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# Papel dos receptores de adenosina e da concentração de glicose na modulação da ativação de macrófagos por antígenos de *Staphylococcus aureus*

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**Índice**

# PARTE I

**Resumo**

As doenças infecciosas estão entre as principais causas de morte no mundo. Nas infecções bacterianas, os componentes das paredes destes organismos são descritos como os principais antígenos. Os macrófagos estão diretamente envolvidos no reconhecimento e ataque de agentes patogênicos, além de atuarem como moduladores da resposta imunológica. Os macrófagos participam do combate às infecções bacterianas através da fagocitose dos agentes patogênicos e da produção de diversos mediadores inflamatórios como citocinas, metaloproteinases (MMP), espécies reativas de oxigênio (ROS) e óxido nítrico(NO). Estes mediadores são importantes na resposta inflamatória, contribuindo para o controle da infecção. Entretanto, a produção exacerbada destas moléculas contribui na patogênese das complicações associadas à inflamação, como a sepse e a falência múltipla de órgão. A maior parte dos estudos tem focado a resposta das células imunes a infecções por bactérias gram-negativas ou ao seu principal antígeno, o lipopolisacarídeo (LPS). No entanto, as infecções por bactérias gram-positivas são freqüentes e resultam em grande taxa de mortalidade. Os receptores de adenosina, em macrófagos, possuem função antiinflamatória em modelos de infecções por bactérias gram-negativas, no entanto, pouco se investigou a ação destes receptores em infecções por bactérias gram-positivas. O diabetes é uma doença metabólica comum que apresenta diversas complicações secundárias, muitas delas associadas aos níveis elevados de glicose. Os pacientes diabéticos apresentam uma ocorrência aumentada de infecções bacterianas, além de o diabetes tipo 2 estar associado com uma inflamação crônica, caracterizada por níveis plasmáticos aumentados de citocinas pró-inflamatórias. Adicionalmente, estes pacientes possuem maior risco de desenvolver doenças cardiovasculares e aterosclerose, o que tem sido relacionado à hiperglicemia e à inflamação crônica. A regulação, pela glicose, da resposta inflamatória em macrófagos expostos a bactérias gram-negativas ou ao seu antígeno principal, o LPS, é descrita, entretanto, a ação da glicose em modelos de infecções por bactérias gram-positivas não é conhecida. Assim, objetivamos estudar o papel dos receptores de adenosina e da concentração de glicose na modulação da ativação de macrófagos por antígenos de *Staphylococcus aureus*, principal bactéria gram-positiva associada a infecções hospitalares. O tratamento dos macrófagos RAW 264.7 com os antígenos ácido lipoteicóico (LTA) e peptideoglicano (PEG) resulta na produção de mediadores inflamatórios. O LTA estimula a produção de ROS, NO, TNF-α e MMP-9, enquanto o PEG aumenta a produção de TNF-α e MMP-9. Ambos os antígenos aumentaram a expressão dos receptores de adenosina A2A e A2B e a degradação de ATP, favorecendo o acúmulo de adenosina. O bloqueio farmacológico, pelo uso de antagonistas, ou a redução da expressão dos receptores A2A e A2B, pelo uso de RNA de interferência, resulta numa ativação aumentada dos macrófagos em reposta ao LTA, enquanto a ativação dos receptores de adenosina por um agonista resulta na inibição da resposta inflamatória aos antígenos de *S. aureus*. O tratamento dos macrófagos RAW 264.7 com concentrações elevadas de glicose aumentaram a resposta inflamatória ao LTA, com o aumento da produção de NO, TNF-α e MMP-9. Estes resultados mostram que tanto os receptores de adenosina como concentrações elevadas de glicose modulam a resposta inflamatória a *S. aureus*. Os receptores de adenosina parecem atuar num mecanismo autócrino de modulação da resposta inflamatória, apontando um possível alvo terapêutico. Já a modulação da resposta inflamatória dos macrófagos por concentrações aumentadas de glicose pode contribuir para complicações associadas ao diabetes, como a aterosclerose e a inflamação crônica.

**Abstract**

Infectious diseases are among the majors causes of mortality around the world. In bacterial infections, the bacterial cell wall components are described as the main antigens. The macrophages are key mediators of inflammatory response, acting in the attack to the pathogens and in the regulation of immune response. These cells act in the phagocytosis of pathogenic agents and in the production of inflammatory mediators, like cytokines, matrix-metalloproteinases (MMP), reactive oxygen species (ROS) and nitric oxide (NO). These mediators participate of inflammatory response, acting in infection control, notwithstanding, exacerbated production of these molecules could contribute to inflammatory complications, like sepsis and multiple organ failure. Several studies has addressed the inflammatory response to gram-negative bacterial infections or lipopolysaccharide (LPS), the main antigen of these microorganisms, however gram-positive bacterial infections are prevalent and associated to high mortality. The adenosine receptors are described to possess anti-inflammatory properties in macrophages in gram-negative bacterial infections models but the role of these receptors in gram-positive bacterial infections is unknown. Diabetes is a prevalent metabolic disorder which presents several associated complications; much of them associated to increased glucose levels. Diabetic patients shown increased occurrence of bacterial infections and type 2 diabetes is associated to a chronic inflammatory state, with increased circulatory levels of pro-inflammatory cytokines. Type 2 diabetic patients possess increased risk of cardiovascular diseases and atherogenesis, which has been associated to hyperglycemia and chronic inflammation. The regulation of macrophages inflammatory response to gram-negative bacterial infections or to its antigen LPS by increased levels of glucose is described, however, the role of increased glucose in gram-positive bacterial infections is unknown. For this reasons, we studied the role of adenosine receptors and increased glucose in macrophages activation by *Staphylococcus aureus* antigens, considering that this bacteria is the major organism in hospital infections. The treatment of RAW 264.7 macrophages with *S. aureus* antigens lipoteichoic acid (LTA) and peptidoglycan (PEG) resulted in the production of pro-inflammatory mediators. LTA stimulated ROS, NO, TNF-α and MMP-9 production, meanwhile PEG increased TNF-α and MMP-9 production. Both LTA and PEG augmented A2A and A2B adenosine receptors expression and ATP degradation, resulting in increased adenosine accumulation. Pharmacological blockade of A2A and A2B adenosine receptors by antagonists resulted in exacerbated activation of macrophages by LTA. Similar results were obtained by the reduction of these receptors expression by RNA interference. In opposition, the stimulation of adenosine receptors by agonist treatment promoted reduced activation of macrophages by *S. aureus* antigens. The exposition of RAW 264.7 macrophages to increased glucose concentration resulted in augmented activation by LTA, with increased NO, TNF-α and MMP-9 production. These results shown that both adenosine receptors and increased glucose alters the inflammatory response to *S. aureus*. Adenosine receptors seem to participate in an autocrine mechanism of inflammatory response regulation, suggesting a putative therapeutic target. The glucose modulation of macrophages inflammatory response could contribute to diabetes associated complications, like atherosclerosis and chronic inflammation.

**Lista de Abreviaturas**

ADP - adenosina difosfato

AMP - adenosina monofosfato

ATP - adenosina trifosfato

ERK - proteína cinase regulada por sinal extracelular

G6PD - glicose-6-fosfato desidrogenase

IDH - isocitrato desidrogenase

iNOS - óxido nítico sintase induzível

JNK - c-jun N terminal proteína cinase

LPS - lipopolissacarídeo

LTA - ácido lipoteicóico

MAPK – proteína cinase ativada por mitógenos

MDH - malato desidrogenase

MEK - proteína cinase ativada por mitógeno

MMP - metaloproteinase

$NADP^+$ - nicotinamida adenina dinucleotídeo fosfato

NADPH - nicotinamida adenina dinucleotídeo fosfato reduzido

NF-kB - fator nuclear kappa B

NO - óxido nítrico

NOD - domínio de oligomerização e ligação de nucleotídeos

NOS - óxido nítrico sintase

PEG - peptideoglicano

PPAR - receptor ativado por proliferadores de peroxissomos

PRR - receptor de reconhecimento de padrão

ROS - espécies reativas de oxigênio

TLR - receptor Toll-like

TNF-$\alpha$ - fator de necrose tumoral alfa

TNF-R - receptor do fator de necrose tumoral alfa

## Introdução

As doenças infecciosas estão entre as principais causas de morte no mundo. Estas infecções são causadas por diferentes agentes patogênicos, incluindo bactérias, fungos, helmintos e protozoários. A patogenicidade e a virulência de cada microorganismo estão relacionadas com os mecanismos de interação existentes entre estes e o hospedeiro. Nos mamíferos, o sistema imune apresenta componentes inatos e adaptativos, envolvidos no reconhecimento e combate dos microorganismos patogênicos. O sistema imune inato atua através de receptores, expressos a partir de genes definidos, que reconhecem padrões comuns existentes em grupos de moléculas presentes nos diferentes tipos de microorganismos. Já o sistema imune adaptativo apresenta receptores codificados a partir de fragmentos gênicos, os quais, por recombinação somática, podem originar uma grande diversidade de receptores que possuem grande especificidade contra antígenos específicos (MEDZHITOV, 2007).

Os componentes das paredes bacterianas são descritos como os principais antígenos nas infecções causadas por estes microorganismos (VAN AMERSFOORT et al., 2003). Nas bactérias gram-negativas, o lipopolisacarídeo (LPS), componente majoritário da parede externa, é o principal antígeno, enquanto que nas bactérias gram-positivas, os principais antígenos são o ácido lipoteicóico (LTA) e o peptídeoglicano (PEG). A ligação destas moléculas a receptores de reconhecimento de padrões (*Pattern Recognition Receptors*, PRRs) existentes nas células do sistema imune inato iniciam as respostas imunológicas às infecções bacterianas (TAKEUCHI E AKIRA, 2001; MEDZHITOV, 2007) (Figura 1). Entre os PRRs, os mais conhecidos são os receptores Toll-like (TLR), receptores transmembranas que se ligam a diferentes moléculas de microorganismos. Os receptores TLR 2, em heterodímeros com TLR 1 ou 6, e TLR 4, em homodímeros, ligam-se a moléculas lipídicas, incluindo o LPS (TLR 4) e o LTA

(TLR 2). Já os receptores TLR 3, 7, 8 e 9 são capazes de reconhecer ácidos nucléicos virais e bacterianos, enquanto os receptores TLR 5 e 11 ligam-se a proteínas de agentes patogênicos, como a flagelina de protozoários (O'NEILL, 2006) (Figura 2). Além destes, outra família de receptores PRRs são os NOD (*Nucleotide-binding Oligomerization Domain* - domínio de oligomerização e ligação de nucleotídeos), receptores citosólicos envolvidos no reconhecimento de peptídeoglicanos bacterianos (MEDZHITOV, 2007). Enquanto os receptores TLR estão envolvidos no reconhecimento de antígenos presentes no espaço extracelular e no interior de endossomos, os receptores NOD são ativados por componentes bacterianos presentes no interior da célula, no citosol (KANNEGANTI et al., 2007).

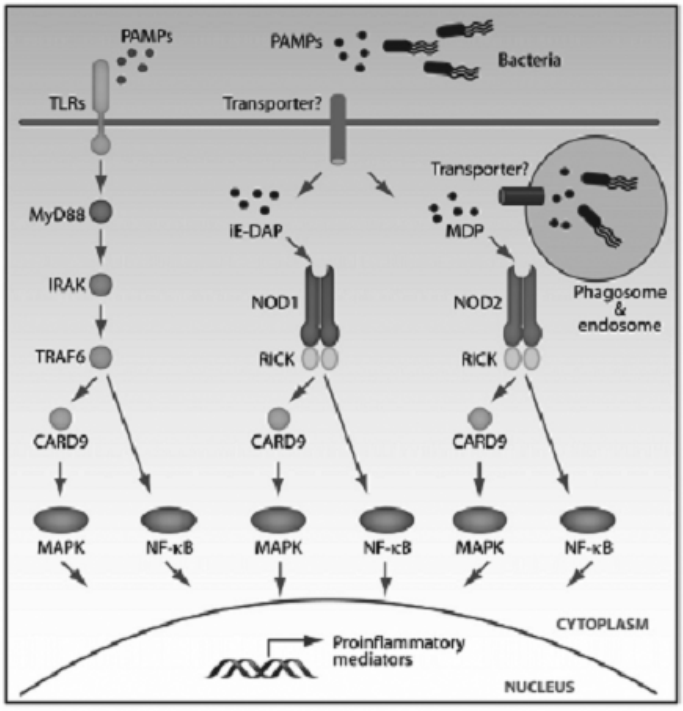


**Figura 1: Ativação de células do sistema imune inato por infecções bacterianas.** Os antígenos bacterianos (PAMPs *pathogen-associated molecular patterns* - padrões moleculares associados a patógenos) ligam-se a receptores de reconhecimento de padrões (*Pattern Recognition Receptors*, PRRs) presentes na superfície - receptores Toll-like (TLRs) - ou no interior - receptores NOD - da célula imune, ativando diferentes rotas de sinalização intracelular. (Imagem de KANNEGANTI et al., 2007)

Os macrófagos constituem uma população heterogênea de fagócitos mononucleares, os quais estão amplamente distribuídos nos tecidos e órgãos. Os macrófagos originam-se a partir dos monócitos presentes na circulação sangüínea, os quais se infiltram nos tecidos e/ou migram para locais de inflamações, diferenciando-se nos macrófagos. Estas células estão diretamente envolvidas no reconhecimento e ataque de agentes patogênicos, além de atuarem como moduladores da resposta imunológica. Os macrófagos atuam diretamente no combate às infecções bacterianas, através da fagocitose dos agentes patogênico (HUME, 2006), adicionalmente, os macrófagos expressam diferentes PRRs, os quais são ativados por antígenos bacterianos, iniciando a resposta inflamatória por intermédio da produção de citocinas, espécies reativas de oxigênio e óxido nítrico (MOSSER, 2003; GORDON e TAYLOR, 2005).

O fator de necrose tumoral alfa (TNF-α) é uma citocina envolvida na regulação de diversos processos fisiológicos e patológicos. Esta citocina atua através da ligação em dois receptores distintos, o TNFR 1 (ou p55) e o TNFR 2 (ou p75), os quais atuam como homotrímeros. A sinalização dos receptores do TNF-α ocorre através da interação com diferentes proteínas adaptadoras citosólicas. Estas, de acordo com o tipo celular, irão desencadear distintas cascatas de sinalização, relacionadas com o controle de processos celulares como ativação inflamatória, proliferação e morte (MACEWAN, 2002). O TNF-α possui grande importância durante a fase aguda da resposta inflamatória a agentes patogênicos, estimulando alterações fisiológicas que contribuem para: a eliminação dos agentes infecciosos, o controle do dano tecidual e a ativação de processos de reparação. No entanto, níveis elevados de TNF-α estão associados com efeitos deletérios presentes em infecções sistêmicas (sepse) e caquexia (HEHLGANS e PFEFFER, 2005). A neutralização da produção do TNF-α tem sido proposta como uma

possível intervenção terapêutica em infecções bacterianas, no entanto, os resultados experimentais têm apresentado baixo êxito (LORENTE e MARSHALL, 2005).

O óxido nítrico (NO) é um radical de nitrogênio altamente reativo e difusível, envolvido na regulação de diversos processos biológicos. Entre estes, estão inclusos o controle da pressão arterial, a regulação da função plaquetária, a defesa imunológica e a transmissão sináptica. O NO é sintetizado a partir da conversão da L-arginina a L-citrulina pelas enzimas óxido nítrico sintases, utilizando NADPH e $O_2$ como co-substratos. São descritas três isoformas de óxido nítrico sintases: duas isoformas constitutivas e cálcio dependentes, as óxido nítrico sintases neuronal e endotelial, e uma isoforma induzível, a qual é cálcio independente. Nas células do sistema imune, a óxido nítrico sintase induzível (iNOS) é responsável pela produção de NO em resposta a estímulos pró-inflamatórios, participando da regulação da resposta imune e possuindo ação microbicida (COLEMAN, 2001; KORHONEN et al., 2005). Entretanto, a produção exacerbada de NO pela iNOS está envolvida no choque hemodinâmico presente na sespe, além de participar de efeitos deletérios oriundos da resposta inflamatória (VINCENT et al., 2000; KORHONEN et al., 2005).

Os macrófagos, bem como outras células fagocitárias, produzem grande quantidade de espécies reativas de oxigênio (ROS – *reactive oxygen species*), as quais possuem poder microbicida (HALLIWELL, 2006). Adicionalmente, as ROS atuam como moléculas sinalizadoras intracelulares, regulando fatores de transcrição envolvidos na resposta inflamatória, como o NF-kB (FORMAN e TORRES, 2001). Em macrófagos expostos a LPS, a produção de ROS está envolvida na expressão e secreção de citocinas pró-inflamatórias como o TNF-$\alpha$ (KIMURA et al., 2008) a interleucina 8 (RYAN et al., 2004) e a interleucina 1$\beta$ (HSU e WEN, 2002). Assim, níveis elevados de ROS, em resposta a infecções bacterianas, contribuem para as complicações associadas

com a resposta inflamatória, incluindo o choque séptico e a falência múltipla de órgãos (RITTER et al., 2004; ANDRADES et al., 2005; VICTOR et al., 2005; HALLIWELL, 2006). Nas células fagocitárias, a principal enzima envolvida na produção de ROS é a NADPH oxidase. Nas células não estimuladas, os componentes da NADPH oxidase estão em diferentes compartimentos celulares, com as subunidades $gp91^{phox}$ e $p22^{phox}$ na membrana plasmática e as subunidades $p47^{phox}$ /$p67^{phox}$/$p40^{phox}$ no citosol, na forma de um complexo. Durante a fagocitose, ou a ativação celular por agentes solúveis, como o LPS, os componentes citosólicos e a GTPase Rac1/Rac2 translocam-se para a membrana plasmática, ligando-se às subunidades $gp91^{phox}$ e $p22^{phox}$, e iniciando a produção de superóxido pela transferência de um elétron do NADPH para o oxigênio (FORMAN e TORRES, 2002; DECOURSEY e LIGETI, 2005).

O NADPH é uma coenzima essencial para diversos processos celulares. Os níveis citosólicos desta coenzima são mantidos pelas desidrogenases citosólicas $NADP^+$-dependentes: a glicose-6-fosfato desidrogenase (G6PD), a isocitrato desidrogenase (IDH) e a malato desidrogenase (MDH). A redução da atividade da G6PD, ocasionando níveis reduzidos de NADPH, ocasiona uma produção aumentada de interleucina 10, dependente da sinalização por ROS, em macrófagos peritoniais de camundongos (WILMANSKI et al., 2005). Além disso, em macrófagos estimulados por LPS, o aumento da atividade da IDH protege estas células da produção de ROS e NO, através da manutenção do balanço redox (MAENG et al., 2004).

As metaloproteinases (MMPs) são uma família de proteases zinco-dependentes que atuam na degradação de proteínas localizadas na membrana citoplasmática e no espaço extracelular, atuando principalmente nos componentes da matriz extracelular. Assim, estas enzimas atuam na migração e infiltração das células imunes. Além disso, as metaloproteinases também atuam na modulação da resposta

inflamatória, através da degradação de citocinas, regulando os níveis plasmáticos destas proteínas sinalizadoras. Entre as metaloproteinases, a MMP 2 e a MMP 9, também conhecidas como gelatinases A e B, respectivamente, formam um grupo específico, pois apresentam um domínio de ligação à gelatina, o qual é fundamental para a ligação ao colágeno desnaturado (PARKS et al., 2004). É sugerido que níveis elevados de metaloproteinases contribuem para o dano tecidual e a falência múltipla de órgãos em um modelo animal de endotoxemia (PAGENSTECHER et al., 2000). Ademais, estas enzimas estão envolvidas na disfunção vascular associada à endotoxemia (LALU et al., 2006).

A resposta inflamatória a infecções bacterianas tem sido alvo de intensa investigação. Entretanto, a maior parte dos estudos tem focado a resposta das células imunes a infecções por bactérias gram-negativas ou ao seu principal antígeno, o LPS. A busca de artigos científicos relacionados a macrófagos e LPS, no banco de dados do PUBMED (http://www.ncbi.nlm.nih.gov/sites/entrez), resulta em mais de 10000 artigos científicos. Entretanto, a busca de artigos relacionados a um dos principais antígenos de bactérias gram-positivas, o LTA, e macrófagos resulta em menos de 200 artigos.

As infecções por bactérias gram-positivas são freqüentes e resultam em grande taxa de mortalidade, superando as infecções por bactérias gram-negativas nas infecções associadas aos ambientes hospitalares (UÇKAY et al., 2007). Nos Estados Unidos, a bactéria gram-positiva *Staphylococcus aureus* é o principal patógeno associado tanto a infecções hospitalares como a infecções adquiridas fora do ambiente hospitalar. Adicionalmente, as infecções causadas por *S. aureus* resistentes a meticilina possuem o maior risco de letalidade entre todos os patógenos (SHORR et al., 2006) e a prevalência de *S. aureus* resistentes a antibióticos tem aumentado mundialmente (UÇKAY et al., 2007). No Brasil, diferentes estudos têm apontado que as infecções por

*S. aureus* são as mais comuns entre as infecções hospitalares, com grande incidência de cepas resistentes a antibióticos (SOUZA et al., 2007; RIBAS et al., 2007). Segundo Guilarde et al. (2006), entre as infecções por *S. aureus* no Hospital da Universidade Federal de Goiás, cerca de 60% são causadas por cepas resistentes à meticilina.

A patogenicidade de *S. aureus* é causada por um repertório de toxinas, exoenzimas, adesinas e outras proteínas imunomoduladoras. Estes antígenos interagem com distintos receptores das células imunes, levando à produção e liberação de diversas moléculas pró-inflamatórias e imunomoduladoras (FOURNIER e PHILPOTT, 2005) (Figura 2). O peptídeoglicano (PEG) e o ácido lipoteicóico (LTA) são os principais componentes da parede bacteriana das bactérias gram-positivas e são descritos modulando a atividade de células imunes principalmente através da ativação dos receptores TLR 2 (SCHWANDNER et al., 1999; WANG et al., 2003), embora o PEG também atue sobre os receptores NOD (MEDZHITOV, 2007; KANNEGANTI et al., 2007). O PEG e o LTA são descritos como responsáveis pela produção de NO, choque e falência múltipla de órgãos provocados por *S. aureus* (DE KIMPE et al., 1995; KENGATHARAN et al., 1998). Em modelos animais, o LTA causa artrite, nefrite, uveíte, encefalomielite, meningite e lesões periodontais, além de ativar cascatas que culminam no choque séptico e falência múltipla de órgãos (GINSBURG, 2002). Ademais, o LTA é descrito como o principal responsável pela produção de TNF-$\alpha$ em macrófagos expostos a bactérias gram-positivas (SEO et al., 2008), além de estimular a produção de ROS em monócitos humanos (LEVY et al., 1990) e células dendríticas de roedores (CHOI et al., 2008). O PEG de *S. aureus* aumenta os níveis de MMP 2 e 9 no plasma e órgãos de ratos (WANG et al., 2004), além de estimular a secreção de MMP 9 em neutrófilos humanos (WANG et al., 2005).

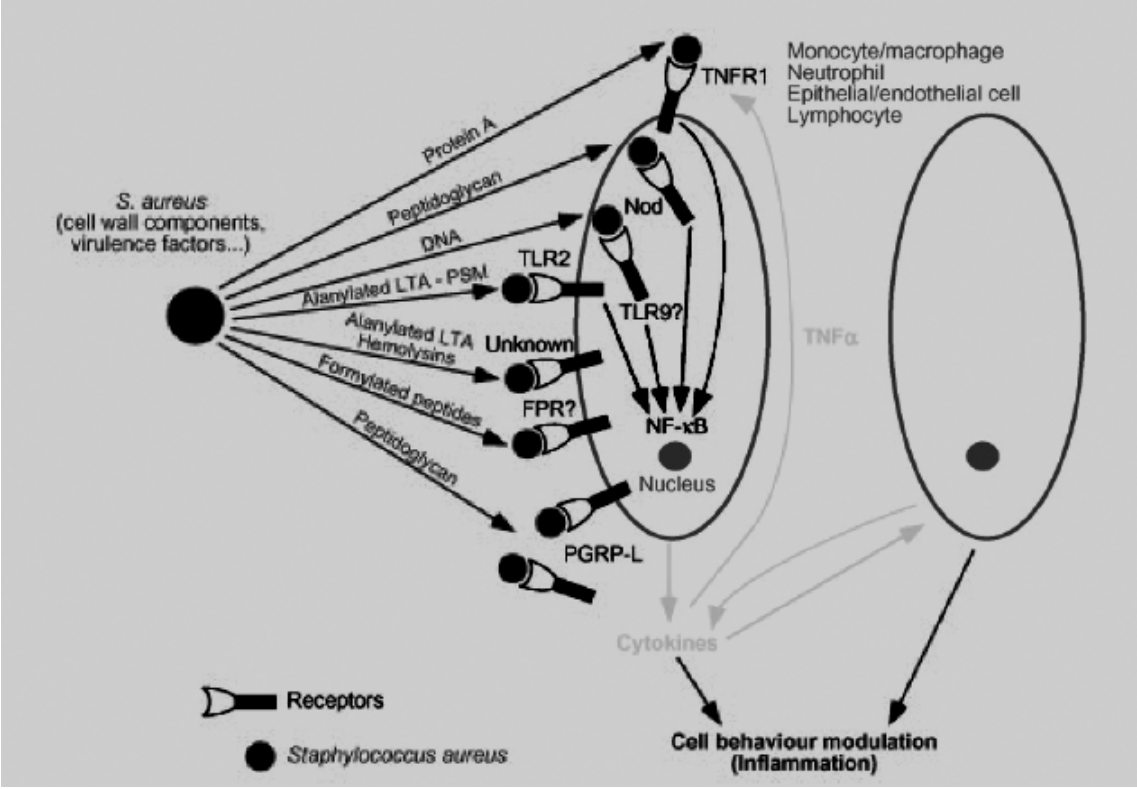


**Figura 2: Ativação de células do sistema imune inato por *Staphylococcus aureus*.** A patogenicidade de *S. aureus* é causada por um repertório de toxinas, exoenzimas, adesinas e outras proteínas imunomoduladoras. Estes antígenos interagem com distintos receptores das células imunes, levando à produção e liberação de diversas moléculas pró-inflamatórias e imunomoduladoras. (Imagem de FOURNIER e PHILPOTT, 2005)

A adenosina é um nucleosídeo de purina envolvido na regulação de diversos processos celulares. Esta molécula, quando no espaço extracelular, atua através de quatro receptores diferentes, chamados de A1, A2A, A2B e A3. Os receptores de adenosina fazem parte da família dos receptores metabotrópicos associados a proteínas G, com sete domínios transmenbranas. Os receptores A1 e A3 estão associados a proteínas G inibitórias, enquanto os receptores A2A e A2B estão associados a proteínas G estimulatórias (FREDHOLM et al. 2001) (Figura 3).

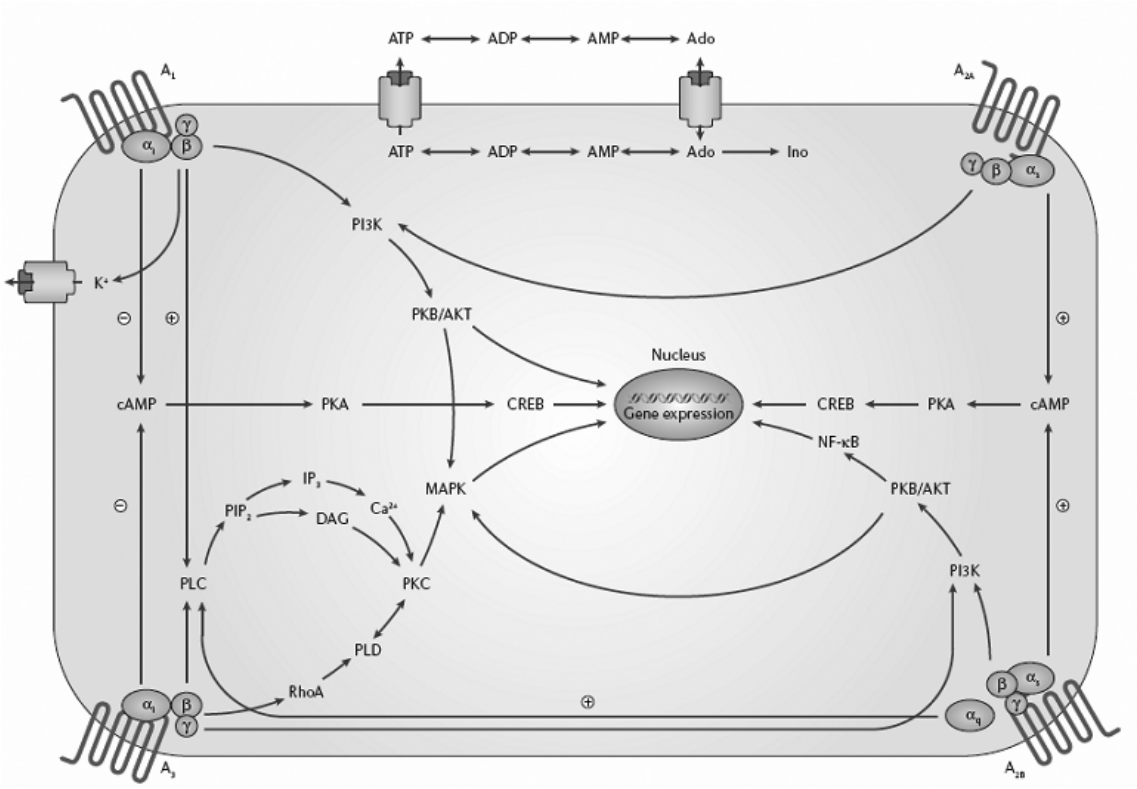


**Figura 3: Adenosina extracelular e seus receptores.** A adenosina, quando no espaço extracelular, atua através de quatro receptores diferentes: os receptores A1 e A3 estão associados a proteínas G inibitórias, enquanto os receptores A2A e A2B estão associados a proteínas G estimulatórias. A concentração extracelular de adenosina é regulada pelo metabolismo extracelular das purinas e pela ação de transportadores específicos presentes na membrana citoplasmática. (Imagem de JACOBSON e GAO, 2006)

Os níveis extracelulares de adenosina são regulados pela ação de transportadores de nucleosídeos, os quais são divididos em equilibrativos, canais de difusão limitada, atuando em ambos os sentidos, e concentrativos, transportadores simporte $Na^+$-dependentes (ROSE e COE, 2008). Adicionalmente, os níveis extracelulares de adenosina são dependentes da atividade de ectonucleotidases, que degradam o ATP a ADP, AMP e adenosina (ZIMMERMANN, 2000) e da adenosina deaminase extracelular, que degrada a adenosina a inosina (FRANCO et al., 1997). Níveis elevados de adenosina e ATP são encontrados em locais de inflamação. Adicionalmente, a adenosina e o ATP possuem papéis antagônicos na inflamação, de

modo que o ATP apresenta efeitos pró-inflamatórios, enquanto a adenosina apresenta efeitos antiinflamatórios. Deste modo, o metabolismo extracelular das purinas pode atuar na modulação de efeitos pró e antiinflamatórios (BOURS et al., 2006).

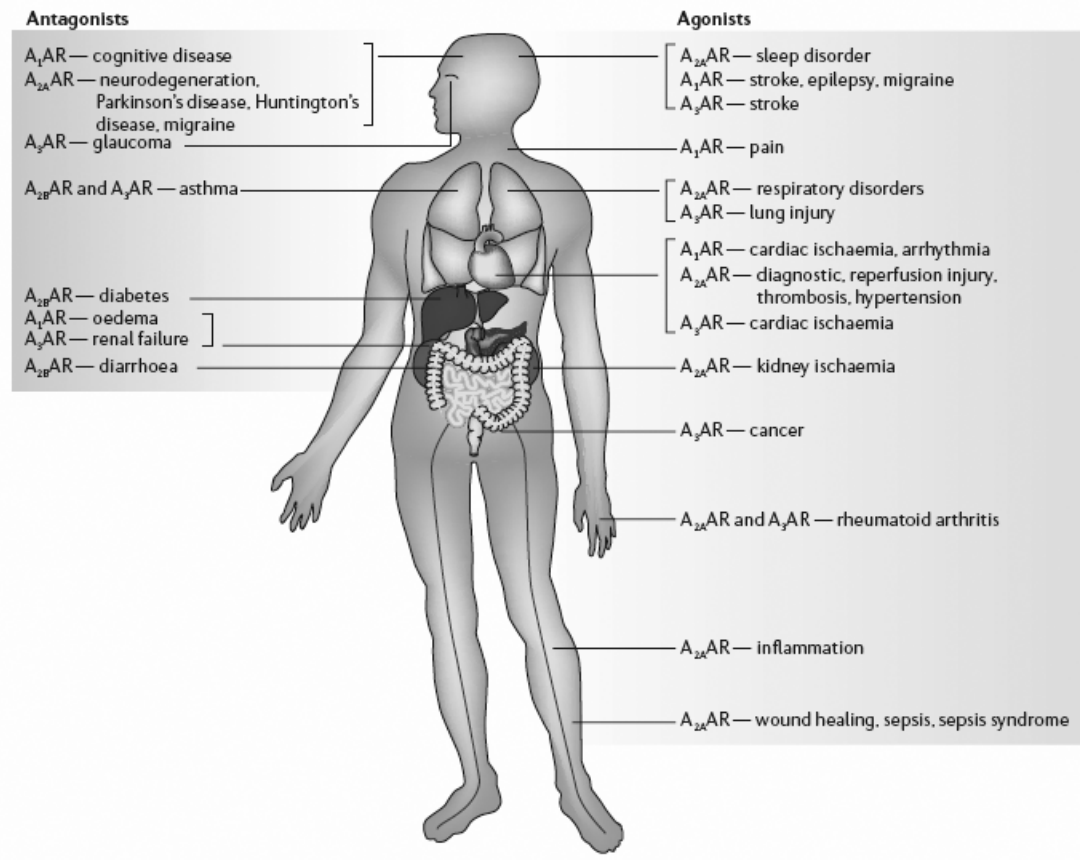


**Figura 4: Alvos terapêuticos de investigações sobre os efeitos farmacológicos de agonistas e antagonistas de receptores de adenosina.** Entre os possíveis alvos terapêuticos para os estudos com os receptores de adenosina estão a inflamação e condições infecciosas, como a sepse. (Imagem de JACOBSON e GAO, 2006)

Um grande esforço tem sido empregado nos estudos referentes ao desenvolvimento de agonistas e antagonistas de receptores de adenosina, bem como na sua aplicação como agentes terapêuticos em diversas condições patológicas, como isquemia cerebral e cardíaca, distúrbios do sono, câncer e doenças imunes (JACOBSON e GAO, 2006) (Figura 4). A ativação de receptores A2A aumenta a sobrevivência em modelos animais de endotoxemia e infecções por bactéria gram-negativas (SULLIVAN et al., 2004). Adicionalmente, roedores nocautes para o receptor A2B apresentam uma resposta inflamatória exacerbada à endotoxemia (YANG et al., 2006).

Diversos estudos mostram efeitos antiinflamatórios dos receptores de adenosina em macrófagos e monócitos em modelos de infecção por bactérias gram-negativas (HASKÓ et al., 2007). Além disso, o receptor A2A modula a produção de TNF-α em macrófagos expostos a diferentes ligantes de receptores TLR (PINHAL-ENFIELD et al., 2003), e a adenosina extracelular aumenta a produção da citocina antiinflamatória interleucina 10 em monócitos estimulados por *S. aureus* (LINK et al., 2000). O receptor A2A modula a expressão de MMP 9 em neutrófilos humanos (ERNENS et al., 2006) enquanto o receptor A2B está envolvido na supressão da produção de MMP 9 pela hipóxia, em células dendríticas derivadas de monócitos humanos (ZHAO et al., 2008). A ativação dos receptores de adenosina também apresenta efeitos antioxidantes (HUANG, 2003; FATOKUN et al., 2007), além de a adenosina reduzir a produção de superóxido em macrófagos ativados por phorbol-éster (SI et al., 1997).

O diabetes é uma doença metabólica prevalente, associada com a sinalização deficiente de insulina, quer seja pela falta de produção de insulina, diabetes tipo 1, ou pelo aumento da resistência à insulina, diabetes tipo 2. Esta doença apresenta diversas complicações secundárias, muitas delas associadas aos níveis elevados de

glicose. Os pacientes diabéticos apresentam uma ocorrência aumentada de infecções bacterianas (SMITHERMAN e PEACOCK, 1995). Além disso, o diabetes tipo 2 está associado com uma inflamação crônica, caracterizada por níveis plasmáticos aumentados de citocinas pró-inflamatórias, o quê está envolvido na patogênese da doença (PICKUP, 2004). Os pacientes diabéticos tipo 2 possuem maior risco de desenvolver doenças cardiovasculares e aterosclerose, o que tem sido relacionado à hiperglicemia e à inflamação crônica (MAZZONE et al., 2008).

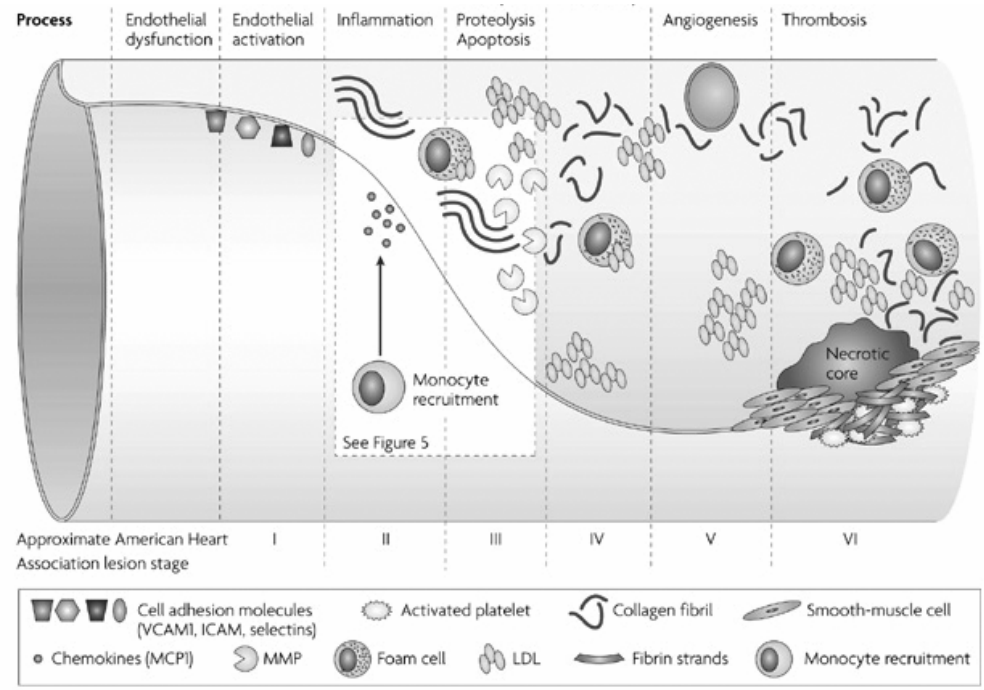


**Figura 5: Participação dos macrófagos na progressão da aterosclerose.** Os macrófagos participam do desenvolvimento da aterosclerose através da sua conversão em células espumosas (*foam cells*) e da regulação das funções das células vasculares, pela produção de citocinas pró-inflamatórias, fatores de crescimento, NO e ROS. Além disso, os macrófagos ativados secretam metaloproteinases que estão envolvidas no aumento da espessura da parede vascular e no rompimento da placa aterosclerótica. (Imagem de LINDSAY e CHOUDHURY, 2008)

Os macrófagos participam do desenvolvimento da aterosclerose através da sua conversão em células espumosas e da regulação das funções das células vasculares, pela produção de citocinas pró-inflamatórias, fatores de crescimento, NO e ROS (YAN e HANSSON, 2007) (Figura 5). Além disso, os macrófagos ativados secretam metaloproteinases que estão envolvidas no aumento da espessura da parede vascular e no rompimento da placa aterosclerótica (NEWBY, 2005). O LPS acelera o desenvolvimento da aterosclerose em coelhos (LEHR et al., 2001) e Kozarov et al. (2006) descreveu a presença de bactérias gram-negativas e gram-positivas em placas ateroscleróticas, incluindo *S. aureus*.

Concentrações elevadas de glicose modificam a resposta de macrófagos ao LPS em modelos animais de diabetes, aumentando a expressão de interleucina 12 (WEN et al., 2006) e TNF-α (SHERRY et al., 2007), além de aumentar a produção de prostaglandina $E_2$, pelo aumento da expressão da ciclooxigenase-II (LO, 2005). Entretanto, a glicose reduz a liberação de interleucina 1β (HILL et al., 1998).

Os receptores ativadores da proliferação de peroxissomos (PPARs - *Peroxisome proliferator–activated receptors*) são receptores nucleares dependentes de ligantes envolvidos na modulação da resposta imune. O PPARγ é um receptor nuclear que possui papel fundamental na adipogênese e no metabolismo da glicose. Nos macrófagos, o PPARγ tem sido descrito como um importante modulador da resposta inflamatória, principalmente através da inibição do fator de transcrição pró-inflamatório NF-kB (ZHANG e CHAWLA, 2004). Adicionalmente, Sartippour e Renier (2000) mostraram que concentrações elevadas de glicose modulam a expressão dos PPARs.

**Objetivos**

Em um trabalho anterior, foi demonstrado que a redução da produção de ROS e citocinas pró-inflamatórias, TNF-α e interleucina 1β, em macrófagos peritoniais está associada a uma maior sobrevivência em um modelo animal de infecção bacteriana (SOUZA et al., 2007). Como descrito anteriormente, diversos trabalhos têm demonstrado o papel antiinflamatório dos receptores de adenosina em modelos de infecção por bactérias gram-negativas, no entanto, pouco se sabe sobre a função destes receptores em infecções por bactérias gram-positivas. Adicionalmente, as alterações ocasionadas na ativação dos macrófagos pelos níveis elevados de glicose parecem estar envolvidos na patogênese do diabetes e na progressão da aterosclerose, entretanto, a ação da glicose sobre a ativação de macrófagos por bactérias gram-positivas é desconhecida. Assim, nessa tese, os objetivos gerais foram:

1. Investigar a possível participação dos receptores de adenosina na modulação da ativação de macrófagos por antígenos (peptídeoglicano e ácido lipoteicóico) de uma bactéria gram-positiva com grande relevância, em número e gravidade, nas infecções hospitalares, o *Staphylococcus aureus*;

2. Investigar a modulação da ativação de macrófagos por antígenos de bactérias gram-negativa (lipopolissacarídeo) e gram-positiva (ácido lipoteicóico) na presença de concentrações elevadas de glicose.

# PARTE II

## Capítulo 1

## Os receptores de adenosina modulam a ativação de macrófagos RAW 264.7 por antígenos de *Staphylococcus aureus*.

**Manuscrito submetido ao periódico The Journal of Immunology**

**Adenosine receptors modulate RAW 264.7 macrophages activation by *Staphylococcus aureus* antigens.**

**Running title: Adenosine receptors and *S. aureus* macrophage activation**

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**Abstract**

Peptidoglycan (PEG) and lipoteichoic acid (LTA) are the main constituents of gram-positive bacteria cell wall and are described to be involved in *Staphylococcus aureus*-induced shock and multiple organ failure. NO and TNF-α are produced by stimulated macrophages and the overproduction of these mediators has been implicated in the genesis of septic and cytokine-induced circulatory shock. Adenosine regulates inflammation and immune response through activation of four distinct receptors (A1, A2A, A2B, and A3). Its extracellular concentration depends on direct adenosine transport and metabolism, and ATP degradation, however, the role of adenosine receptors in gram-positive bacterial infections is poorly known. LTA increased extracellular ATP degradation in macrophages, resulting in extracellular adenosine accumulation. LTA augmented A2A and A2B mRNA levels at 12 hours of treatment and reduced A3 mRNA content at 24 hours. Similar results were obtained with PEG, which also increased extracellular ATP degradation and altered adenosine receptors expression by increasing A2A and A2B mRNA levels at 12 hours and reducing A3 mRNA content at 24 hours. An adenosine receptor non-selective agonist reduced PEG-stimulated TNF-α production and LTA-increased iNOS protein content and NO production. A2A and A2B adenosine receptors blockade by selective antagonists resulted in exacerbated NO production in LTA-treated macrophages. A2A and A2B adenosine receptors gene knockdown and exogenous adenosine deaminase treatment also resulted in augmented LTA stimulation of NO production in RAW 264.7 macrophages. These results suggest an autocrine regulatory role for adenosine signaling in gram-positive antigens stimulation of macrophages.

## Introduction

Gram-positive bacterial infections are frequent and associated with high morbidity and mortality (1). The gram-positive bacterium *Staphylococcus aureus* is a major pathogen in both community-acquired and nosocomial infections and was the most commonly encountered pathogen in all types of early-onset bacteremia in the United States between January 2002 and December 2003, according to Cardinal Health Research Database. Additionally, methicillin-resistant *Staphylococcus aureus* carries the highest relative mortality risk among all pathogens (2).

The pathogenicity of *S. aureus* is associated with a repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins that it produces. These agents interact with distinct receptors in immune cells leading to the production and release of proinflammatory and immunomodulating cytokines (3). Peptidoglycan (PEG) and lipoteichoic acid (LTA) are the main constituents of gram-positive bacteria cell wall and are described to modulate immune functions mainly through Toll-like Receptor 2 activation (4,5). These antigens are described to be involved in *S. aureus*-induced nitric oxide production, shock and multiple organ failure (6,7). NO is a highly reactive nitrogen radical implicated in multiple biological processes. NO is synthesized from L-arginine by nitric oxide synthases. Three nitric oxide synthases are described: the neuronal and endothelial isoforms that are constitutive and calcium-dependent, and the inducible isoform, which is calcium-independent. In stimulated immune cells, the inducible nitric oxide synthase (iNOS) accounts for increased NO production, which is involved in the regulation of immune response and microbicide action (8). Moreover, the production of large amounts of NO by iNOS has been implicated in the genesis of septic and cytokine-induced circulatory shock (9).

Macrophages are a heterogeneous population of mononuclear phagocytes found ubiquitously in the organism. Due to their distribution, these cells are involved in the primary contact with and in the attack against pathogens. Bacterial wall components stimulate Toll-like receptors, initiating macrophage inflammatory response by producing NO, reactive oxygen species and pro-inflammatory cytokines, including TNF-α (10,11). TNF-α is a pleiotropic cytokine involved in the regulation of diverse physiological and pathological responses. TNF-α is known to be important in inducing the acute-phase response, leading to physiological changes that serve to eliminate the infecting organism, limit tissue damage, and activate repair processes. Controversially, increased levels of TNF-α are associated with damaging effects of sepsis and cachexia (12).

Adenosine is an endogenous purine nucleoside that regulates several cellular processes. Extracellular adenosine concentration, which is increased in stressful conditions like inflammation and ischemia, is dependent on direct adenosine transport and metabolism, and extracellular ATP degradation. Extracellular ATP is degraded to ADP, AMP and adenosine by ectonucleotidases (13). There are four types of adenosine receptors (A1, A2A, A2B, and A3) and all adenosine receptors are members of the G-protein-coupled family of receptors (14). There is an increasing interest in the development of selective agonists and antagonists of adenosine receptors and their role as therapeutic agents in a wide range of conditions, including cerebral and cardiac ischemic diseases, sleep disorders, immune and inflammatory disorders and cancer (15). A2A adenosine receptor activation is described to improve survival in models of endotoxemia and gram-negative sepsis (16). Additionally, the knockout of A2B adenosine receptor presented an exacerbated inflammatory response to endotoxemia (17) and several studies have reported anti-inflammatory properties of adenosine

receptors in monocytes and macrophages (18). A2A adenosine receptor is described to modulate TNF-α production in macrophages stimulated by distinct Toll-like receptor agonists (19), and extracellular adenosine increases *S. aureus*-stimulated production of the anti-inflammatory cytokine interleukin 10 in monocytes (20).

Many studies have addressed the anti-inflammatory potential of adenosine receptors in gram-negative infection models, however, the role of these receptors in gram-positive bacterial infections is poorly known. In this work, we evaluated the role of adenosine receptors in *S. aureus* antigens (LTA and PEG) stimulation of RAW 264.7 macrophages.

## Material and Methods

### Chemicals

Lipoteichoic acid and peptidoglycan from *Staphylococcus aureus* were purchased from InvivoGen (San Diego, USA). 5'-N-ethylcarboxamido adenosine (NECA), ZM241385, SCH58261, and MRS1706 were purchased from Tocris Bioscience (Ellisville, USA). Adenosine deaminase was purchased from Sigma (Saint Louis, USA). RPMI 1640 and fungizone were purchased from Gibco$^{TM}$ (Invitrogen, Carlsbad, USA). Anti-mouse iNOS primary antibody was purchased from BD Biosciences (San Jose, USA) and Santa Cruz Biotechnology (Santa Cruz, USA). Fetal bovine serum was purchased from Cultilab (Campinas, Brazil). All other reagents were of analytical grade and were purchased from commercially available sources.

### RAW 264.7 macrophages culture

RAW 264.7 macrophages were obtained from UFRJ Cell Bank, Rio de Janeiro, Brazil. Cells were maintained in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin, and 250 μg/L fungizone at 37 ºC and 5% $CO_2$. Semi-confluent cells were scrapped and plated to 24-well plates (0.4 x $10^6$ cells) or 12-well plates (0.8 x $10^6$ cells for RNA extraction and Western Blot) at a density of 0.8 x $10^6$ cells/ml in RPMI 1640, pH 7.4, supplemented with 10% heat-inactivated FBS, 100 mg/L gentamycin, and 250 μg/L fungizone. After 24 hours, the macrophages were washed with saline and incubated with or without LTA or PEG 1 μg/ml for distinct periods in serum-free RPMI 1640 medium. Adenosine receptors agonist (NECA, 10 μM) and antagonists (A2A – ZM241385 or SCH58261, 10 nM; A2B – MRS1706, 10 nM) as well as adenosine deaminase (1 U/ml), when used, were added 15 minutes prior to LTA or PEG.

### Extracellular ATP and adenosine metabolism

Control, LTA- or PEG-treated RAW 264.7 macrophages were washed three times and then incubated with HBSS (without phenol red, 15 mM HEPES) for different additional periods with ATP (25 μM) or adenosine (25 or 100 μM) in 5% CO2 at 37 ºC. Extracellular ATP and adenosine degradation, as well as its catabolites, were analyzed in a reverse-phase HPLC system equipped with a C-18 column (SupelcosilTM, Supelco®, 25 cm x 4.6 mm) and UV detector. The elution was made with an isocratic gradient of buffer A ($KH_2PO_4$ 60 mM, tetrabutylammonium 5 mM, pH 6.0) and buffer B (buffer A plus methanol 30%) at a flow rate of 1.25 ml/min. UV absorption was measured at 245 nm. Internal standards were used for identification of purines.

**Measurement of TNF-α in culture supernatants**

Cytokine secretion was measured using mouse TNF-α BD OptEIA™ ELISA kit (BD Biosciences), according to manufacturer's directions.

**Nitrite assay**

Nitric oxide production was assayed by quantification of the stable end product of nitric oxide oxidation – nitrite ($NO_2^-$). Briefly, the incubation medium of RAW 264.7 macrophages was collected and was reacted 1:1 with Griess reagent (1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) for 15 min. Nitrite content was measured by absorbance at 540 nm. Nitrite concentration in the samples was calculated using a standard curve prepared with $NaNO_2$.

**Western Blot**

RAW 264.7 macrophages were lysed in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% (w/v) sodium dodecil sulfate (SDS), 10% (v/v) glycerol) and equal amounts of cell protein were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes. Protein loading and electroblotting efficiency were verified through Coomassie Blue staining.

The membrane was blocked in Tween-Tris buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 10% fat-free milk and incubated overnight with primary antibody (anti iNOS). The membrane was washed and incubated for 1 hour with secondary anti-IgG peroxidase conjugate. The immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed with the IMAGE J® software (NIH, USA).

**RNA extraction, cDNA and real-time PCR**

RNA was isolated from 0.8 x $10^6$ RAW 264.7 macrophages using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Approximately 1 μg of total RNA was added to each cDNA synthesis reaction using the M-MLV reverse transcriptase (Invitrogen). Reactions were performed using the primer T23V (5'-TTTTTTTTTTTTTTTTTTTTTTV) as follows: sample RNA (~1 μg), water (to complete 9 μl), and 1 μl of poly-T primer were kept at 70 ºC for 5 minutes; the tubes were transferred to ice, and 30 μl of reaction mix (5 X first strand buffer 6 μl, M-MLV reverse transcriptase 200 U/μl 1 μl, dNTP 5 mM, 2 μl, and water 11 μl) were added. The reaction was carried at 42 ºC for 1 hour. RT-PCR and real-time PCR amplification was carried out using specific primer pairs designed with Primer3 Input (http://frodo.wi.mit.edu/) and synthesized by RW-Genes (RJ, Brazil). The primers utilized were A1R (5'-CTGGACTCTCCTGAGGGACA and 5'-CCACAGGGCTTCACAATCTT), A2AR (5'-TTCCATCTTCAGCCTCTTGG and 5'-CGCAGGTCTTTGTGGAGTTC), A2BR (5'-TTGGCATTGGATTGACTC and 5'-TATGAGCAGTGGAGGAAG), A3R (5'-CTGCCTTTTCATGTCCTGTG and 5'-TTCTATTCCAGCCAAACATGG), iNOS (5'-TCTGCGCCTTTGCTCATGAC and 5'-TAAAGGCTCCGGGCTCTG), β-actin (5'-TACTCCTGCTTGCTGATCCACAT and 5'-TATGCCAACACAGTGCTGTCTGG). RT-PCR products were separated on a 1%

agarose gel and amplification products were visualized by ethidium bromide staining. Real-time PCRs were carried out in an Applied-Biosystems 7500 real-time cycler (Applied Biosystems, Foster City, CA, USA). Reaction settings were composed of an initial denaturation step of 5 min at 94 ºC followed by 45 cycles of 10 s at 94 ºC, 15 s at 60 ºC (A2AR, A3R, TNF and β-actin) or 62 ºC (A2BR and iNOS), and 15 s at 72 ºC; samples were kept for 2 min at 40 ºC for reannealing and were then heated from 55 to 99 $^{o}$C with a ramp of 1 ºC/s to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were made in 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 50 times, 2 µl of Platinum Taq 10 times PCR buffer, 1.2 µl of 50 mM MgCl2, 0.4 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 4.95 µl of water, 1 µl of SYBR (1:100 000 Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen). All results were expressed as a relative ratio between the investigated gene and the β-actin internal control gene.

**Small Interfering RNA**

Predesigned small interfering RNAs (siRNA) against mouse A2A (siRNA IDs 162368, 162369 and 162370) and A2B (siRNA IDs 162156, 162157 and 162158) adenosine receptors, as well as control siRNA (scramble sequence) were obtained from Ambion (Applied Biosystems). RAW 264.7 cells were transfected in 24-well plates with either 10 nM A2AR, A2BR or scramble siRNA in RPMI 1640 10% FBS using siPORT NeoFX transfection agent (according to the manufacturer's instructions; Ambion). Twenty-four hours after transfection, cells were then used for further experimentation.

**Protein quantification**

All the results were standardized with respect to protein content, determined as described by Lowry et al. (21).

### Statistical analysis

Results are expressed as means ± SEM for at least triplicates and are representative of two or more independent experiments. Differences between means were analyzed by Student´s t-test or ANOVA with the Student-Newman-Keuls multiple comparisons test. Statistical significance was defined as $p<0.05$.

**Results**

LTA and PEG stimulated TNF-α secretion in RAW 264.7 macrophages, but LTA presented a higher effect. NECA, a non-selective adenosine receptor agonist, inhibited PEG-stimulated TNF-α secretion, nevertheless, no effect was observed on LTA-stimulated TNF-α production (Figure 1). LTA also stimulated NO production in RAW 264.7 macrophages in a dose-dependent way (Figure 2), however, no effect of PEG was observed (data not shown). NECA reduced NO production in LTA-treated RAW 264.7 macrophages (Figure 3A). NECA also reduced iNOS protein content in LTA-stimulated macrophages (Figure 3B), however, mRNA levels were unaltered (Figure 3C).

A2A, A2B, and A3 adenosine receptors were identified in RAW 264.7 macrophages by RT-PCR, however, A1 adenosine receptor was not observed (data not shown). LTA augmented A2AR and A2BR mRNA levels at 12 hours of treatment, but mRNA levels returned to control values at 24 hours. In opposition, A3R mRNA levels were reduced with 24 hours of incubation (Figure 4).

The A2AR antagonist ZM241385 prevented NECA inhibition of LTA-stimulated NO production (similar result was obtained with A2AR antagonist SCH58261), however, the A2BR antagonist MRS1706 had no effect on NECA inhibition (Figure 5). The selective antagonists for A2A and A2B adenosine receptors increased both basal and LTA-stimulated NO content in supernatants from cultured macrophages (Figure 5), suggesting that adenosine receptors could be involved in an autocrine mechanism of regulation.

Extracellular ATP was degraded by RAW 264.7 macrophages, resulting in extracellular ADP, AMP, and adenosine accumulation. LTA increased extracellular

ATP degradation at 24 hours of treatment, which resulted in augmented extracellular AMP and adenosine concentration (Figure 6).

PEG presented similar results compared to LTA; it also increased extracellular ATP degradation at 24 hour of treatment (Figure 7A) and augmented, in a lesser magnitude, A2AR and A2BR mRNA levels at 12 hours of treatment (Figure 7B). A3R mRNA was reduced at 24 hours of treatment (Figure 7B). No alteration of extracellular adenosine metabolism was observed in 24 hours LTA- or PEG-treated RAW 264.7 macrophages (data not shown).

To investigate if endogenous extracellular adenosine modulates NO production, RAW 264.7 macrophages were incubated in the presence of exogenous adenosine deaminase, with or without LTA. Exogenous adenosine deaminase increased basal and LTA-stimulated NO production (Figure 8).

A2A and A2B adenosine receptors knockdown by small interfering RNA increased LTA-stimulated NO production (Figure 9). The data shown represent the results obtained with siRNA ID 162368, for A2AR, and siRNA ID 162158, for A2BR, which presented the highest effects, compared to others siRNA, in LTA-stimulated NO production. The efficacy of siRNA ID 162368, for A2AR, and siRNA ID 162158, for A2BR, in the reduction of respective adenosine receptors mRNA levels was confirmed by real time PCR (data not shown).

**Discussion**

LTA was described as the major antigen involved in gram-positive stimulated TNF-α production by macrophages, however, others antigens synergistically contribute to total TNF-α secretion (27). In this work, we showed that, in RAW 264.7 macrophages, LTA stimulation of TNF-α secretion was higher than PEG stimulation. Adenosine receptor non-selective agonist NECA reduced the TNF-α secretion of PEG-treated macrophages by 50 %, however, no effect was observed on LTA stimulated TNF-α production. In LPS-treated macrophages, extracellular adenosine and adenosine receptor agonists potently reduce TNF-α secretion (22), evidencing distinct effects of adenosine receptors on gram-negative or gram-positive antigens stimulated TNF-α production.

LTA modulates iNOS expression and NO production in RAW 264.7 macrophages, which is described to be dependent upon the activation of protein kinase C, PI3K, Akt, p38, and NF-κB (28,29). In this work, we showed that NECA inhibited LTA-stimulated NO secretion and iNOS protein expression in RAW 264.7 macrophages. The NECA inhibition of LTA-stimulated NO production was blocked by the A2A receptor antagonist ZM241385, but not by the A2B antagonist MRS1706. Adenosine receptors are described to modulate prostaglandin $E_2$ production in LPS-treated macrophages (22) and Chang *et al.* (30) reported that LTA-stimulated NO production was dependent upon cyclooxygenase 2 expression and prostaglandin $E_2$ synthesis, suggesting a possible mechanism to NECA inhibition of NO production in LTA-treated macrophages.

Extracellular ATP has been described to possess pro-inflammatory properties, in opposition to extracellular adenosine, which is reported to be an anti-inflammatory molecule. Adenosine reduces pro-inflammatory macrophages/monocytes

parameters, as phagocytosis, oxidative burst, NO production, and TNF-α and interleukin 12 secretion (18). Additionally, adenosine increases the production of the anti-inflammatory molecules prostaglandin $E_2$ (22), heme oxigenase 1 (23) and interleukin 10 (24) in stimulated macrophages. Extracellular ATP degradation is increased in LTA-stimulated RAW 264.7 macrophages, which results in increased adenosine accumulation. PEG also stimulates extracellular ATP degradation, suggesting a common mechanism to prevent exacerbated macrophage stimulation, in which the pro-inflammatory signal of ATP is prevented and anti-inflammatory extracellular adenosine is produced. LTA and PEG increased A2A and A2B adenosine receptors mRNA levels in cultured macrophages, but they decreased A3 mRNA levels. These results are similar to those obtained both in human and murine macrophages exposed to LPS, which presented increased A2AR and A2BR RNA expression, while A3R mRNA levels were reduced (25). In interferon-γ-treated macrophages, the A2B receptor is described to be involved in an autocrine mechanism of inflammatory regulation, participating in the deactivation of macrophages (26).

The blockade of A2A and A2B adenosine receptors by selective antagonists increased LTA-stimulated NO production in RAW 264.7 macrophages. Similar result was obtained by small interfering RNA gene knockdown of these receptors. Moreover, extracellular adenosine degradation by exogenously added adenosine deaminase also increased LTA-stimulated NO production. These results suggest an autocrine role for A2A and A2B adenosine receptors in LTA activation of macrophages, where these receptors prevent exacerbated NO production in stimulated macrophages. Although the pharmacological modulation of LTA-stimulated NO production by NECA seems to be dependent of the A2A adenosine receptor, our results indicate that both A2A and A2B are involved in the autocrine regulation of NO production. No additive effect of

antagonist A2A and A2B blockade was observed (data not shown), suggesting that these receptors could be acting by a common mechanism. Several functional heterodimers for adenosine receptors were already described (31), however, the dimerization of A2A and A2B adenosine receptors was unknown.

In this work, we showed that LTA and PEG increased extracellular ATP degradation and altered adenosine receptors expression in RAW 264.7 macrophages. Moreover, the adenosine receptor non-selective agonist modulated PEG-stimulated TNF-α production and LTA increased iNOS protein content and NO production, while A2A and A2B adenosine receptors modulated LTA-stimulation of NO production in cultured macrophages. These results suggest an autocrine regulatory role of adenosine signaling in gram-positive antigens stimulation of macrophages.

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**Figure Legends**

Figure 1. TNF-α secretion by RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA or PEG (1 μg/ml) for 24 hours. When indicated, the adenosine receptor agonist NECA (10 μM) was added 15 minutes prior to LTA or PEG. At the end of the incubation, TNF-α secretion to the culture medium was evaluated by ELISA. Results are shown as mean ± SEM (n = 3). * $p < 0.05$ vs control, # $p < 0.05$ vs PEG and control, $ $p < 0.01$ vs other groups, ANOVA with the Student-Newman-Keuls multiple comparisons test.

Figure 2. Lipoteichoic acid increases NO production in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (0, 0.1, 0.5, 1 or 5 μg/ml) for 24 hours. At the end of the incubation, nitrite accumulation in the incubation medium was measured by the Griess method. Results are shown as mean ± SEM (n = 4). * $p < 0.05$ vs control, ANOVA with the Student-Newman-Keuls multiple comparisons test.

Figure 3. Adenosine receptor agonist modulates lipoteichoic acid-stimulated NO production in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 12 or 24 hours. When indicated, the adenosine receptor agonist NECA (10 μM) was added 15 minutes prior to LTA. (A) Nitrite accumulation in the incubation medium, measured by the Griess method, at 24 hours of incubation. Results are shown as mean ± SEM (n = 3) (B) iNOS protein expression at 12 hours of incubation, evaluated by Western Blot. A representative blot is shown and densitometric analysis results are shown as mean ± SEM (n = 3). (C) iNOS mRNA expression at 12 hours, evaluated by real-time PCR. Results are shown as mean ± SEM (n = 3). * $p <$

0.05 vs control, # $p < 0.05$ vs LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

Figure 4. Lipoteichoic acid modulates adenosine receptors expression in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 12 or 24 hours. A2A (A), A2B (B) and A3 (C) adenosine receptors expression was analyzed by real-time PCR. Results are shown as mean ± SEM (n = 3). * $p < 0.05$ vs control, Student´s t-test.

Figure 5. A2A and A2B adenosine receptors modulate lipoteichoic acid-stimulated NO production in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 24 hours. When indicated, (A) the A2A adenosine receptor antagonist ZM241385 (10 nM) or (B) the A2B adenosine receptor antagonist MRS1706 were added 15 minutes prior to LTA, with or without the adenosine receptor agonist NECA (10 μM),. Nitrite accumulation in the incubation medium was measured by the Griess method. Results are shown as times over control (mean ± SEM, n = 4). * $p < 0.05$ vs control, # $p < 0.05$ vs LTA and control, $ $p < 0.01$ vs other groups, & $p < 0.05$ vs LTA+MRS1706. ANOVA with the Student-Newman-Keuls multiple comparisons test.

Figure 6. Lipoteichoic acid increases extracellular ATP degradation in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 24 hours. At the end of incubation, ATP (25 μM) was added for 30 minutes and ATP degradation and metabolites accumulation were evaluated by HPLC analysis. (A) Extracellular ATP degradation. (B) Extracellular ADP accumulation at 1, 5, 15 and 30

minutes (C) Extracellular AMP accumulation at 15 and 30 minutes. (D) Extracellular adenosine accumulation at 30 minutes. Results are shown as mean ± SEM (n = 3). * $p < 0.05$ vs control, Student's t-test.

Figure 7. Peptidoglycan modulates extracellular ATP degradation and adenosine receptors expression in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were incubated with PEG (1 μg/ml) for 24 hours. At the end of incubation, ATP (25 μM) was added for 30 minutes and extracellular ATP degradation was evaluated by HPLC analysis. (B) RAW 264.7 macrophages were incubated with PEG (1 μg/ml) for 12 or 24 hours. A2A, A2B and A3 adenosine receptors expression was analyzed by real-time PCR. Results are shown as mean ± SEM (n = 3). * $p < 0.05$ vs control, Student´s t-test.

Figure 8. Extracellular adenosine depletion increases lipoteichoic acid-stimulated NO production in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 24 hours. When indicated, exogenous adenosine deaminase (1 U/ml) was added 15 minutes prior to LTA. Nitrite accumulation in the incubation medium was measured by the Griess method. Results are shown as mean ± SEM (n = 4) * $p < 0.05$ vs control, # $p < 0.05$ vs LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

Figure 9. A2A and A2B adenosine receptors knockdown increases lipoteichoic acid-stimulated NO production in RAW 264.7 macrophages. RAW 264.7 macrophages were transfected with either A2A, A2B or scramble siRNA (10 nM) in RPMI 1640 10% FBS for 24 hours. At the end of the transfection, RAW 264.7 macrophages were incubated with LTA (1 μg/ml) in RPMI 1640 for 24 hours. Nitrite accumulation in the

incubation medium was measured by the Griess method. Results are shown as mean ± SEM (n = 4) * $p < 0.05$ vs control, # $p < 0.05$ vs LTA and scramble, ANOVA with the Student-Newman-Keuls multiple comparisons test.

**Figure 1**

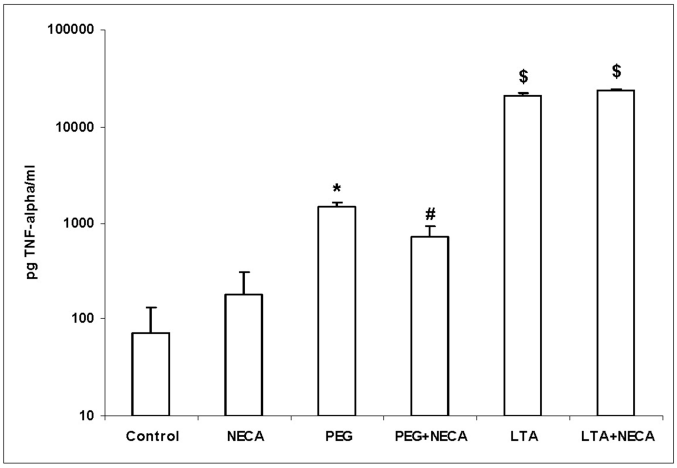

100000
10000
1000
100
10
pg TNF-alpha/ml
*
#
$
$
Control
NECA
PEG
PEG+NECA
LTA
LTA+NECA

**Figure 2**

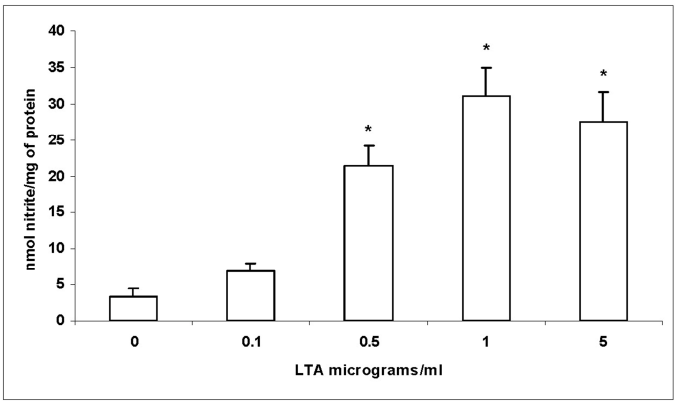

40
35
30
25
20
15
10
5
0
nmol nitrite/mg of protein
*
*
*
0
0.1
0.5
1
5
LTA micrograms/ml

**Figure 3**

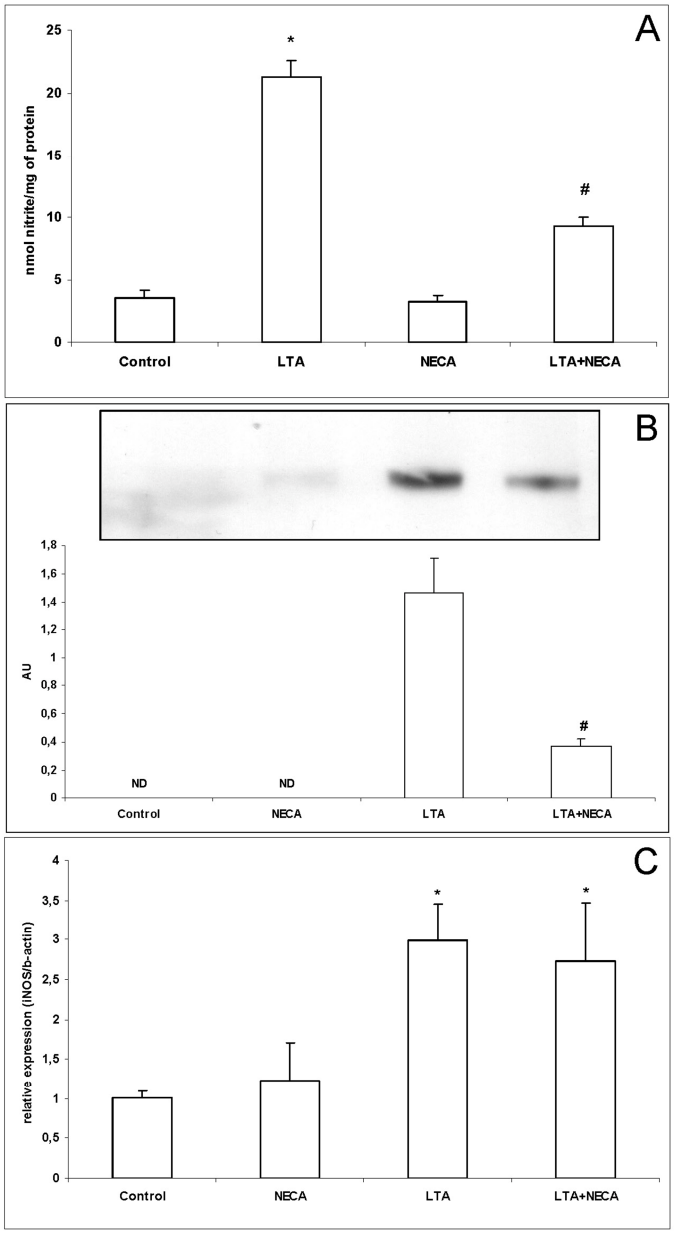

A
nmol nitrite/mg of protein
25
20
15
10
5
0
*
#
Control
LTA
NECA
LTA+NECA
B
AU
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
ND
ND
#
Control
NECA
LTA
LTA+NECA
C
relative expression (iNOS/b-actin)
4
3,5
3
2,5
2
1,5
1
0,5
0
*
*
Control
NECA
LTA
LTA+NECA

**Figure 4**

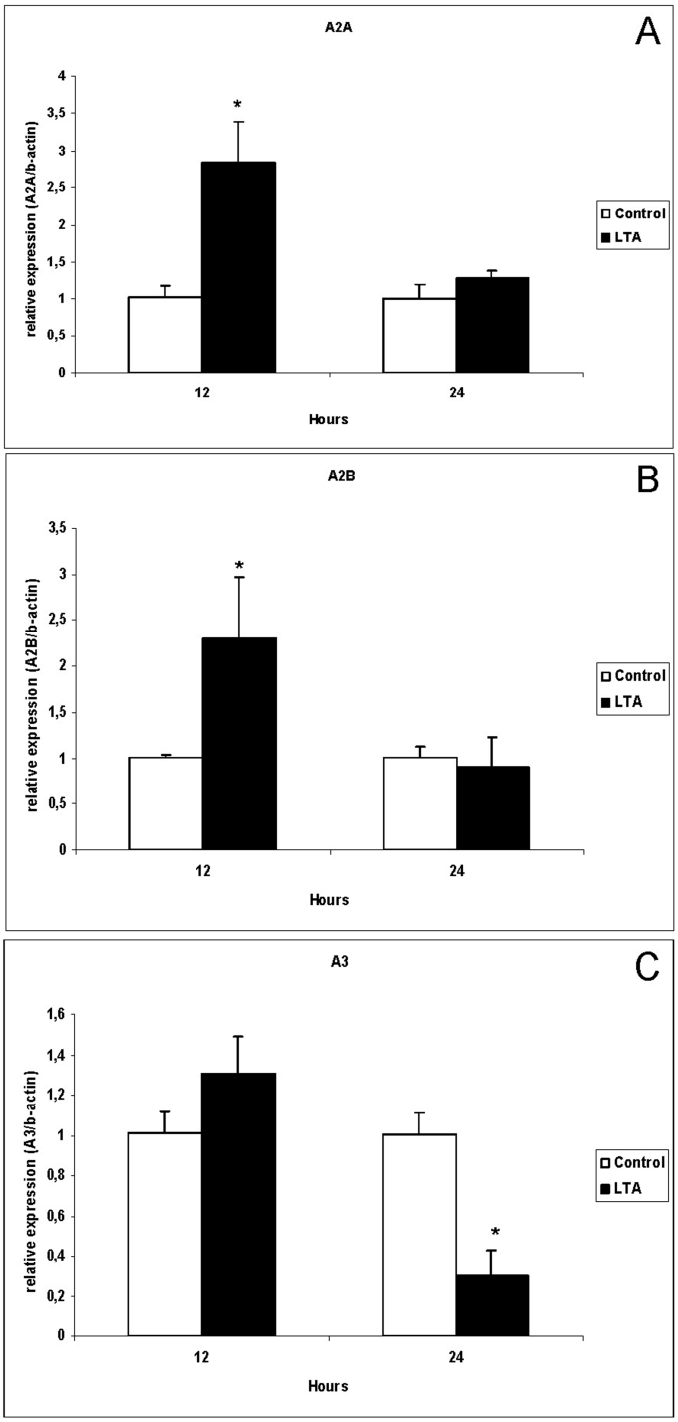
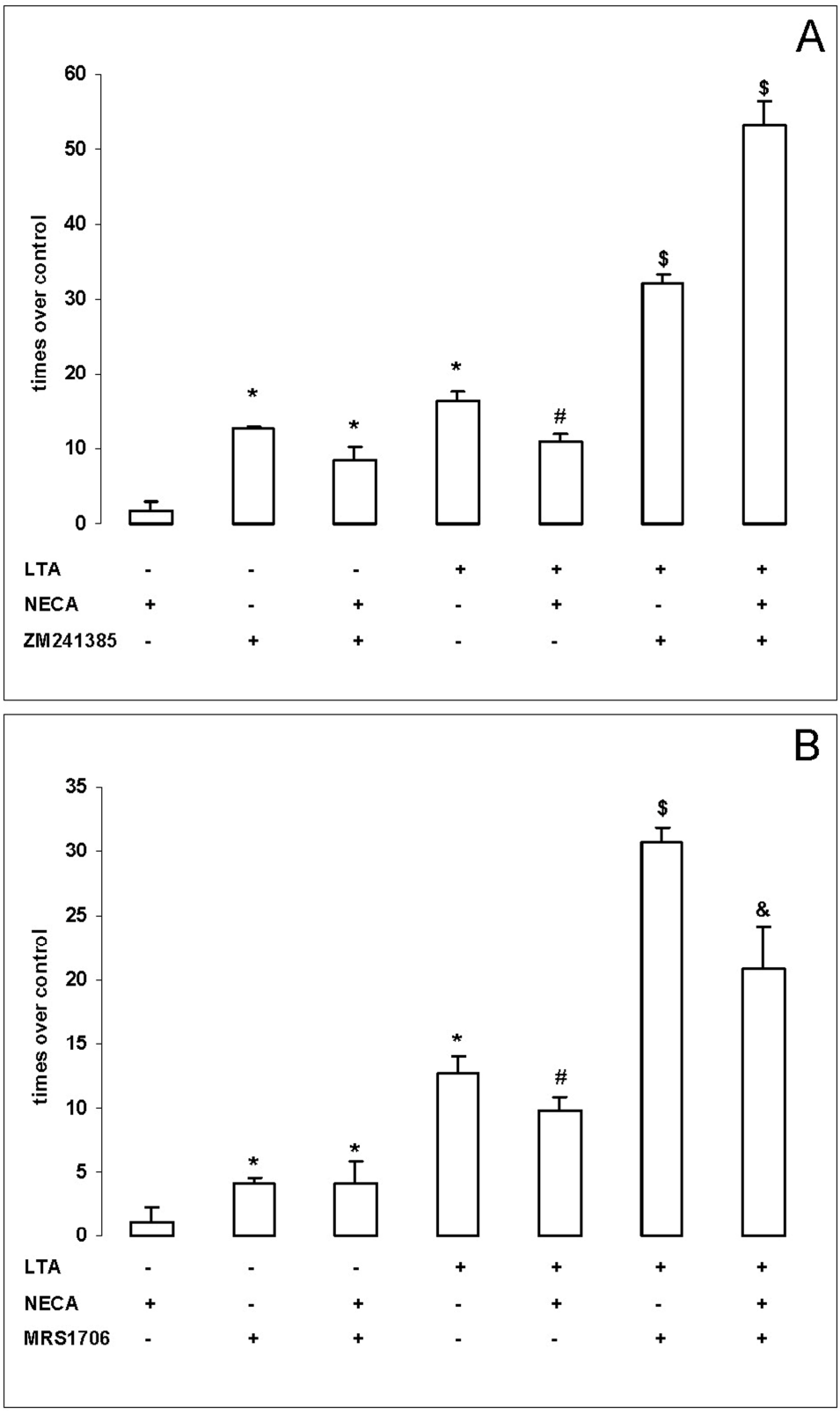

A2A
A
relative expression (A2A/b-actin)
4
3,5
3
2,5
2
1,5
1
0,5
0
*
12
24
Hours
Control
LTA
A2B
B
relative expression (A2B/b-actin)
3,5
3
2,5
2
1,5
1
0,5
0
*
12
24
Hours
Control
LTA
A3
C
relative expression (A3/b-actin)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
12
24
Hours
Control
LTA

**Figure 5**


A
60
50
40
30
20
10
0
times over control
*
*
*
#
$
$
LTA - - - + + + +
NECA + - + - + - +
ZM241385 - + + - - + +
B
35
30
25
20
15
10
5
0
times over control
*
*
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#
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&
LTA - - - + + + +
NECA + - + - + - +
MRS1706 - + + - - + +

**Figure 6**

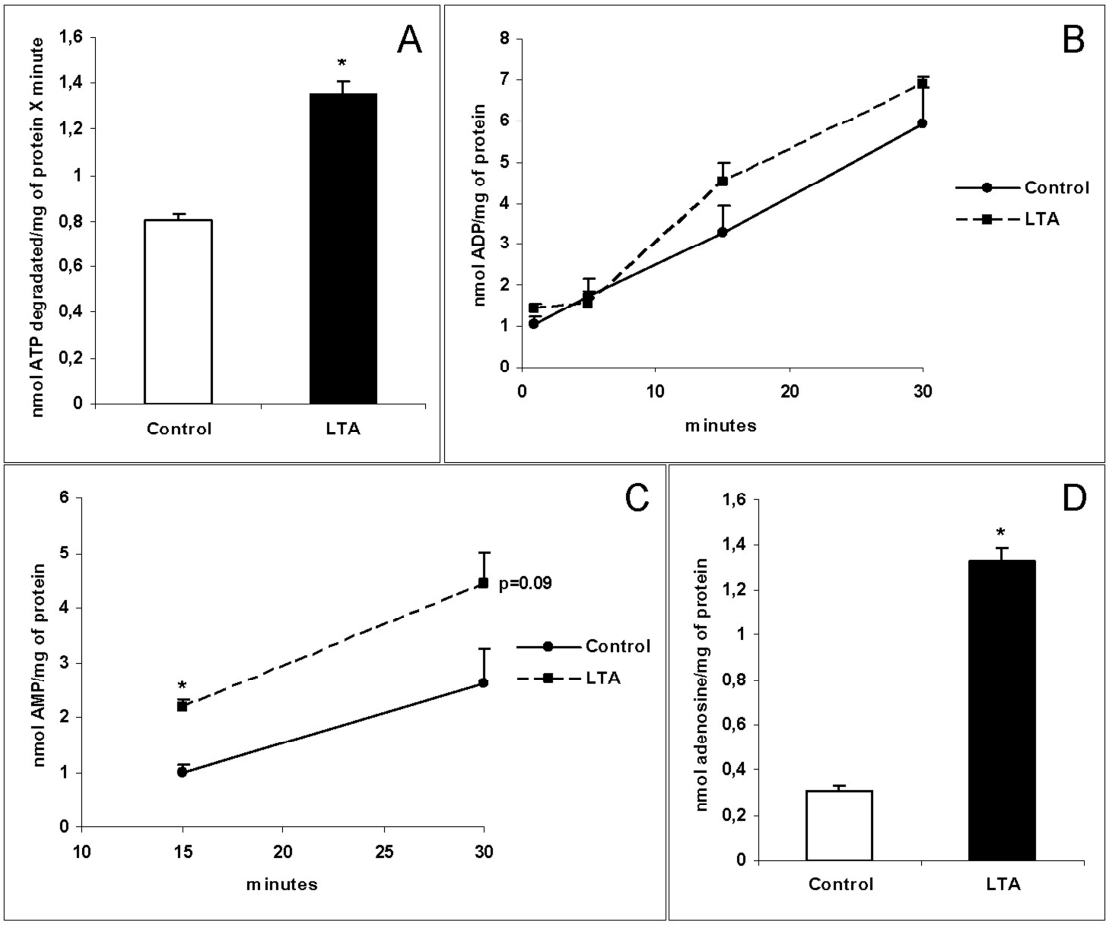
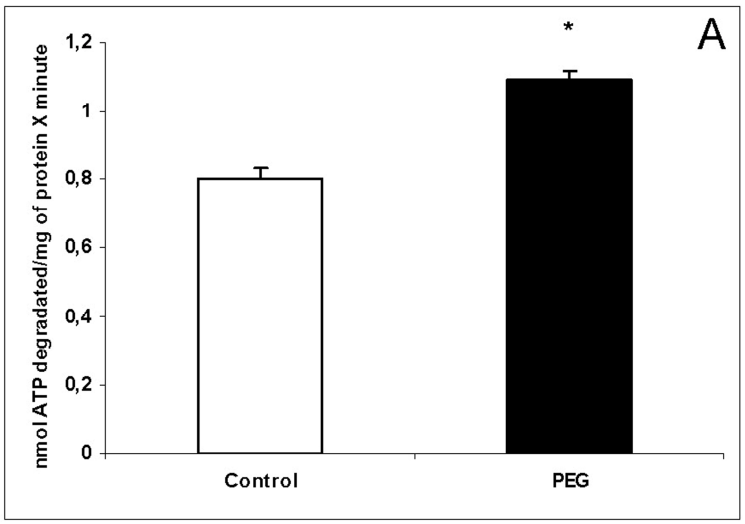
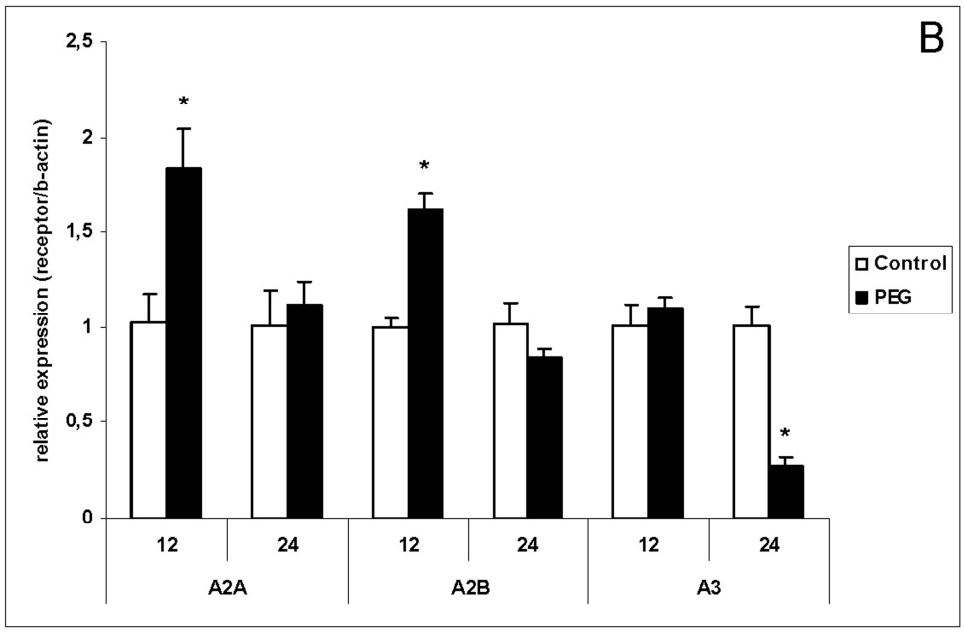

A
nmol ATP degradated/mg of protein X minute
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
Control
LTA
B
nmol ADP/mg of protein
8
7
6
5
4
3
2
1
0
0
10
20
30
minutes
Control
LTA
C
nmol AMP/mg of protein
6
5
4
3
2
1
0
*
p=0.09
10
15
20
25
30
minutes
Control
LTA
D
nmol adenosine/mg of protein
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
Control
LTA

**Figure 7**


A
nmol ATP degradated/mg of protein X minute
1,2
1
0,8
0,6
0,4
0,2
0
*
Control
PEG


B
relative expression (receptor/b-actin)
2,5
2
1,5
1
0,5
0
*
*
*
Control
PEG
12
24
12
24
12
24
A2A
A2B
A3

## Figure 8

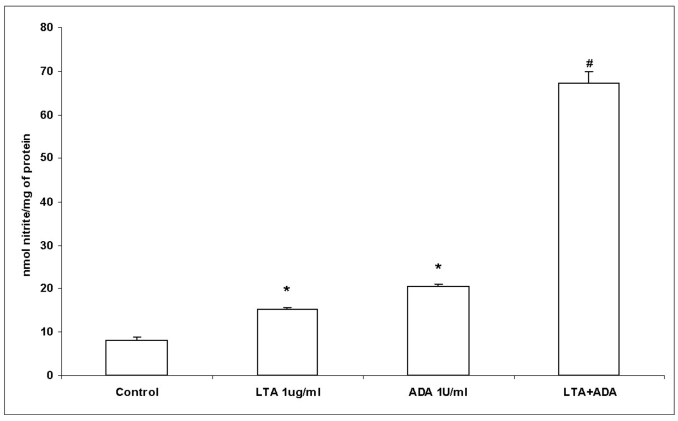
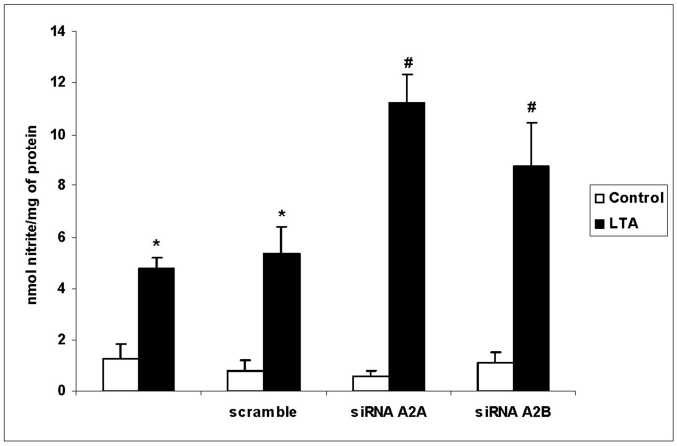

nmol nitrite/mg of protein
80
70
60
50
40
30
20
10
0
*
*
#
Control
LTA 1ug/ml
ADA 1U/ml
LTA+ADA

**Figure 9**


14
12
10
8
6
4
2
0
nmol nitrite/mg of protein
*
*
#
#
scramble
siRNA A2A
siRNA A2B
Control
LTA

## Capítulo 2

# O ácido lipoteicóico de *Staphylococcus aureus* aumenta a expressão de metaloproteinase 9 em macrófagos RAW 264.7: modulação pelos receptores de adenosina A2A e A2B.

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# Lipoteichoic acid from *Staphylococcus aureus* increases matrix metalloproteinase 9 expression in RAW 264.7 macrophages: Modulation by A2A and A2B adenosine receptors

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ABSTRACT

Peptidoglycan (PEG) and lipoteichoic acid (LTA) are the main constituents of Gram-positive bacteria cell wall and are described to modulate immune functions. Increased levels of matrix metalloproteinases (MMPs) were described in endotoxemia, suggesting that they participate to tecidual damage, multiple organs failure and vascular disfunction. *Staphylococcus aureus* PEG is described to increase MMPs 2 and 9 levels in plasma from rat and MMP 9 secretion by human neutrophils, however, the effect of LTA on MMPs is unknown. In this work, was evaluated the modulation of MMPs 2 and 9 expression and secretion in RAW 264.7 macrophages by LTA from *S. aureus*. The role of A2A and A2B adenosine receptors was also investigated. LTA increased MMP 9 expression and secretion at 12 h of treatment. The modulation of MMP 9 secretion was dose dependent, with maximal effect above 1 μg/ml. The inhibitor of mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (U0126, 10 μM) prevented LTA stimulation of MMP 9 secretion; however, the inhibitors of p38 (SB203580, 10 μM) and Jun N-terminal kinase (JNK; SP600125, 10 μM) presented any effect. A2A and A2B adenosine receptors pharmacological blockade or gene knockdown resulted in exacerbated MMP 9 secretion, while an adenosine receptors agonist inhibited LTA-stimulated MMP 9 secretion. These results suggest that LTA increased MMP 9 secretion in macrophages could be involved in complications associated to *S. aureus* infections. Moreover, LTA modulation of MMP 9 is dependent on MEK/ERK pathway and is regulated by A2A and A2B adenosine receptors.



## 1. Introduction

Gram-positive bacterial infections are frequent and associated with high morbidity and mortality (Uckay et al., 2007). The Gram-positive bacterium *Staphylococcus aureus* is a major pathogen in both community-acquired and nosocomial infections and methicillin-resistant *S. aureus* carries the highest relative mortality risk among all pathogens (Shorr et al., 2006). The pathogenicity of *S. aureus* is associated with a repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins that it produces. These agents interact with distinct receptors in immune cells leading to the production and release of pro-inflammatory and immunomodulating cytokines (Fournier and Philpott, 2005). Peptidoglycan (PEG) and lipoteichoic acid (LTA) are the main constituents of Gram-positive bacteria cell wall and are described to modulate immune functions mainly through Toll-like receptor 2 activation (Schwandner et al., 1999; Wang et al., 2003).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are involved in the turnover and degradation of extracellular matrix. These endopeptidases participates in the migration and infiltration of immune cells, additionally, they regulate inflammation and immunity by acting on pro-inflammatory cytokines and chemokines, altering the circulatory concentration of these mediators (Parks et al., 2004). The MMP 2 and MMP 9, also known as gelatinases A and B, respectively, constitutes a specific group of metalloproteinases, presenting a gelatin binding domain, involved in the ligation to denaturated collagen. Increased levels of matrix metalloproteinases were described in endotoxemic mice, suggesting that they participate to tecidual damage and multiple organs failure (Pagenstecher et al., 2000). These enzymes are also involved in endotoxemia associated vascu-

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lar disfunction (Lalu et al., 2006). It was reported that *S. aureus* PEG increased MMPs 2 and 9 levels in plasma and organ homogenates from rat (Wang et al., 2004). Moreover, *S. aureus* PEG enhanced MMP 9 secretion by human neutrophils (Wang et al., 2005).

Adenosine is an endogenous purine nucleoside that regulates several cellular processes. There are four types of adenosine receptors (A1, A2A, A2B, and A3) and all adenosine receptors are members of the G-protein-coupled family of receptors (Fredholm et al., 2001). Human and murine macrophages exposed to lipopolysaccharide (LPS) presented increased A2AR and A2BR RNA expressions (Murphree et al., 2005). Both A2A and A2B adenosine receptors have been described to possess anti-inflammatory properties. A2AR activation is described to improve survival in models of endotoxemia and Gram-negative sepsis (Sullivan et al., 2004) and the knockout of A2BR present an exacerbated inflammatory response to endotoxemia (Yang et al., 2006). Moreover, several studies have reported anti-inflammatory properties of adenosine receptors in monocytes and macrophages (revised in Hasko et al., 2007).

In this work, was evaluated the modulation of MMPs 2 and 9 expression and secretion in RAW 264.7 macrophages by lipoteichoic acid from *S. aureus*. It was also investigated the role of A2A and A2B adenosine receptors on LTA stimulated MMP 9 activity.

## 2. Material and methods

### 2.1. Chemicals

Lipoteichoic acid and peptidoglycan from *S. aureus* were purchased from InvivoGen (San Diego, USA). Lipopolysaccharide from *Escherichia coli* was purchased from Sigma (Saint Louis, USA). 5′-*N*-ethylcarboxamido adenosine (NECA), ZM241385, MRS1706, SP600125, and SB203580 were purchased from Tocris Bioscience (Ellisville, USA). RPMI 1640 and fungizone were purchased from Gibco™ (Invitrogen, Carlsbad, USA). U0126, anti-mouse extracellular signal-regulated kinase (ERK) and p-ERK primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, USA). Fetal bovine serum was purchased from Cultilab (Campinas, Brazil). All other reagents were of analytical grade and were purchased from commercially available sources.

### 2.2. RAW 264.7 macrophages culture

RAW 264.7 macrophages were obtained from UFRJ Cell Bank, Rio de Janeiro, Brazil. Cells were maintained in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/l gentamycin, and 250 μg/l fungizone at 37 °C and 5% $CO_2$. Semi-confluent cells were scrapped and plated to 24-well plates ($0.4 \times 10^6$ cells) or 12-well plates ($0.8 \times 10^6$ cells for RNA extraction and Western Blot) at a density of $0.8 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FBS. After 24 h, the macrophages were washed with saline and incubated with or without LTA, PEG or LPS 1 μg/ml for distinct periods in serum-free RPMI 1640 medium. Adenosine receptors agonist (NECA, 10 μM) and antagonists (A2A—ZM241385, A2B—MRS1706, 10 nM) as well as mitogen-activated protein kinases (MAPKs) inhibitors (ERK—U01261, 10 μM; p38—SB203580, 10 μM; JNK—SP600125, 10 μM), when used, were added 15 min prior to LTA.

### 2.3. Zymography

Gelatin zymography was performed using sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE, 6%) copolymerized with gelatin type B (Sigma—0.3%). Equal amounts of RAW 264.7 macrophages incubation medium were diluted in Laemmli buffer (62.5 mM Tris–HCl, 10% glycerol, 3% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, without b-mercaptoethanol) and subjected to electrophoresis. After this, gels were washed twice for 30 min in 2.5% Triton X-100 at room temperature and were incubated for distinct periods, according to treatment period, in incubation buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 150 mM NaCl, 1% Triton X-100, pH 7.4) at 37 °C. The resulting gels were stained with Coomassie Blue R 250 (Bio-Rad), using a mixture of ethanol–acetic acid–water (0.1% Comassie Blue R 250, 12.5% ethanol, 7.5% acetic acid) for 1 h, and were destained with methanol–acetic acid–water (45% methanol, 10% acetic acid). Areas of enzymatic activity appeared as clear bands over the dark background. Zymographic activity was quantified by densitometric analysis, employing the software ImageJ (NIH, USA), and all results were standardized in relation to control.

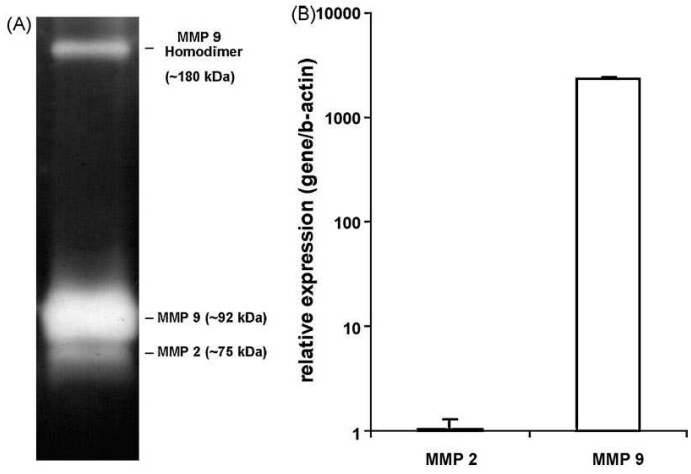


**Fig. 1.** RAW 264.7 macrophages constitutively express and secretes MMPs 2 and 9. (A) RAW 264.7 macrophages were incubated for 24 h and the incubation medium was analyzed for MMPs activities by gelatin zymography. (B) RAW 264.7 macrophages were incubated for 12 h and MMPs 2 and 9 expressions were analyzed by real-time PCR. Results are shown as mean ± S.E.M. ($n = 3$).

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### 2.4. Western Blot

RAW 264.7 macrophages were lysed in Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and equal amounts of cell protein were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. The membrane was blocked in Tween-Tris buffered saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 10% fat-free milk and incubated overnight with primary antibody (anti-ERK or anti-p-ERK). The membrane was washed and incubated for 1 h with secondary anti-IgG peroxidase conjugate. The immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed with the IMAGE J® software (NIH, USA).

### 2.5. RNA extraction, cDNA and real-time PCR

RNA was isolated from $0.8 \times 10^6$ RAW 264.7 macrophages using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Approximately 1 μg of total RNA was added to each cDNA synthesis reaction using the M-MLV reverse transcriptase (Invitrogen). Reactions were performed using the primer T23 V (5′-TTTTTTTTTTTTTTTTTTTTTTTV) as follows: sample RNA (~1 μg), water (to complete 9 μl), and 1 μl of poly-T primer were kept at 70 °C for 5 min; the tubes were transferred to ice, and 30 μl of reaction mix (5× first strand buffer 6 μl, M-MLV reverse transcriptase 200 U/μl 1 μl, dNTP 5 mM, 2 μl, and water 11 μl) were added. The reaction was carried at 42 °C for 1 h. RT-PCR and real-time PCR amplification was carried out using specific primer pairs designed with Primer3 Input (http://frodo.wi.mit.edu/) and synthesized by RW-Genes (RJ, Brazil). The primers utilized were MMP 2 (5′-ATGCCATCCCTGATAACCTG and 5′-TGTGCAGCGATGAAGATGAT), MMP 9 (5′-CATTCGCGTGGATAAGGAGT and 5′-CACTGCAGGAGGTCGTAGGT), and β-actin (5′-TACTCCTGCTTGCTGATCCACAT and 5′-TATGCCAACACAGTGCTGTCTGG). RT-PCR products were separated on a 1% agarose gel and amplification products were visualized by ethidium bromide staining. Real-time PCRs were carried out in an Applied-Biosystems 7500 real-time cycler (Applied Biosystems, Foster City, CA, USA). Reaction settings were composed of an initial denaturation step of 5 min at 94 °C followed by 45 cycles of 10 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C; samples were kept for 2 min at 40 °C for reannealing and were then heated from 55 to 99 °C with a ramp of 1 °C/s to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were made in 20 μl final volume composed of 10 μl of each reverse transcription sample diluted 50 times, 2 μl of Platinum Taq 10 times PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 0.4 μl of 5 mM dNTPs, 0.4 μl of 10 μM primer pairs, 4.95 μl of water, 1 μl of SYBR (1:100 000 Molecular Probe), and 0.05 μl of Platinum Taq DNA polymerase (5 U/μl; Invitrogen). All results were expressed as a relative ratio between the investigated gene and the β-actin internal control gene.

### 2.6. Small interfering RNA

Predesigned small interfering RNAs (siRNA) against mouse A2A (siRNA IDs 162368) and A2B (siRNA IDs 162158) adenosine receptors, as well as control siRNA (scramble sequence) were obtained from Ambion (Applied Biosystems). RAW 264.7 cells were transfected in 24-well plates with either 10 nM A2AR, A2BR or scramble siRNA in RPMI 1640 10% FBS using siPORT NeoFX transfection agent (according to the manufacturer's instructions; Ambion). Twenty-four hours after transfection, cells were then used for further experimentation.

### 2.7. Statistical analysis

Results are expressed as means ± S.E.M. for at least triplicates and are representative of two or more independent experiments. Differences between means were analyzed by Student's *t*-test or ANOVA with the Student–Newman–Keuls multiple comparisons test. Statistical significance was defined as $p < 0.05$.

## 3. Results

Cultured RAW 264.7 macrophages constitutively secrete MMPs 2 and 9 to the incubation medium, as assessed by gelatin zymography. The incubation medium of cultured macrophages also presented a third gelatin degrading band, which posses a molecular weight compatible to a MMP 9 homodimer (~180 kD). The secretion of MMP 9 was higher than MMP 2, which is in accordance with the quantitative real time PCR results, where MMP 9 mRNA levels were higher than MMP 2 (Fig. 1). No effect of LTA was identified in MMP 2 expression and secretion (data not shown).

LTA increased MMP 9 expression and secretion at 12 h of treatment, similarly to PEG and LPS (Fig. 2). LTA effect was already observed at 3 h of treatment and was sustained, at last, until 24 h of

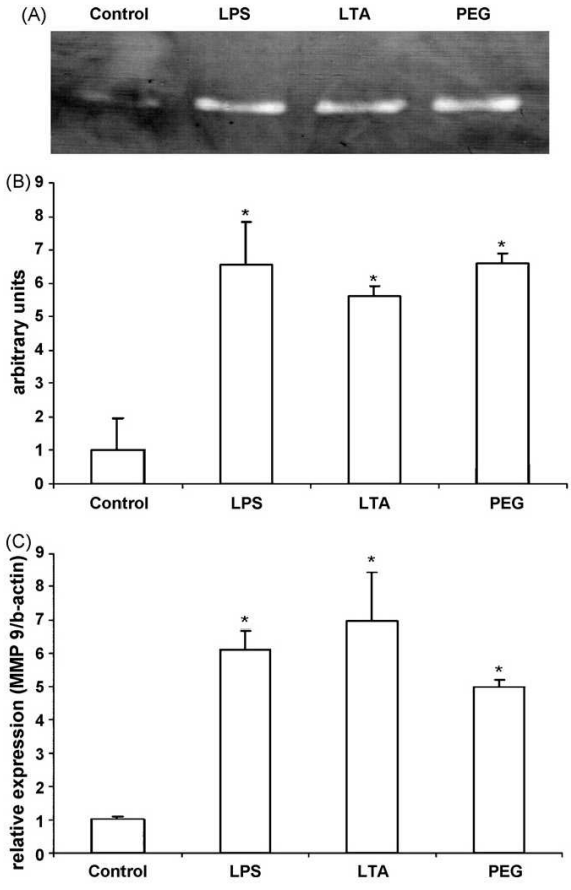


**Fig. 2.** Lipopolysaccharide, lipoteichoic acid, and peptidoglycan increase MMP 9 expression and secretion in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LPS, LTA or PEG (1 μg/ml) for 12 h. (A) Representative zymography of RAW 264.7 macrophages incubation medium. (B) Densitometric quantification of MMP 9 activity. (C) MMP 9 expression analyzed by real-time PCR. Results are shown as mean ± S.E.M. ($n = 3$). *$p < 0.05$ vs. control, ANOVA with the Student–Newman–Keuls multiple comparisons test.

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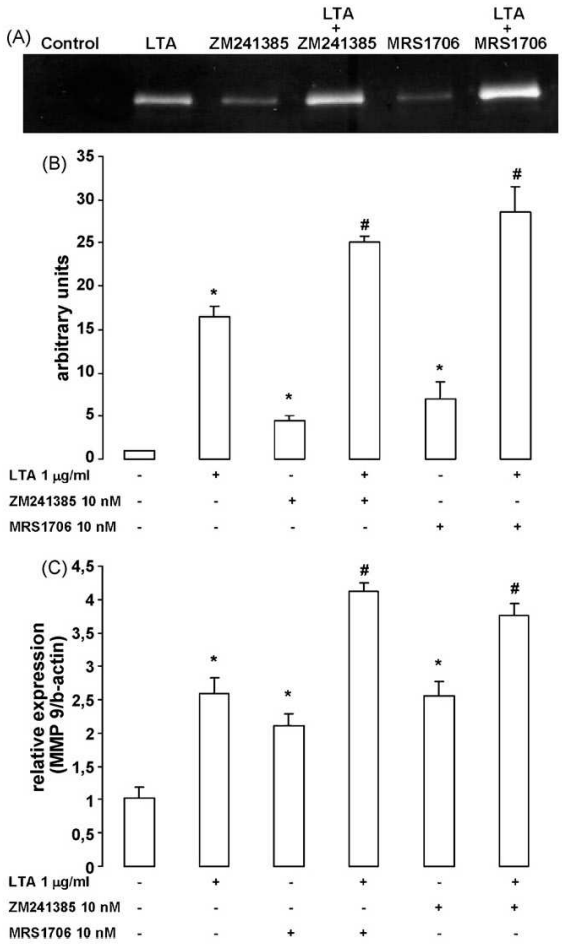


**Fig. 5.** A2A and A2B adenosine receptors modulate lipoteichoic acid-stimulated MMP 9 secretion in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 12 or 24 h. When indicated, the A2A adenosine receptor antagonist ZM241385 (10 nM) or the A2B adenosine receptor antagonist MRS1706 (10 nM) were added 15 min prior to LTA. (A) Representative zymography of RAW 264.7 macrophages incubation medium (24 h). (B) Densitometric quantification of MMP 9 activity (24 h). (C) MMP 9 expression analyzed by real-time PCR (12 h). Results are shown as mean ± S.E.M. ($n = 3$). *$p < 0.05$ vs. control, #$p < 0.05$ vs. control and LTA, ANOVA with the Student–Newman–Keuls multiple comparisons test.

circulatory levels of distinct pro-inflammatory mediators (Parks et al., 2004). In this work, we report that *S. aureus* LTA, as well PEG, increase MMP 9 mRNA expression and secretion in RAW 264.7 macrophages, while no effect was observed in MMP 2. LTA and PEG are described to be involved in *S. aureus*-induced shock and multiple organ failure (De Kimpe et al., 1995; Kengatharan et al., 1998), moreover, the MMPs participate in multiple organs failure (Pagenstecher et al., 2000) and vascular disfunction (Lalu et al., 2006) in endotoxemia, suggesting that LTA and PEG stimulation of MMP 9 in macrophages could be involved in complications associated to *S. aureus* infections.

MAPKs are serine/threonine kinases involved in the regulation of cell cycle progression, proliferation, differentiation, and death. The MAPK family consists of three main subgroups: the ERKs, the stress-activated protein kinases (SAPKs) also know as c-Jun N-terminal kinases (JNKs) and the p38 family. In Kupffer cells, LTA modulation of interleukin 6 is mediated by ERK (Dahle et al., 2004)

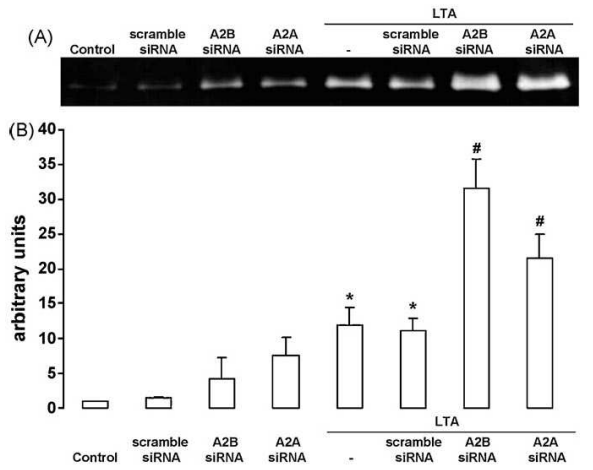


**Fig. 6.** A2A and A2B adenosine receptors knockdown increases lipoteichoic acid-stimulated MMP 9 secretion in RAW 264.7 macrophages. RAW 264.7 macrophages were transfected with either A2A, A2B or scramble siRNA (10 nM) in RPMI 1640 10% FBS for 24 h. At the end of the transfection, RAW 264.7 macrophages were incubated with LTA (1 μg/ml) in RPMI 1640 for 24 h. (A) Representative zymography of RAW 264.7 macrophages incubation medium. (B) Densitometric quantification of MMP 9 activity. Results are shown as mean ± S.E.M. ($n = 3$). *$p < 0.05$ vs. control, #$p < 0.05$ vs. LTA and LTA + scramble siRNA, ANOVA with the Student–Newman–Keuls multiple comparisons test.

and p38 is described to be involved in LTA stimulated inducible nitric oxide expression in RAW 264.7 macrophages (Chang et al., 2006). Additionally, LTA is described to increase phosphorylation of ERK, p38 and JNK in murine macrophages, with these kinases participating in distinct ways in TNF-α and interleukin 1β expression and secretion (Su et al., 2006). In human neutrophils, PEG modulation of MMP 9 expression is dependent of p38 and ERK (Wang et

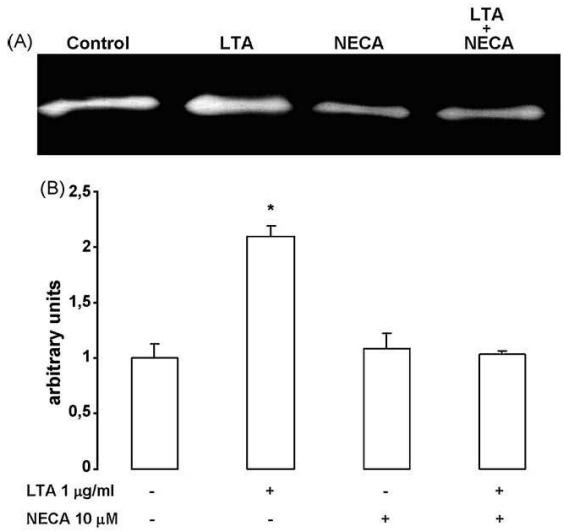


**Fig. 7.** Adenosine receptor agonist modulates lipoteichoic acid-stimulated MMP 9 secretion in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 24 h. When indicated, the adenosine receptor agonist NECA (10 μM) was added 15 min prior to LTA. (A) Representative zymography of RAW 264.7 macrophages incubation medium. (B) Densitometric quantification of MMP 9 activity. Results are shown as mean ± S.E.M. ($n = 3$). *$p < 0.05$ vs. control, ANOVA with the Student–Newman–Keuls multiple comparisons test.

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al., 2005). LTA stimulation of MMP 9 secretion was abolished by the MEK inhibitor U0126, while p38 and JNK inhibitors were ineffective. LTA increased ERK phosphorylation in RAW 264.7 macrophages, which was abolished by U0126. These results points that LTA mediated MMP 9 expression and secretion are dependent on MEK/ERK pathway.

The development of selective agonists and antagonists of adenosine receptors and their role as therapeutic agents in a wide range of conditions, including cerebral and cardiac ischemic diseases, sleep disorders, immune, and inflammatory disorders and cancer are an emerging investigatory field (Jacobson and Gao, 2006). Anti-inflammatory properties of adenosine receptors against Gram-negative antigens in monocytes and macrophages are well described (revised in Hasko et al., 2007) and, in interferon-γ-treated macrophages, adenosine is described to be involved in an autocrine mechanism of inflammatory regulation, participating in the deactivation of macrophages (Xaus et al., 1999). A2AR modulates MMP 9 expression in human neutrophils (Ernens et al., 2006) while A2BR was described to participate in hypoxia suppression of MMP 9 production by human monocyte-derived dendritic cells (Zhao et al., 2008). In LTA treated RAW 264.7 macrophages, the blockade of A2A and A2B adenosine receptors by selective antagonists resulted in exacerbated MMP 9 expression and secretion. Similar results were obtained by A2AR and A2BR gene knockdown. The stimulation of adenosine receptors by a non-selective agonist reduced LTA stimulated MMP 9 secretion. These results suggest that adenosine could be involved in an autocrine regulation of LTA mediated MMP 9 expression and secretion, contributing to reduce tecidual damage in inflammatory conditions. No additive effect of antagonist A2A and A2B blockade was observed (data not shown), suggesting that these receptors could be acting by a common mechanism. Several functional heterodimers for adenosine receptors were already described (Fredholm et al., 2007), however, the dimerization of A2A and A2B adenosine receptors was unknown.

Concluding, in this work we showed that LTA from *S. aureus* stimulates MMP 9 expression and secretion in RAW 264.7 macrophages. This effect is dependent on MEK/ERK pathway and is regulated by A2A and A2B adenosine receptors.

## Acknowledgements

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## Capítulo 3

# Espécies reativas de oxigênio contribuem para a produção de óxido nítrico induzida por ácido lipoteicóico em macrófagos RAW 264.7: modulação por receptores de adenosina.

**Manuscrito a ser submetido**

**Reactive oxygen species participates in lipoteichoic acid induced nitric oxide production in RAW 264.7 macrophages: modulation by adenosine receptors.**

**Running title: ROS and lipoteichoic acid induced NO production**

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**Abstract**

Bacterial wall components stimulate Toll-like receptors, initiating macrophage inflammatory response by producing nitric oxide (NO), reactive oxygen species (ROS) and pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α). Both ROS and NO have antimicrobial activity; moreover, they also act as cellular signaling molecules. Several studies have reported anti-inflammatory properties of adenosine receptors in macrophages and adenosine receptors has been described to possess antioxidant properties. In this work we investigated the modulation of ROS production by adenosine receptors in LTA stimulated RAW 264.7 macrophages. Additionally, we investigated the role of ROS production in LTA stimulated NO and TNF-α production. LTA rapidly increased ROS production in RAW 264.7 macrophages. This effect was blocked by a NADPH oxidase inhibitor. Adenosine, inosine, and the non-selective adenosine receptors agonist 5'-N-ethylcarboxamido adenosine (NECA) prevented LTA stimulated ROS production. LTA increased NO production in RAW 264.7 macrophages, which was also inhibited by NECA. The antioxidant Trolox, a soluble analogue of α-tocopherol, inhibited LTA stimulated ROS and NO production. These results suggests that LTA stimulated ROS production is dependent of NADPH oxidase activity and is involved in LTA augmented NO synthesis. Adenosine receptors activation prevented ROS production by LTA activated macrophages, resulting in reduced NO formation.

**Keywords:** lipoteichoic acid; RAW 264.7 macrophages; adenosine receptors; reactive oxygen species; nitric oxide.

## Introduction

Macrophages are a heterogeneous population of mononuclear phagocytes found ubiquitously in the organism. Due to their distribution, these cells are involved in the primary contact with and in the attack against pathogens. (Mosser, 2003; Gordon and Taylor, 2005). Both ROS and NO have antimicrobial activity; moreover, they also act as cellular signaling molecules (Forman and Torres, 2001; Ferret et al., 2002). Controversially, exacerbated ROS and NO production is related to inflammatory complications, as septic shock and multiple organ failure (Ritter et al., 2004; Andrades et al., 2005; Victor et al., 2005).

The gram-positive bacterium *Staphylococcus aureus* is a major pathogen in both community-acquired and nosocomial infections (Shorr et al., 2006). Lipoteichoic acid (LTA) is one of the main constituents of gram-positive bacteria cell wall and is described to modulate immune functions mainly through toll-like receptor 2 activation (Schwandner et al., 1999; Wang et al., 2003). This antigen is described to be involved in *S. aureus*-induced nitric oxide (NO) production, shock and multiple organ failure (De Kimpe et al., 1995; Kengatharan et al., 1998). Recently, LTA was described as the major antigen involved in gram-positive stimulated TNF-α production by macrophages (Seo et al., 2008). Additionally, LTA is described to increase ROS production in human monocytes (Levy et al., 1990) and murine dendritic cells (Choi et al., 2008), moreover, antioxidant molecules regulates LTA activation of macrophages (Hsiao et al., 2004; Choi et al., 2008).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is expressed by phagocytes and is involved in superoxide production for antimicrobial activity. Upon phagocytosis or stimulation with soluble agents the cytosolic components and the small GTPase Rac1/Rac2 translocate to the plasma membrane binding to

gp91$^{phox}$ and p22$^{phox}$ and initiating superoxide production by transferring one electron from NADPH to oxygen (Forman and Torres, 2002; DeCoursey and Ligeti, 2005). NO is synthesized from L-arginine by nitric oxide synthase using NADPH as a cofactor. Three nitric oxide synthases are described: neuronal and endothelial isoforms that are constitutive and calcium dependent, and the inducible isoform which is calcium independent. In stimulated macrophages, inducible nitric oxide synthase (iNOS) accounts for increased nitric oxide production, which is NADPH dependent (Forstermann and Kleinert, 1995). NADPH is an essential cellular coenzyme and its level is sustained by $NADP^+$ dependent cytosolic dehydrogenases, named glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH).

Adenosine is an endogenous purine nucleoside that regulates several cellular processes. There are four types of adenosine receptors (A1, A2A, A2B, and A3) and all adenosine receptors are members of the G-protein-coupled family of receptors (Fredholm et al. 2001). Several studies have reported anti-inflammatory properties of adenosine receptors in monocytes and macrophages (revised in Haskó et al., 2007) and adenosine receptors has been described to possess antioxidant properties (Huang, 2003; Fatokun et al., 2007). Adenosine is described to reduce superoxide production in phorbol-ester activated peritoneal macrophages (Si et al., 1997) and unpublished results of our group shown that A2A and A2B adenosine receptors regulate NO production in LTA stimulated macrophages.

In this work we investigated the modulation of ROS production by adenosine receptors in LTA stimulated RAW 264.7 macrophages. Additionally, we investigated the role of ROS production in LTA stimulated NO and TNF-α production, as well as the activity of $NADP^+$ dependent cytosolic dehydrogenases.

## Material and Methods

### Chemicals

Lipoteichoic acid from *Staphylococcus aureus* were purchased from InvivoGen (San Diego, USA). 5'-N-ethylcarboxamido adenosine (NECA) was purchased from Tocris Bioscience (Ellisville, USA). Adenosine, trolox, diphenyl iodonium, 2',7'-dichlorofluorescein diacetate and β-$NADP^+$ were purchased from Sigma (Saint Louis, USA). RPMI 1640 and fungizone were purchased from Gibco™ (Invitrogen, Carlsbad, USA). Fetal bovine serum was purchased from Cultilab (Campinas, Brazil). All other reagents were of analytical grade and were purchased from commercially available sources.

### RAW 264.7 macrophages culture

RAW 264.7 macrophages were obtained from UFRJ Cell Bank, Rio de Janeiro, Brazil. Cells were maintained in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin, and 250 μg/L fungizone at 37 ºC and 5% $CO_2$. Semi-confluent cells were scrapped and plated to 24-well plates (0.4 x $10^6$ cells) or 96-well plates (0.08 x $10^6$ cells) at a density of 0.8 x $10^6$ cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FBS. After 24 hours, the macrophages were washed with saline and incubated with or without LTA 1 μg/ml for distinct periods in serum-free RPMI 1640 medium. Adenosine (100 μM), adenosine receptors agonist (NECA, 10 μM), diphenyl iodoniun (DPI, 10 μM) and, trolox (100 μM), when used, were added 15 minutes prior to LTA.

### ROS measurement

Reactive oxygen species were analyzed by the measurement of 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation. Briefly, RAW 264.7 macrophages cultured in 96-well plates were incubated with DCFH-DA (10 μM) for 15 minutes at 37

º C. At the end of incubation, the cells were washed with saline and the treatments added. The rate of DCFH-DA oxidation was analyzed for 1 hour in a multi-well fluorimeter, at 37 º, with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Cytosolic dehydrogenase assay**

RAW 264.7 macrophages were lysed with phosphate buffered saline plus 0.5% Triton X-100, centrifuged at 12,000 x g for 10 minutes and the supernatants were used for enzyme measurements. Dehydrogenase activities were measured by NADPH production at 340 nm at 36 ºC in the presence of specific incubation mediums for each dehydrogenase, as follow:

- Glucose-6-phosphate dehydrogenase (G6PD): Tris-HCl 50 mM, $MgCl_2$ 3 mM, $NADP^+$ 0.2 mM, glucose-6-phosphate 3.2 mM, pH 7.8;

- Isocitrate dehydrogenase (IDH): Tris-HCl 80 mM, $MgCl_2$ 2 mM, $NADP^+$ 2 mM, sodium isocitric acid 5 mM, pH 7.4;

- Malate dehydrogenase (MDH): Tris-HCl 50 mM, $MgCl_2$ 1 mM, $NADP^+$ 0.5 mM, sodium malic acid 0.6 mM, pH 7.4.

**Measurement of TNF-α in culture supernatants**

Cytokine secretion was measured using mouse TNF-α BD OptEIA™ ELISA kit (BD Biosciences), according to manufacturer's directions.

**Nitrite assay**

Nitric oxide production was assayed by quantification of the stable end product of nitric oxide oxidation – nitrite ($NO_2^-$). Briefly, the incubation medium of RAW 264.7 macrophages was collected and was reacted 1:1 with Griess reagent (1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) for 15

min. Nitrite content was measured by absorbance at 540 nm. Nitrite concentration in the samples was calculated using a standard curve prepared with $NaNO_2$.

**Protein quantification**

All the results were standardized with respect to protein content, determined as described by Lowry et al. (1951).

**Statistical analysis**

Results are expressed as means $\pm$ SEM. Differences between means were analyzed by ANOVA with the Student-Newman-Keuls multiple comparisons test. Statistical significance was defined as $p<0.05$.

**Results**

The treatment of RAW 264.7 macrophages with LTA rapidly increased ROS production. This effect was already observable at 10 minutes of treatment and ROS production remains elevated until, at least, 1 hour (Figure 1). Diphenyl iodoniun, a flavoprotein inhibitor described as a NADPH oxidase inhibitor, prevented LTA stimulated ROS production in RAW 264.7 macrophages (Figure 1). Prolonged incubation of RAW 264.7 macrophages with DPI resulted in reduced cell viability (data not shown).

Adenosine and inosine, the product of adenosine deamination by adenosine deaminase, inhibited basal and LTA stimulated ROS production in cultured macrophages (Figure 2). The pre-treatment with non-selective adenosine receptors agonist 5'-N-ethylcarboxamido adenosine (NECA) also prevented LTA stimulated ROS production (Figure 3). Dimethil sulfoxide (DMSO) was utilized as NECA vehicle (0.05%) and increased basal ROS production in RAW 264.7 macrophages, however, no effect was observed in NO production (data not shown).

LTA increased NO production in RAW 264.7 macrophages, which was inhibited by NECA (Figure 4). The antioxidant Trolox, a soluble analogue of α-tocopherol, reduced LTA stimulated ROS and NO production (Figure 5). No effect of Trolox was observed in LTA stimulated TNF-α production (data not shown).

Glucose-6-phosphate dehydrogenase was the most active $NADP^+$ dependent cytosolic dehydrogenase in RAW 264.7 macrophages, followed by isocitrate dehydrogenase and malate dehydrogenase. No effect of LTA was observed on no one of $NADP^+$ dependent cytosolic dehydrogenases (Table I).

**Discussion**

ROS are produced by activated macrophages and participates in antimicrobial activity and cellular signaling. LTA is described to increase ROS production in human monocytes (Levy et al., 1990) and murine dendritic cells (Choi et al., 2008). In cultured RAW 264.7 macrophages, LTA rapidly increased ROS production, which is inhibited by DPI. Hancock and Jones (1987) showed that DPI is selective inhibits NADPH oxidase in macrophages, with no effect on mithochondrial respiration. This suggests that LTA stimulated ROS production is dependent on NADPH oxidase activity.

Adenosine is described to reduce superoxide production in phorbol-ester activated peritoneal macrophages (Si et al., 1997). In RAW 264.7 macrophages, adenosine inhibited basal and LTA stimulated ROS production. Similar results were obtained with inosine, which is formed from adenosine deamination by adenosine deaminase. Inosine, as well as adenosine, is described to present antioxidant properties (Gudkov et al., 2006). The adenosine receptors non-selective agonist also inhibited LTA stimulated ROS production. These results indicate that extracellular adenosine could inhibit ROS production both by adenosine receptors activation and by degradation to inosine.

In LPS stimulated macrophages ROS are involved in tumor necrosis factor-α (Kimura et al., 2008); interleukin-8 (Ryan et al., 2004) and interleukin-1 β (Hsu and Wen, 2002) production. Addittionaly, ROS are linked to NO production and iNOS expression in LPS treated macrophages (Yoo et al., 2004). Antioxidant treatment is described to regulate LTA activation of macrophages, reducing NO production (Hsiao et al., 2004). Adenosine receptors agonist, which reduced ROS production, inhibited LTA stimulated NO production in macrophages. The antioxidant trolox reduced both NO and ROS production in LTA treated macrophages, however, no effect of trolox was observed in LTA increased TNF-α secretion. Similarly, the adenosine receptors agonist had no effect in LTA induced TNF-α secretion (unpublished results). These results suggest that, in RAW 264.7 macrophages, LTA stimulated NO production is, at least in part, dependent of ROS production, while TNF-α is independent. Moreover, adenosine receptors modulation of LTA stimulated NO production seems to be dependent of the inhibition of ROS production. Hsiao et al. (2004) showed that antioxidant treatment of LTA stimulated macrophages resulted in reduced JNK phosphorylation and NF-kB activation, which leads to decreased iNOS expression and NO production, however these authors not investigated the ROS production of macrophages.

NADPH is essential for ROS and nitric oxide production (DeCoursey and Ligeti, 2005; Forstermann and Kleinert, 1995). G6PD, IDH and MDH are essential for the maintenance of cellular NADPH levels and an impaired NADPH production by reduced G6PD activity is associated with augmented IL-10 production in mouse peritoneal macrophages (Wilmanski et al., 2005). IDH activity has been described to be involved in redox buffering in LPS treated macrophages, maintaining NADPH levels for antioxidant enzymes, as glutathione reductase (Maeng et al., 2004). In LTA treated macrophages, no alteration of G6PD, IDH and MDH activities were observed.

Concluding, LTA increased both ROS and NO production in RAW 264.7 macrophages. LTA stimulated ROS production seems to be dependent of NADPH oxidase activity and is involved in LTA augmented NO synthesis. Adenosine receptors activation prevented ROS production by LTA activated macrophages, resulting in reduced NO formation.

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**Figure Captions**

**Figure 1: Lipoteichoic acid increases reactive oxygen species in RAW 264.7 macrophages.** RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 1 hour. Diphenyl iodonium (DPI, 10 μM), when indicated, was added 15 minutes prior LTA. Reactive oxygen species were analyzed by the measurement of 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation. Results are shown as mean ± SEM (n = 6). * $p < 0.05$ vs respective time control, # $p < 0.05$ vs respective time control and LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

**Figure 2: Adenosine and inosine modulates lipoteichoic acid-stimulated reactive oxygen species in RAW 264.7 macrophages.** RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 1 hour. Adenosine or inosine (100 μM), when indicated, was added 15 minutes prior LTA. Reactive oxygen species were analyzed by the measurement of 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation. Results are shown as mean ± SEM (n = 6). * $p < 0.05$ vs respective time control, # $p < 0.05$ vs respective time control and LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

**Figure 3: Adenosine receptors agonist modulates lipoteichoic acid-stimulated reactive oxygen species production in RAW 264.7 macrophages.** RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 1 hour. The adenosine receptors agonist NECA (10 μM), when indicated, was added 15 minutes prior LTA. Reactive oxygen species were analyzed by the measurement of 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation. Results are shown as mean ± SEM (n = 6). * $p < 0.05$

vs respective time DMSO, # $p < 0.05$ vs respective time DMSO and LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

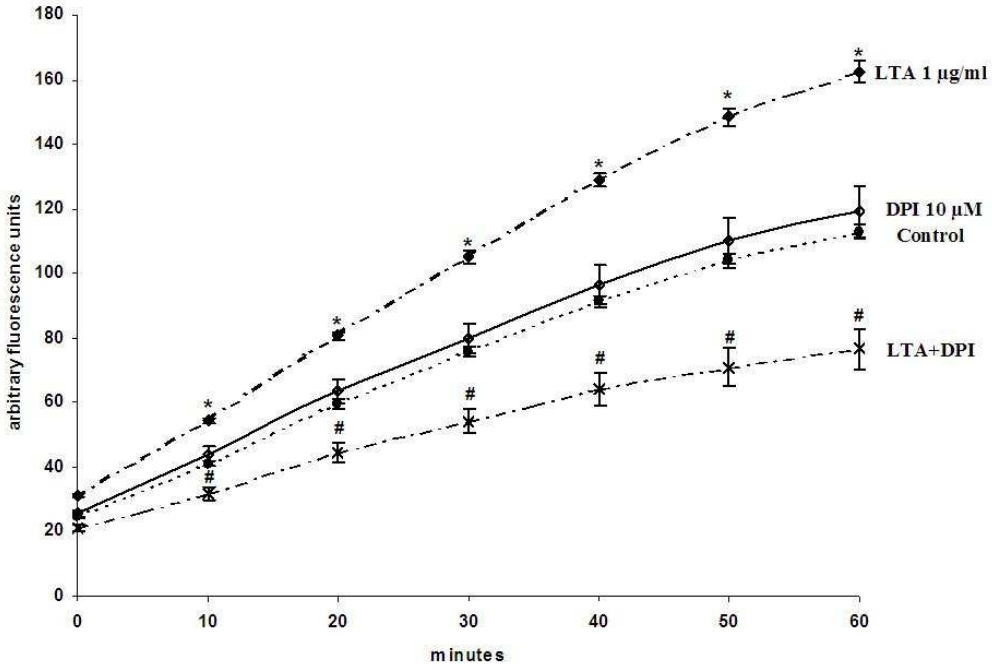
**Figure 4: Adenosine receptors agonist modulates lipoteichoic acid-stimulated nitric oxide production in RAW 264.7 macrophages.** RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 24 hours. When indicated, the adenosine receptors agonist NECA (10 μM) was added 15 minutes prior to LTA. Nitrite accumulation in the incubation medium was measured by the Griess method. Results are shown as mean ± SEM (n = 3). * $p < 0.05$ vs control, # $p < 0.05$ vs control and LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

**Figure 5: Trolox modulates lipoteichoic acid-stimulated reactive oxygen species and nitric oxide production in RAW 264.7 macrophages.** (A) RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 1 hour. Trolox (100 μM), when indicated, was added 15 minutes prior LTA. Reactive oxygen species were analyzed by the measurement of 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation. Results are shown as mean ± SEM (n = 6). * $p < 0.05$ vs respective time control, # $p < 0.05$ vs respective time control and LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test. (B) RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 24 hours. When indicated, Trolox (10 μM) was added 15 minutes prior to LTA. Nitrite accumulation in the incubation medium was measured by the Griess method. Results are shown as mean ± SEM (n = 3). * $p < 0.05$ vs control, # $p < 0.05$ vs control and LTA, ANOVA with the Student-Newman-Keuls multiple comparisons test.

**Table I: Isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PD) and malate dehydrogenase (MDH) activities in RAW 264.7 macrophages.** RAW 264.7 macrophages were incubated with LTA (1 μg/ml) for 12 or 24 hours. Dehydrogenase activities were measured by NADPH production, in the presence of specific incubation mediums for each dehydrogenase. Results are shown as mean ± SEM (n = 4).

| $NADP^+$ cytosolic dehydrogenases activities (nmol/minute X mg of protein) | | | | | | |
|---|---|---|---|---|---|---|
| | IDH | | G6PD | | MDH | |
| | 12 hours | 24 hours | 12 hours | 24 hours | 12 hours | 24 hours |
| **Control** | 29.51 ± 3.45 | 36.18 ± 3.84 | 103.03 ± 5.68 | 186.29 ± 3.22 | 17.00 ± 2.36 | 21.97 ± 5.44 |
| **LTA** | 30.38 ± 1.60 | 38.39 ± 5.49 | 104.24 ± 5.51 | 215.35 ± 19.46 | 12.95 ± 1.00 | 27.58 ± 7.09 |

**Figure 1**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Control
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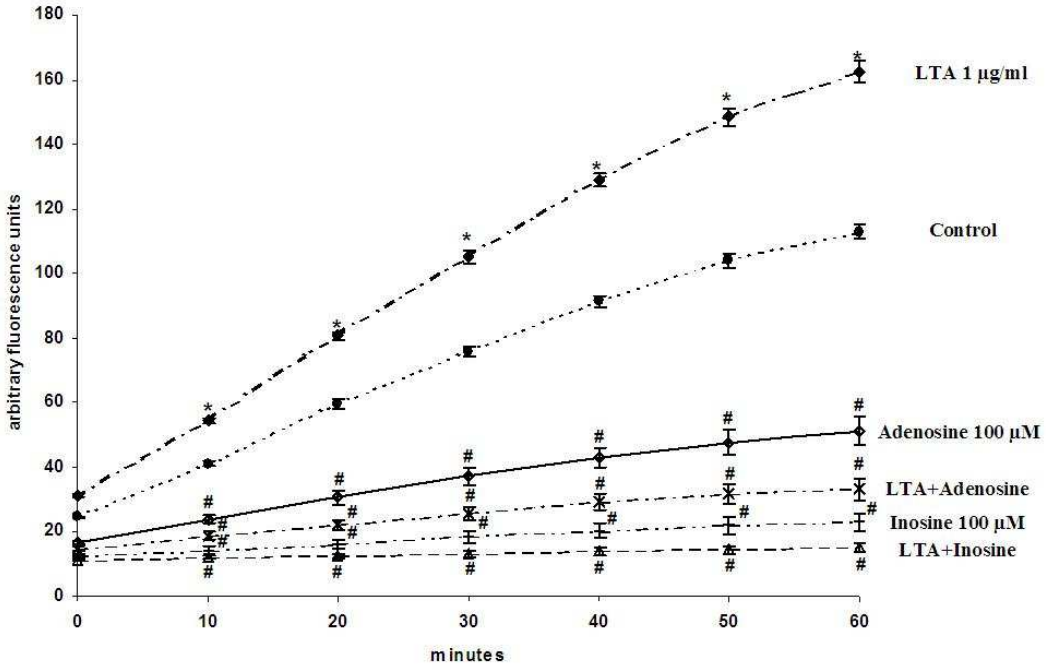
**Figure 2**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LTA+Inosine

**Figure 3**

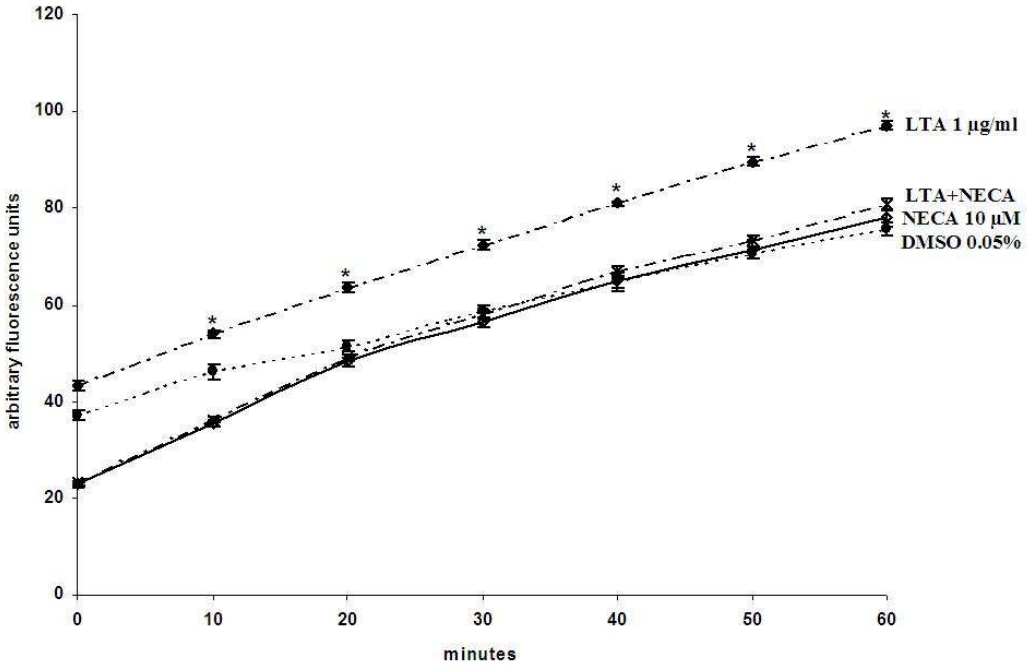
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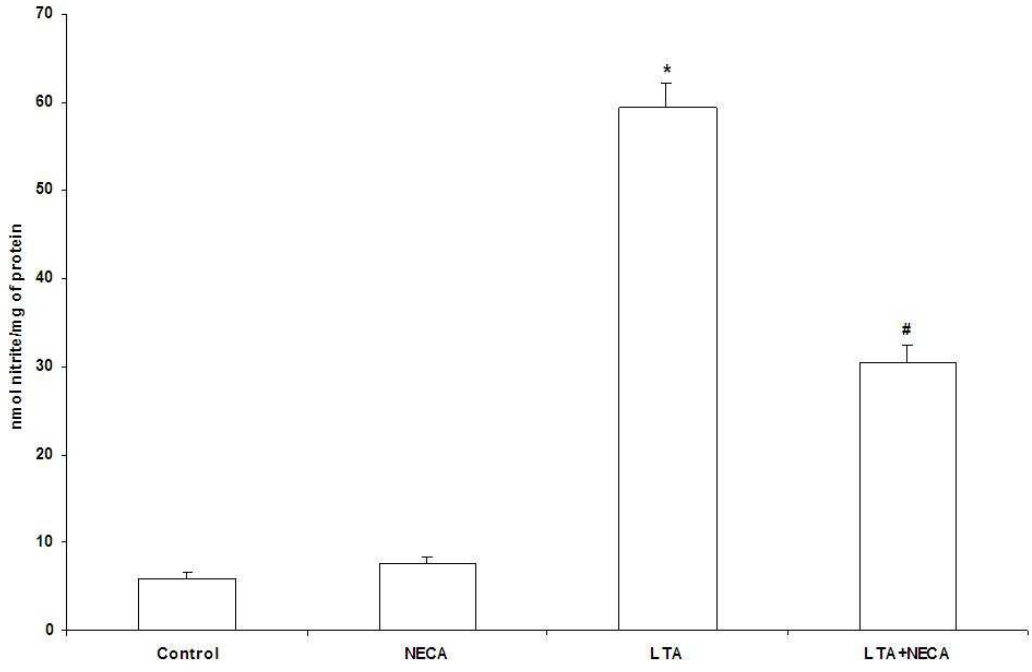
**Figure 4**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Control
NECA
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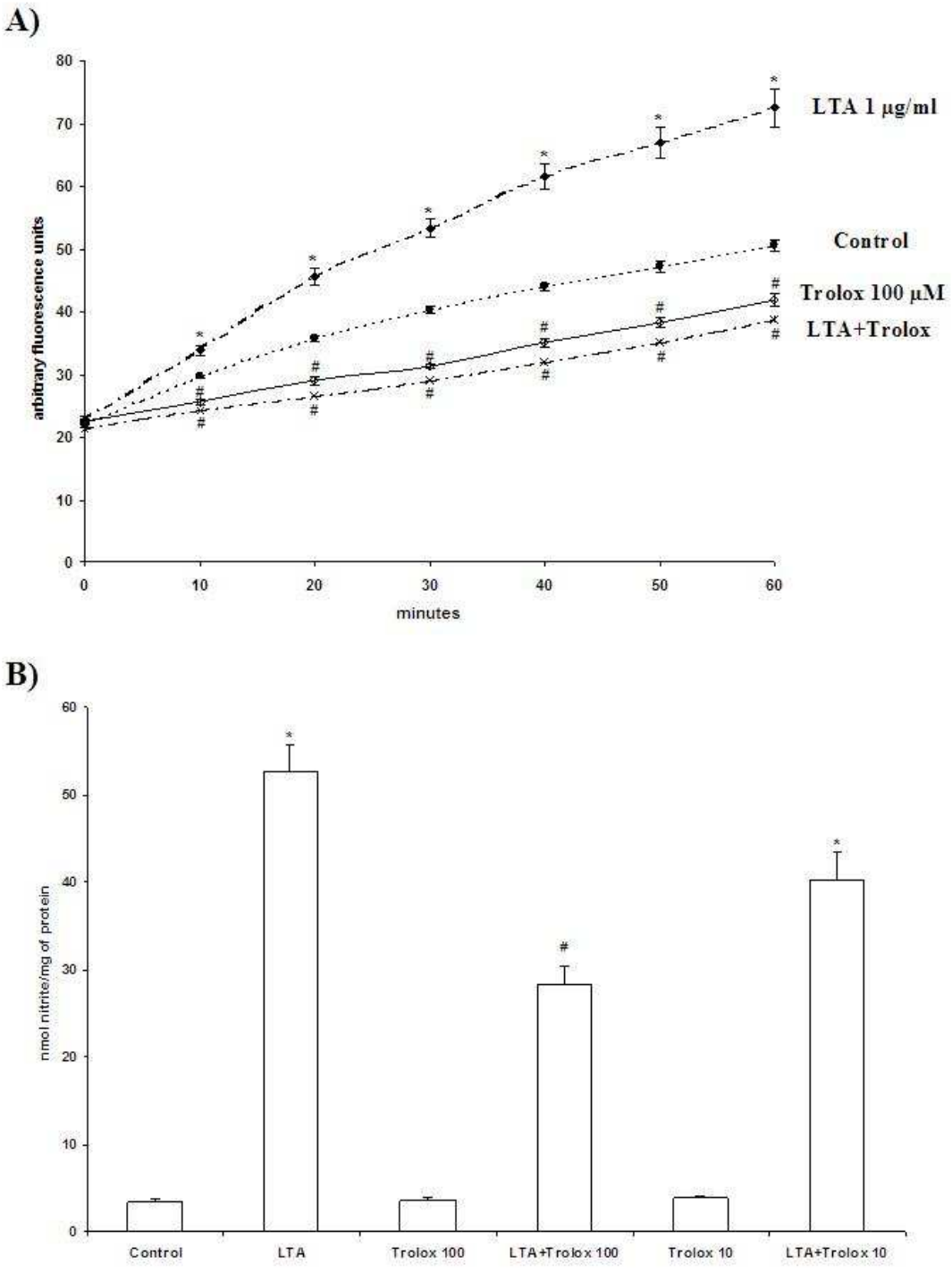
LTA+NECA

**Figure 5**


A)
arbitrary fluorescence units
minutes
LTA 1 µg/ml
Control
Trolox 100 µM
LTA+Trolox
B)
nmol nitrite/mg of protein
Control
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Trolox 100
LTA+Trolox 100
Trolox 10
LTA+Trolox 10

## Capítulo 4

# Regulação da produção de ROS estimulada por LPS em macrófagos peritoniais de ratos diabéticos: participação da glicose e PPARγ

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# Regulation of LPS stimulated ROS production in peritoneal macrophages from alloxan-induced diabetic rats: Involvement of high glucose and PPARγ

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**Abstract**

An increased occurrence of long term bacterial infections is common in diabetic patients. Bacterial cell wall components are described as the main antigenic agents from these microorganisms and high blood glucose levels are suggested to be involved in altered immune response. Hyperglycemia is reported to alter macrophages response to lipopolysaccharide (LPS) and peroxisome proliferators activated receptor gamma (PPARγ) expression. Additionally, glucose is the main metabolic fuel for reduced nicotinamide adenine dinucleotide phosphate (NADPH) production by pentose phosphate shunt. In this work, lipopolysaccharide (LPS) stimulated reactive oxygen species (ROS) and nitrite production were evaluated in peritoneal macrophages from alloxan-induced diabetic rats. Cytosolic dehydrogenases and PPARγ expression were also investigated. LPS was ineffective to stimulate ROS and nitrite production in peritoneal macrophages from diabetic rats, which presented increased glucose-6-phosphate dehydrogenase and malate dehydrogenase activity. In RAW 264.7 macrophages, acute high glucose treatment abolished LPS stimulated ROS production, with no effect on nitrite and dehydrogenase activities. Peritoneal macrophages from alloxan-treated rats presented reduced PPARγ expression. Treating RAW 264.7 macrophages with a PPARγ antagonist resulted in defective ROS production in response to LPS, however, stimulated nitrite production was unaltered. In conclusion, in the present study we have reported reduced nitric oxide and reactive oxygen species production in LPS-treated peritoneal macrophages from alloxan-induced diabetic rats. The reduced production of reactive oxygen species seems to be dependent on elevated glucose levels and reduced PPARγ expression.


*Keywords:* Lipopolysaccharide; Macrophages; Alloxan; Hyperglycemia; PPAR; ROS; Nitric oxide

## Introduction

Diabetes is a prevalent metabolic disorder with several secondary complications. An increased occurrence of long-term bacterial infections is common in diabetic patients, being a major complication in these individuals (Smitherman and Peacock, 1995). It was suggested that high blood glucose levels are involved in reduced bactericidal activity (Nielson and Hindson, 1989) and it has further been demonstrated that high glucose reduces interleukin 1 release from murine macrophages (Hill et al., 1998).

Bacterial cell wall components are described as the main antigenic agents from these microorganisms (Van Amersfoort et al., 2003) and their recognition by Toll-like receptors (TLR) initiates cellular responses to bacterial infections (Takeuchi and Akira, 2001). Lipopolyssacharide (LPS) is a major constituent of the outer membrane of gram negative bacteria and is recognized by animals as a molecular correlate to infection. It binds to TLR 4, triggering multiple signaling cascades (Van Amersfoort et al., 2003; Takeuchi and Akira, 2001). Macrophages are key mediators of innate immunity and their phagocytic activity in response to microbial infections and antigens produces and releases reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). Both ROS and RNS have antimicrobial activity; moreover, they also act as cellular signaling molecules (Forman and Torres, 2001; Ferret et al., 2002). Additionally, exacerbated

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ROS and RNS production in response to bacterial infection is related to inflammatory complications, as septic shock, multiple organ failure and death (Ritter et al., 2004; Andrades et al., 2005; Victor et al., 2005).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is expressed by phagocytes and is involved in superoxide production for antimicrobial activity. In non-stimulated cells, NADPH oxidase components are in different subcellular compartments, with subunits $gp91^{phox}$ and $p22^{phox}$ in the plasma membrane and subunits $p47^{phox}$/$p67^{phox}$/$p40^{phox}$ in the cytosol as a complex. Upon phagocytosis or stimulation with soluble agents, such as LPS, the cytosolic components and the small GTPase Rac1/Rac2 translocate to the plasma membrane binding to $gp91^{phox}$ and $p22^{phox}$ and initiating superoxide production by transferring one electron from NADPH to oxygen (Forman and Torres, 2001; Decoursey and Ligeti, 2005). Nitric oxide is synthesized from L-arginine by nitric oxide synthase using NADPH as a cofactor. Three nitric oxide synthases are described: neuronal and endothelial isoforms that are constitutive and calcium dependent, and the inducible isoform which is calcium independent. In stimulated macrophages, inducible nitric oxide synthase (iNOS) accounts for increased nitric oxide production, which is NADPH dependent (Forstermann and Kleinert, 1995). NADPH is an essential cellular coenzyme and its level is sustained by $NADP^+$ dependent cytosolic dehydrogenases, named glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH). Impaired NADPH production by reduced G6PD activity is associated with augmented IL-10 production and redox dependent signaling in mouse peritoneal macrophages (Wilmanski et al., 2005).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors that have pleiotropic immune modulating effects. Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-dependent nuclear receptor that has a critical role in adipogenesis and glucose metabolism. In macrophages, the role of PPARγ in inflammation response has been studied, showing that this nuclear receptor presents anti-inflammatory properties (Zhang and Chawla, 2004). Additionally, Sartippour et al. (Sartippour and Renier, 2000) showed that PPARs expression are regulated in macrophages by increased glucose.

Considering that increased blood glucose levels are present in diabetes and that this impairs microbial infection response, the aim of the present study was to determine peritoneal macrophage response to LPS in an animal model of diabetes by measuring ROS and nitric oxide production. As glucose is the main metabolic fuel for NADPH production by pentose phosphate shunt, we investigated the activities of cellular dehydrogenases. The modulation of PPARγ expression and the role of this transcription factor in ROS production were also evaluated.

## Materials and methods

### *Chemicals*

Alloxan monohydrate, lipopolyssacharide from *Escherichia coli* (LPS), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), β-$NADP^+$, agarose, ethidium bromide and GW9662 were purchased from Sigma (St. Louis, USA). RPMI 1640 and fungizone were purchased from Gibco™ (Invitrogen, Carlsbad, USA). The SuperScript-II RT preamplification system and dNTPs were purchased from Invitrogen (Carlsbad, USA). All other reagents were of analytical grade and were purchased from commercially available sources.

### *Alloxan-induced diabetic animal model*

Nine-week-old male Wistar rats (190–225 g) were obtained from our breeding stock. Rats were housed in plastic cages, maintained at 22±1 °C and a 12 h light/dark cycle. Animals were supplied with commercial pellet food (Nuvilab® CR-1 Curitiba, PR, Brazil) and water *ad libitum*. Animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian College for Animal Experimentation. Animals were randomly divided into two groups: non-induced and diabetic-induced. Diabetes was induced by an intraperitoneal injection of 150 mg/kg of alloxan monohydrate (0.9% NaCl) after overnight fasting. The non-induced group received only 0.9% NaCl (Prince et al., 1998). Alloxan monohydrate injection leads to the destruction of insulin-secreting β cells of the islets of Langerhans, while other cells (α, γ, δ) are resistant. The destruction of the insulin-secreting β cells was brought about by a redox cycle with the formation of superoxide radicals established by alloxan and the product of its reduction, dialuric acid. Superoxide radicals undergo dismutation to hydrogen peroxide, which produces the reactive hydroxyl radicals by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β cells (Szkudelski, 2001). This is accompanied by typical and permanent hypoinsulinemia and hyperglycemia (Lenzen and Panten, 1988). Blood glucose levels were monitored thereafter by Accu-Chek® Active blood glucose monitor. Ten days later animals from the diabetic-induced group, with glucose levels lower than 300 mg/dL, were not used in this study. Body weight and blood glucose were measured 30 days after alloxan injection and animals were killed by decapitation.

### *Peritoneal macrophages preparation*

Peritoneal macrophages were isolated as described by El-Mahmoudy et al. (El-Mahmoudy et al., 2002) with modifications. Briefly, rats were sacrificed by decapitation, placed with the abdomen facing up and thoroughly wet with 70% ethanol. A transverse cut was made in the inguinal area and the skin was dissected to expose the abdominal wall which was then soaked with 70% ethanol. About 20 ml of cold Hank's balanced salt saline (HBSS) was injected. The needle was removed and the abdomen was gently massaged. The HBSS was drawn back and the peritoneal fluid was dispensed into 50 ml polypropylene tubes. This procedure was repeated two more times. The cells were centrifuged, re-suspended in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone and counted.

Peritoneal macrophages were seeded to 24 well plates ($0.5 \times 10^6$ cells for nitrite and ROS measurements) or 6 well plates ($2 \times 10^6$ cells for cellular dehydrogenase activities and PPAR-γ expression) at a density of $1 \times 10^6$ cells/ml in RPMI 1640, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone. After 2 h, the plates were washed three times with HBSS to remove non-adherent cells. The adherent cells (97% macrophages) were them utilized for cellular dehydrogenase assay and RNA isolation or incubated for 15 h with or without LPS 1 μg/ml, at 37 °C and 5% $CO_2$, for nitrite and ROS measurements.

*RAW 264.7 macrophages culture*

RAW 264.7 macrophages were obtained from UFRJ Cell Bank, Rio de Janeiro, Brazil. Cells were maintained in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone at 37 °C and 5% $CO_2$. Semi-confluent cells were scrapped and plated to 24 well plates ($0.5 \times 10^6$ cells for nitrite and ROS measurements) or 6 well plates ($2 \times 10^6$ cells for cellular dehydrogenase activities) at a density of $1 \times 10^6$ cells/ml in RPMI 1640, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone. After 24 h, the macrophages were washed with saline and incubated with or without LPS 1 μg/ml for 15 h in serum free RPMI 1640 medium. For studies on the influence of PPARγ in the response to LPS, GW9662 – a PPARγ antagonist – was added to cells 2 h before LPS treatment in serum free 1640 medium. For acute high glucose experiments, RAW 264.7 macrophages were washed with saline and incubated for 24 h with serum free RPMI 1640 supplemented with 300 mg/dL glucose (500 mg/dL total glucose concentration) before LPS treatment or cellular dehydrogenase measurement. Normal RPMI 1640 medium contains 200 mg/dL glucose.

*Nitrite assay*

Nitric oxide production was assayed by quantification of the stable end product of nitric oxide oxidation – nitrite ($NO_2$) – as previously described (Souza et al., 2006). Briefly, the incubation medium of peritoneal or RAW 264.7 macrophages were collected after the treatments and centrifuged at 12,000 ×*g* for 10 min. Equal volumes of supernatant and Griess reagent (1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) were reacted for 15 min and the nitrite content was measured by absorbance at 540 nm (Beckman Instruments Spectrophotometer, Fullerton, USA). Nitrite concentration in the samples was calculated using a standard curve prepared with $NaNO_2$.

*ROS measurement*

Reactive oxygen species were measured as previously described (Guimaraes et al., 2006) with minor modifications. Briefly, peritoneal or RAW 264.7 macrophages in 24-well plates were incubated with the treatments and in the last 30 min of treatment 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (10 μM) was added and incubated in the dark at 37 °C. Cells were then washed thrice with PBS, harvested in PBS 0.5% TRITON X-100, centrifuged at 12,000 ×*g* for 10 min and the supernatant collected. The intensity of dichlorofluorescein (DCF) fluorescence in the supernatant was measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Hitachi F2000 Fluorescence Spectrophotometer, Japan).

*Cytosolic dehydrogenase assay*

Peritoneal or RAW 264.7 macrophages were lysed with phosphate buffered saline plus 0.5% Triton X-100, centrifuged at 12,000 ×*g* for 10 min and the supernatants were used for enzyme measurements. Dehydrogenase activities were measured by NADPH production at 340 nm (Beckman Instruments Spectrophotometer, Fullerton, USA) at 36 °C in the presence of specific incubation mediums for each dehydrogenase, as follows:

- Glucose-6-phosphate dehydrogenase (G6PD): Tris-HCl 50 mM, $MgCl_2$ 3 mM, $NADP^+$ 0.2 mM, glucose-6-phosphate 3.2 mM, pH 7,8;
- Isocitrate dehydrogenase (IDH): Tris-HCl 80 mM, $MgCl_2$ 2 mM, $NADP^+$ 2 mM, sodium isocitric acid 5 mM, pH 7,4;
- Malate dehydrogenase (MDH): Tris-HCl 50 mM, $MgCl_2$ 1 mM, $NADP^+$ 0.5 mM, sodium malic acid 0.6 mM, pH 7,4.

*Semi-quantitative RT-PCR*

Total RNA was isolated from peritoneal macrophages using the Trizol Reagent (Invitrogen) as recommended by the manufacturer. Approximately 1 μg of the total RNA was added to each cDNA synthesis reaction using the SuperScript-II RT preamplification system (Invitrogen). Reactions were performed at 42 °C for 1 h using the primer T23V (5′ TTT TTT TTT TTT TTT TTT TTV). Oligonucleotide primers for PPARγ were designed to amplify partial cDNA sequences (501 bp – sense 5′-TTTTCAAGGGTGCCAGTTTC-3′, antisense 5′-TCTGTGACGATCTGCCTGAG-3′). β-actin mRNA expression was determined as an internal control. PCR reactions were performed as follows: denaturation (94 °C, 30 s), extension (72 °C, 30 s) and annealing (60 °C, 30 s). The number of amplification cycles was adjusted to the non-saturated phase and the PCR products were separated on a 1% agarose gel.

Table 1
Blood glucose levels and body weight of normal and alloxan-treated animals

| | Normal ($n$=5) | Alloxan ($n$=5) |
|---|---|---|
| Blood glucose (mg/dL) | 102.08±4.71 | 536.04±27.16 * |
| Body weight (g) | 289.29±8.31 | 230.75±9.59 * |

Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. Blood glucose and body weight were measured 30 days after injection. Values are expressed as mean±SD.

* $p<0.05$ versus normal group (Student's $T$ test).

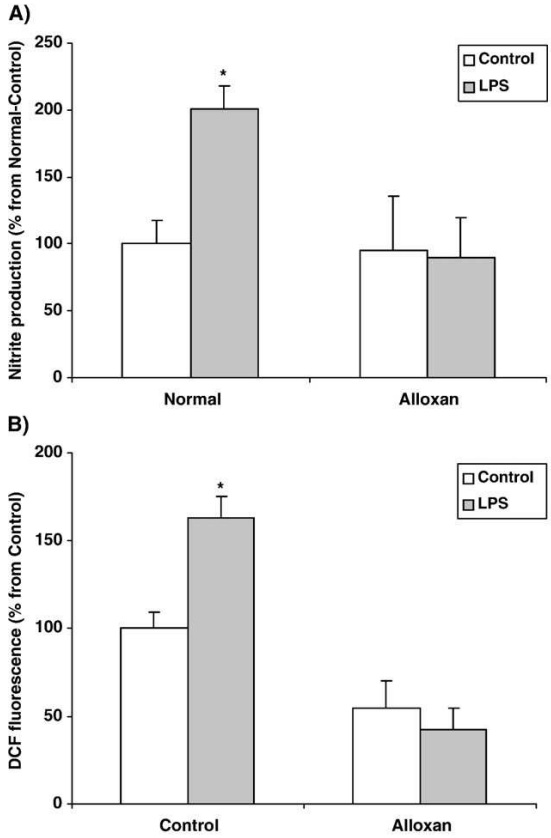


Fig. 1. Nitrite production (A) and DCF oxidation (B) in peritoneal macrophages from normal and alloxan-treated rats. Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. Thirty days after, peritoneal macrophages were isolated and incubated in the presence or not of LPS (1 μg/ml). After 15 h incubation, nitrite production (A) and DCF oxidation (B) were measured as described in Materials and methods. Values are expressed as percentage of control macrophages of normal rats and represent mean±SEM ($n$=5). *$p$<0.05 versus other groups (ANOVA followed by Tukey HSD test).

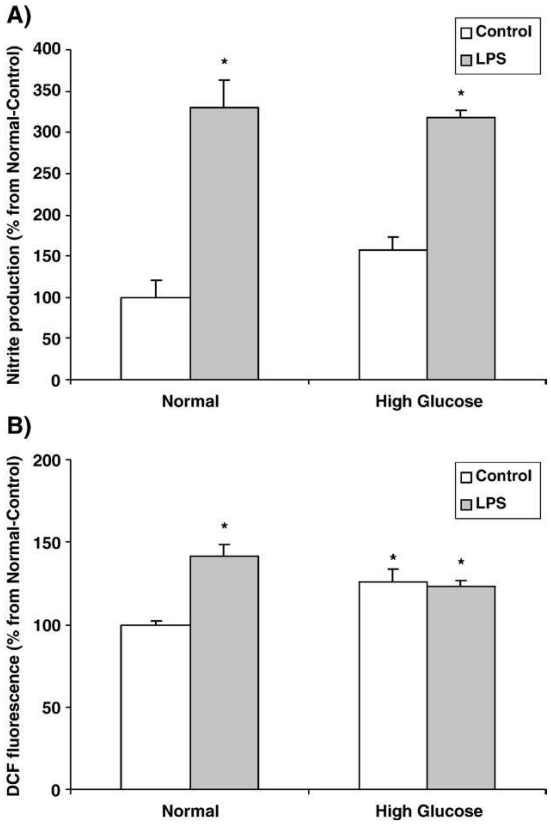


Fig. 2. Nitrite production (A) and DCF oxidation (B) in normal and high glucose-treated RAW 264.7 macrophages. Control (200 mg/dL) or 24 h high glucose (500 mg/dL)-treated RAW 264.7 macrophages were incubated in the presence or not of LPS (1 μg/ml). After 15 h incubation, nitrite production (A) and DCF oxidation (B) were measured as described in Materials and methods. Values are expressed as percentage of control macrophages incubated with normal glucose and represent mean±SEM ($n$=5). *$p$<0.05 versus other groups (ANOVA followed by Tukey HSD test).

Amplification products were visualized by ethidium bromide staining and the quantification was made using ImageJ software (NIH/USA).

*Statistical analysis*

Results are expressed as means+SD or SEM, as indicated in the legends of figures and tables. Differences between means were analyzed by the Student's $T$ test or ANOVA with the Tukey HSD multiple comparisons test. Statistical significance was defined as $p$<0.05.

## Results

Gaulton et al. (Gaulton et al., 1985) reported that the immune dysfunction associated with alloxan-diabetes is a consequence of the diabetic state, in contrast to the immune dysfunction associated with streptozotocin, which seems to be attributable to direct and irreversible impairment of lymphoid cell function and viability. Alloxan monohydrate injection leads to the destruction of insulin-secreting β cells in the islets of Langerhans, while other cells (α, γ, δ) are resistant. This is accompanied by typical and permanent hypoinsulinemia and hyperglycemia (Lenzen

Table 2
Glucose-6-phosphate and malate dehydrogenase activities in peritoneal macrophages from normal and alloxan-treated animals

| | Normal ($n$=4) | Alloxan ($n$=4) |
|---|---|---|
| Glucose-6-phosphate dehydrogenase (μmol/min×mg protein) | 57.11±3.5 | 74.71±5.22 * |
| Malate dehydrogenase (μmol/min×mg protein) | 5.47±1.08 | 7.85±0.5 * |

Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. After 30 days, peritoneal macrophages isolation and dehydrogenase activities assay were performed as described in Materials and methods. Values are expressed as mean±SEM.
* $p$<0.05 versus normal group (Student's $T$ test).

and Panten, 1988). As shown in Table 1, alloxan-treated animals presented increased blood glucose levels and reduced weight. A sustained high glucose level could present both acute and chronic effects, with the later probably related to advanced glycation end products (Vlassara, 2005). LPS increased nitrite (Fig. 1A — ANOVA $f$ value=9.778, $p$=0.026, $n$=5) and ROS (Fig. 1B — ANOVA $f$ value=13.701, $p$=0.026, $n$=5) production in peritoneal macrophages from normal rats. However, in alloxan-treated animals, no effect of LPS was observed on nitrite (Fig. 1A) and ROS (Fig. 1B) production by peritoneal macrophages (Fig. 1B). To investigate if increased glucose levels could be acutely involved in altered response to LPS, we evaluated nitrite and ROS production in the murine macrophage lineage RAW 264.7 with two distinct concentrations of glucose: normal RPMI 1640 concentration, 200 mg/dL (~11 mM) or 500 mg/dL (~27 mM). As shown in Fig. 2A (ANOVA $f$ value=28.19, $p$<0,001, $n$=5), no alteration in basal or LPS stimulated nitrite production was observed in high glucose incubated macrophages. In high glucose-treated RAW 264.7 macrophages, control DCF oxidation was increased compared to normal glucose (Fig. 2B – ANOVA $f$ value=8.643, $p$=0.003, $n$=5); moreover, no effect of LPS on DCF oxidation was observed in high glucose-treated RAW 264.7 macrophages (Fig. 2B).

Glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH) are essential for the maintenance of cellular NADPH levels. In peritoneal macrophages from alloxan-treated animals, G6PD and MDH activities were increased compared to the normal group (Table 2); however, no alteration in IDH activity was observed (data not shown). In high glucose-treated RAW 264.7 macrophages, no alteration of dehydrogenase activities was observed (data not shown).

PPARγ has been described as a regulator of macrophage inflammatory response by modulating the expression of cytokines and inhibiting the activity of pro-inflammatory transcription factors (Pascual et al., 2005). Reduced expression of PPARγ was observed in peritoneal macrophages from diabetic rats (Fig. 3). In order to study reduced PPARγ activity in LPS stimulation of macrophages, RAW 264.7 macrophages were incubated in the presence of GW9662, a specific PPARγ antagonist, before LPS treatment. No effect of GW9662 on LPS stimulated nitrite production was found (Fig. 4A – ANOVA $f$ value=34.092, $p$<0.001, $n$=5). However, the PPARγ antagonist abolished LPS stimulated ROS production compared to the normal group (Fig. 4B — ANOVA $f$ value=10.096, $p$=0.001, $n$=5).

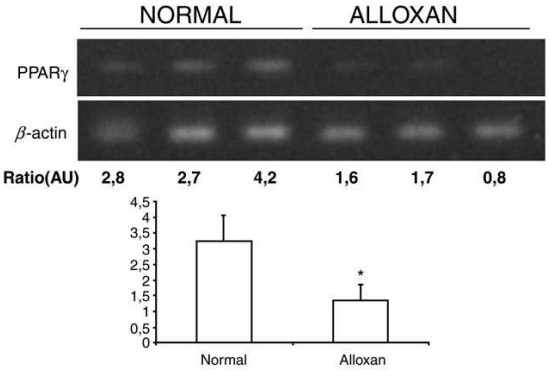


Fig. 3. PPAR-γ expression in peritoneal macrophages from normal and alloxan-treated rats. Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. Thirty days after, peritoneal macrophages were isolated and PPAR-γ expression measured as described in Materials and methods. Values are mean±SD of the ratio between PPAR-γ and β-actin bands in arbitrary units ($n$=3). *$p$<0.05 versus normal group (Student's $T$ test).

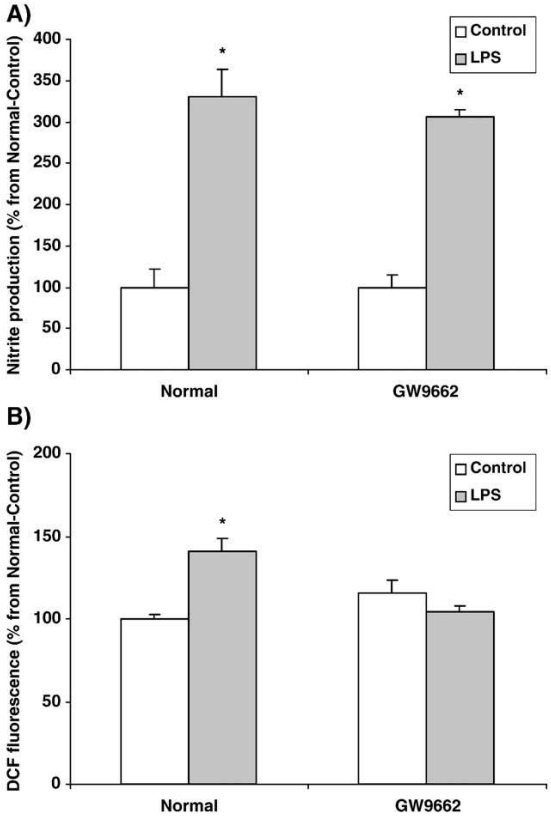


Fig. 4. Nitrite production (A) and DCF oxidation (B) in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated or not for 2 h with PPAR-γ antagonist GW9662 (10 μM). At the end of 2 h, the cells were incubated in the presence or not of LPS (1 μg/ml). After 15 h incubation, nitrite production (A) and DCF oxidation (B) were measured as described in Materials and methods. Values are expressed as percentage from control macrophages and represent mean±SEM ($n$=5). * $p$<0.05 versus other groups (ANOVA followed by Tukey HSD test).

## Discussion

In the present study, we reported reduced nitric oxide and ROS production in LPS-treated peritoneal macrophages from alloxan-induced diabetic rats. Altered macrophage response was already described in diabetic animals, suggesting that these cells could be involved in immune alterations observed in diabetes. Ptak et al. (Ptak et al., 1998) showed that peritoneal macrophages

from alloxan-treated animals presented impaired response to LPS, with altered cytokines and nitrite production. Increased glucose is described to be involved in some alterations of macrophages responses in diabetic animal models, including interleukin-12 expression (Wen et al., 2006) and upregulation of cyclooxygenase-II, leading to increased prostaglandin-$E_2$ production (Lo, 2005). Additionally, increased glucose is described to increase basal ROS production in macrophages (Guha et al., 2000). In high glucose-treated RAW 264.7 macrophages, no effect of LPS was observed on DCF oxidation, as observed in peritoneal macrophages from diabetic rats. ROS are involved in tumor necrosis factor-α (Haddad and Land, 2002); interleukin-8 (Ryan et al., 2004) and interleukin-1 β (Hsu and Wen, 2002) production in LPS stimulated macrophages. Taking into account that increased glucose levels are described to reduce interleukin 1 release from LPS-treated macrophages (Hill et al., 1998), defective ROS stimulation by LPS could be involved in altered cytokine production from macrophages in hyperglycemia and diabetes.

Acute effects of high glucose levels seem to be not involved in reduced nitrite production from LPS-treated peritoneal macrophages, since high glucose treatment presented no effect on LPS stimulated nitric oxide production on RAW 264.7 macrophages. However, chronic effects of higher glucose levels, including advanced glycation end products, could be involved in altered nitrite response to LPS of macrophages from diabetic animals. It was suggested that defective insulin signaling leads to altered nitric oxide production in stimulated macrophages (Stevens et al., 1997), but how this could be involved in alloxan-induced alterations remains to be addressed. NADPH is essential for ROS and nitric oxide production (Decoursey and Ligeti, 2005; Forstermann and Kleinert, 1995). G6PD, IDH and MDH are essential for the maintenance of cellular NADPH levels and an impaired NADPH production by reduced G6PD activity is associated with augmented IL-10 production in mouse peritoneal macrophages (Wilmanski et al., 2005). IDH activity has been described to be involved in redox buffering, maintaining NADPH levels for antioxidant enzymes, as glutathione reductase (Maeng et al., 2004). In peritoneal macrophages from alloxan-treated rats, increased G6PD and MDH activities were observed, but no alteration of cytosolic dehydrogenases was found in high glucose-treated RAW 264.7 macrophages, suggesting that acute effects of glucose altered concentration are not involved in G6PD and MDH regulation. Altered ROS production, observed in both RAW 264.7 and peritoneal macrophages, seems to be not dependent on these dehydrogenase activities, considering that they were regulated in peritoneal macrophages and not in RAW 264.7 cells. Future investigation is necessary to address the role of altered G6PD and MDH activities on altered nitrite production observed in peritoneal macrophages from diabetic rats.

PPARγ was described to regulate macrophages functions (Zhang and Chawla, 2004), and Sartippour et al. (Sartippour and Renier, 2000) showed that PPARγ expression is diminished in macrophages by increased glucose. We observed that in peritoneal macrophages from alloxan-treated rats PPARγ expression was reduced compared to normal rats (Fig. 3). The imbalance of inflammatory cytokines production during the development of diabetes could be involved in this downregulation of PPAR-γ expression. In fact, among the many features induced by alloxan treatment there is the increased plasmatic concentration of TNF-α (Ptak et al., 1998). This cytokine is known to repress PPARγ activity and expression in a variety of cell types (Kim et al., 2005; Gao et al., 2006). Additionally, pharmacological reduction of PPARγ activity in RAW 264.7 macrophages resulted in reduced ROS production in response to LPS, similarly as observed in peritoneal macrophages from diabetic rats. These results suggest that reduced PPARγ expression could be involved in impaired ROS production in LPS-treated peritoneal macrophages from diabetic rats. In opposition to our results, PPARγ agonists have been demonstrated to exert anti-inflammatory properties (Ricote et al., 1998), but many of these effects have been shown to be independent from PPARγ activation (Chawla et al., 2001; Crosby et al., 2005). More studies are necessary to investigate how reduced PPARγ expression could alter macrophage response.

In conclusion, in this work we have reported reduced nitric oxide and reactive oxygen species production in LPS-treated peritoneal macrophages from alloxan-induced diabetic rats. We have shown that reduced ROS production seems to be dependent on acute effects of elevated glucose levels and reduced PPARγ expression.

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## Capítulo 5

# Concentrações elevadas de glicose aumentam a ativação de macrófagos RAW 264.7 por ácido lipoteicóico de *Staphylococcus aureus*

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# High glucose increases RAW 264.7 macrophages activation by lipoteichoic acid from *Staphylococcus aureus*

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ABSTRACT

*Background:* Type 2 diabetes mellitus is associated with an increased risk of cardiovascular diseases and accelerated atherosclerosis, which has been associated to hyperglycemia and chronic inflammation. Activated macrophages are described to participate in atherosclerosis due to foam cell formation and pro-inflammatory mediators production. Bacterial infections are described to accelerate atherosclerosis, moreover, gram-positive and negative bacterial DNA was described in atherosclerotic plaques.
*Methods:* We studied the glucose modulation of RAW 264.7 macrophages activation by the gram-positive bacterial antigen lipoteichoic acid (LTA), evaluating nitrite production, tumor necrosis factor α secretion and matrix metalloproteinase 9 activity.
*Results:* High glucose increased macrophages activation by LTA, evidenced by exacerbated nitric oxide and tumor necrosis factor α production, as well matrix metalloproteinase 9 secretion.
*Conclusions:* These effects could contribute to atherosclerotic risk parameters, like atherome plaque instability, and participate in chronic inflammation present in type 2 diabetes.


## 1. Introduction

Diabetes is a prevalent metabolic disorder with several secondary complications. Type 2 diabetes is associated to a chronic inflammation, characterized by increased circulatory levels of pro-inflammatory cytokines, which has been described to be involved in type 2 diabetes pathogenesis [1]. Type 2 diabetes patients possess increased risk of cardiovascular diseases and accelerated atherosclerosis, which has been associated to hyperglycemia and chronic inflammation (revised in [2]).

Macrophages are a heterogeneous population of mononuclear phagocytes found ubiquitously in the tissues. These cells are involved in innate immune response and regulation. Activated macrophages are described to participate in atherosclerosis progression mediated by foam cell formation and vascular cells regulation due to the synthesis and release of pro-inflammatory cytokines, chemokines, growth factors, reactive oxygen and nitrogen species, and eicosanoids (reviewed in [3]). Additionally, activated macrophages secretes metalloproteinases, which are involved in vascular wall thickening and plaque rupture [4]. Increased glucose is described to be involved in alterations of macrophages responses to lipopolysaccharide (LPS) in diabetic animal models, including interleukin-12 expression [5], upregulation of cyclooxygenase-II, leading to increased prostaglandin-$E_2$ [6] and TNF-α production [7].

The gram-positive bacterium *Staphylococcus aureus* is a major pathogen in both community-acquired and nosocomial infections [8]. LPS, the main gram-negative bacterial antigen, is described to accelerate atherosclerosis in rabbits [9], and Kozarov et al. [10] reported the detection of bacterial DNA in atheromatous plaques, including gram-positive bacteria like *Streptococcus* sp. and *Staphylococcus* sp. Lipoteichoic acid (LTA) is one of the main constituents of gram-positive bacteria cell wall and are described to modulate immune functions mainly through toll-like receptor 2 activation [11,12]. This antigen is described to be involved in *S. aureus*-induced nitric oxide (NO) production, shock and multiple organ failure [13,14]. Recently, LTA was described as the major antigen involved in gram-positive stimulated tumor necrosis factor α (TNF-α) production by macrophages [15].

Several studies has addressed the modulation of macrophages response to lipopolysaccharide and gram-negative bacterial infections by increased glucose concentration, however, the effects of glucose on gram-positive antigen activation of macrophages were unknown. In this work we studied the glucose modulation of RAW 264.7 macrophages activation by the gram-positive bacterial antigen LTA, investigating nitric oxide and TNF-α production, as well as MMP 9 secretion.

## 2. Materials and methods

### 2.1. RAW 264.7 macrophages culture

RAW 264.7 macrophages were obtained from UFRJ Cell Bank, Rio de Janeiro, Brazil. Cells were maintained in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-

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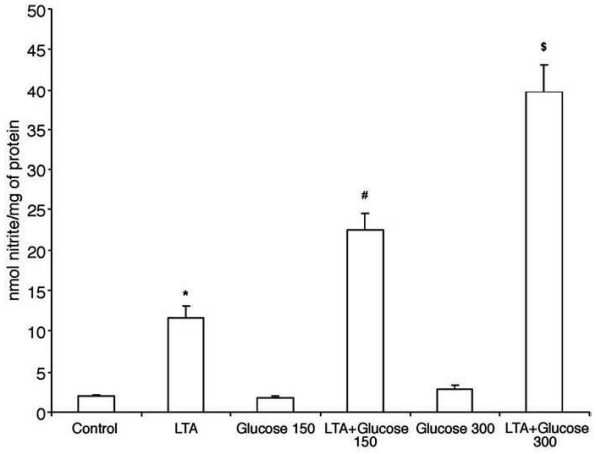


**Fig. 1.** High glucose increases lipoteichoic acid stimulated nitric oxide production in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with normal or increased glucose concentrations (plus 150 or 300 mg/dl) for 48 h. In the last 24 h, the incubation medium was changed and, when indicated, LTA (1 μg/ml) was added. At the end of the incubation, nitrite accumulation in the incubation medium was measured by the Griess method. Results are shown as mean ± SEM ($n=4$). * $p<0.05$ vs control, # $p<0.05$ vs control and LTA, $ $p<0.05$ vs control, LTA and LTA+glucose 150 mg/dl; ANOVA with the Student–Newman–Keuls multiple comparisons test.

inactivated fetal bovine serum, 100 mg/l gentamycin and 250 U/l fungizone at 37 °C and 5% $CO_2$. Semi-confluent cells were scrapped and plated to 24 well plates ($0.4\times10^6$ cells) at a density of $0.8\times10^6$ cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. After 24 h, RAW 264.7 macrophages were washed with saline and incubated for 24 h with serum free RPMI 1640 supplemented or not with 150 or 300 mg/dl D-glucose (350 mg/dl, ~19 mmol/l, or 500 mg/dl, ~27 mmol/l, total D-glucose concentration). The macrophages were maintained an additional period of 24 h with or without LTA (1 μg/ml) in normal or D-glucose supplemented RPMI 1640 medium. Normal RPMI 1640 medium contains 200 mg/dl D-glucose (~11 mmol/l).

### 2.2. Zymography

Gelatin zymography was performed using sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE, 6%) copolymerized with gelatin type B (Sigma—0.3%). Equal amounts of RAW 264.7 macrophages incubation medium were diluted in Laemmli buffer (62.5 mmol/l Tris–HCl, 10% glycerol, 3% sodium dodecyl sulfate, 0.001% bromophenol blue, without β-mercaptoethanol) and subjected to electrophoresis. After this, gels were washed twice for 30 min in 2.5% Triton X-100 at room temperature and were incubated for 8 h in incubation buffer (50 mmol/l Tris–HCl, 5 mmol/l CaCl2, 150 mmol/l NaCl, 1% Triton X-100, pH 7.4) at 37 °C. The resulting gels were stained with Coomassie Blue R 250 (Bio-Rad), using a mixture of ethanol–acetic acid–water (0.1% Comassie Blue R 250, 12.5% ethanol, 7.5% acetic acid) for 1 h, and were destained with methanol–acetic acid–water (45% methanol, 10% acetic acid). Areas of enzymatic activity appeared as clear bands over the dark background. Zymographic activity was quantified by densitometric analysis, employing the software ImageJ (National Institute of Health, Bethesda, MD), and all results were standardized in relation to control.

### 2.3. Other lab assays

Cytokine secretion was measured using mouse TNF-α BD OptEIA™ ELISA kit (BD Biosciences), according to manufacturer's directions. Nitric oxide production was assayed by quantification of the stable end product of nitric oxide oxidation—nitrite ($NO_2^-$). Briefly, the incubation medium of RAW 264.7 macrophages was collected and was reacted 1:1 with Griess reagent (1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) for 15 min. Nitrite content was measured by absorbance at 540 nm. Nitrite concentration in the samples was calculated using a standard curve prepared with $NaNO_2$. All the results were standardized with respect to protein content, determined as described by Lowry et al. [16].

## 3. Statistical analysis

Results are expressed as means + SEM. Differences between means were analyzed by ANOVA with the Student–Newman–Keuls multiple comparisons test. Statistical significance was defined as $p<0.05$.

## 4. Results and discussion

NO is a highly reactive nitrogen radical implicated in multiple biological processes. NO is synthesized from L-arginine by nitric oxide synthases. Three nitric oxide synthases are described: the neuronal and endothelial isoforms that are constitutive and calcium-dependent, and the inducible isoform, which is calcium-independent. In stimulated immune cells, the inducible nitric oxide synthase (iNOS) accounts for increased NO production, which is involved in the regulation of immune response and microbicide action [17]. Moreover, the production of large amounts of NO by iNOS has been implicated in the genesis of septic and cytokine-induced circulatory shock [18]. Glucose enhanced LTA stimulated NO production in RAW 264.7 macrophages in a dose dependent way (Fig. 1). Disequilibrium in NO production has been implicated in altered vascular homeostasis, contributing to the development of atherosclerosis [19].

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases that are involved in the turnover and degradation of extracellular matrix, participating in vascular wall thickening and atherome plaque rupture [4]. These endopeptidases participates in the migration and infiltration of immune cells, additionally, they regulate inflammation and immunity by acting on pro-inflammatory cytokines and chemokines, altering the circulatory concentration of these mediators [20]. The MMP 2 and MMP 9, also known as gelatinases A and B, respectively, constitutes a specific group of metalloproteinases, presenting a gelatin binding domain, involved in the ligation to denaturated collagen. Increased levels of matrix metalloproteinases were described in lipopolysaccharide-induced endotoxemia in mice, suggesting that they contribute to tecidual degradation and multiple organs failure [21]. These enzymes are also involved in endotoxemia associated vascular dysfunction [22]. It was reported that *S. aureus* peptidoglycan (PEG) increased MMP 2 and 9 levels in plasma and organ

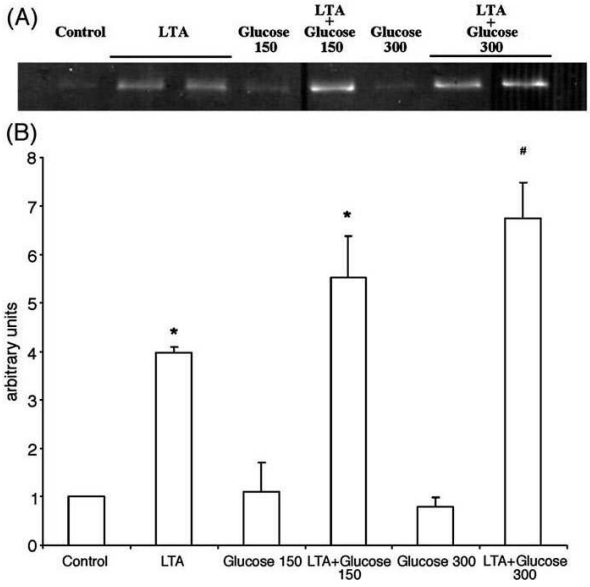


**Fig. 2.** High glucose increases lipoteichoic acid stimulated MMP 9 secretion in RAW 264.7. RAW 264.7 macrophages were incubated with normal or increased glucose concentrations (plus 150 or 300 mg/dl) for 48 h. In the last 24 h, the incubation medium was changed and, when indicated, LTA (1 μg/ml) was added. (A) Representative zymography of RAW 264.7 macrophages incubation medium. (B) Densitometric quantification of MMP 9 activity. Results are shown as mean ± SEM ($n=3$). * $p<0.05$ vs control, # $p<0.05$ vs control and LTA; ANOVA with the Student–Newman–Keuls multiple comparisons test.

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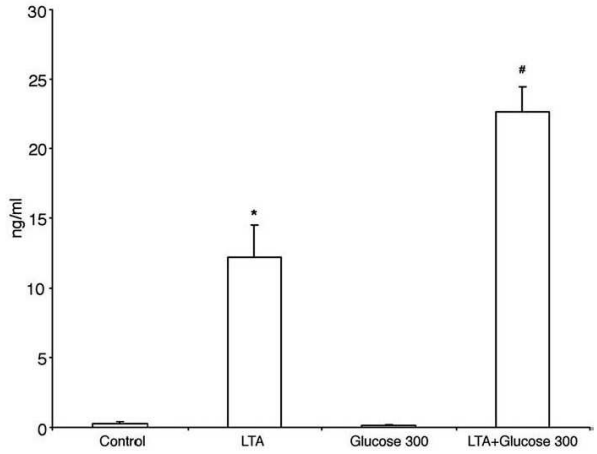


**Fig. 3.** High glucose increases lipoteichoic acid stimulated tumor necrosis factor-alpha secretion by RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with normal or increased glucose concentration (300 mg/dl) for 48 h. In the last 24 h, the incubation medium was changed and, when indicated, LTA (1 μg/ml) was added. At the end of the incubation, TNF-α secretion to the culture medium was evaluated by ELISA. Results are shown as mean±SEM ($n$=3). * $p$<0.05 vs control, # $p$<0.05 vs control and LTA; ANOVA with the Student–Newman–Keuls multiple comparisons test.

homogenates from rat [23]. Moreover, *S. aureus* PEG enhanced MMP 9 secretion by human neutrophils [24]. LTA increased MMP 9 secretion. High glucose concentration augmented LTA stimulated MMP 9 secretion in RAW 264.7 macrophages (Fig. 2).

TNF-α is a pleiotropic cytokine involved in the regulation of diverse physiological and pathological responses. TNF-α is known to be important in inducing the acute-phase response to bacterial infections, leading to physiological changes that support the attack against infecting organism, limit tissue damage, and activate repair processes. However, increased levels of TNF-α are associated with damaging effects of sepsis and cachexia [25]. In chronic inflammatory conditions, like type 2 diabetes, TNF-α promotes atherosclerotic lipids changes and favors insulin resistance development [26]. The effect of LTA on TNF-α production was increased by high glucose concentration, resulting in augmented cytokine secretion by RAW 264.7 macrophages to the incubation medium (Fig. 3).

A sustained high glucose level could cause both acute and chronic effects, with the later probably related to advanced glycation end products [27]. In a previous work, we reported that defective response to LPS by murine macrophages in alloxan induced diabetes is related to increased glucose concentration and reduced peroxisome proliferator-activated receptor gamma (PPARγ) [28]. Additionally, Sartippour et al. [29] showed that PPARs expression is regulated in macrophages by increased glucose, leading to reduced PPARγ expression. PPARγ is described to inhibit inflammatory transcriptional factors, like NF-kB and AP-1 [30], and these transcriptional factors are described to participate in iNOS, MMP 9 and TNF-α expression. This suggests a possible mechanism of high glucose modulation of LTA mediated macrophages activation, in which reduced PPARγ expression/activity, due to high glucose concentration, results in exacerbated inflammatory mediators production, mediated by reduced inhibition of inflammatory transcriptional factors, NF-kB and AP-1.

*Staphylococcus aureus* (*S. aureus*) is described in atheromatous plaques [10], however, Lehr et al. [9] reported that *S. aureus* infections have no effect on arterial wall thickening. In despite of this, our results showed that high glucose increase macrophages activation by LTA from *S. aureus*, which could contribute to others atherosclerotic risk parameters, like atherome plaque instability. An increased occurrence of long-term bacterial infections is common in diabetic patients, being a major complication in these individuals [31]. For this reason, exacerbated macrophage activation could contribute to chronic inflammation present in type 2 diabetes.

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# PARTE III

**Discussão**

Os antígenos peptídeoglicano e ácido lipoteicóico, da bactéria gram-positiva *Staphylococcus aureus*, provocam a ativação dos macrófagos RAW 264.7, evidenciada pela produção de diferentes mediadores inflamatórios (Figura 6). Tanto o PEG como o LTA aumentam a secreção de TNF-α, citocina pró-inflamatória importante na resposta imune, entretanto, o LTA apresenta um efeito mais pronunciado. Isto está de acordo com o descrito por Seo et al. (2008), que demonstram que o LTA é o principal antígeno envolvido na produção de TNF-α em macrófagos expostos a bactérias gram-positivas. Estes autores destacam que outros componentes de bactérias gram-positivas atuam sinergisticamente com o LTA para estimular a produção de TNF-α, no entanto não identificam quais. Nossos resultados indicam que o PEG pode contribuir para a produção de TNF-α estimulada por bactérias gram-positivas em macrófagos.

Nos macrófagos RAW 264.7 o LTA estimulou a expressão da iNOS, aumentando a produção de NO, enquanto o PEG não apresentou efeito na produção de NO. Isto está de acordo com o descrito por De Kimpe et al. (1995) e Kengatharan et al. (1998) que mostraram que o PEG atua sinergisticamente com o LTA para estimular a produção de NO, mas não apresenta efeito quando único ativador dos macrófagos. A produção de NO estimulada por LTA em macrófagos RAW 264.7 é descrita como dependente das proteínas cinases A e C, p38, ciclooxigenase II e NF-kB (KUO et al., 2003; CHANG et al., 2006). O tratamento dos macrófagos com LTA levou a um rápido aumento da produção de ROS, aparentemente dependente de NADPH oxidase. A produção de ROS em resposta ao LTA já havia sido descrita em células dendríticas (CHOI et al., 2008) e monócitos (LEVY et al., 1990). A inibição da produção de ROS por um antioxidante, o trolox, análogo solúvel da vitamina E, acarretou numa diminuição da produção de NO estimulada por LTA, nos macrófagos RAW 264.7.

Hsiao et al. (2004) haviam descrito que o tratamento de macrófagos com um antioxidante inibe a fosforilação de JNK e a ativação de NF-kB estimuladas por LTA, acarretando numa menor produção de NO e expressão de iNOS, entretanto, não investigaram a produção de ROS nestas células. Assim, nossos resultados sugerem que a produção de ROS estimulada por LTA participa da sinalização envolvida na expressão de iNOS e produção de NO nos macrófagos. A produção de ROS está envolvida na expressão e secreção de TNF-α estimulada por LPS em macrófagos (KIMURA et al., 2008), entretanto a inibição da produção de ROS não apresentou qualquer efeito na secreção de TNF-α estimulada por LTA, sugerindo mecanismos diferentes na produção desta citocina pró-inflamatória em resposta a antígenos de bactérias gram-negativas ou gram-positivas. Isto poderia ajudar a explicar os efeitos diversos obtidos nas terapias baseadas na neutralização de TNF-α, nas infecções por bactérias gram-negativas e gram-positivas (LORENTE e MARSHALL, 2005).

O LTA e o PEG aumentaram a expressão e secreção de MMP 9 nos macrófagos RAW 264.7. O PEG já era descrito aumentando MMP 2 e 9 no plasma e órgãos de ratos (WANG et al., 2004), além de aumentar a expressão de MMP 9 em neutrófilos humanos (WANG et al., 2005), entretanto, o efeito do LTA sobre a MMP 9 não havia sido investigado. O efeito do LTA sobre a secreção de MMP 9 nos macrófagos RAW 264.7 é mediado pela ativação de ERK, dependente de MEK. Estes resultados indicam que o aumento da secreção de metaloproteinases estimulada por LTA e PEG em macrófagos pode contribuir na patogênese de infecções por *S. aureus*. Níveis elevados de metaloproteinases estão associados ao dano tecidual, falência múltipla de órgãos e disfunção vascular presentes em modelos de endotoxemia (PAGENSTECHER et al., 2000; LALU et al., 2006).

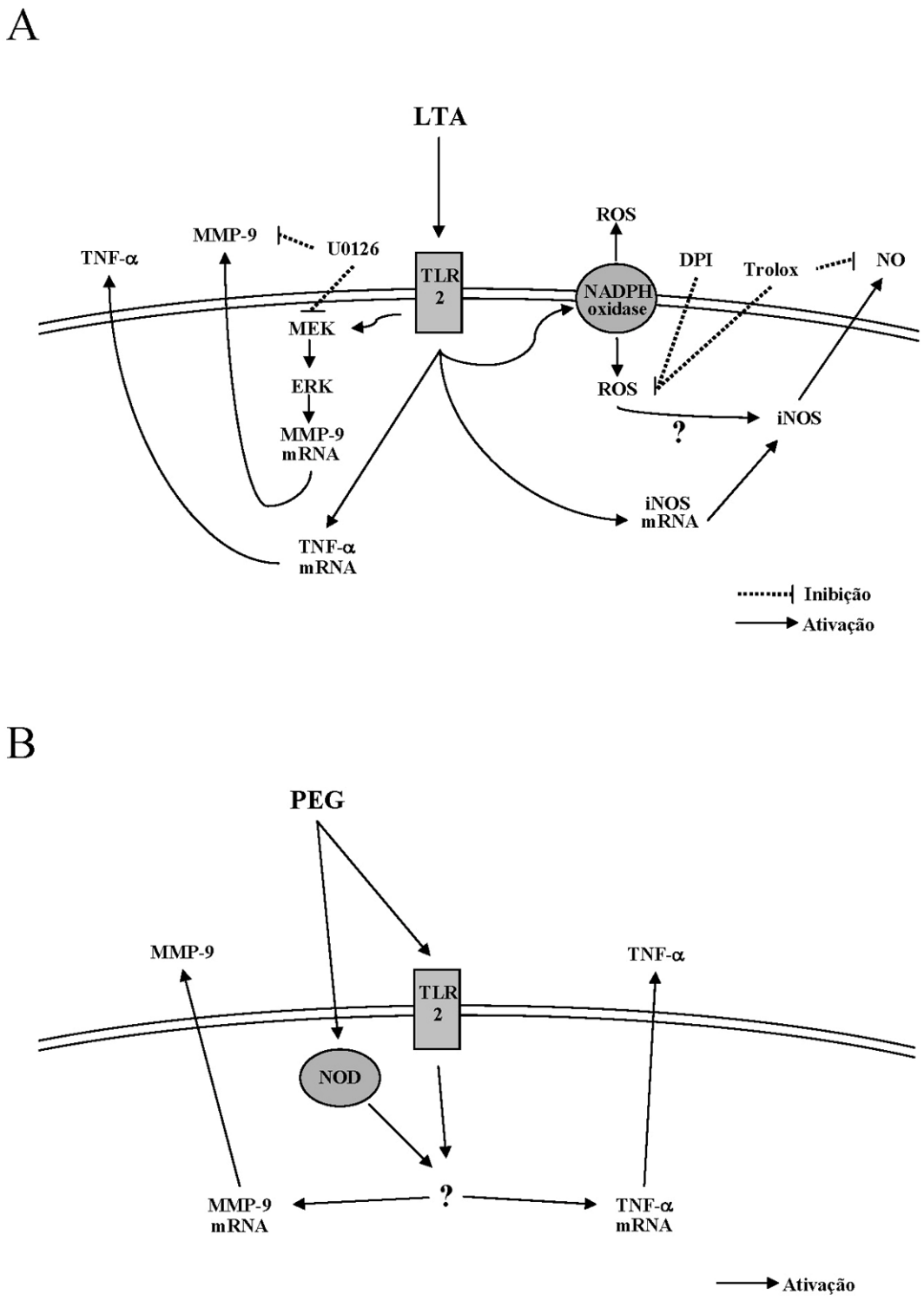


**Figura 6: Ativação dos macrófagos RAW 264.7 pelos antígenos de *Staphylococcus aureus*.** (A) O ácido lipoteicóico (LTA) aumenta a produção de espécies reativas de oxigênio (ROS), óxido nítrico (NO), fator de necrose tumoral alfa (TNF-α) e metaloproteinase 9 (MMP-9). (B) O peptiddeoglicano (PEG) aumenta a produção de fator de necrose tumoral alfa e metaloproteinase 9 (MMP-9).

**Receptores de adenosina**

Os macrófagos RAW 264.7 expressam os receptores de adenosina A2A, A2B e A3. O tratamento dos macrófagos com os antígenos de *S. aureus* LTA ou PEG aumentou a expressão dos receptores A2A e A2B, enquanto que a expressão do receptor A3 foi reduzida (Figura 7). Resultados similares foram obtidos em macrófagos humanos e de roedores tratados com LPS (MURPHREE et al., 2005), evidenciando uma resposta comum na expressão de receptores de adenosina em macrófagos expostos a infecções bacterianas.

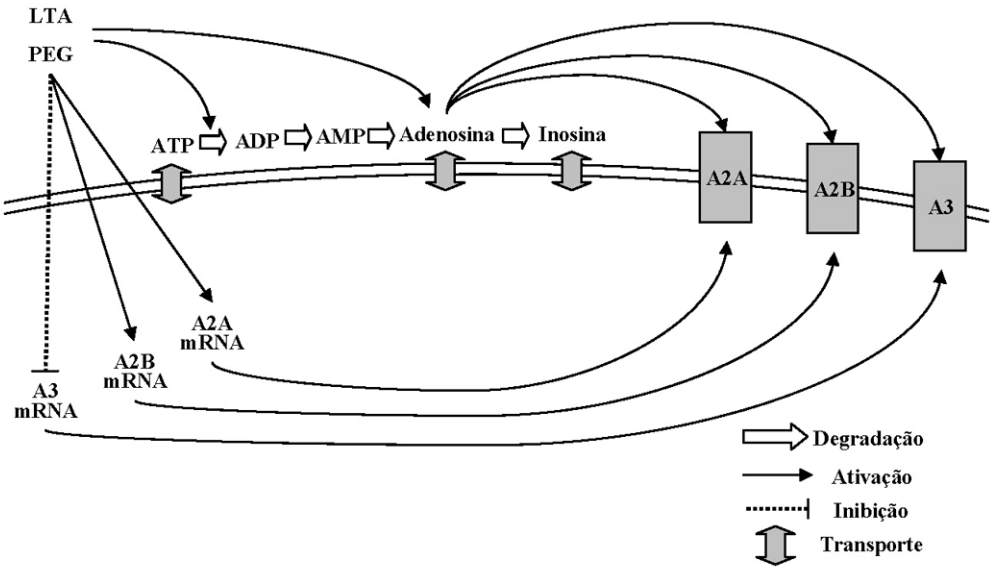


**Figura 7: Modulação do metabolismo purinérgico e da expressão dos receptores de adenosina, nos macrófagos RAW 264.7, pelos antígenos de *Staphylococcus aureus*.** O ácido lipoteicóico (LTA) e o peptideoglicano (PEG) aumentam a degradação de ATP, causando um acúmulo de adenosina. Além disso, estas moléculas aumentam a expressão dos receptores de adenosina A2A e A2B, enquanto reduzem a expressão do receptor A3.

O ATP e a adenosina possuem papéis antagônicos na resposta imune, com o ATP apresentando efeitos pró-inflamatórios e a adenosina apresentando efeitos antiinflamatórios (BOURS et al., 2006). A adenosina e seus receptores reduzem diversos mediadores pró-inflamatórios em macrófagos e monócitos tratados com LPS, incluindo a produção de ROS e NO, e a secreção de TNF-α e interleucina 12 (HASKÓ et al., 2007). Os macrófagos tratados com LTA e PEG apresentaram um aumento na degradação de ATP extracelular, resultando no acúmulo de adenosina extracelular (Figura 7). Por outro lado, a degradação de adenosina extracelular não foi alterada. Com isso, os macrófagos estimulados por antígenos de *S. aureus* apresentam uma situação favorável ao acúmulo de adenosina extracelular, somado ao aumento da expressão de receptores A2A e A2B.

O bloqueio da ação dos receptores A2A e A2B, pela adição de antagonistas seletivos ou pelo silenciamento gênico com o emprego de RNA de interferência, resultou numa resposta aumentada dos macrófagos RAW 264.7 ao LTA (Figura 8). Isto foi observado através do aumento da produção de NO, bem como da expressão e secreção de MMP 9 nos macrófagos tratados com LTA. Adicionalmente, a adição de adenosina deaminase exógena resultou em um aumento da produção de NO nos macrófagos tratados com LTA. Não foram observados efeitos aditivos pelo bloqueio farmacológico simultâneo dos receptores A2A e A2B, sugerindo que o mecanismo de ação destes receptores possa ser comum. Os receptores de adenosina podem atuar como homodímeros e heterodímeros (FREDHOLM et al., 2007), logo, este pode ser um possível mecanismo de ação para os receptores A2A e A2B na modulação da ativação de macrófagos.

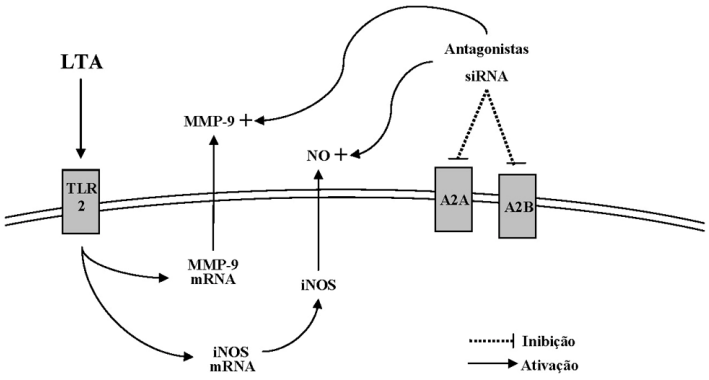


**Figura 8: O bloqueio da ação dos receptores de adenosina aumenta a ativação dos macrófagos RAW 264.7 pelo ácido lipoteicóico de *Staphylococcus aureus*.** O emprego de antagonistas seletivos dos receptores de adenosina A2A e A2B, bem como a inibição da expressão destes receptores pelo uso de RNA de interferência, aumentam a produção de mediadores inflamatórios estimulada por ácido lipoteicóico (LTA): óxido nítrico (NO) e metaloproteinase 9 (MMP-9).

O tratamento dos macrófagos com um agonista não seletivo dos receptores de adenosina resultou numa resposta inflamatória reduzida nos macrófagos estimulados com os antígenos de *S. aureus* LTA e PEG (Figura 9). A ativação dos receptores de adenosina resultou numa redução da secreção de TNF-α nos macrófagos estimulados por PEG. Já nos macrófagos estimulados com LTA, não foi observado efeito sobre a secreção de TNF-α, mas a ativação dos receptores de adenosina resultou numa redução da expressão de iNOS, com a conseqüente redução da produção de NO. O tratamento com o agonista dos receptores de adenosina também reduziu a produção de ROS e a secreção de MMP 9 nos macrófagos estimulados com LTA. Considerando que a produção de ROS parece estar envolvida no aumento da síntese de NO estimulada por LTA, a inibição da produção de ROS pelo agonista dos receptores de adenosina pode ser um dos mecanismos envolvidos na inibição da síntese de NO pelos receptores de adenosina.

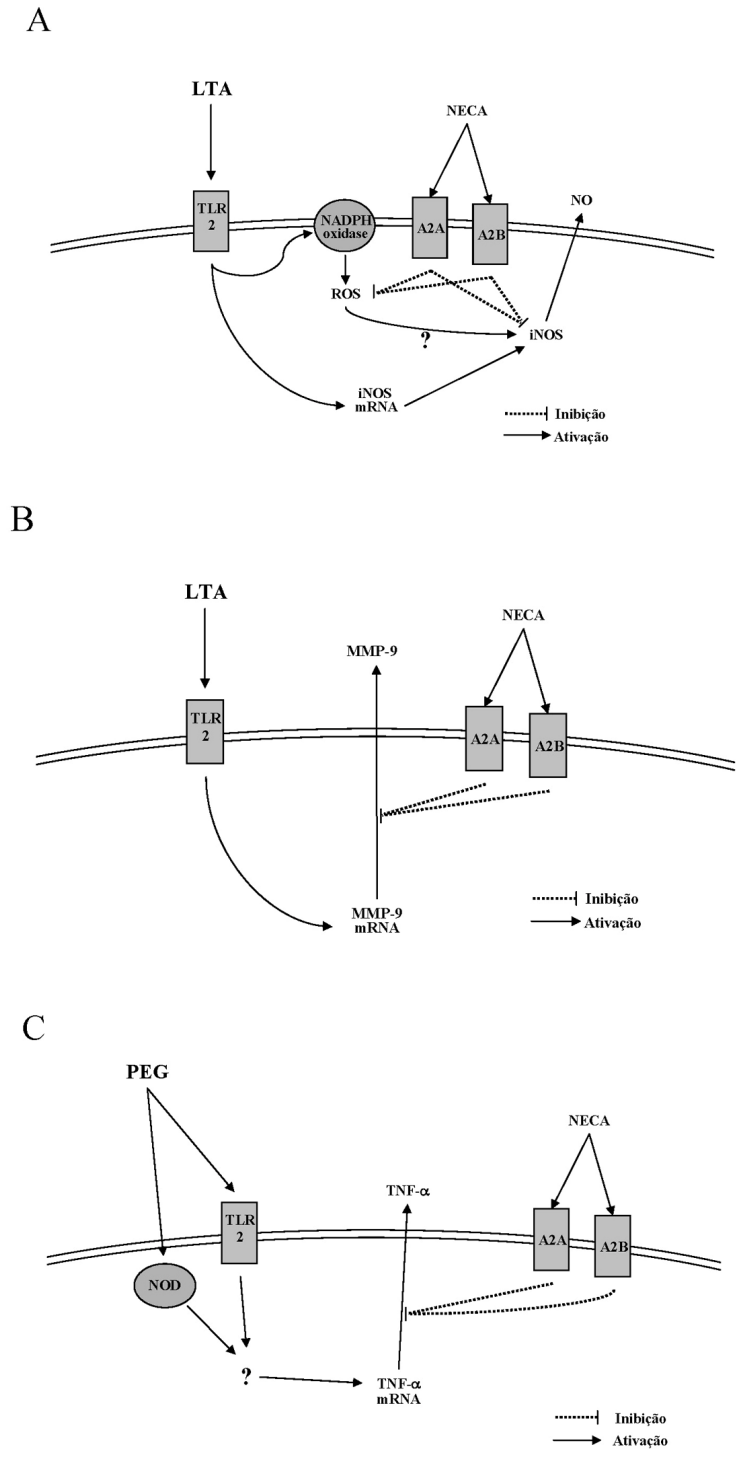


**Figura 9: A estimulação dos receptores de adenosina diminui a ativação dos macrófagos RAW 264.7 pelos antígenos de *Staphylococcus aureus*.** O agonista não seletivo de receptores de adenosina NECA inibe a produção de mediadores inflamatórios estimulada por ácido lipoteicóico (LTA) – [A] óxido nítrico (NO) e espécies reativas de oxigênio (ROS), [B] metaloproteinase 9 (MMP-9) – e por peptideoglicano (PEG) – [C] fator de necrose tumoral alfa (TNF-α).

Estes resultados sugerem que a adenosina, através da ação nos receptores A2A e A2B, possui um papel autócrino na modulação da ativação dos macrófagos por antígenos de *S. aureus*, evitando a ativação exacerbada dos macrófagos (Figura 10). Um papel semelhante foi proposto para o receptor A2B em macrófagos ativados por interferon-γ, nos quais este receptor participa da desativação dos macrófagos (XAUS et al., 1999). Adicionalmente, nossos resultados sugerem que a modulação farmacológica dos receptores de adenosina modifica a resposta inflamatória de macrófagos a *S. aureus*, sugerindo um possível papel terapêutico para estes receptores. Entretanto, resultados antagônicos têm sido obtidos com a modulação farmacológica do receptor A2A em modelos de infecções bacterianas gram-negativas (SULLIVAN et al., 2004) ou polimicrobiais (NÉMETH et al., 2006), o quê pode estar associado aos diferentes efeitos deste receptor na modulação da resposta inflamatória a bactérias gram-negativas e gram-positivas, como a modulação da secreção de TNF-α.

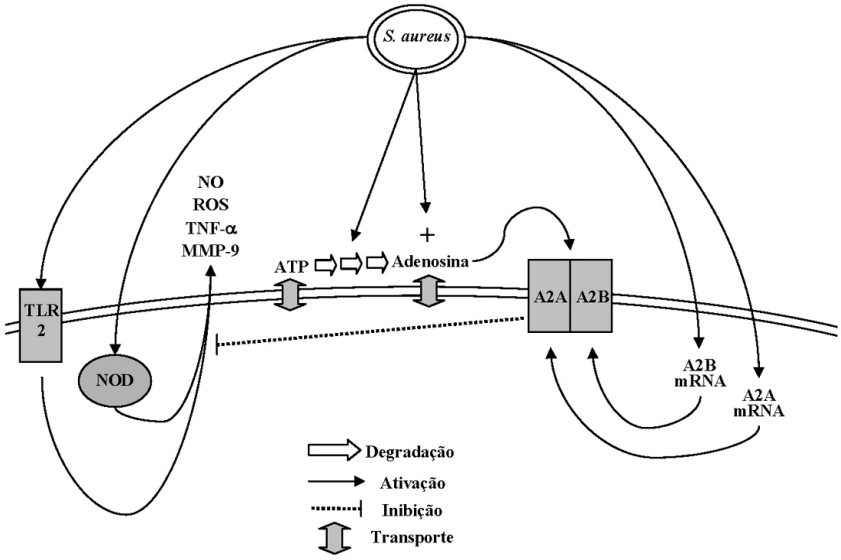


**Figura 10: Papel autócrino da adenosina na modulação da resposta inflamatória de macrófagos a *Staphylococcus aureus*.** Os antígenos de *S. aureus* aumentam a concentração extracelular de adenosina e a expressão dos receptores de adenosina A2A e A2B, os quais regulam a produção dos mediadores inflamatórios.

**Glicose**

Os macrófagos peritoniais de ratos diabéticos, induzidos por injeções de aloxano, apresentaram produções de NO e ROS reduzidas em resposta ao tratamento com LPS. Isto está de acordo com o que havia sido descrito por Ptak et al. (1998), que mostrou que os macrófagos peritoniais de ratos tratados com aloxano possuíam uma produção reduzida de NO e citocinas em resposta ao LPS. Adicionalmente, os macrófagos peritoniais dos ratos diabéticos apresentaram níveis reduzidos de expressão de PPARγ, além do aumento da atividade das desidrogenases citosólicas G6PD e MDH. Concentrações elevadas de glicose têm sido relacionadas com a modificação da resposta ao LPS em macrófagos peritoniais de animais diabéticos (HILL et al., 1998; LO, 2005; WEN et al., 2006; SHERRY et al., 2007), entretanto, o tratamento da linhagem de macrófagos RAW 264.7 com concentrações elevadas de glicose alterou apenas a produção de ROS em resposta ao LPS, não apresentando efeito sobre a produção de NO e sobre a atividade das desidrogenases. Resultados controversos sobre o efeito da glicose na produção de NO em macrófagos estimulados por LPS têm sido publicados. Enquanto Tseng et al. (1997) mostraram que concentrações elevadas de glicose reduzem a produção de NO em macrófagos J774 estimulados com LPS, Chen et al. (2006) descreveram que o tratamento agudo com glicose aumenta a produção de NO em macrófagos estimulados por LPS. Assim, aparentemente, os efeitos da glicose são dependentes da concentração e do tempo de tratamento. Além dos efeitos agudos da elevação da concentração da glicose, não podem ser descartados os efeitos crônicos existentes nos animais diabético, como a glicação de macromoléculas e a formação de intermediários reativos envolvidos na glicação (VLASSARA, 2005), os quais não foram investigados no modelo de cultivo celular.

Sartippour e Renier (2000) mostraram que concentrações elevadas de glicose diminuem a expressão de PPARγ em macrófagos. Nos macrófagos RAW 264.7, a inibição da atividade deste receptor nuclear, pelo uso de um antagonista, também evitou a produção de ROS, em resposta ao LPS. Estes resultados sugerem que a glicose elevada, provocando a diminuição da expressão de PPARγ, pode estar envolvida na redução da resposta ao LPS pelos macrófagos dos ratos diabéticos. Isto é corroborado pelo fato que o PPARγ é descrito como um inibidor de fatores de transcrição inflamatórios, como o NF-kB (SZANTO e ROSZER, 2008), de modo que a redução da sua atividade acarretaria numa maior ativação inflamatória.

Concentrações elevadas de glicose também alteraram a resposta dos macrófagos RAW 264.7 ao tratamento com LTA de *S. aureus*. A incubação dos macrófagos com concentrações elevadas de glicose aumentou a produção de NO, bem como a secreção de MMP 9 e TNF-α (Figura 11). O *Staphylococcus aureus* é descrito em placas ateroscleróticas (KOZAROV et al., 2006), no entanto, Lehr et al. (2001) mostraram que infecções com *S. aureus* não apresentam efeitos no aumento da espessura vascular. No entanto, nossos resultados sugerem que o aumento da resposta ao LTA, pelos macrófagos, pode contribuir para outros fatores de risco associados à aterosclerose, como a instabilidade e ruptura da placa aterosclerótica. A produção aumentada de NO está envolvida na perda da homeostase vascular, contribuindo para o desenvolvimento da aterosclerose (ELAHI et al., 2007). Além disso, as MMPs estão diretamente envolvidas no aumento da espessura vascular e no rompimento da placa aterosclerótica (NEWBY, 2005), assim como níveis elevados de TNF-α contribuem para modificações associadas ao desenvolvimento da resistência à insulina, bem como para o acúmulo lipídico presente na aterosclerose (POPA et al., 2007).

Infecções bacterianas recorrentes são comuns em pacientes diabéticos (SMITHERMAN e PEACOCK, 1995). Ademais, o diabetes tipo 2 está associado com uma inflamação crônica, caracterizada por níveis plasmáticos aumentados de citocinas pró-inflamatórias (PICKUP, 2004), além de os pacientes diabéticos tipo 2 possuírem maior risco de desenvolver doenças cardiovasculares e aterosclerose (MAZZONE et al., 2008). Os resultados apresentados indicam que os efeitos agudos de concentrações elevadas de glicose podem contribuir para diversos fatores relacionados com as complicações associadas ao diabetes, uma vez que alteram a resposta e função dos macrófagos.

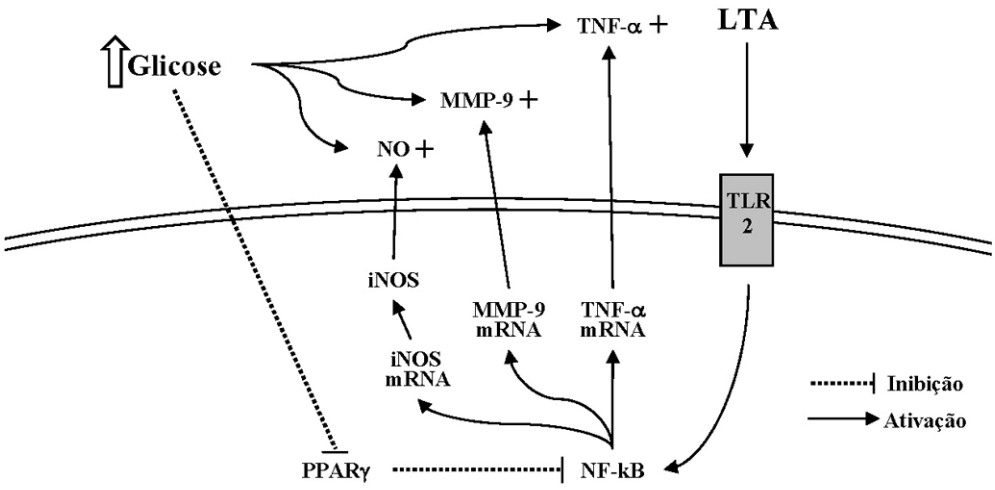


**Figura 11: Concentrações elevadas de glicose aumentam a resposta inflamatória de macrófagos a ácido lipoteicóico de *Staphylococcus aureus*.** Altas concentrações de glicose aumentam a produção de óxido nítrico (NO), metaloproteinase 9 (MMP-9) e fator de necrose tumoral alfa (TNF-α) em macrófagos RAW 264.7 tratados com ácido lipoteicóico (LTA).

## Conclusão

Os resultados obtidos mostram que os receptores de adenosina e concentrações elevadas de glicose alteram a resposta inflamatória a antígenos de *Staphylococcus aureus*. Os receptores de adenosina modulam a ativação dos macrófagos pelo LTA e pelo PEG, diminuindo a produção de mediadores pró-inflamatórios, como NO, ROS, TNF-α e MMP-9, atuando em um sistema autócrino de regulação da resposta inflamatória. Assim, estes receptores constituem possíveis alvos de intervenções terapêuticas em infecções causadas por bactérias gram-positivas. Já a glicose, quando em concentrações elevadas, aumenta a resposta inflamatória dos macrófagos ao LTA, o que pode estar envolvido em complicações associadas ao diabetes e à hiperglicemia, nas quais fatores inflamatórios estão envolvidos, como a aterosclerose e a inflamação crônica.

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