas ou antagonistas seletivos deste receptor como uma ferramenta terapêutica continua sendo uma questão a ser investigada.

Palavras-chave: ATP, receptor P2X7; *Mycobacterium tuberculosis*, camundongos.

ABSTRACT

Tuberculosis (TB) remains a leading cause of death worldwide, due to the great adaptability of the bacillus, which can survive in various conditions inside and outside the human host. Previous studies showed evidence that polymorphisms in P2X7 receptor are e associated with increased risk of TB. The present study aimed to analyze the role of purinergic P2X7 receptor in *M. tuberculosis* infection and host interaction mechanisms in vitro and in vivo. The macrophage murine cell line RAW 264.7 was used for in vitro experiments. . Our results demonstrated that treatment of RAW 264.7 cells with ATP (3 and 5 mM) resulted in a statistically significant reduction of counting colony forming units (CFUs). Male wild-type C57BL/6 (wild-type) and P2X7 receptor KO (P2X7R$^{-/-}$) mice (25–30 g) were used throughout this study for in vivo. Immunohistochemistry showed that the purinergic P2X7 receptor expression was found significantly augmented in the lungs of mice infected with *M. tuberculosis*. Furthermore, *M. tuberculosis*-infected P2X7R$^{-/-}$ mice showed an increase of *M. tuberculosis* burden in lung tissue, when compared to infected wild type mice. Infected mice showed a marked increase in the spleen weight, in comparison to non-infected animals, indicating the occurrence of splenomegaly. In P2X7R$^{-/-}$ spleens, we observed a significant decrease in the populations of Treg (CD4+Foxp3+), T cells (CD4+, CD8+CD25+ and CD4+CD25+), dendritic cells (CD11c+) and B220+ cells. However, a significant increase in CD11b+ cells was observed in P2X7R$^{-/-}$ mice, when compared to wild-type animals. In the lungs, P2X7R$^{-/-}$ *M. tuberculosis*-infected mice exhibited pulmonary infiltrates containing an increase of Treg cells (CD4+Foxp3+), T cells (CD4+ and CD8+) and a decrease in the B220+ cells, when compared with wild-type *M. tuberculosis*-infected mice. The findings observed in the present study provide novel evidence on the role of P2X7 receptors in the pathogenesis and control of tuberculosis. Whether selective agonists or antagonists of this receptor might be useful for improving TB complications remains a matter to be investigated.

Keywords: ATP, P2X7 receptor; *Mycobacterium tuberculosis*, mouse.

LISTA DE ABREVIATURAS

ADP – adenosina 5’- difosfato

AMP – adenosina 5’-monofosfato

AMPc - adenosina 5’-monofosfato cíclico

ATP – adenosina 5’- trifosfato

BCG - Bacillus Calmette-Guérin

BzATP – 2’,3’,-O-(4-benzoil)benzoil-adenosina-5’-trifosfato

COX-2 - ciclooxigenase-2 (COX-2),

e5’NT – ecto-5’-nucleotidase

HIV – vírus da imunodeficiência humana

IL-1β – interleucina 1 beta

iNOS - óxido nítrico siintase induziívelda

MDR-TB - tuberculose resistente a múltiplas drogas

NDPK – nucleosídeos difosfoquinase

NPP – nucleotídeo pirofosfatase

NTPDase - ecto-nucleosídeo trifosfato difosfohidrolase

OMS – Organização Mundial da Saúde

SNC – Sistema Nervoso Centralsistema nervoso central

TB – tuberculose

TDR-TB – tuberculose totalmente resistente

TNFα – fator de necrose tumoral alfa

Treg – células T regulatórias

UDP – uridina 5’-difosfato

UFC – unidade formadora de colônia

UTP - uridina 5’-trifosfato

XDR-TB – tuberculose extensivamente resistente

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1. INTRODUÇÃO

1.1 Tuberculose

A tuberculose (TB) humana é uma doença infecto-contagiosa causada principalmente pelo bacilo *Mycobacterium tuberculosis*, uma bactéria aeróbia que causa infecção usualmente nos pulmões (Cole *et al.* 1998). A TB foi responsável por milhões de mortes no passado, quando ainda não existia tratamento adequado para a doença e se desconhecia seu agente causal. No século XIX, a TB apresentou-se como uma doença avassaladora, com altas taxas de transmissão e que levava a um número de mortes muito elevado. Esta época corresponde ao início da revolução industrial, onde houve o surgimento dos aglomerados urbanos, muitas vezes sem estruturas de higiene e habitação, o que colaborava para a disseminação e o estabelecimento de inúmeras doenças, dentre elas a TB (Dormandy 2002).

Somente em 1882, o médico e bacteriologista alemão Robert Koch tornou pública a identificação do *M. tuberculosis* como agente etiológico da TB, durante o IV Congresso Mundial de Tuberculose (Kaufmann 2003). O *M. tuberculosis* é geralmente transmitido entre os indivíduos por aerossóis que contêm o bacilo. Essas pequenas gotículas podem permanecer por longos períodos de tempo em ambientes fechados. Quando inalada, a micobactéria é fagocitada pelos macrófagos alveolares nos bronquíolos respiratórios e nos alvéolos. O bacilo inalado poderá ou não estabelecer a infecção e isso dependerá da virulência bacteriana e da capacidade bactericida dos macrófagos do hospedeiro (ATC

2000). Sob condições normais, a infecção é combatida pelo sistema imune do hospedeiro e, em 90 % dos indivíduos infectados, a infecção não progride para a doença ativa (Russel 2001).

A descoberta da estreptomicina, em 1944, foi um marco no desenvolvimento de fármacos para tratar a TB; a seguir, outros fármacos usados atualmente no tratamento da doença foram introduzidos, incluindo: ácido p-aminosalicílico (1946), isoniazida (1952), ciclosserina (1955), canamicina (1957), rifampicina (1965), etionamida (1966), etambutol (1968) e pirazinamida (1970) (Duncan 2003). O desenvolvimento destes fármacos e o emprego de medidas profiláticas proporcionaram uma diminuição no número de mortes, que se manteve por algumas décadas (Bloom e Murray 1992; Daniel 1997). Contudo, a TB ressurgiu com força e, é atualmente, considerada a principal causa de morte devido a um único agente infeccioso (Enarson e Murray 1996). A epidemia do vírus da imunodeficiência humana (HIV), a deterioração dos programas de saúde pública visando o controle da TB, a multiplicação de aglomerados urbanos sem estrutura sanitária e o número elevado de pessoas sem moradia são alguns dos fatores apontados como responsáveis pelo ressurgimento TB em muitos países, inclusive desenvolvidos (Bloom e Murray 1992).

Em 1993, a Organização Mundial da Saúde (OMS) declarou a TB como emergência de saúde global, estimando-se que um terço da população humana esteja infectada pelo bacilo e que na última década 30 milhões de pessoas morreram desta doença (Dye *et al.* 1999; Corbett *et al.* 2003). Dados epidemiológicos globais do ano de 2010 indicam a incidência de 128 casos de

TB a cada 100.000 habitantes, o que corresponde a 8,8 milhões de doentes no mundo (Figura 1), levando 1,4 milhões dos pacientes a óbito. Dentro desta estimativa, 1,1 milhões dos casos de TB são referentes à co-infecção TB-HIV (WHO 2011). O Brasil ocupa o 19º lugar na relação dos 22 países nos quais se concentram 80 % dos casos de TB; em 2010, foram registrados 85 mil novos casos (incluíndo 18 mil casos de coinfecção HIV-TB), ou seja, uma incidência de 43 casos por 100.000 habitantes, levando 5 mil dos pacientes, ao óbito, excluindo os casos de morte por coinfecção HIV-TB (WHO 2011). Assim, a TB permanece como uma das principais causas de morte em todo o mundo, em virtude da grande capacidade de adaptação do bacilo, que consegue sobreviver em diversas condições dentro e fora do hospedeiro humano.

O *M. tuberculosis* tem sido apontado como o patógeno de maior sucesso no planeta, de todos os tempos, conseguindo muitas vezes permanecer silencioso e latente dentro do hospedeiro, sendo capaz de escapar das defesas do sistema imune (Wickelgren 2000; Enserink 2001). O bacilo pode permanecer dormente até que as defesas do hospedeiro sejam diminuídas como no caso da infecção pelo HIV e por imunossupressão por fármacos. Com o aumento no número de infectados com o HIV, torna-se preocupante o número de hospedeiros ativamente contaminados e capazes de transmitir a doença (Manabe e Bishai 2000).

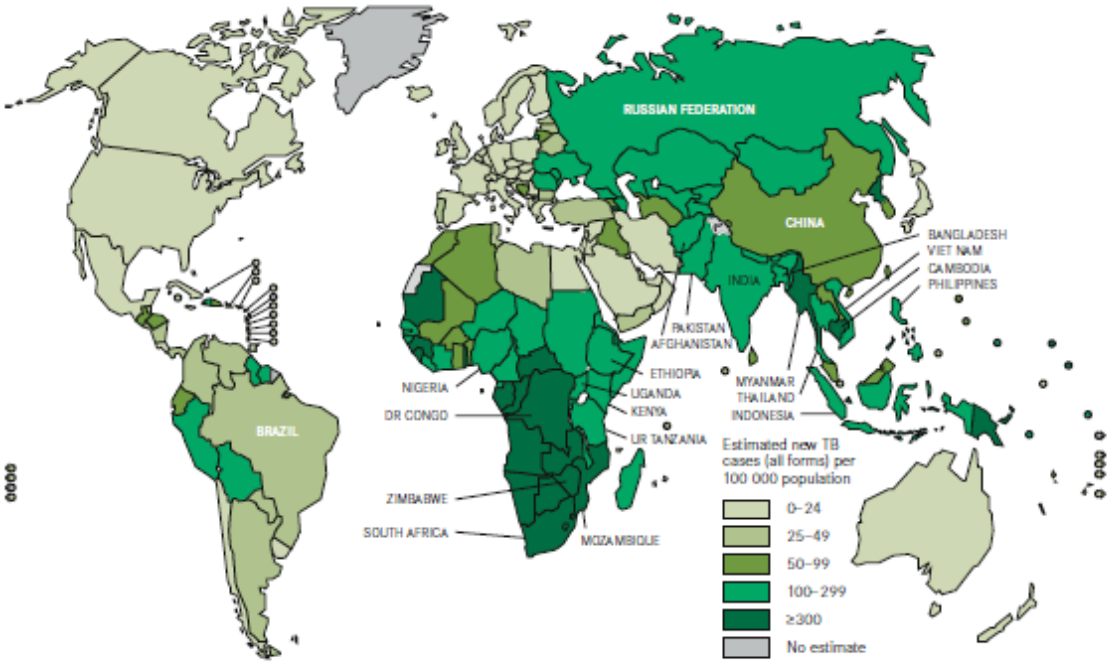

Figura 1. Mapa mostrando as estimativas de incidência de TB no mundo em 2010, de acordo com a Organização Mundial da Saúde (WHO 2011).

TB e HIV são tão proximamente relacionados, que o termo co-epidemia tem sido usado para descrever sua relação. A taxa de reativação da TB latente em pessoas imunocompetentes é de 0,2 % por ano. Já, em pacientes com HIV, este risco de reativação da TB latente pode chegar até 13,3 % ao ano, dependendo do nível de imunossupressão (Knigge *et al.* 2000). Pacientes HIV-positivos que são infectados com TB desenvolvem a doença ativa em uma taxa de 37 % nos primeiros seis meses; já nos pacientes imunocompetentes, essa taxa é de até 5 % nos dois primeiros anos. Além disso, pacientes HIV-positivos apresentam deficiência na absorção de alguns fármacos, talvez resultante da enteropatia relacionada ao HIV, o que pode comprometer o tratamento da TB e

também levar ao surgimento de cepas resistentes a fármacos usados para tratar a TB (Sepkowitz *et al.* 1995).

Atualmente, o tratamento recomendado pela OMS consiste na administração combinada dos fármacos isoniazida, rifampicina, pirazinamida e etambutol durante dois meses. O tratamento deve prosseguir por mais quatro meses, quando se administra isoniazida e rifampicina. Infelizmente, alguns países não conseguem empregar tal programa terapêutico para a totalidade dos seus pacientes, reforçando a hipótese de que tratamentos mais curtos e com menores efeitos colaterais melhorariam a adesão do paciente, levando a uma maior eficácia no tratamento da TB (Duncan 2003).

De fato, regimes de tratamento inapropriados e a não adesão do paciente ao tratamento são comumente associados com a emergência de cepas de TB resistentes a múltiplas drogas (MDR-TB), cujos isolados são resistentes a pelo menos isoniazida e rifampicina, dois dos principais fármacos usados no tratamento padrão da TB (Basso e Blanchard 1998; Duncan 2003). Pacientes com MDR-TB devem ser tratados com uma combinação de fármacos de segunda linha que, além de serem significativamente mais caras, possuem maior toxicidade e são menos efetivas que os fármacos de primeira linha (O'Brien e Nunn 2001). Em países industrializados, o tratamento normal custa em torno de 2.000 dólares por paciente, mas pode atingir 250.000 dólares para pacientes com MDR-TB (Pasqualoto e Ferreira 2001).

Casos de co-infecção com HIV e MDR-TB alcançam taxas de mortalidade próximas a 100 %, sendo esta definida como a infecção oportunista de maior

morbidade associada à AIDS (Fatkenheuer *et al.* 1999). Cerca de 300.000 novos casos de MDR-TB são diagnosticados a cada ano; 4 a 20 % destes são classificados como TB extensivamente resistente (XDR-TB). Esta é definida como casos de TB cujos isolados são resistentes à isoniazida, rifampicina e a pelo menos três das seis principais classes de fármacos de segunda linha (aminoglicosídeos, polipetídeos, fluoroquinolonas, tiamidas, ciclosserina e ácido p-aminosalicílico) . XDR-TB é relatada em todo o mundo, inclusive nos Estados Unidos, onde a TB estava sendo considerada sob controle. XDR-TB é a forma de TB que deixa o paciente sem opção de tratamento de acordo com os padrões internacionais. Dados divulgados pela OMS revelaram que, em 2008, ocorreram 30.000 casos de XDR-TB, os quais foram detectados em 45 países e, até o mês de novembro de 2008, 57 países já registravam pelo menos um caso de XDR-TB (CDC 2009). Em paralelo às cepas MDR-TB e XDR-TB, uma nova linhagem de *M. tuberculosis* foi identificada recentemente e denominada TDR-TB, ou seja, uma cepa totalmente resistente aos fármacos atualmente disponíveis para tratar a TB. O pouco êxito na terapia e a ocorrência já difundida destas cepas resistentes levam a discussões sobre a drástica situação de casos de TB virtualmente incuráveis e aponta para a necessidade urgente de se introduzir fármacos anti-TB novos e mais eficazes (Duncan 2003; Dorman e Chaisson 2007).

1.2 O Sistema Purinérgico

Purinas extracelulares (adenosina, ADP, e ATP) e pirimidinas (UDP e UTP) são moléculas sinalizadoras importantes que medeiam diversos efeitos biológicos, através da ativação de receptores purinérgicos (Ralevic e Burnstock 1998)**.**

Embora tenha sido recebida com certo ceticismo, a função de neurotransmissor do ATP foi sugerida no fim dos anos 50 (Holton 1959), mas somente em 1972, Burnstock propôs o primeiro modelo de sinalização purinérgica (Burnstock 1972). Este modelo de neurotransmissão não adrenérgica e não colinérgica, explicava como o ATP era estocado, liberado, ligava-se em receptores específicos e ainda tinha sua ação finalizada por enzimas que o hidrolisavam (Figura 2) . Ao longo do tempo, receptores e enzimas foram identificados, clonados e caracterizados, estabelecendo-se então o sistema purinérgico de sinalização celular não somente de neurônios, mas de diferentes tipos celulares (Burnstock 2006).

Existem duas famílias principais de receptores purinérgicos: receptores de adenosina ou P1 e, os receptores P2, que reconhecem principalmente ATP, ADP, UTP, e UDP. Os receptores P1/Adenosina são acoplados à proteína G e se subdividem em quatro subtipos, A_1, A_{2A}, A_{2B}, e $A_{3,}$ de acordo com a estrutura molecular, bioquímica e caracterização farmacológica. Baseado em diferentes estruturas moleculares e mecanismos de transdução de sinal, os receptores P2 se dividem em duas famílias de receptores: receptores P2X, ionotrópicos, e P2Y,

metabotrópicos (Ralevic e Burnstock 1998; Burnstock 2006). Atualmente, sete subtipos de receptores P2X (P2X1 à P2X7) e oito subtipos de receptores P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) já foram identificados (Burnstock 2006). Exemplos do potencial terapêutico dos receptores P2Y incluem: receptores $P2Y_1$ envolvidos na agregação plaquetária e, receptores $P2Y_2$, responsáveis pelo aumento do fluxo de íons cloro, sendo alvos para o tratamento de fibrose cística (von Kugelgen e Wetter 2000).

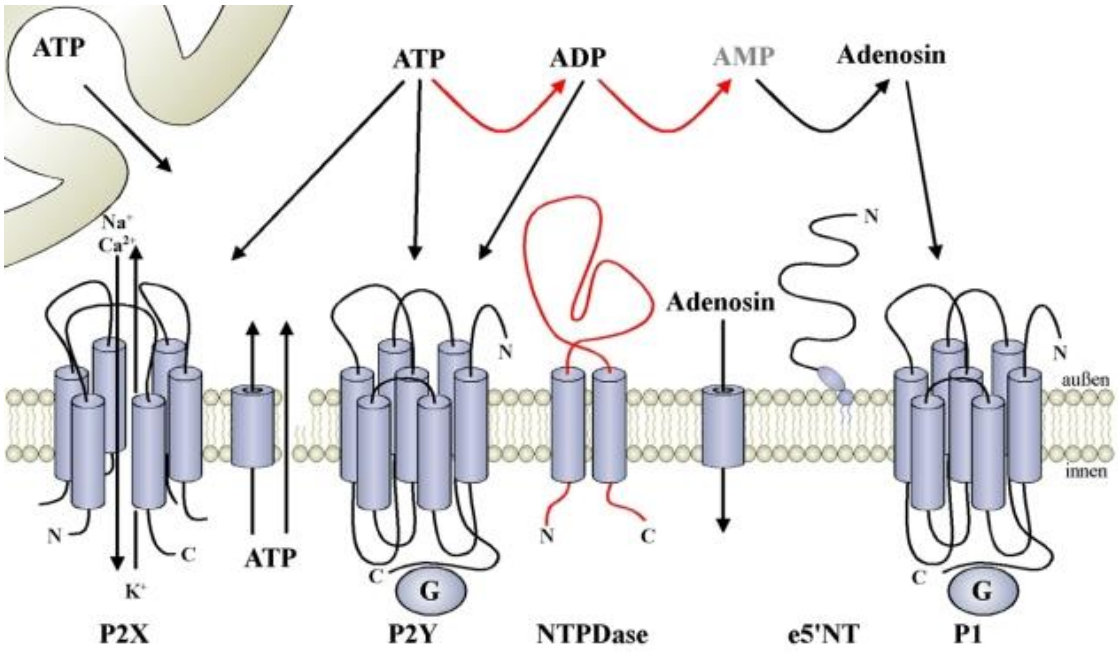

Figura 2. Sinalização Purinérgica. O ATP exerce seus efeitos ligando-se aos receptores P2X e P2Y, sendo hidrolisado pelas NTPDases em ADP, que também liga-se aos receptores P2Y, o ADP por sua vez também é hidrolisado pelas NTPDases em AMP, no qual seu último fosfato é retirado pela e-5'NT. A adenosina formada se liga aos receptores P1 e pode ser captada por transportadores. Figura retirada e adaptada do site http://www.uni-leipzig.de/~straeter/research/ntpdase.html de autoria do Prof. Dr. Norbert Sträter.

Os sete subtipos de receptores P2X apresentam-se como homo ou hetero oligômeros ($P2X_{2/3}$, $P2X_{4/6}$, $P2X_{1/5}$, $P2X_{2/6}$, $P2X_{4/7}$) e, quando ativados, permitem a passagem de íons tais como Ca^{2+}, Na^{2+} e K^{+}, despolarizando a membrana plasmática. Já, os subtipos de receptores $P2Y_{1,2,4,6,11\text{-}14}$ ativam cascatas de sinalização envolvendo fosfolipase C, inositol 3-fosfato e liberação de Ca^{2+} dos estoques intracelulares ou, então afetam a adenilil ciclase, alterando os níveis de AMPc (Ralevic e Burnstock 1998; Burnstock 2007; Burnstock 2008).

Há importantes mecanismos envolvidos no controle da concentração de ATP extracelular. As ecto-nucleotidases são enzimas envolvidas na degradação de nucleotídeos e na formação de nucleosídeos, possuindo, portanto, um papel chave na regulação purinérgica. As ecto-nucleotidases compreendem diversas famílias de enzimas. As NTPDases (nucleosídeo trifosfato difosfohidrolases) são capazes de hidrolisar tri- e di-fosfonucleotídeos extracelulares e, já tem pelo menos 8 enzimas bem caracterizadas em mamíferos (NTPDases 1-8). Alterações relacionadas à atividade de NTPDases têm sido descritas em várias doenças, assim como em células tumorais de diferentes origens. Há ainda enzimas da família NPP (nucleotídeo pirofosfatases), as fosfatases alcalinas, as ecto-5´-nucleotidases, e as nucleosídeos difosfoquinases (NDPK) (Wink *et al.* 2003; White e Burnstock 2006). Esta sinalização pode ser encontrada em praticamente todos os sistemas fisiológicos: cardíaco, sanguíneo, imunológico, digestivo, endócrino, nervoso central e periférico, pulmonar e renal de humanos e murinos, desempenhando as mais diferentes funções (Burnstock 2006). Com ampla participação dessa via de sinalização nos diferentes processos

fisiológicos, é fácil perceber que qualquer descontrole em qualquer uma das etapas de sinalização pode levar a diferentes condições patológicas. Exatamente por isso, antagonistas e agonistas purinérgicos têm servido como base para o desenvolvimento de fármacos empregados no tratamento de diferentes doenças.

1.3 O Receptor P2X7

Entre os receptores P2X, o subtipo P2X7 destaca-se dentre os demais, porque além de abrir canais catiônicos na célula, ele é capaz de dilatar poros na membrana plasmática em resposta a altas concentrações de agonista ou exposição prolongada, o que torna a célula permeável a moléculas de peso molecular de até 900 Da (North 2002).

Os receptores P2X7 têm sido descritos em diversos sistemas, tais como cardiovascular, neurológico, sistema imune, gástrico, urogenital e respiratório, assim como em dermatologia e oncologia (Burnstock 2006). Os receptores P2X7 existem em um número limitado de tipos celulares, mas são facilmente detectáveis em linhagens de células hematopoiéticas incluindo monócitos, macrófagos e linfócitos (Labasi *et al.* 2002; Yoon *et al.* 2007). Dentro do sistema nervoso central (SNC), receptores P2X7 são localizados na microglia e células de Schwann, bem como em astrócitos (Donnelly-Roberts e Jarvis 2007).

Os receptores P2X7 são ativados por altas concentrações de ATP e com maior potência pelo 2',3'-O-(4-benzoilbenzoil)-ATP (BzATP) (Donnelly-Roberts e

Jarvis 2007). Vários antagonistas dos receptores P2X7 têm sido identificados a fim de avaliar as funções deste receptor. Recentemente, foi publicado um estudo com a caracterização completa de um antagonista do receptor P2X7, AZ11645373, que mostrou alta afinidade pelo receptor de humanos, mas apresentou baixa afinidade por receptores de ratos (Stokes *et al.* 2006). Outros antagonistas seletivos do receptor P2X7 e com afinidade comparável em humanos, ratos e camundongos têm sido desenvolvidos e caracterizados, incluindo o A438079 e o A740003 (Donnelly-Roberts e Jarvis 2007).

A ativação do receptor P2X7 resulta em abertura rápida e reversível de canais que são permeáveis a Na+, K+ e Ca^{2+} (Donnelly-Roberts e Jarvis 2007). O receptor serve também como um regulador da inflamação, pois está envolvido na produção de citocinas pró-inflamatórias, tais como TNFα e IL-1β, levando à indução de ciclooxigenase-2 (COX-2), metaloproteinases, óxido nítrico sintase induzida (iNOS) e produção de superóxidos (Labasi *et al.* 2002; Donnelly-Roberts e Jarvis 2007).

De maneira interessante, o receptor P2X7 tem sido alvo de estudos para o desenvolvimento de novos analgésicos (King 2007). Assim, dados recentes obtidos com camundongos *knockout* para o receptor P2X7 ou com novos antagonistas potentes e seletivos deste receptor indicam um papel crucial para o receptor P2X7 no início e na persistência de certos tipos de dor crônica (Donnelly-Roberts e Jarvis 2007) e também em processos inflamatórios como a cistite hemorrágica (Martins *et al.* 2011).

Foi demonstrado que a ausência deste receptor torna as células epiteliais menos apoptóticas, sugerindo que a ativação dos receptores P2X7 poderia estar envolvida com a regulação de apoptose (Goncalves *et al.* 2006). Neste contexto, tem sido proposto que a exposição prolongada de receptores P2X7 a agonistas leva à formação de um poro citolítico na membrana celular, permitindo assim a entrada de partículas maiores, de até 900 Da e, consequente morte celular (Burnstock 2006; Donnelly-Roberts e Jarvis 2007).

O gene do receptor P2X7 é altamente polimórfico e a maioria das substituições confere perda de função da proteína, embora em alguns casos possam tornar o receptor mais sensível (Cabrini *et al.* 2005; Fuller *et al.* 2009).

1.4 Receptor P2X7 e Tuberculose

Como já descrito anteriormente, a estimulação de células através do receptor P2X7 causa uma abertura de canais catiônicos que permite o influxo de Ca^{2+} e o efluxo de K^{+}, fenômenos envolvidos na indução de apoptose e na liberação de IL-1β (Verhoef *et al.* 2003). A ativação contínua do receptor P2X7 induz a formação de poros na membrana permeáveis a solutos hidrofílicos e a apoptose. Já foi bem caracterizado que a indução de apoptose, através da ativação do receptor P2X7 em macrofágos infectados com *M. tuberculosis*, também resulta na morte da micobactéria (Canaday *et al.* 2002; Shemon *et al.* 2006).

Um polimorfismo comum no éxon 13 (A1513C) resulta na substituição do ácido glutâmico na posição 496 pela alanina (E496A) (Gu *et al.* 2001). Este polimorfismo ocorre na região do gene que codifica a cadeia carboxiterminal da proteína. Diferentes estudos têm demonstrado que a homozigose para o alelo C (C/C) leva à perda quase completa da função do receptor P2X7, com a ausência da morte de micobactérias induzida por ATP nesses indivíduos. Outro polimorfismo observado é o 489C-T, que substitui o aminoácido histidina da posição 155 por uma tirosina (His155 para Tyr), esse alelo tem uma frequência de 0.455 em caucasianos. O polimorfismo His155 para Tyr produz um ganho de função no receptor P2X7, na formação de poros e no influxo de Ca^{++} (Cabrini *et al.* 2005; Fuller *et al.* 2009).

De forma interessante, o receptor P2X7 parece estar relacionado em outras doenças infecto-contagiosas como a Leishmaniose e a doença de Chagas (Coutinho-Silva *et al.* 2007; Cascabulho *et al.* 2008; Chaves *et al.* 2009; Marques-da-Silva *et al.* 2011).

2. OBJETIVOS

2.1 Objetivo Geral

Estudos recentes têm sugerido que o sistema purinérgico pode estar envolvido no desenvolvimento e controle da TB. Neste contexto, o presente estudo teve como objetivo investigar o papel do receptor P2X7 em um modelo *in vivo* e *in vitro* de infecção por *M. tuberculosis*, a fim de encontrar um possível alvo farmacológico para tratar ou melhorar a qualidade de vida de indivíduos acometidos pela TB.

2.2 Objetivos Específicos

a. Investigar o efeito do ATP sobre a viabilidade do *M. tuberculosis* infectado na linhagem murina de macrófagos RAW 264.7;
b. Investigar a modulação do receptor P2X7 em pulmões de camundongos infectados com *M. tuberculosis*, através de análise imuno-histoquímica;
c. Avaliar o efeito da deleção gênica dos receptores P2X7, sobre o desenvolvimento da TB em camundongos, através da quantificação de Unidades Formadoras de Colônias;
d. Avaliar, através da técnica de citometria de fluxo, as alterações das populações de células do sistema imune, induzidas pela infecção por *M. tuberculosis* nos baços e pulmões de camundongos selvagens e *Knockout* para o receptor P2X7.

3. ARTIGO CIENTÍFICO

Os resultados do presente trabalho foram submetidos ao periódico *Immunobiology.*

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Implication of purinergic P2X7 receptor in *M. tuberculosis* infection and host interaction mechanisms: a mouse model study

Running Head: Implication of P2X7 receptor in tuberculosis.

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Abbreviations: APC (Antigen-Presenting Cell), ATP (Adenosine-5'-Triphosphate), BCG (Bacille Calmette-Guerin), CFU (Colony Forming Unit), FBS (Fetal Bovine Serum), HIV (Human Immunodeficiency Virus), MDR (Multidrug-resistant), OADC (Oleic Acid, Albumin, Dextrose, Catalase), PBS (Phosphate Buffered Saline), SDS (Sodium Dodecyl Sulfate), TB (Tuberculosis), TDR (Totally Drug-Resistant), Treg (Regulatory T cell), WHO (World Health Organization), XDR (Extensively Drug-Resistant).

Abstract

In the present study, we analyzed the role of purinergic P2X7 receptor in *M. tuberculosis* infection and host interaction mechanisms *in vitro* and *in vivo.* For experimental procedures, a macrophage murine cell line RAW 264.7, and male Swiss, wild-type C57BL/6 and P2X7 receptor knockout ($P2X7R^{-/-}$) mice were used throughout this study. We have demonstrated that treatment of RAW 264.7 cells with ATP (3 and 5 mM) resulted in a statistically significant reduction of *M. tuberculosis*-colony-forming units. The purinergic P2X7 receptor expression was found significantly augmented in the lungs of mice infected with *M. tuberculosis* H37Rv. Infected wild-type mice showed a marked increase in the spleen weight, in comparison to non-infected animals. Furthermore, *M. tuberculosis*-infected $P2X7R^{-/-}$ mice showed an increase of *M. tuberculosis* burden in lung tissue, when compared to infected wild-type mice. In $P2X7R^{-/-}$ spleens, we observed a significant decrease in the populations of Treg (CD4+Foxp3+), T cells (CD4+, CD8+CD25+ and CD4+CD25+), dendritic cells (CD11c+) and B220+ cells. However, a significant increase in CD11b+ cells was observed in $P2X7R^{-/-}$ mice, when compared to wild-type animals. In the lungs, $P2X7R^{-/-}$ *M. tuberculosis*-infected mice exhibited pulmonary infiltrates containing an increase of Treg cells (CD4+Foxp3+), T cells (CD4+ and CD8+) and a decrease in the B220+ cells, when compared with wild-type *M. tuberculosis*-infected mice. The findings observed in the present study provide novel evidence on the role of P2X7 receptors in the pathogenesis of tuberculosis.

Introduction

Tuberculosis (TB) is a worldwide public health priority and remains the leading cause of mortality due to a single bacterial pathogen, *Mycobacterium tuberculosis*. The World Health Organization (WHO) has estimated 8.8 million incident cases of TB, and approximately 1.4 million people died of TB in 2010 (World Health Organization, 2011) . *M. tuberculosis* has been identified as the most successful pathogen of all times, because it remains silent and latent within the host, being able to escape from the immune defenses (Enserink, 2001, Wickelgren, 2000). By arresting phagosomal maturation (Malik, et al., 2000, Pethe, et al., 2004), it persists dormant until the host defenses are suppressed, as seen in human immunodeficiency virus (HIV) infection. The growing rate of HIV-TB co-infection (Fatkenheuer, et al., 1999, World Health Organization, 2010, World Health Organization, 2011), the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and more recently, totally drug-resistant (TDR) strains of *M. tuberculosis* (World Health Organization, 2010, World Health Organization, 2011), increased the need for identifying different therapeutic options to treat TB, as well as to better understand the pathophysiology of this insidious disease.

Extracellular purines, including ATP, display a series of roles in several physiological processes, such as vascular tonus, pain sensation, neurotransmission, cell proliferation, differentiation, and cell death (Burnstock, 2006). The purinergic receptors mediating these effects are classified into P1 (A_1, A_{2A}, A_{2B}, and A_3), which are activated by nucleosides, and P2 receptors that

respond to nucleotides. These receptors are further subdivided into metabotropic P2Y ($P2Y_{1, 2, 4, 6, 11-14}$) and ionotropic P2X subtypes (P2X1-7) (Burnstock, 2006).

The P2X7 receptor subtype presents a series of particularities. First, its activation requires high concentrations of ATP (up to 300 µM), while the other P2X receptors display a very high sensitivity for ATP. Next, P2X7 receptor is highly expressed in immune and inflammatory cells, throughout the central or peripheral nervous systems (Burnstock, 2011, Donnelly-Roberts and Jarvis, 2007, Ferrari, et al., 1997).

P2X7 receptor activation results in a rapid and reversible opening of channels that are permeable to Na^+, K^+ and Ca^{2+} (Donnelly-Roberts and Jarvis, 2007). This receptor also serves as a regulator of inflammation, and it is involved in the production of pro-inflammatory cytokines such as TNF-α and IL-1 β, leading to the induction of cyclooxygenase-2 (COX-2), metalloproteinases, inducible nitric oxide synthase (iNOS) and production of superoxide anion (Donnelly-Roberts and Jarvis, 2007, Labasi, et al., 2002). This receptor has been involved in the activation of peripheral macrophages and glia, neutrophil infiltration, and prostaglandin production (Burnstock, 2006, Di Virgilio, 2007, Donnelly-Roberts and Jarvis, 2007, Goncalves, et al., 2006, Labasi, et al., 2002). Recent studies have demonstrated the influence of P2X7 receptor in a wide range of experimental models of disease, such as depression, epilepsy, Parkinson's disease, arthritis, cancer, hemorrhagic cystitis, and chronic pain, by using either selective P2X7 receptor antagonists or knockout (KO) mice to P2X7 receptors (Basso, et al., 2009, Chessell, et al., 2005, Donnelly-Roberts

and Jarvis, 2007, Honore, et al., 2006, Li, et al., 2006, Marcellino, et al., 2010, Teixeira, et al., 2010).

It was shown that absence of P2X7 receptor make epithelial cells less apoptotic, suggesting that its activation might be involved in the regulation of apoptosis (Goncalves, et al., 2006). In this context, it has been proposed that prolonged exposure of P2X7 receptor to agonists leads a formation of a cytolytic pore in the cell membrane, allowing the entry of larger particles up to 900 Da, and consequent cell death (Burnstock, 2006, Donnelly-Roberts and Jarvis, 2007). Noteworthy, ATP was found to induce both cell death and killing of intracellular mycobacteria within BCG-infected human macrophages (Lammas, et al., 1997, Molloy, et al., 1994). The cytotoxic and mycobactericidal effects of ATP have been shown to be mediated by the specific interaction of ATP with macrophage surface P2X7 receptors (Lammas, et al., 1997).

Recent studies have demonstrated that the polymorphism 1513A→C of P2X7 receptor gene causes an amino acid change from glutamic acid at amino acid position 496 to an alanine in the C-terminus (Gu, et al., 2001), impairing multiple P2X7 receptor functions, including cation fluxes in a variety of cells, the release of IL-1β, IL-18, and matrix metalloproteinase (MMP)-9 from macrophages, or shedding of CD23 and CD62L from lymphocytes (Gu, et al., 2001, Saunders, et al., 2003, Sluyter, et al., 2004, Sluyter, et al., 2004, Sluyter and Wiley, 2002). This polymorphism is associated with impaired ability to kill *Mycobacterium bovis* BCG via ATP *in vitro* (Fernando, et al., 2005, Saunders, et

al., 2003), and increased susceptibility to extra pulmonary tuberculosis (Fernando, et al., 2007).

In the present study, we analyzed the mycobactericidal activity of ATP in *M. tuberculosis*-infected murine macrophages, as well as the *in vivo* expression of P2X7 receptors in a murine model of *M. tuberculosis*-infection. We have also assessed, for the first time, the effects of P2X7 receptor gene deletion on splenomegaly and colony forming unit (CFU) counts in lungs and spleens of *M. tuberculosis*-infected mice. Attempts have also been made to evaluate the immune profile of P2X7 receptor KO mice infected with the laboratorial *M. tuberculosis* H37Rv strain, by means of an extensive flow cytometry analysis.

Materials and Methods

Murine cell line culture and macrophage infection

The macrophage murine cell line RAW 264.7 (obtained from Banco de Células do Rio de Janeiro – BCRJ) was cultured in RPMI-1640 (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin–Streptomycin at 37 ºC with 5% CO_2. For infection of macrophages, we used the method previously described by Mascarello et al (Mascarello, et al., 2010) with some modifications. Briefly, *M. tuberculosis* H37Rv strain was grown in Middlebrook 7H9 (Difco) containing 10% oleic acid/albumin/dextrose/catalase (OADC) enrichment at 37 ºC. RAW 264.7 cells were seeded in 12-well tissue culture plates (Corning) at 1 X 10^5 cells per well in RPMI-1640 medium (supplemented with 10% FBS) for 24 h at 37 ºC with 5% CO_2. The cells were then washed three times with RPMI-1640 to remove non-adherent cells. Infection of RAW 264.7 cells was performed at a multiplicity of infection (MOI) of 10:1 (Bacteria/macrophage) for 3 h at 37 ºC with 5% CO_2. Infected RAW cells were washed three times with RPMI-1640 to remove extracellular bacteria and replaced with 1 mL fresh RPMI (supplemented with 10% FBS), with ATP in concentrations of 3 mM and 5 mM. This was defined as time 0 h. At 2h and 20h the infected macrophages were lysed with 0.025% SDS, and the number of viable bacteria was determined by plating serial dilutions of each well on Middlebrook 7H10 Agar supplement with 10% OADC. The plates were incubated at 37 ºC for 21 days before counting procedures. All experiments were performed in triplicates and the results are expressed in CFU per well.

Animals

Male Swiss, wild-type C57BL/6 (wild-type) and P2X7 receptor KO (P2X7R$^{-/-}$) mice (25–30 g) were used throughout this study. Swiss and C57BL/6 mice were obtained from Universidade Federal de Pelotas (UFPEL; Pelotas, RS, Brazil), and P2X7R$^{-/-}$ mice were donated by Dr Robson Coutinho-Silva, Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil). The KO mice were generated by the method developed by Dr James Mobley (PGRD, Pfizer Inc, Groton, CT, USA). The KO mice used in the present study were C57BL/6 inbred. The animals were housed in groups of six per cage and maintained in controlled temperature (22 ± 2°C) and humidity (60-70%), under a 12 h light-dark cycle, with food and water *ad libitum*. All the experimental protocols were approved by the Local Animal Ethics Committee (CEUA 10/00203-PUCRS).

Experimental infection in mice

Mice were anaesthetized by intraperitoneal (i.p.) injection of a mixture containing ketamine (100 mg/kg) and xilazine (10 mg/kg) (Cristália, Itapira, SP, Brazil and Vetbrands, Jacareí, SP, Brazil, respectively), and subsequently infected with ~10^7 *M. tuberculosis* H37Rv strain suspended in 0.2 ml of saline solution intravenously (i.v.), through retro-orbital venous sinus, according to the technique described previously (Cynamon, et al., 1999, Klemens, et al., 1994), with some modifications. Non-infected control group received saline by i.v. retro-orbital route. The efficiency of infection was confirmed by acid-fast staining of lung slices using the specific Ziehl-Neelsen staining. After 4 weeks, the animals

were euthanized under deep isoflurane inhalation. The lungs and spleens were aseptically removed and used to perform immunohistochemistry analysis, CFU counts and flow cytometry analysis.

Immunohistochemistry

The expression of P2X7 receptors in lungs was assessed by immunostaining, according to method previously described (Goncalves, et al., 2006), with some modifications. For these experiments, the lungs were collected 4 weeks post *M. tuberculosis* infection and fixed in buffered neutral formalin. Paraffin-embedded sections (3 µm) were cut onto glass slides. The sections were de-waxed in xylene three times for 15 min, rehydrated in decreasing concentrations of ethanol for 5 min and washed three times in phosphate-buffered saline for 10 min. Endogenous peroxidase was quenched for 30 min with 0.3% hydrogen peroxide: methanol solution (v/v). After washing in distilled water and phosphate-buffered saline, a blocking step was included using either, extract of mouse liver or 5% bovine serum albumin in phosphate-buffered saline for a total of 30 min. *Rabbit anti-P2X7 receptor-purified polyclonal antibody* (1:200 in PBS; Alomone, Jerusalem, Israel, catalogue number APR-004) was incubated overnight at 4ºC in a humidified chamber. Negative controls were carried out with bovine serum albumin instead of primary antibody. After achieving room temperature, sections were washed in 0.25% phosphate-buffered saline-tween solution for 5 min. *Universal immuno-peroxidase polymer for mouse tissue sections anti-rabbit primary antibody* (N-Histofine – Simple Stain Mouse

MAX PO code 414341F), was incubated for 2 h. Reactions were revealed using diaminobenzidine chromogen substrate (Liquid DAB, DAKO cat number K3466, USA). After washing the slides, counterstaining was done with Harris hematoxylin for 1 to 2 min. The images were captured by Zeiss AxioImager M2, and analysed through the Image-Pro Plus 4.0 software (Media Cybernetics L.P). The lung P2X7 receptor surface density was determined by considering the percentage of stained areas in the total histological fields. For this experimental set, we have used 8 wild-type Swiss mice per group.

CFU determination protocol

For evaluation of bacterial load, the right lungs and spleens were aseptically removed and homogenized in 3 ml of phosphate-buffered saline (PBS). For each organ, the suspension was serially diluted 10-fold, and 0.1 ml of each appropriate dilution was cultured in duplicate by plating onto 7H10 Middlebrook agar supplemented with 10% OADC. The colonies were counted after 28 days of incubation at 37ºC.

Spleen weight determination

The spleens were dissected free from connecting tissues. The weight of each spleen was determined, and the result was expressed as milligram per gram of animal.

Flow cytometry analysis.

Following euthanasia, the spleens and lungs were excised and disrupted against a nylon screen in media containing Collagenase D (Roche). Single cell suspensions were obtained, and the cells were counted with Trypan blue. Subsequently, the cells were stained with antibodies against CD4-PECy5, CD25-PE, Foxp3-Alexa 647, CD8a-FITC, Gr1-PE, CD11b-APC, CD11c-PECy7 e B220-FITC (BD Biosciences). We investigated the following population subsets of immune cells: CD4+, CD8+, CD4+Foxp3+, CD4+CD25+Foxp3, CD4+CD25+, CD8+CD25+, CD11b+, CD11c+ and B220+. Cells were analyzed in a FACSCanto II flow cytometer (BD Biosciences). Data was corrected using the correspondent (non-infected) control group and expressed in fold variations between P2X7R$^{-/-}$ and wild-type mice.

Statistical analysis

To compare the viable *M. tuberculosis* cell counts recovered from infected macrophages, or spleens and lungs of mice, the numbers were firstly converted into logarithms of CFU (Log_{10} CFU). Data from spleen and lung CFU, and flow cytometry analysis were evaluated using Student's unpaired *t*-test. The spleen weight was previously corrected with the body weight from each animal (spleen weight/body weight). Data of spleen weight and macrophages CFU, was evaluated by a one-way analysis of variance (ANOVA), followed by Dunnett's post-test, using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Differences were considered significant at the 95% level of confidence.

Results

Effects of ATP on macrophage infection

In this study, we initially investigated whether the activation of P2X7 receptors is related to colony formation in the macrophage murine cell line RAW 264.7 infected with *M. tuberculosis*. The incubation of *M. tuberculosis*-infected RAW 264.7 cells with high concentrations of ATP (3 and 5 mM), which is known to selectively activate P2X7 receptors, resulted in a statistically significant reduction of CFU counts by 2.211Log_{10} and 2.205 Log_{10} units respectively, at 2 h (P<0.001) and, by 0.832 Log_{10} and 1.084 Log_{10} units respectively at 20 h post treatment (P < 0.05) (Figure 1).

Mouse *M. tuberculosis*-infection and lung P2X7 receptor expression

To extend *in vitro* evidence, we performed the infection of wild-type Swiss mice with *M. tuberculosis* H37Rv strain. After 4 weeks, the animals were euthanized and the lungs were harvested for determining the lung expression of P2X7 receptors. Interestingly, the infection with ~10^7 bacilli per mouse led to a significant increase of P2X7 receptor expression in lung tissues, as it can be observed from the representative immunohistochemistry images in Figure 2A. Semi-quantitative analysis revealed that infection of mice with H37Rv *M. tuberculosis* was able to increase the expression of P2X7 receptors in 2.8-fold, in relation to non-infected mice (P<0.001) (Figure 2B). We have additionally performed Ziehl-Neelsen staining from the histological sections of lungs used to

immunohistochemistry procedures, which confirmed the success of infection (Figure 2C).

CFU determination in lungs and spleens

P2X7R$^{-/-}$ and wild-type mice were infected with ~10^7 *M. tuberculosis* H37Rv bacilli, by i.v, route. Following 4 weeks, the lungs and spleens were harvested for determining the *M. tuberculosis* counts. In two independent experiments, there was significant difference *M. tuberculosis* CFU in the lungs. The bacterial load in this organ was 0.881 Log_{10} unit higher in P2X7R$^{-/-}$ mice, when compared to wild-type group (P<0.001) (Figure 3A). In the spleens, there was no statistically significant difference in *M. tuberculosis* CFU comparing the infected-P2X7R$^{-/-}$-group and the wild-type infected-control, 4 weeks post-infection (Figure 3B).

Spleen weight determination

To define splenomegaly grade, we weighed the spleens of infected mice and compared with non-infected animals. We observed an augmentation of mouse spleens at 4 weeks post-infection with *M. tuberculosis*, characterizing splenomegaly in all groups. Remarkably, wild-type infected mice showed an increase of 6.6-fold in relation to non-infected wild-type mice (P<0.001), while P2XR7$^{-/-}$ infected mice showed an increase of 6.9-fold in relation to non-infected P2X7R$^{-/-}$ mice (P<0.001) (Figure 4).

Flow cytometry analysis.

We next investigated to what extent the differences observed between P2X7R$^{-/-}$ and wild-type *M. tuberculosis*-infected mice might be associated with distinct patterns of immune cell infiltration. Therefore, we performed an extensive flow cytometry analysis of both spleen and lung samples. In spleens obtained from infected P2X7R$^{-/-}$ mice, there was a significant decrease in the populations of Treg cells (CD4^{+}CD25^{+}Foxp3^{+}), which corresponded to 0.54-fold (P<0.05) when compared to wild-type *M. tuberculosis*-infected animals (Figure 5A). T cells (CD4^{+}; CD8^{+}CD25^{+}; and CD4^{+}CD25^{+}) from infected P2X7R$^{-/-}$ mice decreased by 0.36-fold, 0.63-fold and 0.93-fold, respectively (P<0.05) (Figure 5C, F and E respectively). For dendritic cells (CD11c^{+}), there was a decrease of 0.69-fold in P2X7R$^{-/-}$ mice, when compared to wild-type mice (P<0.001) (Figure 5H), whilst a reduction of 0.59-fold was seen in B220^{+} cells (P<0.001) (Figure 5I). However, a significant increase in CD11b^{+} cells (0.36-fold) (P<0.01) (Figure 5G) was observed in P2X7R$^{-/-}$ mice when compared to wild-type animals.

Concerning the lungs, P2X7R$^{-/-}$ *M. tuberculosis*-infected mice exhibited pulmonary infiltrates containing an increased number of Treg cells CD4^{+}Foxp3^{+} (4.56-fold) (P<0.01) (Figure 6B). T cells (CD4^{+} and CD8^{+}) increased by 7.52-fold and 3.47-fold, respectively (P<0.01) (Figure 6C and D), whereas B220^{+} cells decreased in 0.87-fold (P<0.05), when compared with wild-type *M. tuberculosis*-infected mice (Figure 6I).

Discussion

The purinergic P2X7 receptor is a ligand-gated cation channel expressed in cell membrane, which is activated by high concentrations of ATP (Burnstock, 2006, Burnstock and Knight, 2004, Rassendren, et al., 1997). It has been demonstrated that activation of macrophage P2X7 receptors can induce death of different intracellular microorganisms (Biswas, et al., 2008, Chaves, et al., 2009, Coutinho-Silva, et al., 2007, Coutinho-Silva, et al., 2001, Coutinho-Silva, et al., 2003, Marques-da-Silva, et al., 2011). Relevantly, this receptor is widely expressed in mycobacteria-infected macrophages, and it was found to be related to the killing mechanisms of these cells by ATP (Lammas, et al., 1997). Therefore, this study aimed at further investigating the role of P2X7 receptors during *M. tuberculosis* infection, both *in vitro* and *in vivo*.

The *in vitro* stimulation of a macrophage murine cell line RAW 264.7 with ATP, an agonist of P2X7 receptor at high concentrations, caused a decreased *M. tuberculosis* load. This evidence confirms data of previous studies, in which murine and human macrophages were infected with Bacille Calmette-Guerin (BCG) and treated with ATP (Fairbairn, et al., 2001, Lammas, et al., 1997). The mycobatericidal activity of ATP is likely due to activation of P2X7 receptors (Fairbairn, et al., 2001, Lammas, et al., 1997, Smith, et al., 2001) leading to phagosome-lysosome fusion (Fairbairn, et al., 2001) and dependent on phospholipase D (Kusner and Adams, 2000) and Ca^{2+} signaling (Kusner and Barton, 2001, Malik, et al., 2000). Our work shows a statistically significant reduction in CFU counts after exposing infected macrophages with both tested

concentrations of ATP (3 and 5 mM). Therefore, it is feasible to suggest that P2X7 receptor is implicated in the mechanisms of mycobacteria-killing of this murine cell line.

In order to investigate the role of the P2X7 receptor in the immune responses to *M. tuberculosis,* we established a murine model of *in vivo* infection using C57BL/6 mice. The model of infection used herein was different from usual methods, in which the animals were exposed to aerosol and a low dose of *M. tuberculosis* was absorbed via inhalation (Myers, et al., 2005, Saini, et al., 2011). In our model, *M. tuberculosis* is directly injected in the blood stream in a high concentration (~10^7 bacilli). Our results showed a high burden of *M. tuberculosis* observed in lungs of either $P2X7R^{-/-}$ or wild-type C57BL/6 mice, through Ziehl-Neelsen staining (Figure 2C) and CFU counting (Figure 3). *M. tuberculosis* inoculation in mice resulted in a marked inflammatory response of both lungs, characterized by marked macrophage infiltration and typical tuberculosis-related granulomes (results not shown). Additionally, our data revealed that, in normal conditions, P2X7 receptor is constitutively expressed in lungs of wild-type mice, in accordance to previous studies conducted by Barth et al. (Barth, et al., 2007), and by Chen et al. (Chen, et al., 2004), in mice and rats, respectively. Strikingly, in the present study, the lungs of mice infected with *M. tuberculosis* showed a significant increase in P2X7 receptor immunoreactivity, corresponding to about 2.8-fold in relation to non-infected mice. This evidence supports previous literature data showing that activation of P2X7 receptors in macrophages is able to induce the apoptosis and kill intracellular mycobacteria (Saunders, et al.,

2003). The increase of P2X7 receptor expression was also observed in rodent models of inflammation such as hemorrhagic cystitis induced by cyclophosphamide (Martins, et al., 2011), chronic inflammatory airway disease (Lucattelli, et al., 2011, Muller, et al., 2011), and lipopolysaccharide-induced lung injury (Moncao-Ribeiro, et al., 2011). Of high interest, the increase of P2X7 receptor immunopositivity was localized especially around the granuloma region, strengthening the possible relevance of this receptor subtype for *M. tuberculosis* infection mechanisms. Therefore, we might well infer that in the presence of *M. tuberculosis*, the immune cells in lungs increase the membrane expression of P2X7 receptor in an attempt to control the infection spread.

In the present study, we showed the results of two independent experiments, in which the burden of *M. tuberculosis* in the lungs of $P2X7R^{-/-}$ were statistically different from the control group. The $P2X7R^{-/-}$ mice showed an increase of *M. tuberculosis* load in the lungs compared to infected wild-type mice. This data suggests that P2X7 receptor has an important role in the control of pulmonary tuberculosis, corroborating the studies in which genetic variation in P2X7 receptor is associated with significant differences in the capacity of human macrophages to kill mycobacteria (Fernando, et al., 2005), and also that P2X7 receptor gene polymorphisms in mexican mestizo patients are related to increased susceptibility for *M. tuberculosis* infection (Nino-Moreno, et al., 2007).

Notably, our results demonstrate that *M. tuberculosis* infection induces severe splenomegaly. Of high interest, *M. tuberculosis*-infected $P2X7R^{-/-}$ mice showed a significant increase in the spleen weight when compared to infected

wild-type mice. Martins de Sousa et al. (Martins de Sousa, et al., 2010) previously demonstrated splenomegaly in mice infected with *Mycobacterium massiliense*. Their results point out evidence indicating that splenomegaly in infected animals could be due to hemolysis or inflammation. Besides, a previous study showed that mycobacteria infection exhibits an increase in the total number of cells in the liver and spleen, as a result of numerous focal granulomatous lesions and an immune inflammatory (Martins de Sousa, et al., 2010). Of note, it has been formerly shown that spleens from P2X2/P2X3 KO animals were larger and weighed significantly more than the corresponding wild-type spleens (Coutinho-Silva, et al., 2005). On the other hand, our results did not show significant difference between the spleens of wild-type and $P2X7R^{-/-}$ non-infected mice. Nevertheless, in our set of experiments the enlarged spleens of the *M. tuberculosis*-infected $P2X7R^{-/-}$ mice may be due to the chronic infection and inflammation found in these animals. Strikingly, flow cytometry analysis of $P2X7R^{-/-}$ spleens showed a significant decrease in the populations of Treg (CD4+CD25+Foxp3+), T cells (CD4+, CD8+CD25+ and CD4+CD25+), dendritic cells (CD11c+) and B220+ cells, when compared to wild-type animals.

The spleen is a highly structured lymphoid organ (Mebius and Kraal, 2005). As microorganisms enter the marginal sinus of the spleen, they approach in contact with a variety of splenic phagocytes, including macrophages, dendritic cells, and granulocytes (Kraal, 1992). Dendritic cells play an important role in carrying and presenting antigen from peripheral tissues to lymphatic organs to trigger the adaptive immune response (Angeli, et al., 2006). We showed that

infection of wild-type mice with *M. tuberculosis* lead to an increase of phagocytes (CD11b+) and dendritic cells (CD11c+) in spleen, but the P2X7R$^{-/-}$ mice had a diminished dendritic cell and augmented phagocytes, when compared to wild-type mice. Thus, we infer that in P2X7R$^{-/-}$ mice, the diminished dendritic cells impair the antigen presentation and the immune cells recruit other phagocytic cells in attempt to control the infection.

The interaction of T cells and infected macrophages is central to protective immunity to *M. tuberculosis*. This pathology requires contributions by multiple T cell subsets, which include a dominant role for CD4+ cells and significant roles for CD8+. Therefore, after initial innate phase, adaptive immune response is necessary to control dividing bacilli (Wiley, et al., 2011). Despite P2X7R$^{-/-}$ mice display impaired fusion of lysosome with paghossome (Fairbairn, et al., 2001), we observed a higher percentage of T cells (CD4+ and CD8+) in the pulmonary infiltrate of P2X7R$^{-/-}$, when compared to wild-type *M. tuberculosis*-infected mice. This increase in T cells number could be explained by the fact that antigen presentation is not completely impaired in P2X7R$^{-/-}$ mice (Sluyter and Stokes, 2011). Furthermore, Schenk et al (Schenk, et al., 2011) have verified that the increase in extracellular ATP at the inflammatory site could affect the activation state of effector T cells. This study demonstrated that activation of P2X7 receptor, via ATP, inhibits the suppressive function of Treg cells. Accordingly, our experiments showed an expressive increase of Tregs cells in P2X7R$^{-/-}$, when compared to wild-type mice in the lung. Therefore, the lack of P2X7 receptor in

Treg cells might increase the host susceptibility to infections, such as *M. tuberculosis*.

Corroborating our data, a study of Vordermeier et al (Vordermeier, et al., 1996) demonstrated that B cell-deficient mice infected with *M. tuberculosis* had higher bacterial loads. They considered several mechanisms by which B cell could influence the course of *M. tuberculosis* infections. Because of their functions as antigen-presenting cells (APCs), B cells could influence the specificity and phenotype of protective CD4+ T cell response, and take up antigens by their antigen-specific immunoglobulin receptors presenting processed epitopes to T cells. According to the previous study, this antigen-specific response may determine which of the possible T cell determinants will be eventually presented, thus modulating the specificity of T cell anti-mycobacterial response. Our results confirm this explanation demonstrating that P2X7R$^{-/-}$ *M. tuberculosis*-infected mice present a diminished population of B cells (B220+) in lungs and spleens, and an augmentation of *M. tuberculosis* CFU in lungs. Nonetheless, other defects cannot be excluded. This assembly of results are clearly indicating that P2X7R$^{-/-}$ mice have a major susceptibility to mycobacterial infections.

In summary, the evidence presented herein provides novel evidence on the relevance of P2X7 receptors as target molecules for the pathogenesis of TB. Whether selective agonists or antagonists of this receptor might be useful for improving TB complications remains a matter to be investigated.

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Figure Legends

Figure 1. Effect of ATP in murine macrophages RAW 264.7 infected with *M. tuberculosis* H37Rv. ATP induced the killing of *M. tuberculosis* in the murine macrophages RAW 264.7. Each *column* represents the mean of triplicates, and the *vertical lines* show the S.E.M. **P*<0.05 and ****P*<0.001 denote the significant level in comparison to non-treated group.

Figure 2. (A) Representative images of immunostaining for P2X7 receptor in the lung into the following groups: Non-infected (upper) show a lower immunoreactivity for P2X7 receptor, while *M. tuberculosis*-infected mice (below) show a higher P2X7 receptor immunoreactivity. **(B)** Effect of infection with *M. tuberculosis* on P2X7 receptor expression in the lung from mice. Each *column* represents the mean of eight animals, and the *vertical lines* show the S.E.M. ****P*<0.001 denote the significant level in comparison to non-infected group. **(C)** Representative image from Ziehl-Neelsen staining of *M. tuberculosis* infected mice confirming the success of infection model.

Figure 3. CFU counts in lungs and spleens of *M. tuberculosis*-infected mice **(A)** *M. tuberculosis* burden in lungs of $P2X7R^{-/-}$ and wild–type-infected animals. **(B)** Load of *M. tuberculosis* in spleens of $P2X7R^{-/-}$- and wild–type-infected animals. Each *column* represents the mean of eight animals, and the *vertical lines* show the S.E.M. ****P*<0.001 denote the significant level in comparison to wild type infected group.

Figure 4. Effect of *M. tuberculosis* infection in weight of spleens. The spleen weights were determined, and the results were expressed as milligram per gram of animal. Each *column* represents the mean of eight animals, and the *vertical lines* show the S.E.M. ***P<0.001 denote the significant level in comparison to wild type non-infected (WT) group, $^{###}P$<0.001 denote the significant level in comparison to P2X7R$^{-/-}$ non-infected group, and $^{\&\&\&}P$<0.001 denote the significant level in comparison to wild type infected group (WT+MT).

Figure 5. Differences in Immune cells subsets in spleen of *M. tuberculosis*-infected mice. (A) CD4+CD25+Foxp3+, (B) CD4+Foxp3+, (C) CD4+, (D) CD8+, (E) CD4+CD25+, (F) CD8+CD25+, (G) CD11b+, (H) CD11c+ and (I) B220+ cells. Data was expressed in fold variations between infected and non-infected correspondent group. Each *column* represents the mean of four animals, and the *vertical lines* show the S.E.M. *P<0.05, **P<0.01and ***P<0.001 denote the significant level in comparison to wild type infected group.

Figure 6. Differences in Immune cells subsets in lungs of *M. tuberculosis*-infected mice. (A) CD4+CD25+Foxp3+, (B) CD4+Foxp3+, (C) CD4+, (D) CD8+, (E) CD4+CD25+, (F) CD8+CD25+, (G) CD11b+, (H) CD11c+ and (I) B220+ cells. Data was expressed in fold variations between infected and non-infected correspondent group. Each *column* represents the mean of four animals, and the *vertical lines* show the S.E.M. *P<0.05 and **P<0.01 denote the significant level in comparison to wild type infected group.

Figure 1.

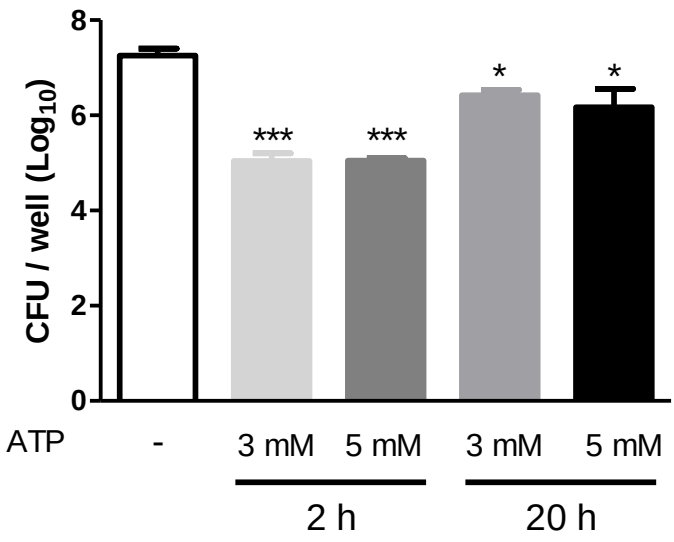


Figure 2.

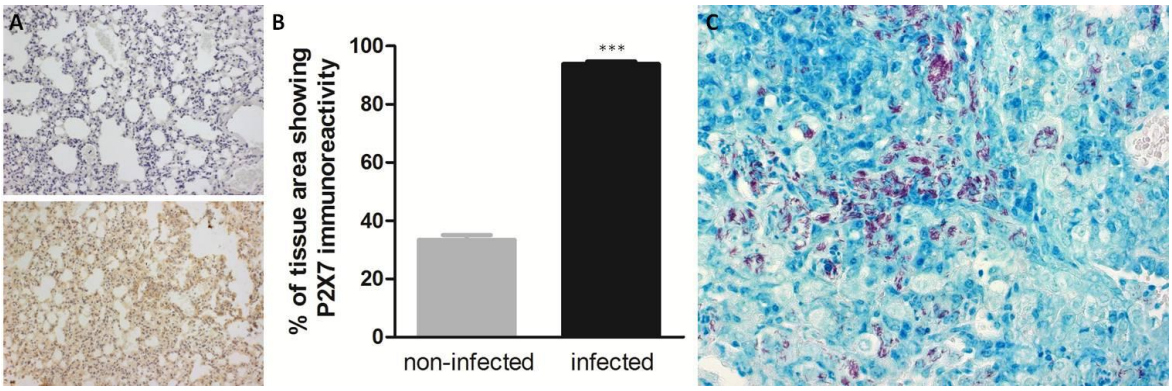

A
B
% of tissue area showing P2X7 immunoreactivity
100
80
60
40
20
0
***
non-infected
infected
C

Figure 3.

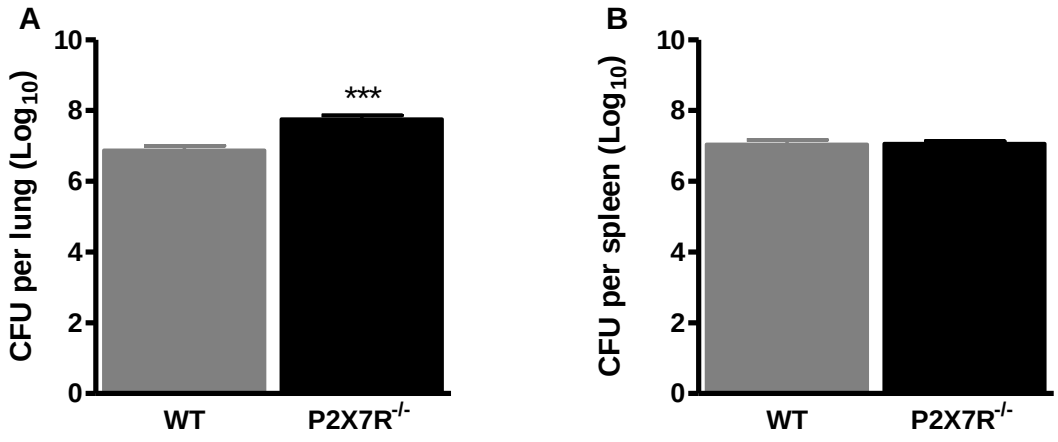

A
CFU per lung (Log10)
10
8
6
4
2
0
***
WT
P2X7R-/-
B
CFU per spleen (Log10)
10
8
6
4
2
0
WT
P2X7R-/-

Figure 4.

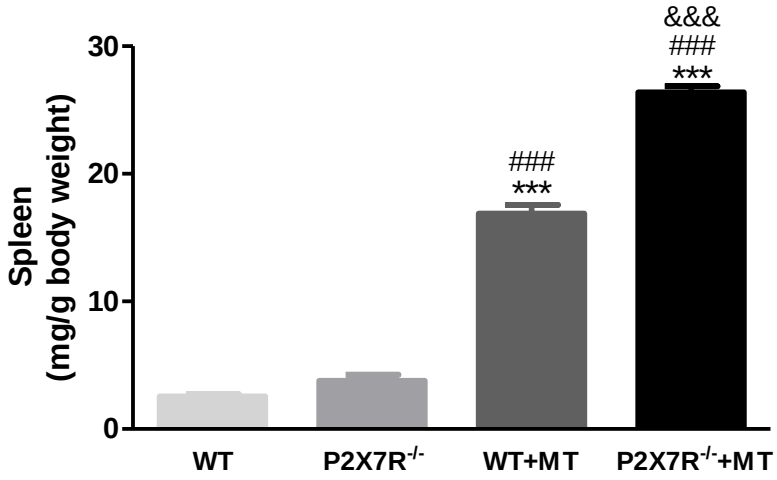
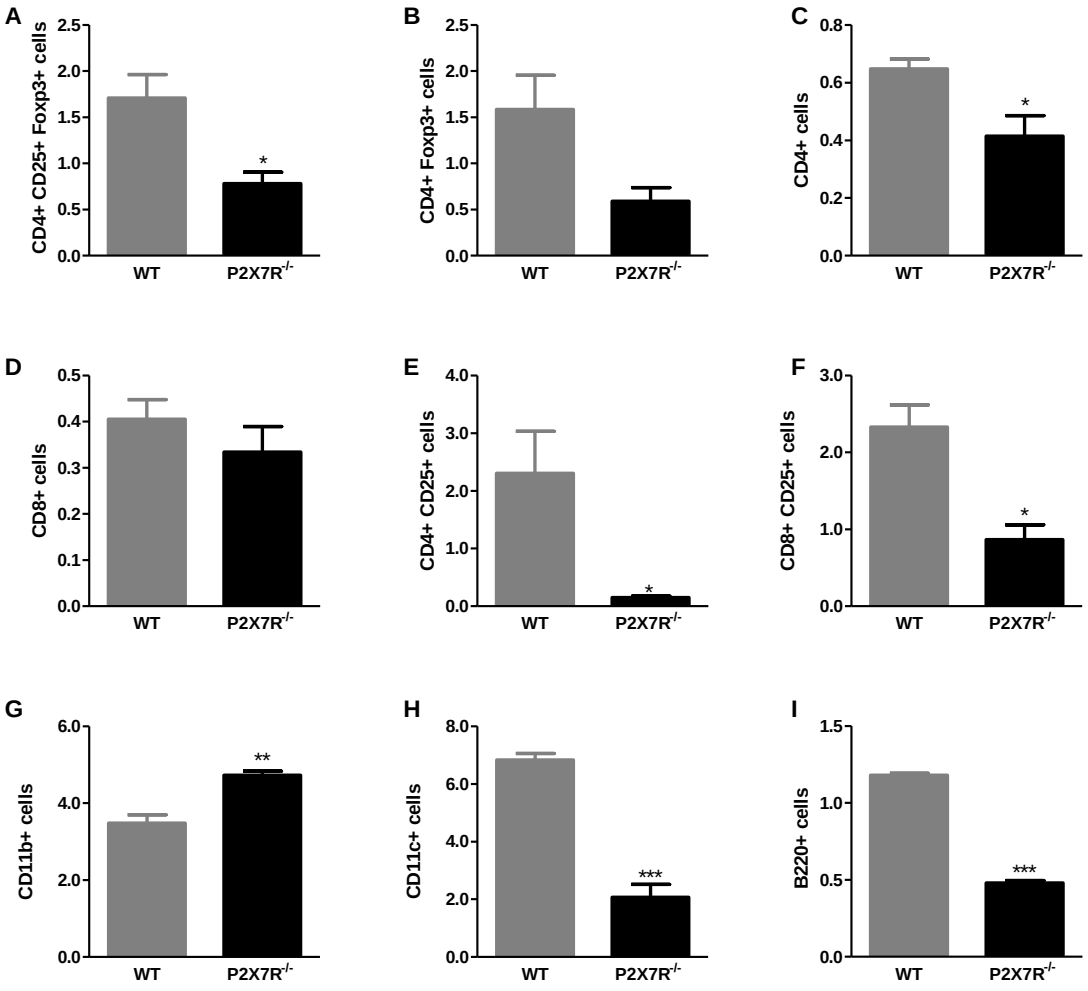
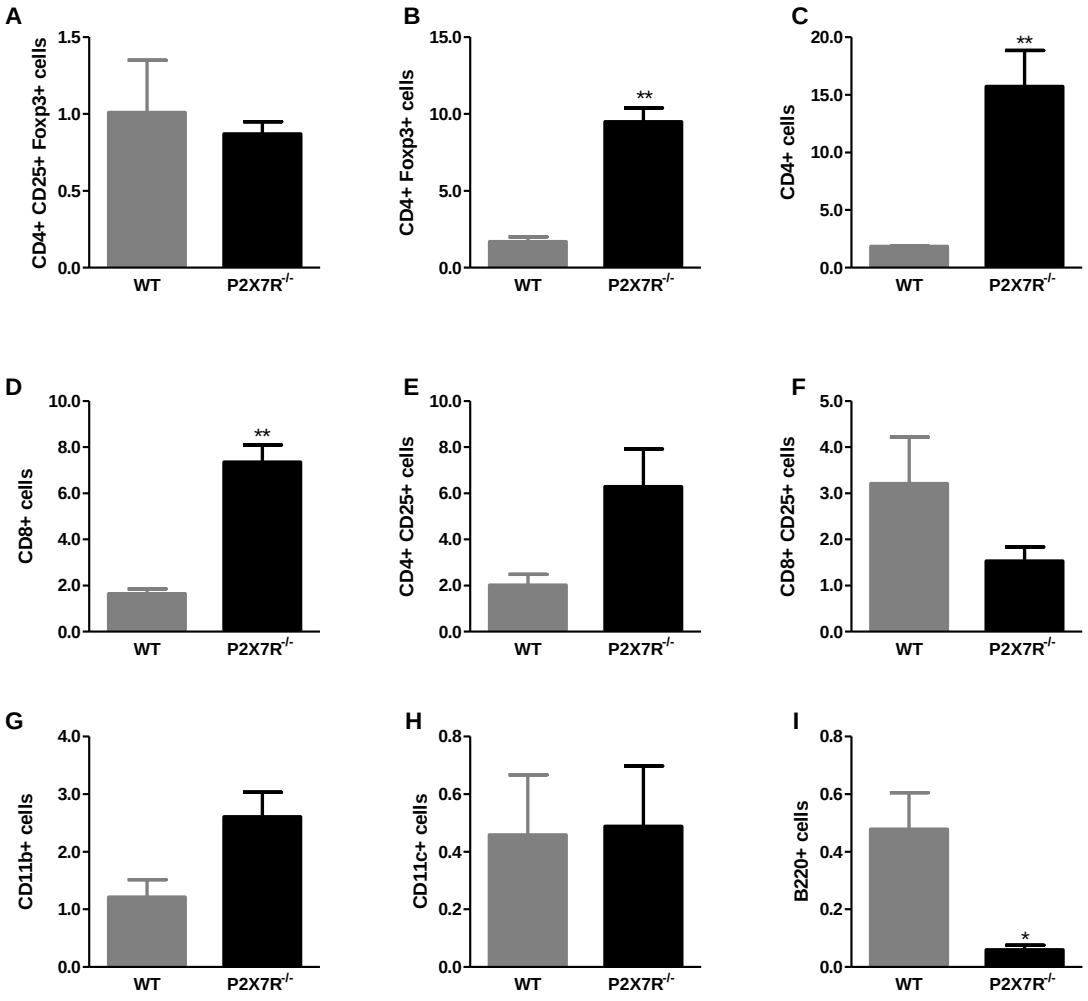


Figure 5.


A
CD4+ CD25+ Foxp3+ cells
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
P2X7R-/-
B
CD4+ Foxp3+ cells
2.5
2.0
1.5
1.0
0.5
0.0
WT
P2X7R-/-
C
CD4+ cells
0.8
0.6
0.4
0.2
0.0
*
WT
P2X7R-/-
D
CD8+ cells
0.5
0.4
0.3
0.2
0.1
0.0
WT
P2X7R-/-
E
CD4+ CD25+ cells
4.0
3.0
2.0
1.0
0.0
*
WT
P2X7R-/-
F
CD8+ CD25+ cells
3.0
2.0
1.0
0.0
*
WT
P2X7R-/-
G
CD11b+ cells
6.0
4.0
2.0
0.0
**
WT
P2X7R-/-
H
CD11c+ cells
8.0
6.0
4.0
2.0
0.0
***
WT
P2X7R-/-
I
B220+ cells
1.5
1.0
0.5
0.0
***
WT
P2X7R-/-

.

Figure 6.


A
CD4+ CD25+ Foxp3+ cells
1.5
1.0
0.5
0.0
WT
P2X7R-/-
B
CD4+ Foxp3+ cells
15.0
10.0
5.0
0.0
**
WT
P2X7R-/-
C
CD4+ cells
20.0
15.0
10.0
5.0
0.0
**
WT
P2X7R-/-
D
CD8+ cells
10.0
8.0
6.0
4.0
2.0
0.0
**
WT
P2X7R-/-
E
CD4+ CD25+ cells
10.0
8.0
6.0
4.0
2.0
0.0
WT
P2X7R-/-
F
CD8+ CD25+ cells
5.0
4.0
3.0
2.0
1.0
0.0
WT
P2X7R-/-
G
CD11b+ cells
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H
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P2X7R-/-

4. CONSIDERAÇÕES FINAIS

A tuberculose foi declarada como problema de emergência de saúde global pela OMS. Sabe-se que na última década, 30 milhões de pessoas morreram desta doença e, estima-se que um terço da população humana esteja infectado pelo bacilo da TB. (Dye *et al.* 1999; Corbett *et al.* 2003). O *M. tuberculosis* tem sido apontado como o patógeno de maior sucesso no processo de infecção de todos os tempos, conseguindo permanecer latente dentro do hospedeiro e sendo capaz de escapar das defesas do sistema imune (Wickelgren 2000; Enserink 2001). Assim, a TB permanece como uma das principais causas de morte em todo o mundo, devido à grande capacidade de adaptação do bacilo, que consegue sobreviver em diversas condições dentro e fora do hospedeiro humano.

Já se passaram quase 40 anos desde a introdução do último fármaco para tratar a TB. Estima-se que o desenvolvimento completo de uma nova substância anti-TB custe de 100 a 800 milhões de dólares (Duncan 2003). Considerando-se que 95% dos casos de TB ocorrem em países em desenvolvimento, a indústria farmacêutica parece não estar suficientemente atraída, em termos financeiros, para o desenvolvimento de novos fármacos para tratar esta doença (O'Brien e Nunn 2001). Entretanto, a re-emergência da TB como um problema de saúde mundial, associada à grande susceptibilidade de pessoas infectadas com o HIV à doença, e a proliferação de cepas MDR e XDR-TB indicaram a necessidade urgente da pesquisa por novos alvos para o tratamento da TB.

O receptor purinérgico P2X7 tem sido descrito em diversos sistemas, sendo ativado por altas concentrações de ATP (Burnstock 2006). De fato, a ativação do receptor P2X7 induz à formação de poros na membrana e à apoptose. O receptor purinérgico P2X7 está implicado na regulação de uma série de processos nos macrófagos; entre eles, ativação do inflamossomo, secreção de IL-1β, modulção e regulação de células T, entre outros (Coutinho-Silva *et al.* 2003; Ferrari *et al.* 2006; Coutinho-Silva *et al.* 2009). Além disso, Biswas e colaboradores (2008) mostraram que o tratamento com ATP em macrófagos humanos infectados com *Mycobacterium bovis*-BCG induz à morte por um mecanismo dependente do receptor P2X7.

Na literatura, há alguns estudos demonstrando que indivíduos que apresentam certos polimorfismos no gene que codifica o receptor purinérgico P2X7, possuem uma perda da função desse receptor (Saunders *et al.* 2003; Sluyter *et al.* 2004; Sluyter *et al.* 2004). Tendo em vista que estudos têm demonstrado que este receptor está relacionado com o aumento da suscetibilidade à tuberculose (Nino-Moreno *et al.* 2007; Sambasivan *et al.* 2010; Tekin *et al.* 2010; Singla *et al.* 2011), o presente estudo objetivou investigar a importância desse receptor no modelo murino de infecção por *M. Tuberculosis*.

Na etapa inicial do presente estudo constatou-se que macrófagos murinos (RAW 264.7) infectados com *M. tuberculosis,* quando tratados com o agonista do receptor P2X7, ATP (3 e 5 mM), apresentaram uma redução significativa na viabilidade da micobactéria intracelular. Estes dados demonstraram que a ativação dos receptores purinérgicos P2X7 produz uma ação micobactericida

nos macrófagos, o que levou-nos a avaliar a expressão do receptor P2X7 em pulmões de animais infectados com o *M. tuberculosis*.

Um segundo achado importante deste estudo foi mostrado através da técnica de imuno-histoquímica , sendo que os tecido de pulmões de camundongos selvagens (*wild-type*) infectados com *M. tuberculosis* apresentaram um aumento pronunciado na imunorreatividade para o receptor P2X7, quando comparados ao grupo controle (não infectados). Estes resultados nos permitem inferir que as células presentes no pulmão ao serem infectadas com *M. tuberculosis,* aumentam a expressão do receptor P2X7 em uma tentativa de controle da infecção, o que poderia estar relacionado ao processo inflamatório existente. De fato, estudos demonstraram que a inflamação induzida por lipopolissacarídeo no modelo de lesão pulmonar, aumenta a imunoexpressão do receptor P2X7 no tecido pulmonar (Moncao-Ribeiro et al. 2011).

Outro resultado interessante diz respeito à avaliação do número unidades formadoras de colônias (UFCs) nos órgãos dos animais infectados. Os pulmões dos animais *knockout* para o receptor P2X7 ($P2X7R^{-/-}$) infectados com *M. tuberculosis* apresentaram um aumento nas UFCs quando comparados aos animais *wild-type*.

De maneira interessante, Fairbairn e colaboradores (2001) mostraram que macrófagos derivados de camundongos $P2X7R^{-/-}$ são incapazes de matar efetivamente o BCG em resposta ao ATP (Fairbairn *et al.* 2001). Portanto, os resultados por nós encontrados nos levam a postular que o receptor purinérgico

P2X7 está diretamente ligado ao controle da tuberculose nos pulmões destes animais.

Além disso, o aumento da carga pulmonar de *M. tuberculosis* e o aumento de peso do baço observado em camundongos P2X7R$^{-/-}$ infectados com o *M. tuberculosis* sugerem que esse receptor tem um papel fundamental também para a compreensão da patogênese da tuberculose. De fato, nossos resultados mostraram que a infecção com *M. tuberculosis* levou a uma esplenomegalia acentuada e, a distintos padrões nas populações das células do sistema imune nos pulmões e no baço. Os baços dos animais P2X7R$^{-/-}$ apresentaram uma diminuição nas populações das células T regulatórias (Treg), células T, células dendríticas e células B, e um aumento na população de células fagocíticas, quando comparados ao grupo controle (animais *wild-type*). Nos pulmões, a infecção com *M. tuberculosis* causou um aumento nas células Treg e células T e, uma diminuição nas células B, quando comparados ao grupo controle. Essas alterações permitem inferir que a resposta immune nos animais P2X7R$^{-/-}$ difere da resposta encontrada nos animais *wild type*. Essa diferença na resposta imune mostra a importância do receptor P2X7 na orquestração da resposta imune contra o *M. tuberculosis* e pode explicar os dados obtidos na contagem de UFCs.

Deste modo, os dados obtidos no presente estudo, somados aos dados da literatura, reforçam as evidências de que o receptor P2X7 exerce uma função crucial na patogênese e controle da tuberculose. As evidências aqui apresentadas indicam o receptor P2X7 como um possível alvo para a

compreensão da TB. Porém, o uso de agonistas ou antagonistas seletivos deste receptor como uma ferramenta terapêutica continua sendo uma questão a ser investigada.

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