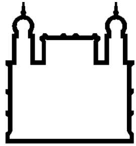


Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

Doutorado em Biologia Celular e Molecular

Perla Villani Borges da Silva

ESTUDO DOS MECANISMOS DE AÇÃO DO TETRANORTRITERPENÓIDE DE ORIGEM NATURAL, GEDUNINA, NA SINALIZAÇÃO DE RECEPTORES TIPO TOLL E INFLAMASSOMA

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Celular e Molecular

Orientadora: Dra. Carmen Penido

RIO DE JANEIRO
Fevereiro, 2017

Ficha catalográfica elaborada pela
Biblioteca de Ciências Biomédicas/ ICICT / FIOCRUZ - RJ

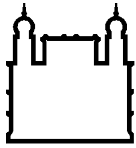
S586
Silva, Perla Villani Borges da

Estudo dos mecanismos de ação do tetranortriterpenóide de origem natural, gedunina, na sinalização de receptores tipo Toll e inflamassoma / Perla Villani Borges da Silva. – Rio de Janeiro, 2017. xii, 85 f. : il. ; 30 cm.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Celular e Molecular, 2017. Bibliografia: f. 72-82

1. Gedunina. 2. Receptor do tipo Toll. 3. Inflamassoma. 4. Macrófagos. 5. Inflamação. I. Título.

CDD 616.07995



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Rio de Janeiro, 9 de fevereiro de 2017

"Com a conclusão deste trabalho só posso dizer que tudo que almejamos nesta vida há de ser realizado porque não haverá outra oportunidade. Sou uma privilegiada, pois tive a chance e uma força de vontade incrível de ter realizado tudo que desejei e não deixarei retalhos pendentes para serem costurados na minha alma. Este doutorado engrandeceu minha história e complementa meus conhecimentos, que não se resumem a conceitos mas numa sucessão de momentos, aprendizados, convivência com pessoas maravilhosas, e espero que tenho finalizado mais esta etapa de vida com a expectativa de novos sonhos a serem conquistados".

(Perla Villani)

AGRADECIMENTOS

Agradeço à minha orientadora Dra. Carmen Penido, pelo belíssimo projeto que me possibilitou desenvolver este trabalho e por sua excelente orientação durante estes quatro anos, que não só incluem elaboração de experimentos na bancada, como também escrita e elaboração de projetos e artigos para publicação. Considero-a não só uma excelente pesquisadora como também uma grande amiga e companheira.

À Dra. Maria das Graças Henriques, pela oportunidade de trabalhar na equipe do Laboratório de Farmacologia Aplicada.

À minha família, principalmente meu marido Renato Einicker Garrido, que me deu todo suporte necessário (emocional e financeiro) para que eu não desistisse e ao meu filho que me deu inspiração de vida para que eu tivesse forças para mais esta conquista. Não poderia deixar de mencionar minha querida mãe, Irma Villani, que sempre me deu todo apoio emocional e conforto monitorando sempre o neto nos momentos em que estive ausente, além de sempre acreditar em mim. Também incluo o meu segundo pai Gilvan Ferreira que sempre esteve junto a mim para fortalecer ainda mais este apoio. Ao meu pai Manoel Borges que serve de inspiração e exemplo de vida para que minhas metas sempre sejam realizadas no tempo certo, direcionando os caminhos com palavras de sabedoria. Aos meus irmãos Lisa e Douglas Villani, pela confiança e admiração conferida, espero não desapontá-los. Aos meus sogros Lili e Marcial que com grande carinho sempre acreditaram na minha dedicação e interesse de concluir mais este trabalho.

Aos meus queridos amigos Janaina Machado, Jennifer Lowe, Renata Vilela, Marcelo Einicker, Carlos Einicker, Adriana Pugliese, Nilson Nunes Tavares e Dra Aída Hassón-Voloch (*in memoriam*, minha primeira orientadora) por estarem presentes e saberem da grande importância de mais etapa na minha vida e que há muito tempo atrás ficou pendente.

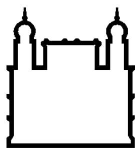
Aos meus companheiros e amigos do Laboratório de Farmacologia Aplicada Tatiana Pádua, Maria Fernanda Souza, Magaiver Andrade, Fátima Vergara, André Cadéa, Thadeu Costa, Luana Correa, Leonardo Seitto, Érika Cunha e Márcia Rami pela compreensão, colaboração e contribuição nestes quatro anos seja em experimentos, no suporte psicológico, cada qual com

sua participação especial. Sem esquecer de minha companheira de trabalho Katelim Hottz que no tempo em que estivemos juntas foi com grande alegria e espírito de equipe que desenvolvemos parte deste projeto.

Às Dra. Patrícia Pacheco, Dra. Mariana Souza e Dra. Elaine Rosas pelo experiência e profissionalismo, neste tempo aprendi muito com vocês.

Às colaborações e parcerias que fiz na Fiocruz que foram importantes para o desenvolvimento do meu trabalho e publicações, cada qual com sua participação e orientação em momentos distintos – Dr. Paulo Ricardo Batista, Dr. Ernesto Raúl Caffarena e Dr. Raghavendra Nulgumnalli Manjunathaiah (experimentos *in silico*), Dr. Franklin Souza-Silva e Dr. Carlos Roberto Alves (experimentos de SPR), Dra. Clarissa Menezes Maya-Monteiro (experimentos de western blotting), Dra. Ana Paula Monteiro e Dr. Alan Brito Carneiro (experimentos de FLICA e inflamassomas), Dr. Dumith Chequer Bou Habib e MSc Jairo Ramos Temerozo (experimentos TLR2 e TLR3) e Dr. Thomas E. Krahe por todo suporte na revisão de nossos artigos.

Ao programa de Pós-Graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz e ao CNPq, CAPES, FAPERJ e FIOCRUZ pelo apoio financeiro.



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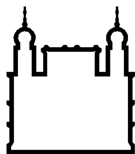
ESTUDO DOS MECANISMOS DE AÇÃO DO TETRANORTRITERPENÓIDE DE ORIGEM NATURAL, GEDUNINA, NA SINALIZAÇÃO DE RECEPTORES TIPO TOLL E INFLAMASSOMA

RESUMO

TESE DE DOUTORADO

Perla Villani Borges da Silva

A ativação dos receptores do tipo toll (TLR) por padrões moleculares associados a patógenos (PAMPs) desencadeia uma resposta imune inata, através da produção de citocinas e da ativação de inflamassoma. Neste trabalho, nós investigamos o efeito da gedunina, um tetranortriterpenóide de origem natural, sobre a ativação de TLR *in vitro* e *in vivo*. O pré e o pós-tratamento de camundongos C57BL/6 com gedunina (i.p.) inibiu a migração de células mononucleares, eosinófilos e neutrófilos para a cavidade pleural estimulada com lipopolisacarídeo bacteriano (LPS), assim como reduziu a produção do fator de necrose tumoral (TNF)-α, interleucina (IL)-6 e óxido nítrico (NO). O pós-tratamento *in vitro* de macrófagos murinos imortalizados com gedunina também modulou negativamente a produção destes mediadores inflamatórios induzida por LPS. O tratamento com gedunina também reduziu a ativação do inflamassoma NLRP3 induzida por LPS em leucócitos pleurais *in vivo* e em macrófagos imortalizados *in vitro.* Além disto, a gedunina inibiu a ativação de caspase-1 e, consequentemente, a produção de IL-1β *in vivo* e *in vitro*, após estímulo com LPS. De forma interessante, além de inibir a produção de mediadores pró-inflamatórios, a gedunina induziu a expressão dos fatores anti-inflamatórios heme oxigenase-1 (HO-1), Hsp70 e IL-10. Também demonstramos que o efeito da gedunina não é restrito à sinalização de TLR4. Esta substância também reduziu a produção de mediadores TNF-α, IL-6, NO, IL-1 β e a expressão de NLRP3, assim como induziu o aumento de IL-10 e HO-1, em macrófagos estimulados com os agonistas de TLR2 e TLR3, PAM3CSK4 e POLY I:C. Estudos de modelagem *in silico* revelaram que a gedunina pode se ligar de forma eficiente a caspase-1, TLR2, TLR3 e ao MD-2 do complexo receptor TLR4. Em conjunto, os nossos dados demonstram que a gedunina modula a respostas mediadas por TLR4, TLR3 e TLR2 e ainda revelam novos alvos moleculares deste tetranortriterpenóide.



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ABSTRACT

TESE DE DOUTORADO

Perla Villani Borges da Silva

Activation of toll-like receptors (TLRs) by pathogen-associated molecular patterns (PAMPs) triggers an innate immune response, via cytokine production and inflammasome activation. Herein, we have investigated the effect of gedunin, a natural tetranortriterpenoid, on the activation of TLR *in vitro* and *in vivo.* Intraperitoneal (i.p.) pre- and post-treatments of C57BL/6 mice with gedunin impaired the influx of mononuclear cells, eosinophils and neutrophils, as well as the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and nitric oxide (NO), triggered by lipopolysaccharide (LPS) in mouse pleura. Accordingly, *in vitro* post-treatment of immortalized murine macrophages with gedunin also impaired LPS-induced production of such mediators. Gedunin treatment also diminished LPS-induced expression of NLRP3 inflamassome in pleural leukocytes *in vivo* and in macrophages *in vitro*. In line with this, gedunin inhibited both LPS-induced caspase-1 activation and IL-1β production *in vivo* and *in vitro*. In addition, gedunin treatment triggered the generation of the anti-inflammatory factors heme oxigenase-1 (HO-1), Hsp70 and IL-10. Here, we also demonstrate that gedunin effect is not restricted to TLR4-mediated response. This compound diminished TNF-α, IL-6, NO and IL-1 β production, reduced the expression of NLRP3, as well as enhanced IL-10 and HO-1 by macrophages stimulated with the TLR2 and TLR3 agonists, PAM3CSK4 and POLY I:C. *In silico* modeling studies revealed that gedunin efficiently docked into caspase-1, TLR2, TLR3 and to MD-2, component of TLR4. Overall, our data demonstrate that gedunin modulates TLR4, TLR3 and TLR2-mediated responses, and reveal new molecular targets for this compound.

LISTA DE SIGLAS E ABREVIATURAS

17-AAG	17-alil amino-17-demetoxi geldanamicina
AP-1	proteína ativadora-1
APS	persulfato de amônio
ASC	*apoptosis-associated speck-like protein containing a CARD*
ATP	adenosina trifosfato
CARD	domínio de recrutamento da caspase
CD14	conjunto de diferenciação 14
Cdc37	ciclo de divisão celular 37
COX-2	ciclooxigenase-2
DMEM	*Dulbecco's modified Eagle*
DMSO	dimetilsulfóxido
DTT	ditiotreitol
ECL	*enhanced chemiluminescence*
EDTA	ácido etileno diamino-tetra acético
EIA	*enzyme immunoassay*
ELISA	*enzyme linked immunosorbent assay*
EPM	erro padrão da média
ERK	*extracelular signal-regulated kinase*
HO-1	heme oxigenase-1
HRP	*horseradish peroxidase*
HSR	resposta de choque térmico
HSF-1	fator de choque térmico-1
Hsp	proteína de choque térmico
IFN	interferon
IgG	imunoglobulina G
IKK	*IκB kinase*
IL	interleucina
iNOS	*inducible nitric oxide synthase*
i.p.	intraperitoneal
i.pl.	intrapleural

IRAK	*Interleukin-1-receptor associated kinase*
IRF3	*Interferon regulatory transcripitional fator 3*
JNK	*C-Jun N-terminal kinase*
KO	*knockout*
LBP	proteína ligante de LPS
LPS	lipopolissacarídeo
LRR	repetições ricas em leucina
MAL	*MyD88-adapter-like*
MD-2	proteína mielóide de diferenciação-2
MyD88	fator de diferenciação mielóide 88
NACHT	*central nucleotide-binding domain*
NLR	receptores do tipo *Nod*
NLRP3	"*nucleotide-binding domain and leucine-rich repeat protein-3*"
NO	óxido nítrico
NP-40	*Nonidet P-40*
NFκB	fator nuclear κB
OPD	orofenilenodiamina
PAM3CSK4	*palmitoyl-3-Cys-Ser-(Lys)4*
PAMP	padrão molecular associado a patógenos
PBS	salina tamponada com fosfato
PG	prostaglandina
POLY I:C	*polyriboinosinic:polyribocytidylic acid*
PRR	receptores padrão de reconhecimento
PVDF	difluor polivinilideno
PYD	domínio pirina
SDS	dodecil sulfato de sódio
SGT1	supressor do alelo G2 de skp1
SPR	ressonância plasmônica de superfície
TAK1	*transforming growth factorβ-activated kinase-1*
TEMED	tetrametiletilenodiamina
TIR	receptor Toll/IL-1
TIRAP	proteína adaptadora contend domínio TIR

TLR	receptor do tipo toll
TNF-α	fator de necrose tumoral
TRAM	molécula adaptadora relacionada à TRIF
TRIF	*TIR-domain-containing adapter-inducing interferon-beta*
TRAF	fator associado ao receptor de TNF
Tris	trisaminometano
WT	*wild type*

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1. INTRODUÇÃO

1.1 Padrões de reconhecimento e sinalização de TLR em macrófagos

O sistema imune inato representa a primeira linha de defesa contra patógenos e sua resposta é mediada por células fagocíticas e por células apresentadoras de antígenos (APC), tais como granulócitos, macrófagos e células dendríticas. Esta resposta não específica contra patógenos depende do reconhecimento de estruturas conservadas distintas, denominadas padrões moleculares associados a patógenos (PAMPs), e se dá através de um número limitado de receptores padrão de reconhecimento (PRRs), tais como receptores do tipo toll (TLRs), receptores do tipo *Nod* (NLR) citoplasmático e receptor do tipo gen I induzido por ácido retinóico (RLRs) (1, 2). Dentre os receptores padrões de reconhecimento, podemos destacar o TLR, que são receptores sentinelas do sistema imune inato descobertos no final da década de 1990 em mosca-da-fruta do gênero *Drosophila* (3, 4). Vale ressaltar que a importante descoberta do gene toll rendeu um prêmio nobel de medicina em 2011 para Bruce Beutler e Jules Hoffman.

Os TLRs são proteínas transmembranares do tipo I, presentes em uma variedade de populações celulares e são compostos por diferentes domínios (5, 6). Dentre os principais domínios constituintes do TLR podemos destacar: um extracelular rico em leucinas (LRRs), um transmembranar e um intracelular com alta semelhança com o receptor IL-1 humano, essencial à sua sinalização, conhecido como TIR (domínios de resistência a Toll/IL-1) e que é conservado entre todos os membros da família de receptores Toll (1, 7). Os TLRs podem ser encontrados em compartimentos celulares distintos, presentes na porção extracelular da bicamada lipídica ou na porção intracelular (8). Até a presente data já foram caracterizados 10 diferentes tipos de TLR em humanos (1 ao 10) e 11 em murinos, sendo estes receptores então divididos em dois grupos, caracterizados por sua localização e especificidade: i) os presentes na superfície celular, TLR1, TLR2, TLR4, TLR5, TLR6 e TLR11, que reconhecem principalmente componentes bacterianos, como lipopeptídeos, lipoproteínas, lipopolissacarídeos e componentes fúngicos e ii) os expressos em vesículas intracelulares como retículo endoplasmático (RE), endossomas, lisossomas e endolisossomas, TLR3, TLR7, TLR8 e TLR9, que reconhecem ácidos nucléicos microbianos, tais como DNA dupla fita, RNA fita simples e DNA oligodesoxinucleótidos CpG (cadeias curtas de DNA). Além disto, os TLRs também são capazes de reconhecer padrões moleculares associados a dano (DAMP), que são

moléculas liberadas por células em situações de estresse, como as proteínas de choque térmico (“heat shock proteins”, Hsps) (9).

Quando ativados, os TLRs recrutam moléculas adaptadoras intracelulares, contendo domínios TIR, sendo descritas quatro moléculas adaptadoras que orquestram esta sinalização intracelular. Essas proteínas incluem a MyD88 (*Myeloid differentiation primary response gene 88*) (10), a MAL (*MyD88 adaptor-like*), também conhecida como TIRAP (TIR *domain-containing adaptor protein*) (11, 12), a TRIF (TIR *domain-containing adaptor inducing* IFN-β) (13, 14) também conhecida como TICAM-1 (TIR *domain-containing adaptor molecule-1*) e a TRAM (TRIF-*related adaptor molecule*) ou TICAM-2 (15-17). A sinalização de TLR está dividida em duas vias intracelulares distintas: i) via dependente de MyD88, utilizada pela maioria dos TLRs com exceção de TLR3, e ii) via independente de MyD88, que compreendem a via de TLR3 e a via tardia de TLR4 (18). A via dependente de MyD88 se dá com a associação desta com a proteína MAL, que recruta e ativa proteínas quinases IRAK1 e IRAK4, que então interage com TRAF6, ativa TAK1 (*transforming growth factorβ-activated kinase-1*) e se associa à proteína TAB (proteína ligante de TAK). A proteína TAB1 então ativa o complexo IKK, que fosforila IκB e permite a translocação de NF-κB, antes inativo no citoplasma, para o núcleo da célula. Este por sua vez induz a transcrição de genes associados à resposta inflamatória, tais como o do TNF-α (fator de necrose tumoral), das interleucinas (IL)6, IL-1β, do interferon gama (IFN-γ), de quimiocinas, de moléculas de adesão e de peptídeos antimicrobianos (β-defensinas, catelicidinas). TAK1 também ativa membros da família de *mitogen-activated protein* (MAP) quinases, como JNK, proteína quinase ativada por mitógeno (p38) e ERK, que levam à ativação de outro fator transcricional, AP-1, que induz a transcrição de citocinas pró-inflamatórias (FIGURA 1.1) (19-20).

As proteínas de choque térmico são chaperonas intracelulares envolvidas na manutenção de funções celulares e apresentam papel citoprotetor quando as células são expostas a algum tipo de estresse. Além disto, estas proteínas participam da resposta imune inata, tal como a Hsp70, que ao ser liberada para o meio extracelular é reconhecida principalmente por TLR2 e TLR4 em células apresentadoras de antígeno, exercendo efeitos imunoreguladores, participando desta forma da resposta imune inata (21). A via de sinalização destes TLRs sob situação de estresse físico e oxidativo, por exemplo, pode resultar na ativação de NADPH oxidase e na produção de espécies reativas de oxigênio (ROS) em neutrófilos e macrófagos (22, 23).

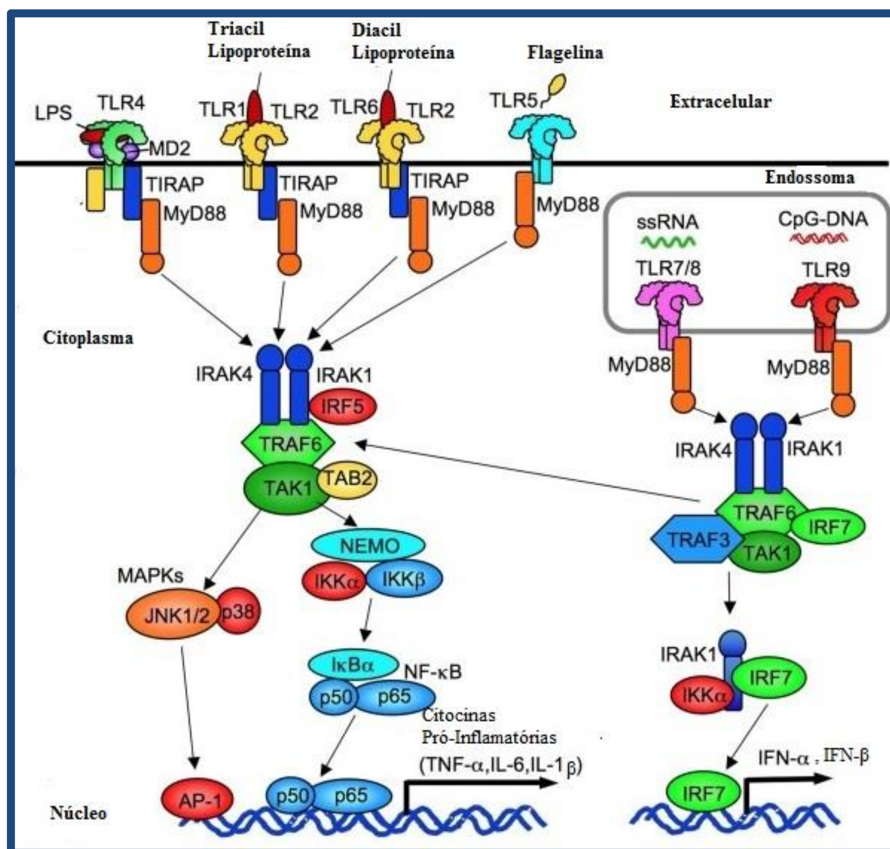

Figura 1.1. Via de sinalização de TLR dependente de MyD88. A sinalização de TLR1, -2, -4, -5, ou -6 dependente de MyD88 se inicia pela ligação de agonistas, que resulta na ativação de vias IKK e MKK. Cada uma destas vias induz a expressão de citocinas pró-inflamatórias, como TNF-α, IL-6, e IL-1β, pelos fatores de transcrição NF-kB e AP-1. Além disto, TLR7/8 e TLR9 endossomais ativam as mesmas vias, levando à expressão de IFNs do tipo I (IFN-α e IFN-β), mediada por IRF7, em adição a outras citocinas pró-inflamatórias induzidas por NF-κB e AP-1. Adaptado de Yang & Seki, 2012 (2).

A via de sinalização independente de MyD88, induzida por TLR3 e pela via tardia de TLR4, leva ao recrutamento de uma proteína adaptadora TRIF, que resulta na dimerização e na ativação do inibidor de quinase NF-κB (IKKi) e da quinase de ligação do ativador de NF-κB associado ao membro da família TRAF (TBK1). Dados da literatura demonstram que TRIF recruta TRAF3 para ativar TANK (membro da família TRAF associado a ativação de NF-κB)/TBK1/IKKi, que são importantes na dimerização e translocação do fator de transcrição IRF3. IRF3 junto com NF-κB ativam a transcrição de genes relacionados ao interferon do tipo 1 (FIGURA 1.2) (24).

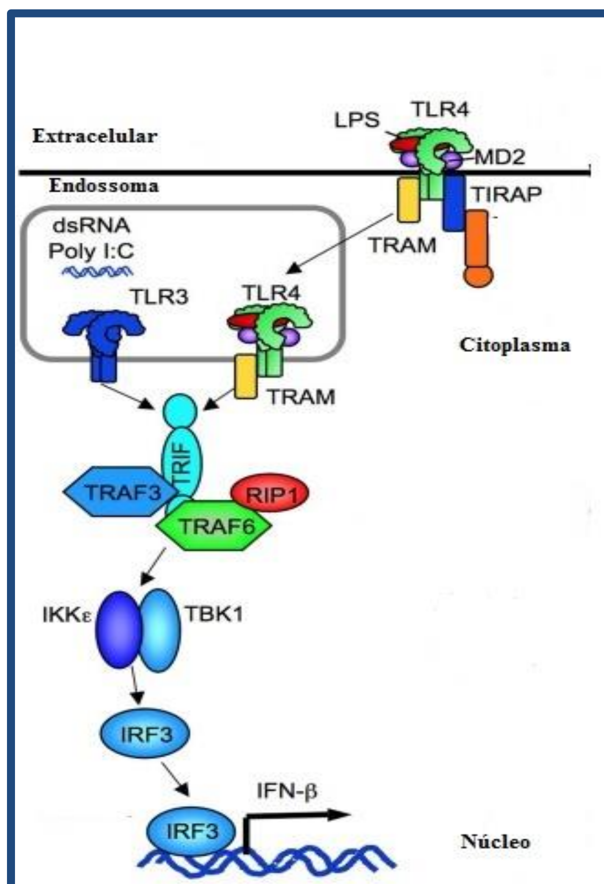

Figura 1.2. Via de sinalização de TLR independente de MyD88. A via independente de MyD88 é induzida por TLR3 endossomal e pela via de sinalização tardia de TLR4. O reconhecimento de agonistas leva ao deslocamento de TRIF, que irá mediar o recrutamento de TRAF3 e RIP1, com consequente ativação do fator de transcrição IRF3, NF-κB ou AP-1, e consequente expressão de IFNs do tipo 1. Adaptado de Yang & Seki, 2012 (2).

Vale ainda ressaltar que estudos cristalográficos de TLRs junto aos seus ligantes demonstraram que estes receptores são capazes de formar heterodímeros entre si, como por exemplo: TLR1/TLR2 e TLR2/TLR6. Estes heterodímeros são especialmente importantes para o reconhecimento de lipopeptídios e lipoproteínas de bactérias gram positivas. Além disso, tem-se também a formação de homodímeros, tais como TLR3/TLR3 que reconhecem RNA de fita dupla. Também foi demonstrado que para a sinalização de TLR4 é importante a sua interação com moléculas acessórias, como MD2 (fator mielóide de diferenciação-2) e CD14 (conjunto de diferenciação 14), para que ocorra a formação de seu homodímero (26).

1.1.1 Via de sinalização de TLR4

A especificidade de reconhecimento do TLR a estruturas microbianas conservadas inclui o reconhecimento de lipopolissacarídeo bacteriano (LPS), endotoxina presente na membrana externa de bactérias Gram-negativas, pelo TLR4, que é majoritariamente expresso por macrófagos (27-30). O reconhecimento do LPS por macrófagos depende de um complexo receptor que compreende (FIGURA 1.3): i) TLR4; ii) duas glicoproteínas solúveis secretadas MD-2 (29) - que funciona como um co-receptor extracelular do TLR4 e iii) o CD14 - proteína

encontrada tanto na forma solúvel quanto ancorada na superfície de células pelo glicosilfosfatidilinositol (31). Este reconhecimento se dá inicialmente pela ligação do LPS à proteína ligante de LPS (*LPS-binding protein*, LBP) presente no soro, formando complexos de alta afinidade. Em seguida, a LBP transfere o LPS ao seu receptor CD14, que finaliza a sequência de transferência para o complexo TLR4/MD-2 (10, 32-37). Vale ressaltar que as chaperonas Hsp90 e Hsp70, juntamente com o CD14, estão presentes constitutivamente em *lipid rafts* na superfície celular fazendo parte deste complexo de receptores, formado após estímulo com LPS (173, 174). Na ausência de LPS, o MD-2 forma um heterodímero com o domínio extracelular do TLR4, apresentando uma estrutura similar à de uma ferradura que, após a ligação do LPS ao complexo, induz a dimerização do receptor, composta por TLR4, MD-2 e LPS, na proporção de 2:2:2, e que leva à subsequente ativação da cascata de sinalização (38-40). O LPS é composto por três componentes: o lipídio A (porção principal hidrofóbica do LPS que responde pela maior parte dos efeitos biológicos imunomoduladores e que se liga ao MD-2), o núcleo de oligossacarídeo e o antígeno O (41, 42).

O reconhecimento do LPS pelo complexo TLR4 leva ao recrutamento das proteínas adaptadoras MAL/MyD88 e TRAM/TRIF, que se associam a este receptor através de interações homofílicas entre os domínios TIR presentes no TLR4 e nas proteínas adaptadoras, que em resposta leva à ativação de duas vias de sinalização distintas, que apresentam cinéticas sequenciais, denominadas via aguda e via tardia, conforme demonstrado na FIGURA 1.3 (2, 43). Inicialmente, TLR4 recruta MAL/MyD88, que leva à ativação da via aguda, e é então endocitado e entregue a vesículas intracelulares, para então formar um complexo com as proteínas adaptadoras TRAM/TRIF, levando à ativação da fase tardia (5-7). Estas duas etapas de sinalização são requeridas para translocação significativa de NF-kB e ativação de (MAP) quinase, com consequente indução de citocinas pró-inflamatórias como TNF-α, IL-6, IL-1β, como também de óxido nítrico sintase induzida (iNOs) e de IFN do tipo I (15, 44-47). A superprodução destes mediadores durante a infecção pode levar ao dano tecidual, disfunção múltipla dos órgãos e choque séptico (48-51).

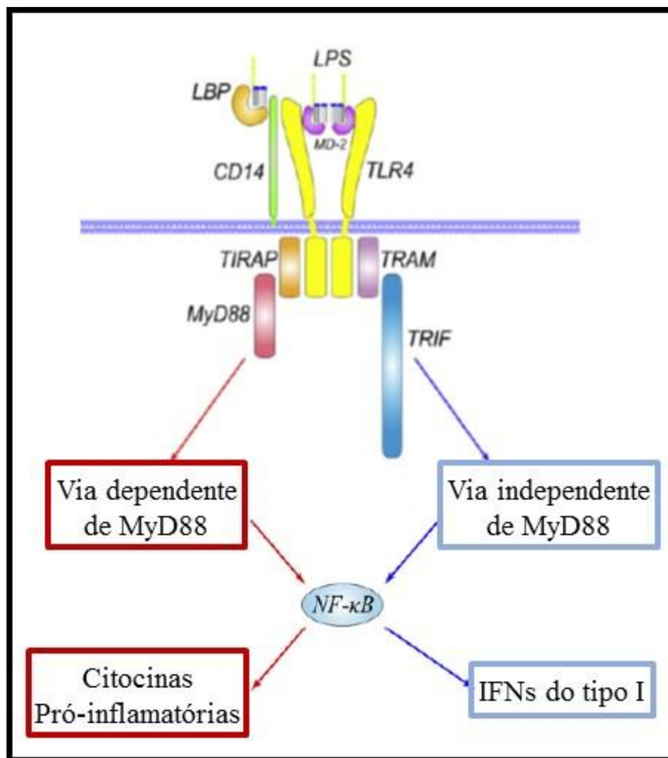

Figura 1.3. Via de sinalização de TLR4. Sinalização de TLR4 após o reconhecimento de LPS, via LBP e CD14, mediada por MD-2. Vias de sinalização dependente de MyD88 (aguda) e independente de MyD88 (tardia), levam à produção de mediadores pró-inflamatórios e de interferon do tipo I, respectivamente.
Adaptado de Yong-Chen Lu, Wen-Chen Yeh, 2008 (25).

1.1.1.1 Indução do LPS na ativação de macrófagos

Diferentes tipos celulares reconhecem o LPS que leva à indução da produção de diversos mediadores inflamatórios. Dentre estes tipos celulares capazes de reconhecer o LPS, podemos destacar os macrófagos, leucócitos que apresentam um papel central na resposta inflamatória e na regulação da resposta imune (52). Os macrófagos são células centrais que participam do sistema imune inato, por serem versáteis no reconhecimento e resposta a uma ampla variedade de estímulos, por expressarem diferentes receptores de superfície e intracelulares, além de participarem da resposta imune adapatativa, atuando como células apresentadoras de antígenos (53). Estas células quando ativadas possuem um alto potencial endocítico, fagocítico e secretor, sendo capazes de modular outros tipos celulares, através da produção de mediadores pró-inflamatórios, como por exemplo TNF-α, IL-1β, IL-12, IL-6, o NO e prostaglandinas e anti-inflamatórios como por exemplo a IL-10 que desempenham diferentes papéis ao longo da resposta inflamatória (53, 57). Podem ser caracterizados fenotipicamente em: i) macrófagos M1, resposta Th1, classicamente ativados que produzem mediadores pró-inflamatórios e que regulam a defesa do hospedeiro e a imunidade antitumoral, e ii) macrófagos M2, resposta Th2, alternativamente ativados que produzem mediadores anti-inflamatórios e que apresentam uma resposta supressora ou reguladora da imunidade (53).

A produção de mediadores inflamatórios por macrófagos estimulados com LPS tem papel fundamental em inúmeros processos, como por exemplo o TNF-α que modula, por exemplo, a produção de outras citocinas, a expressão de moléculas de adesão, proliferação celular e diferenciação e, consequentemente, contribui para processos fisiopatológicos, como o choque séptico (54). A produção da citocina IL-6, pelo macrófago ativado, também está envolvida em diversas doenças inflamatórias, tem papel importante na indução da febre (via hipotálamo), participa da migração e ativação de leucócitos, como também da proliferação e diferenciação de linfócitos B e T, além de induzir hepatócitos na liberação de proteínas de fase aguda (como proteína C reativa, fibrinogênio) (54). Assim como as citocinas pró-inflamatórias, o macrófago ativado induz a produção de NO, intermediário reativo de nitrogênio, via enzima sintase de óxido nítrico induzível, iNOS, que é induzida por ativação de TLR em resposta a PAMPs, como o LPS, principalmente na presença de IFN-γ. O NO é um radical livre de ação citotóxica e antimicrobiana, além de potente vasodilatador da musculatura lisa (55). Outro importante mediador produzido durante a resposta inflamatória inicial é a PGE_2 (prostaglandina E_2), um eicosanóide de ação vasodilatadora, sintetizado a partir do ácido araquidônico, pela ação da enzima ciclo-oxigenase (COX)-2, com função reguladora de dor, febre e facilitador do influxo de leucócitos no local inflamado, o que causa edema (56). Dentre os mediadores anti-inflamatórios produzidos pelos macrófagos estimulados com LPS podemos destacar a IL-10, que é um regulador importante da resposta inflamatória, que atua na inibição da produção de citocinas pró-inflamatórias, quimiocinas (como CCL2, CCL3, CCL5, CCL11) e de receptores de quimiocinas (57). Modelos experimentais de resposta inflamatória induzida pelo LPS se caracterizam pela atração de leucócitos para o foco inflamatório, com o influxo inicial de neutrófilos, seguido pelo acúmulo de eosinófilos e células mononucleares durante a fase posterior da reação. Esta resposta inflamatória é mediada principalmente por macrófagos residentes, que são as principais células que reconhecem o LPS, e que através da produção de fatores quimiotáticos levam então ao recrutamento destas ouras populações celulares, como neutrófilo e eosinófilos (58).

Vale ressaltar que outros PAMPs, além do LPS, podem ativar a via de sinalização de TLR4 como proteína de fusão (F) de vírus sincicial respiratório (RSV) e o envelope protéico de vírus de tumor mamário de ratos (MMTV) (59). Em adição, moléculas endógenas como proteínas de choque térmico (Hsps), ácido hialurônico e β-defensina 2 também podem interagir direta ou indiretamente com TLR4 (60-62).

1.1.2 Vias de sinalização de TLR2 e TLR3

O TLR2 reconhece uma grande variedade de PAMPs de leveduras, bactérias Gram-positivas e Gram-negativas, sendo expresso em células imune, endoteliais e epiteliais. Além disto, é o único receptor que forma heterodímeros com outros receptores do tipo toll, como TLR1 ou TLR6. A estrutura cristalográfica de ambos heterodímeros TLR2/TLR1 e TLR2/TLR6 demonstram que o ectodomínio rico em leucina (LRR), além de ser responsável pelo reconhecimento de patógenos, confere uma estrutura em forma de "ferradura" quando não complexados com o ligante e se apresentam na forma de "m" quando complexados, estabilizando o receptor (64, 64).

Os principais ligantes de TLR2 compreendem frações de diacil e triacil de lipoproteínas/lipopeptídeos (LPs), altamente expressas na membrana externa de bactérias Gram-positivas. As frações de triacil LPs são reconhecidas majoritariamente pelo heterodímero TLR2/TLR1, enquanto que frações de diacil LPs pelo heterodímero TLR2/TLR6. Dentre os agonistas de TLR2/TLR1 mais amplamente estudados, podemos destacar o PAM3CSK4 "palmitoyl-3-Cys-Ser-(Lys)4", que é um lipopeptídeo triacilado, enquanto que PAM2CSK4 "palmitoyl-2-Cys-Ser-(Lys)4" é um lipopeptídeo diacilado e agonista de TLR2/TLR6, e ambos mimetizam o terminal amino acilado de lipoproteinas bacterianas (65). Apesar dos ligantes determinarem diferentes cinéticas, suas vias de sinalização são comuns, independente de qual heterodímero seja ativado (66-67). A diferença cinética se dá pelo ligante, como por exemplo os ligantes de diacil LPs precisam de CD36 para se ligar e desta forma serem transferidos para CD14, que por sua vez os transfere para o heterodímero TLR2/TLR6 ou TLR2/TLR1 e assim formam o complexo de ligação com o TLR2. Este complexo de transferência do ligante ocorre dentro de *lipid rafts* na membrana, aos quais CD36 e CD14 estão ancorados (68). Porém, o CD36 não é sempre requerido para a transferência do ligante para o CD14, principalmente quando este ligante é um triacil LPs. Neste caso, o reconhecimento do ligante para sua transferência ao CD14 é feito pela vitronectina (glicoproteína de matriz extracelular presente no sangue) que permite a formação do complexo TLR2/TLR1 com o LPs (69). Seguindo a formação do complexo TLR2 / ligante, o heterodímero geralmente inicia a cascata de sinalização via proteína adaptadora MAL/MyD88, que desencadeia a transcrição de genes relacionados à inflamação, via NFκB e proteína ativadora-1 (AP-1), e consequente produção de citocinas pró-inflamatórias (70, 71).

Os receptores intracelulares do tipo TLR3 e TLR7 representam os principais receptores

da resposta imune desencadeada por infecções virais. O TLR3 reconhece ácidos nucléicos virais de fita dupla de RNA (dsRNA), principal intermediário de replicação viral, enquanto que o TLR7 reconhece fita simples de RNA (ssRNA) restrita a alguns tipos de vírus (72). Dentre os principais agonistas de TLR3 utilizados experimentalmente, podemos destacar um análogo sintético de dsRNA que é o POLY I:C "*polyriboinosinic:polyribocytidylic acid*", que está comumente associado à infecção viral e induz uma resposta imune anti-viral. Em adição ao reconhecimento de POLY I:C, TLR3 também reconhece RNAs genômicos de reovírus, dsDNA produzidos pela replicação de ssRNA, vírus sincicial respiratório, vírus da encefalomiocardite, vírus do Oeste do Nilo e pequenos RNAs de interferência (73).

O reconhecimento de dsRNA se dá no retículo endoplasmático de células não-estimuladas pelo TLR3, através de seu ectodomínio que tem a forma de ferradura, e que permite uma maior superfície de contato do receptor com o agonista. O dsRNA é reconhecido por dois sítios distintos da cadeia lateral convexa de TLR3, no seu domínio N e C terminal, que após o estímulo sofre dimerização e transita para o endossoma por um mecanismo dependente de pH (73-74). A dimerização de TLR3 e a fosforilação da tirosina do seu domínio citoplasmático no endossoma leva então ao recrutamento de proteínas adaptadoras TRAM/TRIF, que induz a ativação de fatores de transcrição de interferon do tipo I (IFNs) e outras citocinas, via fator regulatório de interferon 3 (IRF3), NFκB e AP-1 (75). A potencialização da produção de IFNs mediado por IRF3 e o aumento da expressão de TLR3, em células epiteliais respiratórias, pode ser mediada pela geração de espécies reativas de oxigênio (ROS), que são induzidas rapidamente por infecções virais (76-77). A modulação destes efeitos mediados por estresse oxidativo induzido por infecções virais, sobre a via de sinalização de TLR pode ser um alvo terapêutico para estas diversas doenças, inclusive de doença pulmonar obstrutiva crônica (2).

Dados da literatura têm demonstrado que a via de sinalização de TLR, induzida por seus respectivos agonistas, representa o primeiro sinal necessário para a ativação da sinalização de NLRs (receptores do tipo *Nod*) e que permitem a atividade de inflamassomas. Este primeiro sinal desencadeado pela via de sinalização de TLR é dependente da translocação nuclear de NFκB e consequente produção de pró-citocinas pró-inflamatórias como pró-IL-1 e pró-IL-18 (não induzido), necessárias para via do inflamassoma. Vale ressaltar que a integração da via de sinalização de TLR com a de NLRs representa um importante mecanismo de prevenção de respostas desreguladas associadas aos inflamassomas, como doenças inflamatórias hereditárias e adquiridas, desta forma a modulação integrada destas vias representam importantes alvos

farmacológicos para fármacos anti-inflamatórios (78-79, 82).

1.2 Inflamassomas

Inflamassomas são complexos protéicos oligoméricos intracelulares que são importantes componentes da imunidade inata e que estão estreitamente associados à detecção intracelular de patógenos ou de produtos microbianos que eventualmente conseguem chegar ao interior da célula. Sabe-se que existem inflamassomas distintos, cada qual ativado por diferentes estímulos, porém os principais sinais comuns requeridos para a formação do complexo são: i) via ativação de TLR, através de PAMPs dentre os quais se incluem LPS, RNA viral e bacteriano, que aumentam a transcrição de IL-1β; e ii) através da detecção de seus agonistas, como espécies reativas de oxigênio em estresse metabólico.

Os NLR são constituídos principalmente por um domínio efetor e variável N-terminal contendo pirina (PYD), um domínio de recrutamento e ativação de caspase (CARD), e um domínio de nucleotídeo NACHT (também conhecido como domínio NOD) seguido de um domínio C-terminal de repetições ricas em leucina (LRRs). Os NLRs estão envolvidos no reconhecimento de padrões moleculares associados a patógenos ou de sinais associados ao hospedeiro e/ou ao ambiente. Dos 22 membros da família NLR podemos destacar NLRP1, NLRP3 e NLRC4 que sob ativação formam um complexo conhecido como inflamassoma. NLRP3 é ativado por uma ampla variedade de patógenos, como produtos bacterianos e RNA viral e é um dos inflamassomas mais bem caracterizados. A sua ativação pode também se dar por DAMPs, o que indica uma clara correlação entre o sistema imune inato e a resposta fisiológica relacionada ao stress celular e dano (78). A estrutura de NLR determina sua terminologia conforme FIGURA 1.4, existindo por exemplo: NLRP3 *(nucleotide-binding domain and leucine-rich repeat protein-3*), que é o receptor do tipo NOD (NLRP) que possui o domínio PYD (pirina) na porção N-terminal e NLRC4, que possui o domínio efetor CARD na sua porção variável N-terminal (79). Direcionamos nosso estudo ao inflamassoma induzido NLRP3, também conhecido como criopirina, que é o único dentre os NLRs cuja expressão basal não é suficiente para ativação de inflamassoma. Na grande maioria dos casos a ativação dos receptores do tipo NOD leva à liberação de caspase-1 ativada, que pode ser por uma via dependente da proteína adaptadora ASC (*apoptosis-associated speck-like protein containing a CARD)* ou não, sendo que o NLRP3 necessita de ASC para esta ativação (80).

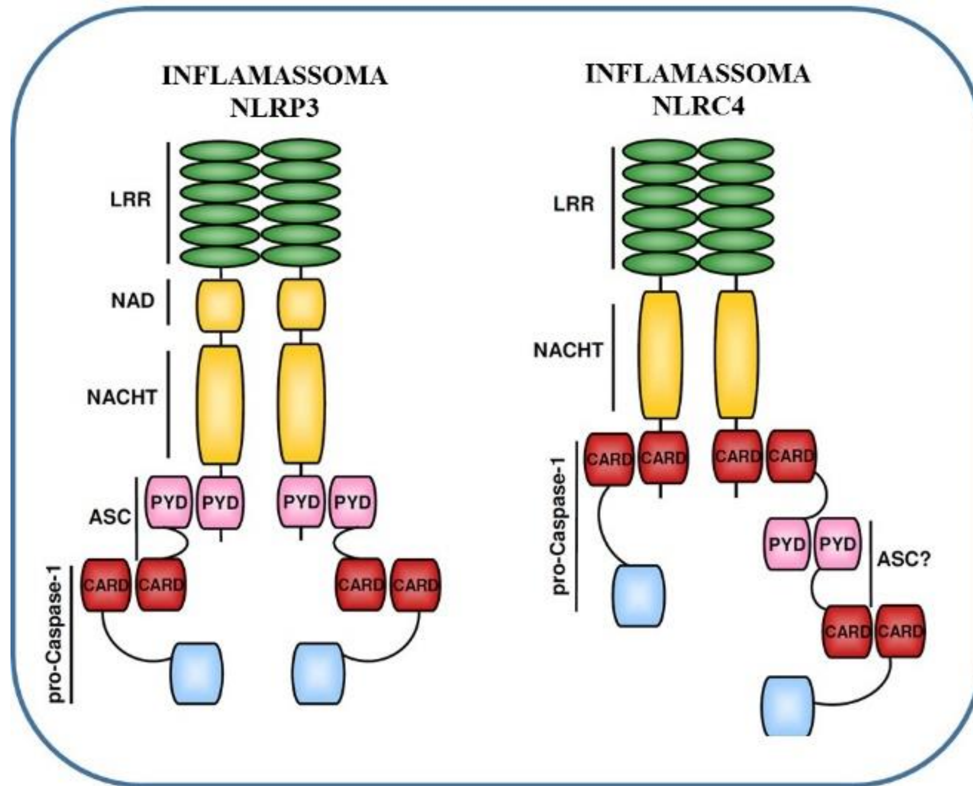

Figura 1.4. Inflamassoma NLRP3 e NLRC4 – O inflamassoma NLRP3 consiste nos domínios LRR (repetições ricas em leucina), NACHT (domínio de ligação ao nucleotídeo), ASC (proteína adaptadora contendo pirina (PYD), CARD e caspase-1. O inflamassoma NLRC4 pode interagir diretamente com pró-caspase 1, podendo ou não requerer ASC para tal interação. Seus domínios consistem em LRR, NACHT, CARD e PYD. Adapatado de Eitel et al., 2011 (181).

1.2.1 NLRP3

O inflamassoma NLRP3 é associado a diversas doenças infecciosas, autoimune e síndromes auto-inflamatórias. A formação do NLRP3 é induzida por diversos estímulos como por exemplo ATP extracelular, glicose, ácido úrico, β-amilóide e ocorre após a oligomerização de NLR, via acoplamento da molécula adaptadora ASC, proteína composta de um domínio de pirina (PYD) e domínio de recrutamento e ativação de caspase-1 (CARD) (81, 82). A região promotora deste complexo ativado contém um sítio de ligação para AP-1, e sua expressão é aumentada após translocação nuclear de NFκB. O complexo NLRP3 então formado leva à auto-clivagem de caspase-1 o que resulta na sua dimerização, consequente proteólise de pró-IL-1β e pró-IL18 e subsequente liberação de IL-1β e IL-18 (79-80). Ao contrário da pró-IL18 que é constitutiva e que a sua expressão pode ser aumentada após ativação celular, a transcrição de pró-IL-1β só ocorre via translocação de NF-κB para o núcleo (83).

1.2.1.1 Regulação de NLRP3

A formação do complexo de inflamassoma NLRP3 pode ser desencadeada por diversos estímulos, incluindo moléculas associadas a patógenos, situações de estresse celular, ativação por fluxo de íons, estresse oxidativo e autofagia (79). A primeira via de regulação de NLRP3 se dá pela indução transcricional que se inicia pelo reconhecimento de moléculas microbianas como LPS, PAM3CSK4 e CpG, pelos receptores do tipo toll, etapa esta importante e que se constitui na expressão dos componentes que serão utilizados na montagem e ativação do inflamassoma, como pró-caspase-1, pró-IL-1β e o próprio NLRP3. A segunda via é um dos principais reguladores da formação de inflamassomas desencadeado pelo sinal extracelular de ATP (via receptor $P2X_7$), assim como por moléculas formadoras de poros, tais como toxinas bacterianas. Este sinal se caracteriza por efluxo de íons, sendo a sua ativação dependente de baixas concentrações de K^+ intracelular, que promovem a formação do complexo ASC, conforme FIGURA 1.5. Outro modelo de regulação e ativação de NLRP3 está baseado em estresse oxidativo, através da produção de espécies reativas de oxigêncio (ROS) que podem ser geradas tanto pela sinalização de ATP extracelular via NADPH-oxidase durante a fagocitose, quanto pela mitocôndria. Além disto, as ROS podem desencadear outro fluxo de íons neste processo de ativação de NLRP3, que é o influxo de Ca^{+2} (78, 82).

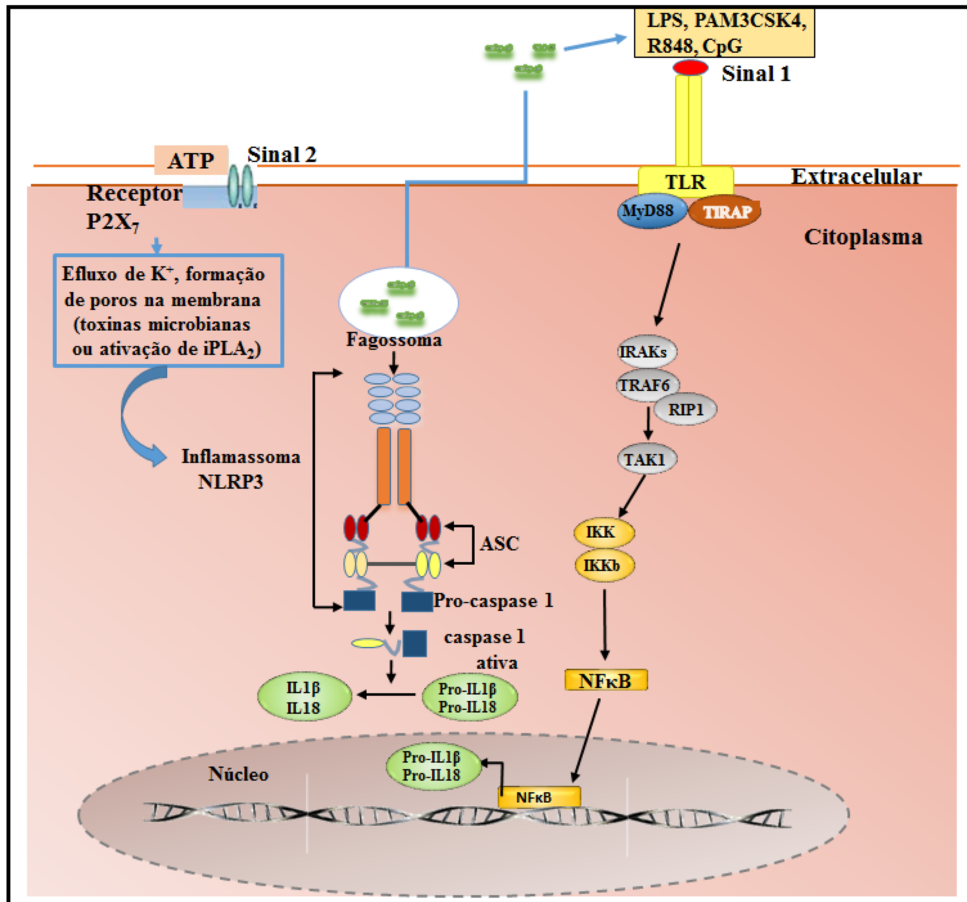

Figura 1.5. Inflamassoma, ativação de caspase-1 e liberação de IL-1β. Via 1: Estímulo de macrófagos por agonistas de TLR (LPS, dinucleotídeos CpG e lipopeptídeos PAM3CSK4) induzem a síntese de pró-ILβ. Este sinal então dispara uma segunda resposta que estimula ativação de caspase-1, lise de pró-IL1β, pró-IL-18, NLRP3 e consequente liberação de citocinas IL1β e IL-18. **Via 2**: Ocasionado pelos principais agentes de alterações iônicas na célula como: efluxo de K^+, ATP via receptor purinérgico $P2X_7$, agentes formadores de poros na membrana (nigericina – ionóforo de K^+), que agem via ativação de fosfolipase A2 independente de cálcio ($iPLA_2$) como mediadores do processamento de IL-1β. Adaptado de Mariathasan e Monack, 2007 (78).

A terceira via de regulação da formação do complexo de inflamassoma se dá através do processo catabólico de autofagia, que ocorre pela remoção de moléculas de pró-IL-1β ou pela prevenção da liberação de ROS pela mitocôndria. Além disto, a autofagia também pode regular a apoptose, que constitui um outro modo de regulação de inflamassoma, pois regula a ativação ou liberação de IL-1β (80, 84).

Por último, um outro tipo de regulação da ativação de inflamassoma, que vem sendo descrito na literatura, são os reguladores protéicos do hospedeiro, que formam plataformas de sinalização e que contribuem para a resposta de uma maneira geral. Dentre estes podemos destacar as proteínas de choque térmico (Hsps), como a Hsp90 e sua co-chaperona ubiquitina

ligase associada à proteína SGT1 (supressor do alelo G2 de skp1) (79-80, 107).

A ativação de inflamassoma NLRP3 é altamente dependente da concentração de seus agonistas. A formação de NLRP3 é consequente de sua oligomerização em um único complexo citosólico que contrasta com a maioria das sinalizações via receptor de superfície celular, como os TLRs. Os inflamassomas, além de terem uma localização citossólica estratégica, têm como principal característica a participação da resposta contra patógenos e moléculas endógenas, sendo, desta forma, um importante alvo para o tratamento de doenças não-infecciosas relacionadas à inflamação crônica, tais como artrite, obesidade, diabetes do tipo 2, aterosclerose e doenças neurodegenerativas (79-82).

Vale ressaltar que, mesmo que diferentes funções do inflamassoma venham sendo descritas desde 2002, a sua principal característica é a produção e a liberação de IL-1β e IL-18 no meio extracelular (81). A produção destas citocinas têm importantes papéis na resposta imune inata, visto que a IL-18 é uma citocina produzida por macrófagos e que induz a produção de IFN-γ em células T. A liberação de IFN-γ também é responsável pelo recrutamento de neutrófilos para o tecido inflamado e que, em conjunto, ajudam a restringir os patógenos intracelulares (79). Em adição, a IL-1β é um dos mediadores pró-inflamatórios solúveis mais produzidos durante a resposta inflamatória por diversos tipos celulares, principalmente por macrófagos. O reconhecimento desta citocina se dá via receptor de IL-1, amplamente distribuído por células do sistema imune e que induz a expressão de vários genes pró-inflamatórios, incluindo a IL-6, IL-8, IL-12, COX-2, quimiocinas, peptídeos antimicrobianos e a própria IL-1β (80). O efeito das citocinas IL-1β e IL-18 e de outros fatores endógenos, sugerem uma forte ligação entre a ativação de inflamassoma e o sistema imune adaptativo, tendo em vista que atuam via diferenciação de células T (84).

Além da produção de citocinas, a ativação de inflamassomas leva a um fenômeno de morte programada na célula hospedeira, chamada piroptose (80). Este processo é desencadeado por caspase-1 ativada que induz a formação de poros na membrana resultante de pressão osmótica, é caracterizado pela rápida liberação de conteúdo citossólico e principalmente descrito em macrófagos e células dendríticas (78-80). Isto demonstra que inflamassomas podem atuar de modo independente de caspase-1, IL-1β e IL-18, intervindo na resposta imune por vias alternativas, para a restrição intracelular bacteriana (84). É importante ressaltar que os inflamassomas apresentam a capacidade de auto-inibição quando o patógeno está presente, prevenindo sinais pró-inflamatórios inapropriados e prejudiciais. Esta autoinibição é mediada

pelo domínio LRR (que reconhece padrões moleculares específicos) dos NLRs, que bloqueia a sua oligomerização por um mecanismo ainda desconhecido (85).

Considerando os inflamassomas como ferramentas importantes da resposta inflamatória, que é crítica para a efetividade do sistema de defesa, esses complexos proteicos representam um alvo de escolha para a busca de novas terapias promissoras, por um mecanismo de modulação desta importante via (48-51).

1.3 Envolvimento da Hsp90 na via de sinalização de TLR e na ativação de inflamassoma

A proteína de choque térmico (Hsp) 90 é uma chaperona muito abundante nas células eucarióticas e procarióticas (1-3%) e altamente conservada (86-88). Esta chaperona é induzida em células submetidas a diferentes condições de estresse (ex.: calor, frio ou privação de oxigênio) e possui um papel essencial no enovelamento, na estabilização e na ativação de um conjunto de mais de 200 proteínas denominadas "clientes". Muitas destas proteínas clientes estão relacionadas a diversos processos celulares, tais como transdução de sinais (ex.: tirosina quinases, serina/treonina quinases), ciclo celular (ex.: fatores de transcrição, fatores apoptóticos) e transporte intracelular, além de processos homeostáticos e fisiopatológicos (89-92, 175). A Hsp90 desempenha suas funções à custa de adenosina trifosfato (ATP), com auxílio de co-chaperonas, tais como p23 e ciclo de divisão celular (Cdc)37, que são importantes para o reconhecimento de protéinas clientes e para atividade desta chaperona (93, 110-111). Estruturalmente, a Hsp90 é descrita como um homodímero de forma flexível, que contém três diferentes domínios: i) domínio N-terminal ligado ao nucleotídeo, no qual a atividade ATPase está localizada; ii) domínio médio de regulação no qual se ligam as proteínas clientes e as co-chaperonas, e onde ocorre a hidrólise de ATP e iii) domínio C-terminal, responsável pela dimerização da proteína e por sua estabilização (94). São descritas duas isoformas da Hsp90, uma isoforma induzida - Hsp90α - e uma isoforma constitutiva - Hsp90β (95).

Dados publicados na literatura demonstram que a sinalização de TLR4 induzida por LPS em macrófagos é modulada em parte por Hsp90, uma vez que a inibição seletiva da sua atividade inibe a translocação nuclear do fator de transcrição NFκB e a produção das citocinas TNF-α e IL-1β (96-98). Foi proposto que Hsps são importantes chaperonas envolvidas na sinalização de TLR4, por manter a integridade estrutural do complexo receptor multimérico TLR4/MD-2/CD14 durante reconhecimento do LPS, e também por regular membros de MAP quinases que fazem parte da sinalização (99-100). Em relação ao seu envolvimento no

complexo receptor de TLR4, foi sugerido que o LPS é transferido do CD14 para as chaperonas Hsp90 e Hsp70, que se apresentam constitutivamente na membrana plasmática em *lipid rafts* e que fazem parte deste complexo de receptores. Destes, o LPS é então transferido para o sítio hidrofóbico do MD-2, se ligando por interações hidrofóbicas (37-38, 100). Dentre as proteínas clientes de Hsp90 envolvidas na cascata de sinalização do TLR4 encontram-se proteínas quinases, tais como ERK (*extracelular signal-regulated kinases*), IRAK (*interleukin-1-receptor associated kinase*), JNK (*C-Jun N-terminal kinase*), IKK (*IκB kinase*), p38 e TAK1 (*TGF-β activated kinase 1*), fatores de transcrição, tais como IRF3 (*Interferon regulatory transcripitional fator 3*) e proteínas reguladoras do ciclo celular que desempenham papéis fundamentais em doenças neurodegenerativas, infecções, câncer e respostas inflamatórias. Em conjunto, estes dados evidenciam a Hsp90 como um alvo promissor para abordagens terapêuticas para diferentes patologias (101-105).

Além de participar da via de sinalização de TLR, foi proposto que a atividade da chaperona Hsp90 é essencial para a resposta imune mediada por sensores NLR, tais como NOD1 (domínio de oligomerização de ligação de nucleótido 1), NOD2 e NLRP3 (106, 107). Ensaios de co-imunoprecipitação demonstram que os sensores de NLR formam complexos estáveis com a Hsp90 e com sua co-chaperona SGT1 (que tem sua ação independente do *turnover* de ATP e dependente de ligação de proteína cliente), através de seu domínio de repetição rico em leucina (LRR) e com a NACHT. Estes dados indicam que a atividade chaperona da Hsp90 é importante para a maturação, estabilização e ativação de NLRP3 na via de inflamassoma (FIGURA 1.6, letra C) (106-107).

1.3.1 Inibidores de Hsp90

Existem diversos inibidores da atividade de Hsp90 de origem natural, sintética e semi-sintética disponíveis comercialmente e com mecanismos de ação distintos. Dentre eles, podemos destacar os inibidores de Hsp90 que interagem primariamente com os dois domínios principais da proteína (N- e C- terminal) e os que interagem com proteínas acessórias chamadas de co-chaperonas. Todos os inibidores que se ligam ao domínio N-terminal, de ligação de ATP, induzem a uma super expressão de Hsps, como Hsp32 (HO-1) Hsp40, Hsp70 e Hsp90, fenômeno este chamado de resposta de choque térmico (HSR). O fenômeno se inicia com a liberação do fator-1 de transcrição de *heat shock* (HSF-1), que normalmente se encontra ligado a Hsp90 e na forma monomérica. Em seguida, ocorre a sua translocação para o núcleo na forma

ativada, trimérica e fosforilada, que se liga a regiões promotoras de genes Hsp no DNA, ocasionando então a super expressão das Hsps, conforme FIGURA 1.6, letra A (108).

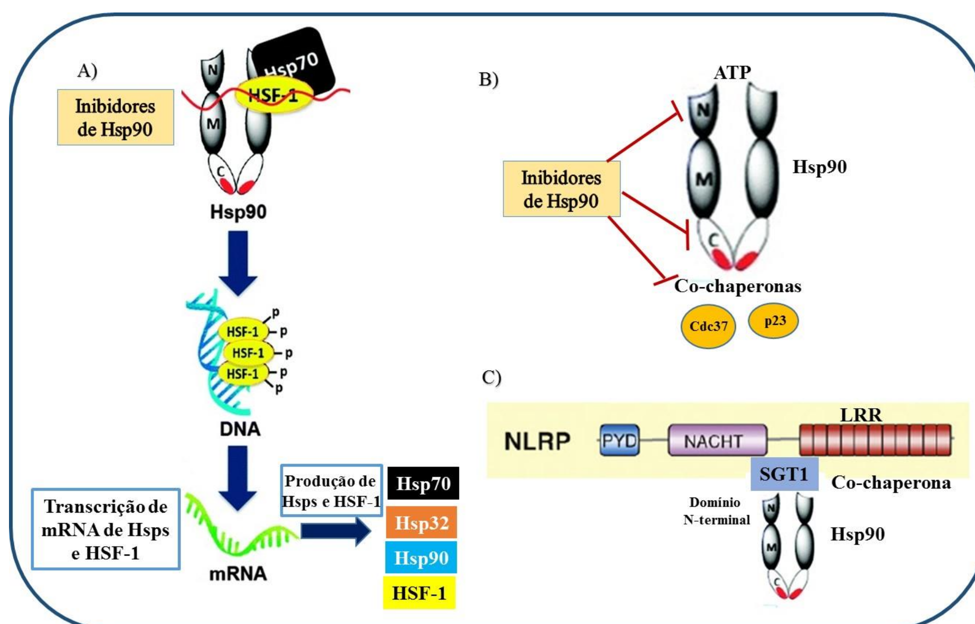

Figura 1.6. Representação esquemática da modulação da atividade de Hsp90. A) Os inibidores de Hsp90 induzem um resposta de choque térmico (HSR) por ativar o fator de transcrição HSF-1, que se encontra incialmente ligado à Hsp90 e, quando ativado, se desliga da chaperona e assume sua forma trimérica ativa, sendo então translocado para o núcleo onde é fosforilado e ativado. Em seguida, o HSF-1 se liga aos promotores dos genes Hsp, induzindo a expressão de Hsps, como Hsp90, Hsp70, Hsp32, e de HSF-1. B) Diferentes tipos de moduladores de Hsp90 induzem HSR, por se ligarem diretamente à chaperona via domínio N-terminal ou C-terminal ou por interagir com as suas co-chaperonas, p23 e Cdc37, inibindo sua interação com a proteína cliente. C) Representação esquemática da interação entre o domínio N-terminal da Hsp90 com sua co-chaperona SGT1 (supressor do alelo G2 de skp1) e domínios NACHT e LRR de NLRP3, importante para estabilização e maturação de NLR. Moduladores de Hsp90 diretos e indiretos podem impedir a formação deste complexo inflamassoma com Hsp90, aumentando a degradação desta proteína cliente. Adaptado de Wang e McAlpine, 2015 (108).

Dentre os principais inibidores da Hsp90 do tipo N-terminal encontram-se a geldanamicina e seus análogos 17-alil amino-17-demetoxi geldanamicina (17-AAG) e 17-dimetilamino-17-demetoxi geldanamicina (17-DMAG), que levam a um aumento da degradação de suas proteínas clientes. Tem-se também outros tipos inibidores, desta chaperona, como a novobiocina e cisplatina, que se ligam ao domínio C-terminal, e que são considerados inibidores fracos (109). Ainda, outros inibidores atuam de forma indireta, se ligando a co-chaperonas como a Cdc37 ou p23, impedindo assim a interação destas com a chaperona, levando a um aumento da degradação de proteínas clientes, tais como o receptor de glicocorticóide (para p23) e algumas proteínas quinases (para Cdc37). Dentre estes inibidores,

podemos citar o celastrol e a gedunina, cujo mecanismo se assemelha ao demonstrado na FIGURA 1.6, letra B (106, 110-113). Os inibidores diretos e indiretos da Hsp90 podem também impedir a interação da sua co-chaperona SGT-1, via domínio N-terminal, inibindo desta forma a formação do complexo Hsp90-SGT-1 com NLRP3, levando então à desestabilização desta proteína e consequente inibição de inflamassoma (como demonstrado na FIGURA 1.6, letra C) (107). Piaz e colaboradores (2012) demonstraram utilizando como ferramenta ressonância plasmônica de superfície (SPR) que a interação da gedunina com a chaperona Hsp90 não se dá de forma direta, tendo em vista que nenhum complexo de ligação foi observado quando a gedunina foi injetada sobre a Hsp90 imobilizada, confirmando que a sua inibição não é direta e sim via ligação específica à co-chaperona p23 (112), que auxilia na sua modulação conformacional. Em diversos modelos experimentais, tem sido demonstrado que inibidores de Hsp90 suprimem diferentes vias de sinalização e exibem uma potente atividade antiproliferativa, citoprotetora e anti-inflamatória (114-118).

1.4 Gedunina

A gedunina é um metabólito secundário presente no óleo obtido das sementes de espécies vegetais da família Meliaceae (*Carapa guianensis* – popularmente conhecida como andiroba - e *Azadirachta indica* – popularmente conhecida como Neem) (119-120). A gedunina, em conjunto com diferentes limonóides com quem apresenta similaridade estrutural, é quimicamente classificada como tetranortriterpenóides, que são triterpenos metabolicamente modificados que possuem um esqueleto básico precursor 4,4,8-trimetil-17-furanil esteróide (121-122).

Sua estrutura química, representada na FIGURA 1.7, pertence à classe de nortriterpenóides altamente oxigenados denominados limonóides, que possuem um núcleo principal de fusão trans de 4 anéis de seis membros com um furano anelado. Além disto, a gedunina possui dois confôrmeros estruturais representados pela α- e β-gedunina, e que diferem na rotação do grupo furano.

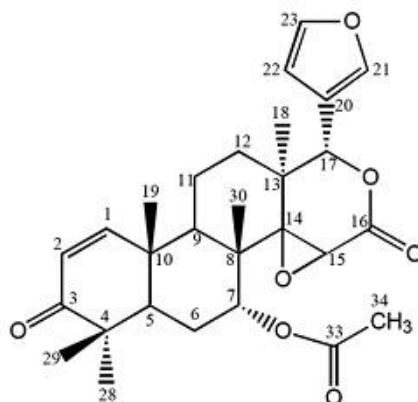

Figura 1.7. Estrutura química da gedunina.

A diferença de energia de ambos confôrmeros está entre 0,88 kcal/mol, indicando que ambos são estáveis e são igualmente possíveis no estado solúvel. Os confôrmeros são fortemente ligados e possuem geometria e energia similares, sendo orientados pela complementariedade de interações eletrostáticas. Dados da literatura demonstram que o potencial eletrostático molecular (MEP - associação entre densidade eletrônica, reatividade e interações) da gedunina assimétrica é representada por regiões de alta densidade eletrônica negativa com átomos de oxigênio próximos (átomos eletronegativos) e regiões potencialmente positivas, representados por átomos de hidrogênio (123).

Considerando as atividades tradicionalmente conhecidas do óleo extraído das sementes de *C. guianensis* Aublet, que incluem atividade anti-inflamatória, analgésica, antibacteriana (121, 180) e anti-malárica (124), nosso grupo de pesquisa vem comprovando a eficácia deste óleo e de uma fração enriquecida com estes limonóides quanto às suas propriedades antialérgica e anti-inflamatória em diferentes modelos experimentais *in vivo* e *in vitro* (125-128). Trabalhos prévios publicados pelo nosso grupo demonstram uma importante atividade anti-inflamatória, anti-alérgica e imunomoduladora da gedunina, inibindo diferentes parâmetros em modelos experimentais *in vitro* e *in vivo*, tais como: i) a formação de edema em modelo murino induzido por zimosan; ii) a migação e ativação de leucócitos no modelo de pleurisia induzida por diferentes estímulos; iii) a proliferação de linfócitos T na resposta alérgica *in vivo*; iv) a produção de mediadores inflamatórios, incluindo IL-5, ligante de quimiocina C-C (CCL)11/eotaxina, TNF-α, IL-1β, assim como mediadores lipídicos (LTB4, PGE2), corpúsculo lipídico e endotelina; v) hiperalgesia em modelo de inflamação articular aguda induzida por zimosan, e vi) a translocação nuclear de fatores de transcrição como NFκB e NFAT (126-130).

Em conjunto, estes resultados obtidos em nosso laboratório foram de extrema relevância para determinar a eficácia deste limonóide em diferentes modelos *in vitro* e *in vivo.*

Considerando que substâncias isoladas de produtos naturais constituem fontes de novos fármacos, podemos destacar a gedunina como uma destas substâncias que apresentam potencial uso terapêutico. Dentre os diversos usos terapêuticos deste limonóide, relatados na literatura, podemos destacar a sua atividade antitumoral, através da inibição do ciclo celular e do crescimento de células tumorais, além da indução da apoptose em diferentes linhagens tumorais (tais como, MCF-7, NCI-H460 e A375-C5) (131).

Valendo-se do fato de que a gedunina é uma molécula pequena, e que por motivos biofísicos, moléculas pequenas interagem com facilidade com múltiplas proteínas (proteínas transportadoras e receptores), podemos justificar desta forma, os seus mecanismos multialvos de interação com essas proteínas. A falta de seletividade de muitos fármacos na interação em uma vasta variedade de alvos, é de maneira geral, universal e muito comum (132). Sendo assim, para o esclarecimento do possível mecanismo de ação destes compostos a elucidação dos alvos proteicos tem grande impacto sobre a estratégia no processo de descoberta de novas drogas. O efeito descrito para a gedunina é principalmente relacionado à sua modulação da Hsp90. Desta forma, pretendemos, neste estudo, avaliar os efeitos desta modulação sobre a expressão de outras proteínas relacionadas ao estresse e a sua relação com o reconhecimento de patógenos.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo deste projeto foi avaliar o efeito da gedunina sobre a resposta inflamatória induzida por ligantes de TLR4, TLR3 e TLR2, e investigar novos alvos moleculares deste tetranortriterpenóide relacionados às vias de sinalização de TLRs e do inflamassoma NLRP3.

2.2 Objetivos específicos

- Investigar o efeito da gedunina sobre a ativação de TLR4/MD-2/CD14, TLR3 e TLR2 em macrófagos estimulados com LPS, PAM3CSK4 e POLY I:C, avaliando a produção de mediadores inflamatórios e a formação de inflamassoma;

- Investigar o efeito da gedunina sobre as vias de sinalização aguda (dependente de MAL/MyD88) e tardia (dependente de TRAM/TRIF) de TLR4/MD-2/CD14 em macrófagos estimulados com LPS;

- Investigar o efeito da gedunina no modelo de pleurisia murina induzida por LPS, avaliando o acúmulo de leucócitos, a produção de mediadores inflamatórios e a via do inflamassoma NLRP3;

- Avaliar o efeito da gedunina sobre a indução de fatores anti-inflamatórios em macrófagos estimulados ou não com LPS, PAM3CSK4 e POLY I:C;

- Estudar o mecanismo de interação molecular da gedunina com a caspase-1, TLR2, TLR3 e MD-2 (co-receptor de TLR4), utilizando modelagem *in silico,* e avaliar a afinidade de ligação da gedunina ao MD-2.

3. ARTIGOS

3.1 Artigo 1

Gedunin Binds to Myeloid Differentiation Protein 2 and Impairs Lipopolysaccharide-Induced Toll-Like Receptor 4 Signaling in Macrophages

Autores: Perla Villani Borges, Katelim Hottz Moret, Clarissa Menezes Maya-Monteiro, Franklin Souza-Silva, Carlos Roberto Alves, Paulo Ricardo Batista, Ernesto Raúl Caffarena, Patrícia Pacheco, Maria das Graças Henriques e Carmen Penido.

Mol Pharmacol 88:949–961, 2015

Trabalhos prévios publicados pelo nosso grupo demonstram que a gedunina apresenta uma atividade anti-inflamatória e imunomoduladora em diferentes modelos experimentais *in vitro* e *in vivo* (125-129). Compreendendo a importância dos macrófagos como células centrais da resposta inflamatória de origem infecciosa ou alérgica, a identificação de novas substâncias capazes de modular a ativação destas células é de grande relevância para o controle da resposta imune. Neste trabalho, tivemos como objetivo estudar o mecanismo de ação da gedunina sobre a ativação de TLR4 em macrófagos murinos ativados por LPS. Desta forma, avaliamos o efeito biológico da gedunina sobre a ativação de macrófagos induzida por LPS, investigando a modulação das vias MAL/MyD88 e TRAM/TRIF, a produção de mediadores pró- e anti-inflamatórios, e a translocação nuclear do fator de transcrição NFκB. Em adição, investigamos – através de ensaios *in silico* e de ressonância plasmônica de superfície (SPR) – a ligação da gedunina no sítio de interação hidrofóbica do MD-2, componente do complexo receptor de TLR4. Em conjunto, nossos resultados demonstram que a gedunina modula a resposta induzida por LPS em macrófagos, inibindo diferentes parâmetros da resposta inflamatória e induzindo fatores anti-inflamatórios. Ainda, nossos resultados demonstram que a gedunina se liga ao MD-2, inibindo a formação do complexo LPS/TLR4/MD-2/CD14, o que sugere que o seu mecanismo de ação não se restringe à modulação da atividade de Hsp90, como foi previamente demonstrado na literatura.

MOLECULAR PHARMACOLOGY

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Dear Dr. Penido:

I want to congratulate you and your co-authors on your excellent article entitled "Gedunin Binds to Myeloid Differentiation Protein 2 and Impairs Lipopolysaccharide-Induced Toll-Like Receptor 4 Signaling in Macrophages", which was published in Molecular Pharmacology (October 2015, vol. 88, pp. 949-961). Your article was selected as one of the journal highlights that was posted this month on the ASPET website (www.aspet.org). The paper was also announced on Twitter (Mol Pharm (ASPET) @MolPharmJournal) and announced on Molecular Pharmacology's Facebook page (www.facebook.com/molpharmaspetjournal). Thank you for submitting your work to Molecular Pharmacology!

Sincerely,

Stephen Traynelis,
Editor-in-Chief, Molecular Pharmacology

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1521-0111/88/5/949–961$25.00
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http://dx.doi.org/10.1124/mol.115.098970
Mol Pharmacol 88:949–961, November 2015

Gedunin Binds to Myeloid Differentiation Protein 2 and Impairs Lipopolysaccharide-Induced Toll-Like Receptor 4 Signaling in Macrophages [S]

Perla Villani Borges, Katelim Hottz Moret, Clarissa Menezes Maya-Monteiro, Franklin Souza-Silva, Carlos Roberto Alves, Paulo Ricardo Batista, Ernesto Raúl Caffarena, Patrícia Pacheco, Maria das Graças Henriques, and Carmen Penido

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Received March 13, 2015; accepted August 26, 2015

ABSTRACT

Recognition of bacterial lipopolysaccharide (LPS) by innate immune system is mediated by the cluster of differentiation 14/Toll-like receptor 4/myeloid differentiation protein 2 (MD-2) complex. In this study, we investigated the modulatory effect of gedunin, a limonoid from species of the Meliaceae family described as a heat shock protein Hsp90 inhibitor, on LPS-induced response in immortalized murine macrophages. The pretreatment of wild-type (WT) macrophages with gedunin (0.01–100 μM, noncytotoxic concentrations) inhibited LPS (50 ng/ml)–induced calcium influx, tumor necrosis factor-α, and nitric oxide production in a concentration-dependent manner. The selective effect of gedunin on MyD88-adapter–like/myeloid differentiation primary response 88– and TRIF-related adaptor molecule/TIR domain–containing adapter-inducing interferon-β–dependent signaling pathways was further investigated. The pretreatment of WT, TIR domain–containing adapter-inducing interferon-β knockout, and MyD88 adapter–like knockout macrophages with gedunin (10 μM) significantly inhibited LPS (50 ng/ml)–induced tumor necrosis factor-α and interleukin-6 production, at 6 hours and 24 hours, suggesting that gedunin modulates a common event between both signaling pathways. Furthermore, gedunin (10 μM) inhibited LPS-induced prostaglandin E_2 production, cyclooxygenase-2 expression, and nuclear factor κB translocation into the nucleus of WT macrophages, demonstrating a wide-range effect of this chemical compound. In addition to the ability to inhibit LPS-induced proinflammatory mediators, gedunin also triggered anti-inflammatory factors interleukin-10, heme oxygenase-1, and Hsp70 in macrophages stimulated or not with LPS. In silico modeling studies revealed that gedunin efficiently docked into the MD-2 LPS binding site, a phenomenon further confirmed by surface plasmon resonance. Our results reveal that, in addition to Hsp90 modulation, gedunin acts as a competitive inhibitor of LPS, blocking the formation of the Toll-like receptor 4/MD-2/LPS complex.

Introduction

Recognition of lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, by the immune system, involves at least three receptor molecules: cluster of differentiation 14 (CD14), Toll-like receptor (TLR) 4, and myeloid differentiation protein 2 (MD-2), which are mostly expressed by macrophages (Wright et al., 1990; Ulevitch and Tobias, 1995; Shimazu et al., 1999; Viriyakosol et al., 2000). LPS recognition by the TLR4 complex leads to the recruitment of the adaptor proteins MyD88-adapter-like/Myeloid differentiation primary response 88 (MAL/MyD88) and TRIF-related adaptor molecule/TIR domain–containing adapter-inducing interferon-β (TRAM/TRIF), which in turn activate two distinct signaling pathways that present different kinetics. TLR4 initially recruits MAL/MyD88, leading to early phase activation, and is further endocytosed and delivered to intracellular vesicles to only then form a complex

This research was supported by the Carlos Chagas Filho Foundation for Research Support of the State of Rio de Janeiro and by the National Council for Scientific and Technological Development. P.V.B. and K.H.M. were supported by fellowships from the Coordination for the Improvement of Higher Education Personnel Foundation and the National Council for Scientific and Technological Development as students of the Oswaldo Cruz Institute (Fiocruz) Graduate Program in Cellular and Molecular Biology.
P.V.B and K.H.M. are co-first authors of this work.
dx.doi.org/10.1124/mol.115.098970.
[S] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; CD14, cluster of differentiation 14; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HO-1, heme oxygenase-1; HSF-1, heat shock factor-1; IL, interleukin; LPS, lipopolysaccharide; MAL KO, MyD88 adapter–like knockout; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; NO, nitric oxide; PGE_2, prostaglandin E_2; RU, resonance unit; SPR, surface plasmon resonance; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF KO, TIR domain–containing adapter-inducing interferon-β knockout; WT, wild type.

with TRAM/TRIF, leading to the late phase activation (Hwang et al., 1997; Kagan et al., 2008; Kawai and Akira, 2011). These two pathways are required to drive robust nuclear factor κB (NFκB) and mitogen-activated protein kinase (MAPK) activation and the subsequent induction of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and nitric oxide (NO) (Byrd-Leifer et al., 2001; Fitzgerald et al., 2003; Sato et al., 2003; Li et al., 2005a; Gais et al., 2010). The overproduction of these mediators during inflammation can lead to tissue damage, multiple organ dysfunction, and septic shock (Dos Santos and Slutsky, 2000; Marshall, 2001; Su, 2002; Shen et al., 2004).

It has been proposed that the heat shock protein (Hsp) chaperone machinery is implicated in TLR4/MD-2/CD14 signaling by maintaining the structural integrity of the multimeric LPS receptor complex and by regulating MAPK members (Triantafilou et al., 2001; Echeverría et al., 2011). In addition, it has been suggested that LPS is transferred from CD14 to Hsp70 and Hsp90, and then interacts with a large hydrophobic pocket of MD-2 (da Silva Correia et al., 2001; Triantafilou et al., 2001; Park et al., 2009). Hsp90 is an abundantly and ubiquitously expressed chaperone that helps to maintain, at the expense of ATP, the structure of several membrane, cytoplasmic, and endoplasmic reticulum–associated client proteins (Pratt and Toft, 1997; Picard, 2002; Zhao and Houry, 2005; McClellan et al., 2007). Hsp90 chaperoning activity can be inhibited by geldanamycin, 17-allylamino-17-demethoxy-geldanamycin (17-AAG), and celastrol, which leads to increased degradation of Hsp90 client proteins (Matts et al., 2011). In experimental models, Hsp90 inhibitors have been shown to suppress different signaling pathways and display potent antiproliferative, cytoprotective, and anti-inflammatory activities (Lewis et al., 2000; Poulaki et al., 2007; Ambade et al., 2012; Chow et al., 2013; Leung et al., 2015). These effects result from nonfunctional conformational changes as well as from the induction of heat shock response via the activation of heat shock factor-1 (HSF-1), which leads to increased expression of Hsp90 and other Hsps, including Hsp70, Hsp40, and Hsp32 [heme oxygenase-1 (HO-1)] (Pritchard et al., 2001; Shamovsky and Nudler, 2008; Trott et al., 2008; Chow et al., 2013).

Gedunin and its analogs are an important bioactive limonoid-type tetranortriterpene isolated from the Meliaceae family and are reported to display a wide range of biologic activities, including antitumor, antimalarial, antiallergic, and anti-inflammatory activity in different experimental models (Brandt et al., 2008; Kamath et al., 2009; Ferraris et al., 2011, 2012; Miranda Júnior et al., 2012; Henriques and Penido, 2014; Conte et al., 2015). The molecular mechanism of action related to the biologic effects of gedunin relies on the inhibition of Hsp90 activity via specific binding to the cochaperone p23 and by disrupting the cochaperone Cdc37-Hsp90 interaction (Matts et al., 2011; Patwardhan et al., 2013). However, here we demonstrate that gedunin mechanisms of action go beyond Hsp90 modulation. Using in silico and surface plasmon resonance (SPR) analysis, we show that gedunin binds to MD-2, impairing TLR4/MD-2/CD14 signaling and decreasing LPS-induced inflammatory response in murine macrophages.

Materials and Methods

Cell Culture. Immortalized bone marrow–derived macrophage cell lines generated from wild-type (WT), MyD88 adapter–like knockout (MAL KO), and TIR domain–containing adapter-inducing interferon-β (TRIF KO) C57BL/6 mice (a kind gift from Douglas Golenbock, University of Massachusetts Medical School, Worcester, MA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (FBS) (10%; Gibco/Life Technologies, Carlsbad, CA), sodium pyruvate (1 mM, Sigma-Aldrich), and ciprofloxacin (10 μg/ml, Fresoflox; Fresenius Kabi, Barueri, Brazil).

Cytotoxicity Assay. To perform in vitro assays with gedunin, we first examined the cytotoxic effects of this substance (and gedunin vehicle, dimethylsulfoxide) on WT immortalized macrophages. Viable cells were seeded in a flat bottom 96-well plate (2×10^5 cells/well, in quadruplicate) and cultured for 24 hours in the presence of different concentrations of gedunin (0.001–1000 μM; 5% CO_2 at 37°C). The assay was assessed by the resazurin reduction method. The absorbance was read at 555/585 nm ($\lambda_{ex}/\lambda_{em}$) using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA) and results are expressed as percentages of viable cells (Table 1). The compound concentrations that induced ≥10% of cell death were considered cytotoxic and were not used in the biologic assays. It is noteworthy that dimethylsulfoxide (≤0.1%, maximal concentration used) and LPS (≤1 μg/ml) induced ≤10% of macrophage death.

Treatments and Stimulation. WT, MAL KO, and TRIF KO macrophages (10^6 cells/well) were plated in 6- or 24-well plates in DMEM (supplemented with 2% FBS, 1 mM sodium pyruvate, and 10 μg/ml ciprofloxacin) for 24 hours and then treated with gedunin (0.01–100 μM; Gaya Chemical Corporation, New Milford, CT). After 1 hour, macrophages were stimulated with LPS (50–1000 ng/ml, from *Escherichia coli* O111:B4, Sigma-Aldrich) and repurified by a repeated phenolchloroform extraction (Hirschfeld et al., 2000) for 6 or 24 hours, and the cell-free supernatants were recovered for analysis. Hsp90 inhibitor 17-AAG (1 μM) and dexamethasone (100 nM), used as reference inhibitors, were purchased from Sigma-Aldrich and induced ≤10% of macrophage death at the concentrations used.

Calcium Mobilization Assay. Intracellular calcium concentrations on preplated WT immortalized macrophage cells (2×10^5 per well) were pretreated with gedunin (0.01–100 μM) and exposed to LPS (1 μg/ml) over 600 seconds using the FLIPR Calcium Plus Assay Kit on a FlexStation II fluorometric microplate reader (Molecular Devices), with fluorescence intensity ratios at 485/525 nm ($\lambda_{ex}/\lambda_{em}$) recorded up to 5 minutes and analyzed using SoftMax Pro (Molecular Devices).

Nitrite Determination. Cells were seeded on 24-well plates in a final concentration of 1×10^6 cells/well (DMEM supplemented with

TABLE 1

Macrophage viability after exposure to gedunin

Results are expressed as the percentage of cell viability from quadruplicate wells (2×10^5 cell/well), after incubation of macrophages with gedunin from 24 hours (37°C, 5% CO_2). Cell viability was assessed by the resazurin reduction method, as described in *Materials and Methods*.

Medium	Tween 20 (3%)	Gedunin						
		0.001	0.01	0.1	1	10	100	1000
		μM						
100 ± 2.90	2.7 ± 0.05	100 ± 1.23	100 ± 3.46	100 ± 0.22	100 ± 1.97	100 ± 4.74	91.5 ± 3.96	86.9 ± 0.39

2% FBS, in 5% CO_2 at 37°C) and allowed to grow to confluence. Confluent cells were pretreated with gedunin (0.01–10 μM) and exposed to LPS (50 ng/ml) for 24 hours. The supernatant was collected and nitrite, a stable metabolite of NO in aqueous solutions, was measured by Griess reaction, after the addition of 100 μl modified Griess reagent (Sigma-Aldrich) to the wells for 15 minutes at room temperature. Absorbance was read at 562 nm using a Spectramax M5 microplate reader (Molecular Devices). The concentration of nitrite was calculated from a sodium nitrite standard curve (range, 1.5–100 μM).

Cytokine Analysis. TNF-α, IL-6, and IL-10 levels were evaluated in the supernatants of stimulated immortalized macrophages by an enzyme-linked immunosorbent assay using matched antibody pairs (Quantikine; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Results are expressed as picograms per milliliter.

Prostaglandin E_2 Quantification. Concentrations of prostaglandin E_2 (PGE_2) were measured in the supernatants of LPS (50 ng/ml)–stimulated macrophages pretreated or not with gedunin (10 μM), 17-AAG (1 μM), or dexamethasone (100 nM) using an EIA kit (Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer's instructions.

Western Blot Analysis. Total protein content in cytoplasmic and nuclear extracts was determined by the Bradford reagent (Sigma-Aldrich). Cell lysates were denatured in Laemmli's sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and heated at 95°C for 3 minutes. Aliquots containing 30 μg protein were resuspended in SDS-PAGE loading buffer, resolved on 12% SDS acrylamide gels, and transferred onto polyvinyl difluoride (PVDF) Hybond membranes (Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% nonfat dry milk/Tris-buffered saline containing 0.1% Tween 20 for 2 hours at room temperature, the membranes were probed overnight at 4°C with specific primary antibodies followed by horseradish peroxidase–labeled secondary antibodies. Rabbit polyclonal anti-mouse HO-1 (1:5000) and horseradish peroxidase–labeled goat polyclonal anti-rabbit antibodies (1:2500) were obtained from Enzo Life Sciences (Farmingdale, NY). Mouse monoclonal anti-goat Hsp70 (1:5000), mouse monoclonal anti-goat COX-2 (1:5000) and mouse monoclonal anti-rabbit NFκB p65 (1:5000) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). PVDF sheets were incubated with streptavidin-conjugated horseradish peroxidase (1:10,000) for 1 hour and developed by an ECL-Plus reagent (Enhanced Chemiluminescence; Amersham Biosciences) and visualized on Hyperfilm (Amersham Biosciences). The bands were quantified by densitometry, using the ImageJ software program (National Institutes of Health, Bethesda, MD).

In Silico Docking Simulations. The structures of the human MD-2 and the cochaperone p23 were taken from the Protein Data Bank codes 2E56 (Ohto et al., 2007) and 1EJF (Weaver et al., 2000), respectively. Before the docking simulation, the protein structure was prepared adding hydrogens and the protonation of titrable residues were calculated with the PROPKA program (Li et al., 2005b), inside the pdb2pqr program (Dolinsky et al., 2004, 2007), considering a pH of 7.

All molecular docking calculations were performed using the Autodock Vina program (Trott and Olson, 2010), in a two-step approach: 1) the blind docking procedure and 2) the pocket search procedure. The blind docking procedure consisted of searching the entire protein surface to determine the potential binding pocket(s). This was achieved using the grid center as the center of each protein, using a grid size big enough to cover the entire protein surface. After finding the binding pockets, we centered the grid center within the discovered binding pocket and performed a more accurate search by the pocket search procedure, using the following parameters: energy_range, 10; num_modes, 20; and exhaustiveness, 800.

Physicochemical Binding Assays of Gedunin toward MD-2. The SPR analysis was performed on carboxyl sensor chips coated with nickel-nitrilotriacetic acid (HisCap; ICx Nomadics Inc., Stillwater, OK) previously treated with an activation solution (500 μM $NiCl_2$) in a constant flow (10 μl/min) at 37°C for 10 minutes, for each binding assay cycle. Prior the binding assay, the recombinant human rhMD-2 fusion protein (R&D Systems) was immobilized (0.1750 μg/ml, flow rate of 5 μl/min, for 10 minutes) on the chip surface with reaction buffer (150 mM NaCl, 100 mM HEPES, pH 7.4). It is important to note that due to the lack of a commercially available recombinant murine MD-2, rhMD-2 was used in this study. rhMD-2 shares 56% of identity with recombinant murine MD-2 at the amino acid level, and their common residues are essential for TLR4 activation by LPS (Zimmer et al., 2008; Park et al., 2009). The differences in surface charge distribution (electrostatic potential) of human and murine MD-2 binding pockets do not alter the hydrophobic interaction between LPS and MD-2 inside the cavity. The binding of gedunin and of LPS to immobilized MD-2 was performed using different concentrations in reaction buffer (gedunin: 0.001–1000 μM and 0.482–482.0 μg/ml; LPS: 0.482–482.0 μg/ml). At the end of each interaction step, the HisCap chip was treated with regenerating buffer (0.7 M imidazole, pH 8.0) at 37°C for 3 minutes (50 μl/min). All binding assays were registered in real time using a sensorgram, and changes in the SPR angle (θ spr) were expressed as arbitrary resonance units (RU). To avoid artifacts, RU values from the reference channel were subtracted from the RU values of test samples. All SPR analyses were performed in a SensíQ Pioneer optical transduction biosensor (ICx Nomadics Inc.).

The association and dissociation rates of complex formation were calculated based on the analysis of sensorgram graphs. Kinetic values were obtained using Qdat software (ICx Nomadics Inc.). Data for the affinity constant (K_{eq}) and of Gibbs free energy (θ G°) for the complexes formed were derived from the K_a and K_d data (Souza-Silva et al., 2014). The reliability of the data was confirmed by double-reciprocal plots (1/response versus 1/free analyte), as previously described (Pastushok et al., 2005; Samoylov et al., 2005). Inhibitory modes of gedunin for LPS (9.64 μg/ml) binding to immobilized MD-2 was performed using different concentrations of gedunin (0.482–482.0 μg/ml) in reaction buffer. The binding of gedunin (14.5 μg/ml) to MD-2 in the presence of LPS (0.482–482.0 μg/ml) was also compared.

Statistical Analysis. Data are reported as the mean ± S.E.M. and were analyzed by means of analysis of variance, followed by the Student–Newman–Keuls test. Values of $P < 0.05$ were regarded as significant.

Results

Concentration Effect of Gedunin on LPS-Induced Macrophage Activation. The effect of different concentrations of gedunin was evaluated in vitro on LPS-induced calcium influx and on TNF-α and nitrite production by WT macrophages. As observed in Fig. 1, A and B, the stimulation of macrophages with LPS (1 μg/ml) induced a sustained increased in the levels of intracellular calcium, which was inhibited by the pretreatment with gedunin, from 0.01 to 100 μM. Gedunin pretreatment also impaired LPS (50 ng/ml)–induced TNF-α production by macrophages, in a concentration-dependent manner ($R^2 = 0.96$; $P < 0.001$; Fig. 1C). In addition, as shown in Fig. 1D, LPS (50 ng/ml) plus interferon-γ (200 IU/ml) increased nitrite production by macrophages, which was also inhibited by gedunin pretreatment from 0.01 to 10 μM. Since 10 μM gedunin resulted in 100% viability (Table 1) and significantly impaired different parameters of macrophage activation, this concentration was used in all subsequent experiments.

Gedunin Impairs LPS-Induced Cytokine Production of Early and Late Phase Responses. LPS recognition by

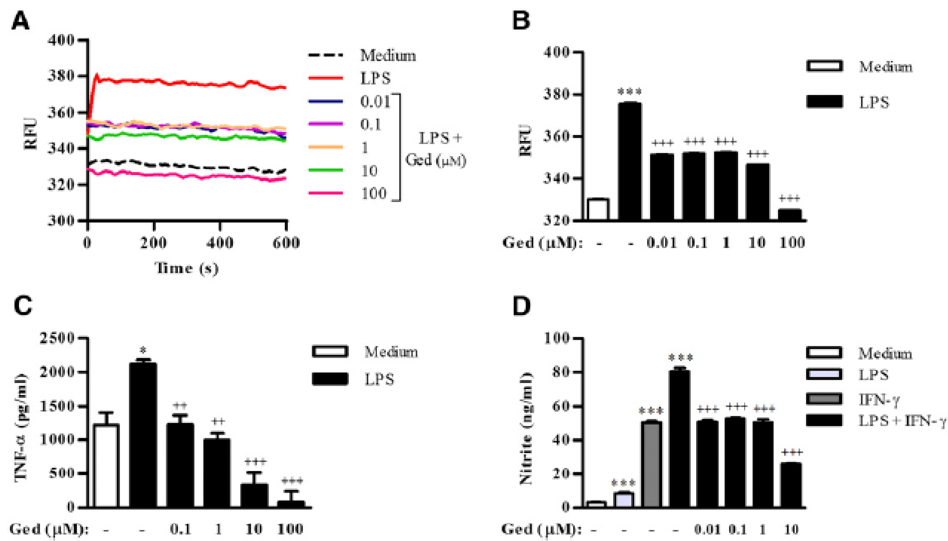


Fig. 1. Concentration-dependent inhibition of LPS-induced macrophage activation. In vitro pretreatment of WT immortalized macrophages (10^6 cells/well) with gedunin (Ged; 0.1–100 μM) for 1 hour impaired intracellular calcium influx (A and B), TNF-α (C), and NO production (D) by macrophages stimulated with LPS. (A) Kinetics of calcium influx of LPS (1 μg/ml)–stimulated macrophages over 600 seconds measured by the FLIPR Calcium Plus Assay Kit and (B) means of values obtained within 600 seconds for each group. (C) TNF-α levels were determined by an enzyme-linked immunosorbent assay in the supernatants of macrophages 6 hours after LPS (50 ng/ml) stimulation. (D) NO was determined in supernatants of macrophages stimulated with LPS (50 ng/ml) plus interferon (IFN)-γ (200 IU/ml) at 24 hours by the colorimetric Griess reagent. (C and D) Results are expressed as the mean ± S.E.M. for triplicate wells per group from three independent experiments and were statistically analyzed by means of analysis of variance, followed by the Student-Newman–Keuls test. Statistically significant differences between stimulated and nonstimulated groups are indicated by asterisks ($^{*}P < 0.05$; $^{***}P < 0.001$), whereas plus signs ($^{++}P < 0.01$; $^{+++}P < 0.001$) represent differences between treated and stimulated groups.

the TLR4/MD-2/CD14 complex induces a signaling cascade that culminates in the production of cytokines, such as TNF-α and IL-6, via early (MAL/MyD88-dependent) and late (TRAM/TRIF-dependent) pathways (Akira and Takeda, 2004). To investigate the selective effect of gedunin on the early and late activation triggered by LPS, we evaluated the production of cytokines by TRIF KO and MAL KO macrophages 6 and 24 hours after stimulation, respectively. As shown in Fig. 2, A–D, TNF-α production through early and late pathways was significantly inhibited by gedunin, as well as by the reference inhibitors 17-AAG and dexamethasone pretreatments. Similarly, as shown in Fig. 2, E–H, the pretreatment with gedunin, 17-AAG, and dexamethasone inhibited IL-6 production via early and late phases.

Gedunin Diminishes PGE_2 Production and Cyclooxygenase-2 Expression Induced by LPS. We further evaluated the effect of gedunin on LPS-induced PGE_2 production through early and late pathways. As shown in Fig. 3, A and B, the pretreatment with gedunin, 17-AAG, and dexamethasone inhibited LPS-induced PGE_2 production by WT macrophages 6 and 24 hours after stimulation. The expression of cyclooxygenase-2 (COX-2), the enzyme responsible for the production of prostanoids including PGE_2 (Alhouayek and Muccioli, 2014), is shown to be induced after stimulation with LPS (Hwang et al., 1997; Rhee and Hwang, 2000) and IL-1β (Arias-Negrete et al., 1995; Endo et al., 2014). Here, we show that the pretreatment with gedunin decreased LPS (50 ng/ml)–induced COX-2 protein expression in WT immortalized macrophages (Fig. 3C). Decreased COX-2 expression was also observed for 17-AAG and dexamethasone.

Gedunin Inhibits Nuclear Translocation of NFκB. NFκB activation is critical for the synthesis of inflammatory mediators, including cytokines (D'Acquisto et al., 2002). Thus, we investigated the ability of gedunin to inhibit NFκB activation in vitro by analyzing its translocation into the nucleus by Western blot analysis. NFκB/p65 protein levels were determined in nuclear extracts of WT immortalized macrophages stimulated with LPS (50 ng/ml) for 24 hours. LPS stimulation enhanced the presence of the p65 subunit in the nucleus, compared with nonstimulated WT immortalized macrophages (Fig. 3D). NFκB translocation was inhibited by gedunin, 17-AAG, and dexamethasone pretreatments.

Gedunin Triggers Anti-Inflammatory Mechanisms in Macrophages. The induction of anti-inflammatory and pro-resolving mechanisms may be an additional means by which anti-inflammatory substances exert their effects. Moreover, it has been demonstrated that some Hsp90 modulators can induce the expression of anti-inflammatory factors (Shamovsky and Nudler, 2008; Trott et al., 2008; Chow et al., 2013; Der Sarkissian et al., 2014). Here we show that gedunin pretreatment enhanced HO-1 expression on LPS (50 ng/ml)–stimulated WT immortalized macrophages, whereas 17-AAG and dexamethasone pretreatment did not enhance the expression of HO-1 (Fig. 4A). The fact that LPS failed to increase HO-1 expression 6 hours after stimulation is in accordance with previous reports (Song et al., 2003; Rushworth et al., 2005). Incubation of unstimulated macrophages with gedunin induced more prominent expression of HO-1 compared with cells incubated with gedunin and LPS ($P \leq 0.05$). In addition, gedunin and 17-AAG pretreatments were able to induce Hsp70 expression on WT immortalized macrophages that

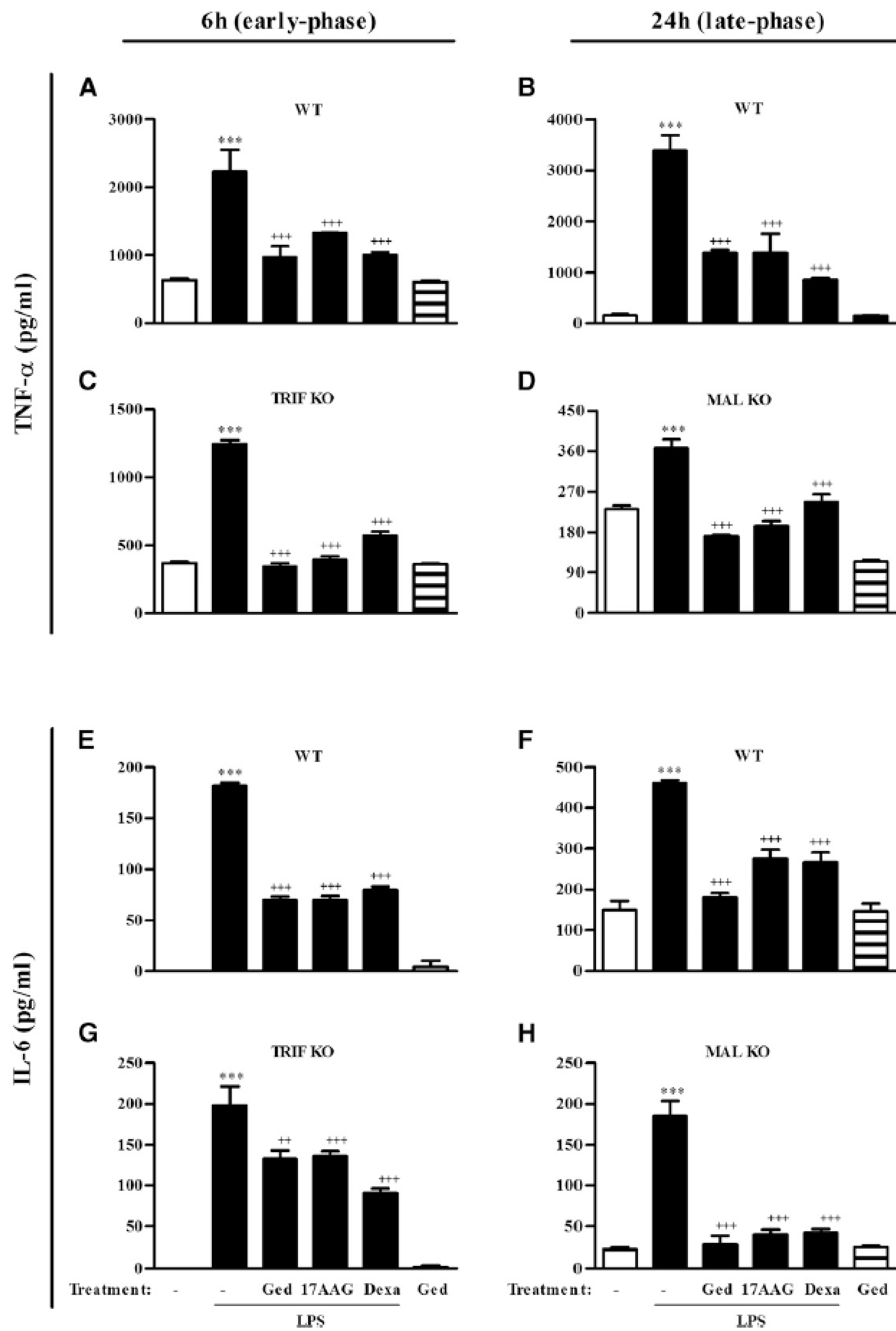


Fig. 2. Gedunin impairs LPS-induced cytokine production during early and late phase activation. Pretreatment of WT (A, B, E, and F), TRIF KO (C and G), and MAL KO (D and H) macrophages (10^6 cells/well) with gedunin (Ged; 10 μM), 17-AAG (1 μM), or dexamethasone (Dexa; 100 nM) for 1 hour impaired LPS (50 ng/ml)–induced TNF-α (A–D) and IL-6 (E–H) at 6 hours (left-column graphs) and 24 hours (right-column graphs), as determined by an enzyme-linked immunosorbent assay in cell-free supernatants. Results are expressed as the mean ± S.E.M. for quadruplicate wells per group from three independent experiments and were statistically analyzed by means of analysis of variance, followed by the Student-Newman–Keuls test. Statistically significant differences between stimulated and nonstimulated groups are indicated by asterisks ($^{***}P < 0.001$), whereas plus signs ($^{++}P < 0.01$; $^{+++}P < 0.001$) represent differences between treated and stimulated groups.

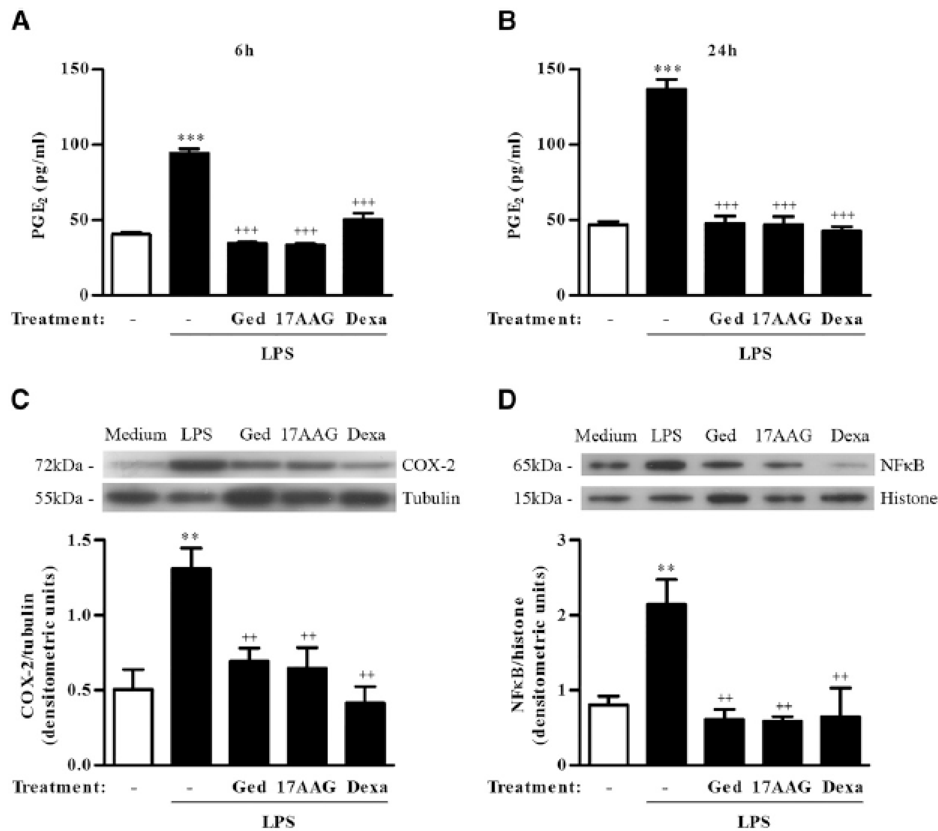


Fig. 3. Gedunin diminishes LPS-induced PGE_2 production, COX-2 protein expression, and NFκB nuclear translocation. In vitro pretreatment of WT immortalized macrophages (10^6 cells/well) with gedunin (Ged, 10 μM), 17-AAG (1 μM), or dexamethasone (Dexa, 100nM) for 1 hour impaired LPS (50 ng/ml)–induced PGE_2 levels in cell-free supernatants at 6 and 24 hours (A and B), COX-2 expression in whole cell extracts at 24 hours (C), and NFκB expression in nuclear fractions 24 hours after stimulation (D). (C and D) Representative Western blots are shown on top, whereas densitometric analyses are shown in the graphs. Results are expressed as the mean ± S.E.M. for quadruplicate wells per group from at least three independent experiments and statistically analyzed by means of analysis of variance, followed by the Student-Newman–Keuls test. Statistically significant differences between stimulated and nonstimulated groups are indicated by asterisks ($^{**}P < 0.01$; $^{***}P < 0.001$). whereas plus signs ($^{++}P < 0.01$; $^{+++}P < 0.001$) represent differences between treated and stimulated groups.

were stimulated with LPS (Fig. 4B). Similarly, the increase in Hsp70 expression observed in macrophages that were pretreated with dexamethasone was not statistically significant compared with LPS-stimulated macrophages. Furthermore, LPS-induced IL-10 production was enhanced in WT immortalized macrophages by pretreatments with gedunin, 17-AAG, and dexamethasone (Fig. 4C). Interestingly, the incubation of unstimulated macrophages with gedunin was able to induce increased HO-1 and IL-10 expression. Thus, our results show that gedunin is also capable of inducing anti-inflammatory factors in macrophages.

Gedunin Docks to the MD-2 Protein Surface at the LPS Binding Site. The complexity of the TLR4 signaling pathway (e.g., numerous proteins acting at different levels) hinders the identification of molecular targets of gedunin. We first analyzed the structure and interaction of known targets of gedunin. It was recently demonstrated that gedunin is able to bind and inactivate p23, avoiding the formation of its complex with Hsp90 (Patwardhan et al., 2013). Structurally, p23 presents an antiparallel β-sandwich fold and the gedunin binding site is located within the region that forms the complex to Hsp90. Using a binding docking scheme, we were able to find the same previously reported binding site that allowed us to obtain an equivalent binding mode of gedunin to p23 (Supplemental Fig. 1).

Among the potential candidates of proteins of the TLR4 pathway, we considered proteins that directly bind and recognize LPS, including the TLR4 accessory protein MD-2 (Meng et al., 2010). Considering that gedunin is a very hydrophobic molecule, we tested whether it would bind to MD-2.

Comparison between the sequences and crystal structures of MD-2 and p23 revealed that these two proteins possess a similar fold, as represented by the structural alignments of their crystal structures (Fig. 5). Thus, both MD-2 and p23 present an antiparallel β-sandwich fold with a well defined hydrophobic pocket (p23 being more compact).

Mapping Gedunin Binding Sites in MD-2. We then used an in silico docking approach to identify putative gedunin binding sites in the MD-2 structure. Our hypothesis is based

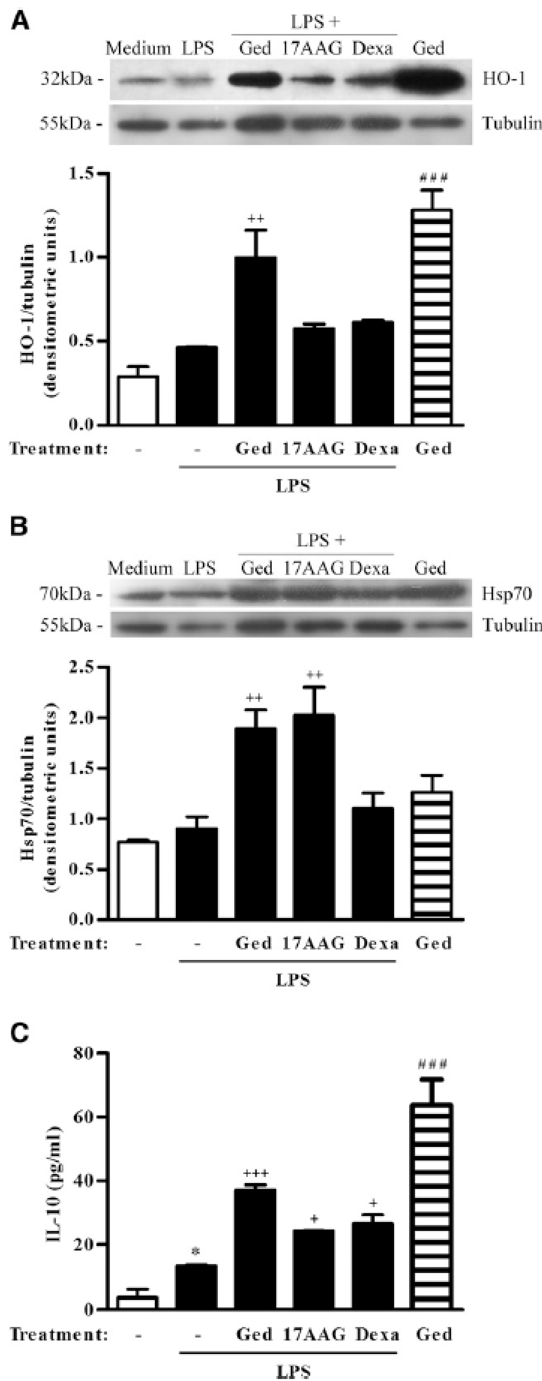


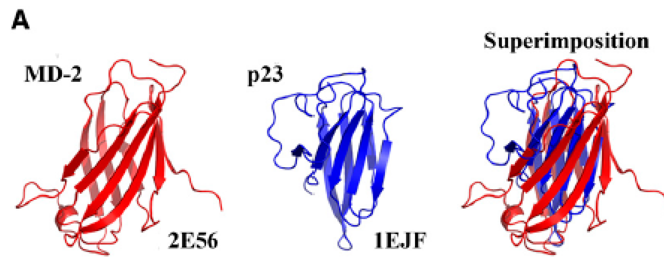
Fig. 4. Gedunin triggers anti-inflammatory mechanisms in macrophages. Expression of HO-1 (A) and Hsp70 (B) in whole WT immortalized macrophage extracts (10^6 cells/well), 6 and 24 hours after LPS (50 ng/ml) stimulation, respectively, evaluated by Western blot analysis. Cell were pretreated for 1 hour with gedunin (Ged; 10 μM), 17-AAG (1 μM), or dexamethasone (Dexa; 100 nM) before stimulation. Representative Western blots from three independent experiments are shown (top panels). (C) Effect of gedunin pretreatment on LPS (50 ng/ml)–induced IL-10 production by WT macrophages, evaluated in 24-hour cell-free supernatants by an enzyme-linked immunosorbent assay. Results are represented as the mean ± S.E.M. for at least triplicate samples per group from three independent experiments. Statistical analysis was performed by means of analysis of variance, followed by the Student-Newman–Keuls test. Statistically significant differences between treated and stimulated groups are indicated by plus signs ($^{+}P < 0.05$; $^{++}P < 0.01$), whereas pound signs ($^{\#\#\#}P < 0.001$) represent differences between treated and non-stimulated groups and asterisks ($^{*}P < 0.1$) represent differences between stimulated and non- stimulated groups.

on two assumptions: 1) p23 and MD-2 share some structural similarity; and 2) since gedunin binds to p23, it probably binds to MD-2.

Our results show that gedunin docked to the large hydrophobic site that overlaps to the LPS binding site (Fig. 6). As illustrated in Fig. 6A, all 16 high-score poses were docked at the most hydrophobic region of the MD-2 protein surface, within the LPS binding pocket (the hydrophobic pocket). Moreover, the MD-2 residues interacting with the higher-score docked conformation of gedunin are all hydrophobic, including Val24, Ile32, Ile46, Val48, Ile52, Leu61, Leu78, Phe119, Phe121, Cys133, Val135, Phe151, and Ile153 (with the exception of the polar Ser120) (Fig. 6, B and C). Thus, according to our predictions, the binding of gedunin to the MD-2 surface would impair LPS binding, avoiding the formation of the complex between MD-2 and LPS.

Physicochemical Binding Assays to MD-2 Protein. The biosensor analysis performed here was useful to prove the binding property of MD-2 with its ligand, gedunin. The kinetics of the interaction was evaluated after activation of the HisCap sensor chip upon immobilization of the complexes, which was activated to recognize and bind to the histidine region of the MD-2. The binding of MD-2 protein to the HisCap chip exhibited a significant binding rate of 452 ± 18 RU/s for the interaction with nickel-nitrilotriacetic acid. Therefore, the binding site of MD-2 was accessible to interact with gedunin in solution. The time RU/s variation in 890 seconds was used to evaluate the interactions between MD-2 and gedunin (Fig. 7A).

The dissociation values of MD-2 binding (in RU/s) were 1.3 ± 0.1, 1.6 ± 0.2, 3.4 ± 0.4, 4.0 ± 0.2, 5.7 ± 0.6, 13 ± 0.1, and 33.4 ± 0.3 for gedunin concentrations (in μM) of 0.001, 0.01, 0.10, 1.0, 10.0, 100.0, and 1000.0, respectively (Fig. 7, A and B). The kinetic values of gedunin interaction with MD-2 was assessed by the affinity constant, as K_d 280 ± 29 μM. Gedunin was further analyzed using a series of concentrations, which demonstrated an increase in the SPR signal (in RU/s), indicating that this limonoid directly binds to immobilized MD-2 in a concentration-dependent fashion (R^2 = 0.925; Fig. 7B). Double-reciprocal plot linearization shows that gedunin binding to MD-2 occurs in a concentration-dependent fashion (Fig. 7C). Figure 7D shows the dose-response curves of gedunin and LPS binding to MD-2, demonstrating RU_{max} values of 38.653 (R^2 = 0.952) for gedunin and 69.953 (R^2 = 0.9846) for LPS. The double-reciprocal plot was performed to calculate 50% of saturation of binding of gedunin and LPS to immobilized MD-2 and revealed values of 14.5 μg/ml and 9.64 μg/ml, respectively. The addition of gedunin (14.5 μg/ml) to LPS previously added to immobilized MD-2 at different concentrations diminished RU values of LPS binding (R^2 = 0.9883), evaluated 20 seconds after dissociation time (Fig. 7E). A similar binding inhibition curve was observed when



B

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                 ..........+.........+.........+.........+.........+.....
2E56  SSA 1   17 ---------------------CCCCCEEEEEECEEEEEEEEEECCCCCC-EEEEEC
1EJF  SSA 2    1 CEEEEEEEEEECEEEEEEEEE------------------------CC-CECCEEE-
2E56        1 17 ---------------------EAQKQYWVCNSSDASISYTYCDKMQYPI-SINVNP
1EJF        2  1 MQPASAKWYDRRDYVFIEFCV------------------------ED-SKDVNVN-

                 ..........+.........+.........+.........+.........+.....
2E56  SSA 1   51 CCCCCCEEEEEEEEEECCCCCCCCEEEEEEEEEEECEEECCEEEEECCCCCCCCCC
1EJF  SSA 2   31 -EE-CEEE-EEEEEE-C---------------------------------------
2E56        1 51 CIELKGSKGLLHIFYIPRRDLKQLYFNLYITVNTMNLPKRKEVICRGSDDDYSFCR
1EJF        2 31 -FE-KSKL-TFSCLG-G---------------------------------------

                 ..........+.........+.........+.........+.........+.....
2E56  SSA 1  107 CECCECEEEEEEEEEECCCCCC--EEEEEEEEEEECCCCECCEEEEEEEEEECCCC
1EJF  SSA 2   44 ---CCCEEEEEEEEEC-CC-CCCCE-EEEEEECE---------EEEEEEEC----C
2E56        1 107 ALKGETVNTTISFSFKGIKFSK--GKYKCVVEAISGSPEEMLFCLEFVILHQPNSN
1EJF        2 44 ---SDNFKHLNEIDLF-HC-IDPND-SKHKRTDR---------SILCCLRK----G

                 ..........+.........+.........+.........+.........+.....
2E56  SSA 1      ------------------------------
1EJF  SSA 2   81 CCCCCCCCCCCCCCCCCCEEECCCCCCCCC
2E56        1    ------------------------------
1EJF        2 81 ESGQSWPRLTKERAKLMWLSVDFNNWKDWE

Alignment Score:  0.808
RMSD:  4.495 Angstrom
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Fig. 5. Structural alignment of the crystal structures of the human MD-2 and the cochaperone p23. (A) Representation of human MD-2 (Protein Data Bank identifier 2E56) in red on the left, p23 (Protein Data Bank identifier 1EJF) in blue in the middle, and the superposition of both structures on the right. (B) Secondary structure-based alignment of the sequences from MD-2 and p23 structures using the Protein Structure Alignment module of Maestro software (Suite 2012; Schrödinger LLC, New York, NY).

LPS (9.64 μg/ml) was added to gedunin at different concentrations (R^2 = 0.8511), suggesting that gedunin might compete with LPS for the same binding region of MD-2. Figure 7F shows the percentage of inhibition calculated using the values demonstrated in Fig. 7E, considering 100% as the resonance signal of $1/RU_{max}$ of gedunin (RU = 19.33) and LPS (RU = 35.00).

Discussion

In this study, we demonstrate that gedunin has a remarkable suppressive effect on macrophage activation induced by LPS. We also provide evidence that gedunin binds to the MD-2 component of the TLR4 complex and, by impairing the upstream activation of LPS signaling cascade, gedunin inhibits both early (MAL/MyD88-dependent) and late (TRAM/TRIF-dependent) pathways.

Our group previously demonstrated that gedunin presents important anti-inflammatory and immunomodulatory effects in different experimental models in vivo, including experimental arthritis, allergic pleurisy, and allergic lung inflammation, in which macrophages play a pivotal role (Penido et al., 2005, 2006a,b; Henriques and Penido, 2014; Conte et al., 2015). Here, we demonstrate that gedunin directly modulates macrophages in vitro, in a concentration-dependent manner, inhibiting early and classic parameters of macrophage activation after pathogen-associated molecular pattern recognition. The fact that different pathways are involved in calcium influx and TNF-α and NO production reinforces that gedunin blocks an upstream and common mechanism involved in these three responses, most likely due to MD-2 binding. However, the modulation of Hsp90 by gedunin (Brandt et al., 2008; Patwardhan et al., 2013) also explains the impairment of LPS-induced NO production, since NO synthesis has been shown to be modulated by Hsp90, through Hsp90–inducible nitric oxide synthase interaction (Yoshida and Xia, 2003; Luo et al., 2011). Indeed, it was previously reported that Hsp90 inhibition by geldanamycin also impaired NO production by macrophages stimulated with LPS (Luo et al., 2011).

The recognition of LPS by the TLR4/MD-2/CD14 complex culminates in the production of TNF-α and IL-6 via both MAL/MyD88- and TRAM/TRIF-dependent pathways (Wax et al., 2003; Akira and Takeda, 2004). Hsp90 (in association with Hsp70 and other chaperones) maintains the conformation and activity of MAL/MyD88- and TRAM/TRIF-dependent kinases involved in TNF-α and IL-6 production, such as p38, extracellular signal-regulated kinase 1/2, and c-Jun N-terminal kinase (Davis and Carbott, 1999; Richter and Buchner, 2001;

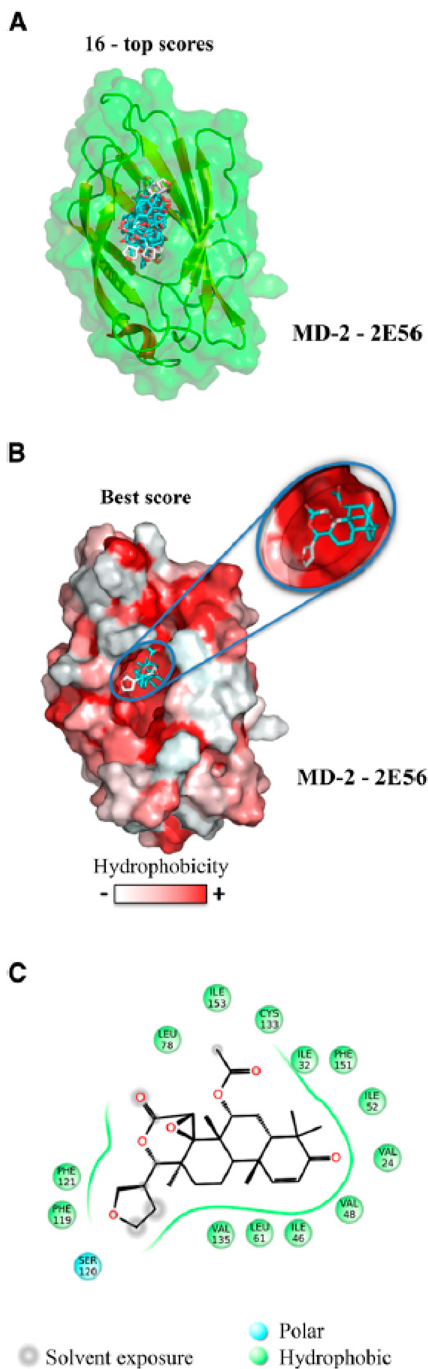

Fig. 6. In silico molecular docking reveals the binding mode of gedunin on the MD-2 structure (Protein Data Bank identifier 2E56). (A) The 16 top high-score conformations of gedunin within the hydrophobic pocket. Representation plus molecular surface of human MD-2. (B) Best-score predicted pose of gedunin on the molecular surface of MD-2, colored according to the Eisenberg normalized consensus hydrophobicity scale (Eisenberg et al., 1984). (C) Two-dimensional ligand interactions diagram of gedunin within the hydrophobic pocket, generated using Maestro software (Suite 2012; Schrödinger LLC, New York, NY). Polar and hydrophobic amino acids are illustrated in blue and green circles.

Beutler et al., 2003; Bode et al., 2003; Yamamoto et al., 2003; Echeverría et al., 2011). Indeed, geldanamycin has been shown to inhibit TNF-α and IL-6 mRNA as well as protein levels in LPS-stimulated macrophages (Vega and De Maio, 2003; Wax et al., 2003). Whether gedunin distinctly modulates MAL/MyD88 and TRAM/TRIF pathways was investigated here by means of TRIF and MAL KO macrophages. The fact that this limonoid impaired cytokine production via both signaling cascades supports our assumption that gedunin acts upstream in LPS-induced macrophage activation. This effect is likely to occur either via MD-2 blockade (as revealed by our in silico and SPR experiments) or via modulation of TLR4-associated Hsp90, as previously proposed (Latz et al., 2002; Triantafilou and Triantafilou, 2004; Triantafilou et al., 2004, 2008). These authors have suggested, by means of fluorescence recovery after photobleaching analysis, that Hsp90 is part of the multimeric LPS receptor complex (Triantafilou et al., 2001). It is noteworthy that the ability of gedunin to modulate both MAL/MyD88 and TRAM/TRIF pathways independently of TLR4/MD-2 was also observed, since gedunin treatment impaired TNF-α production by macrophages stimulated with Pam3 (a TLR2 selective agonist) and polyinosinic/polycytidylic acid (a TLR3 selective agonist) (Supplemental Fig. 2). These data reinforce the role of Hsp90 in this phenomenon, since several kinases involved in MAL/MyD88 and TRAM/TRIF pathways are regulated by Hsp90 chaperoning activity (Yang et al., 2006; Hinz et al., 2007; Shi et al., 2009; Yun et al., 2011).

In addition to cytokines, LPS in vitro stimulation triggers increased COX-2 expression and PGE_2 production by macrophages via the TRIF signaling pathway (Endo et al., 2014). In accordance, we demonstrate here that WT macrophages produced higher amounts of PGE_2 24 hours, rather than 6 hours, after LPS stimulation, a time point in which the TRIF signaling pathway is most prominent. Nonetheless, gedunin pretreatment diminished PGE_2 production to basal levels during early and late responses. As mentioned above, Hsp90 plays an important role in the regulation of MAPK family members and, by stabilizing transforming growth factor β-activated kinase 1, triggers MAPKs and NFκB activation, involved in COX-2 expression (Eliopoulos et al., 2002; Shi et al., 2009; Echeverría et al., 2011; Bode et al., 2012). There are different mechanisms proposed for Hsp90 modulation of NFκB. It has been shown that geldanamycin reduces LPS-mediated NFκB nuclear translocation in murine macrophages (Byrd et al., 1999). Malhotra et al. (2001) demonstrated that modulation of Hsp90 activity by geldanamycin did not impair inhibitor of κB degradation or NFκB translocation into the nucleus; however, it reduced the formation of the NFκB/DNA complex and, therefore, inhibited activation of cytokine promoter. Other reports have demonstrated that the inhibition of Hsp90 activity by geldanamycin reduced the stability of LPS-induced TNF-α and IL-6 transcripts, a phenomenon partially dependent of p38 (Wax et al., 2003). Our results demonstrate that, in our model, gedunin impaired LPS-induced NFκB translocation in macrophages, reinforcing the additional role of this limonoid in modulating Hsp90 in

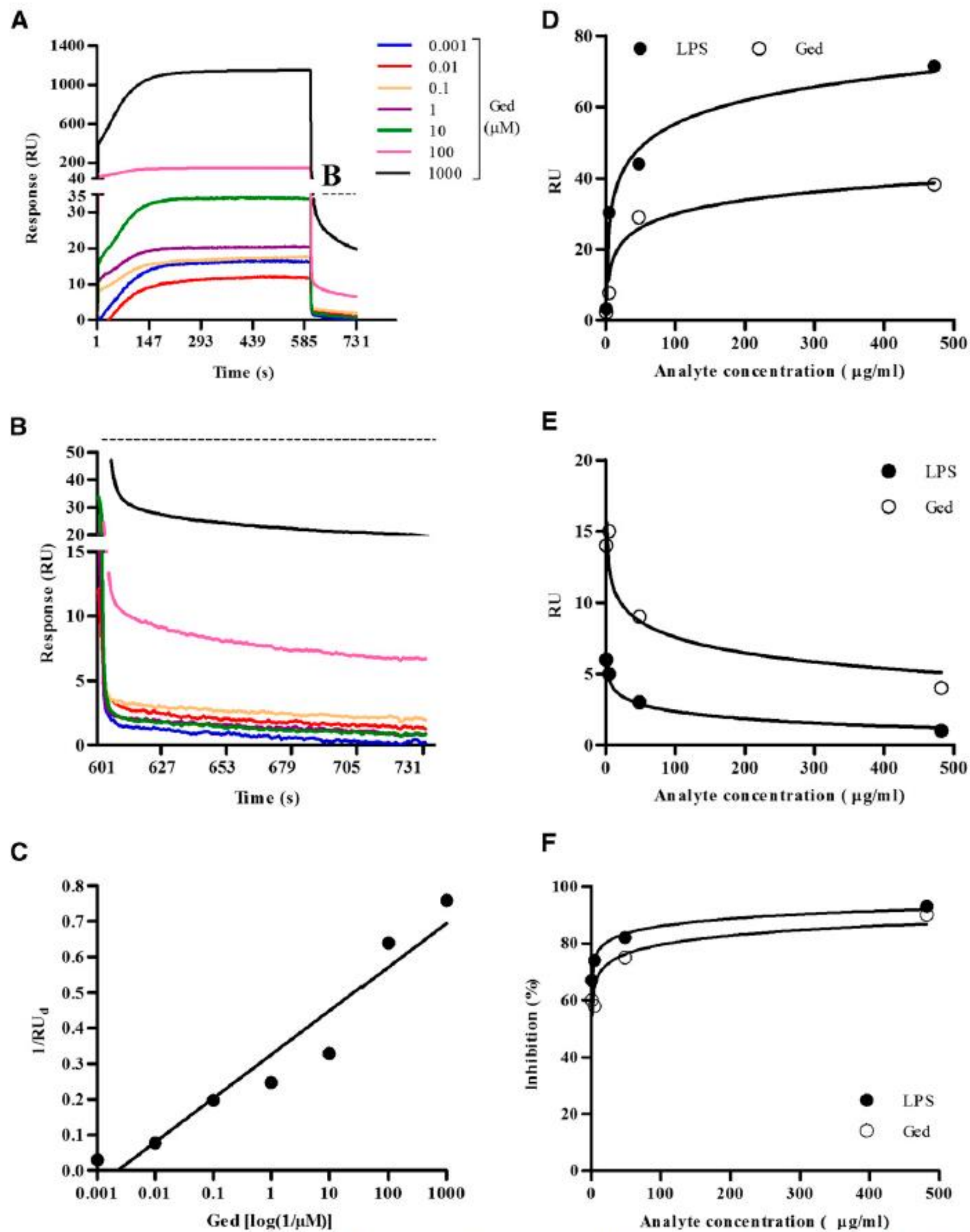

Fig. 7. Biosensing surface assays to assess the binding of gedunin and LPS to immobilized MD-2. After covering the HisCap chip with MD-2, the binding assay of gedunin (Ged; 0.001–1000 μM) was followed by the variation of response throughout 890 seconds. (A and B) Resonance signals are represented by sensorgram in arbitrary RU analyzed after subtraction of a reference line using Qdat software. (C) Double-reciprocal plot linearization of gedunin binding to MD-2. (D) Concentration curves of binding of gedunin and LPS (0.482–482 μg/ml) to immobilized MD-2. (E) Binding inhibition curves of gedunin by LPS ($R^2 = 0.9883$) and of LPS by gedunin ($R^2 = 0.8511$), analyzed using 14.5 μg/ml gedunin and 9.64 μg/ml LPS, after 20 seconds of dissociation time. (F) Percentage of binding inhibition. The degree of inhibition was measured considering 100% as the resonance signal of $1/RU_{max}$ of gedunin (RU = 19.33) and LPS (RU = 35.00). Data are representative of five experiments.

LPS-triggered response (i.e., blocking the upstream signaling of TLR4/MD-2).

In addition to the ability to inhibit LPS-induced proinflammatory mediators, gedunin also triggered anti-inflammatory factors, namely HO-1 (Hsp32), Hsp70, and IL-10. Supporting our data, the induction of Hsps, including HO-1, by the Hsp90 inhibitor celastrol was previously demonstrated in vitro and in vivo (Trott et al., 2008; Chow et al., 2013; Der Sarkissian et al., 2014). HO-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation, which is widely expressed during cellular stress and inflammation, triggered by diverse stimuli, including LPS (Camhi et al., 1995, 1998). During inflammation, HO-1 plays roles in anti-inflammatory and immunomodulatory responses, mediated by the degradation of proinflammatory free heme, as well as via the production of bilirubin and carbon monoxide, which present anti-inflammatory properties (Gozzelino et al., 2010; Naito et al., 2014). As described in the literature, LPS in vitro stimulation triggers increased HO-1 expression, induced by IL-10, via a p38 MAPK-dependent pathway (Lee and Chau, 2002). In addition, it has been demonstrated that carbon monoxide attenuates Hsp90 activity and promotes dissociation of its client proteins (Lee et al., 2014). Here, we demonstrate that this stress-inducible protein was increased by gedunin incubation in the presence or absence of LPS, suggesting that, in our experimental model, gedunin effects are likely a result of both MD-2 binding and Hsp90 modulation. The fact that HO-1 expression was higher in gedunin pretreated macrophages compared with medium- or LPS-stimulated cells reinforces that gedunin can modulate macrophage function independently of TLR4/MD-2 activation, likely via Hsp90.

The inhibition of Hsp90 activity has been shown to induce increased expression of Hsp70 and Hsp90 via HSF-1 activation and is related to cell protection during stress (Sharp et al., 2013; Paul and Mahanta, 2014; Leung et al., 2015). Accordingly, we observed the induction of Hsp90 expression in LPS-stimulated macrophages pretreated with gedunin, 17-AAG, and dexamethasone (which also modulates HSF-1 activity; Knowlton and Sun, 2001) (Supplemental Fig. 3). Overexpression or induction of intracellular Hsp70 (by stress or by Hsp70-inducing compounds) has been shown to decrease nuclear NFκB translocation, inducible nitric oxide synthase expression, and production of TNF-α, IL-6, IL-1β, and NO (Shi et al., 2006; Dokladny et al., 2010; Kim et al., 2012; Muralidharan et al., 2014). Accordingly, it has been demonstrated that extracellular Hsp70 diminishes LPS-induced TNF-α by bone marrow–derived dendritic cells in vitro and induces IL-10 production by synovial cells from patients with arthritis (Detanico et al., 2004). The anti-inflammatory cytokine IL-10 suppresses LPS-induced macrophage activation by inhibiting the expression of specific TLR-induced proinflammatory genes referred to as "IL-10 counter-regulated genes" (Moore et al., 1993; Cardwall and Weaver, 2014). It is noteworthy that IL-10–mediated anti-inflammatory response is also mediated by HO-1 expression (Lee and Chau, 2002). Interestingly, our study revealed that gedunin acts as an Hsp70 inducer and also increases the production of IL-10, supporting its anti-inflammatory and immunomodulatory effects.

Based on the assumptions that p23 and MD-2 share some structural similarity and that gedunin binds to p23, we hypothesized that gedunin might bind to MD-2. Supporting the biologic effects observed with LPS-activated macrophages pretreated with gedunin, our in silico results showed that gedunin docked to the MD-2 large hydrophobic site that overlaps to the LPS binding site, avoiding the formation of the complex between CD14/MD-2/TLR4 and LPS. Furthermore, the binding of gedunin to MD-2 proposed by our docking experiments was proved by the biosensing surface assays we performed. Even though the physicochemical conditions in the SPR assays might not exactly reflect that of the cellular microenvironment, the affinity of gedunin to MD-2 corroborates our in vitro data. The measurement of kinetic values strongly indicates that gedunin is capable of forming complexes with MD-2 and therefore acts as anti-inflammatory in LPS-stimulated macrophages. On the basis of our SPR data, the Gibbs free energy of the formed complexes suggests that gedunin spontaneously binds to MD-2. This finding is physicochemical proof that gedunin interferes with the binding of LPS to MD-2 as a competitive inhibitor and therefore impairs the formation of TLR4/MD-2/LPS complex. Overall, our data suggest that, in addition to Hsp90 modulation, gedunin impairs the TLR4 signaling pathway by inhibiting the binding of LPS to MD-2 in macrophages.

Acknowledgments

The authors thank Thomas Krahe for critical reading of the manuscript and Thadeu Costa, Leonardo Seito, Tatiana Pádua, and Kennedy Bonjour for technical assistance.

Authorship Contributions

Participated in research design: Pacheco, Penido.

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Contributed new reagents or analytic tools: Maya-Monteiro, Alves, Caffarena, Pacheco, Henriques, Penido.

Performed data analysis: Borges, Moret, Maya-Monteiro, Souza-Silva, Batista, Penido.

Wrote or contributed to the writing of the manuscript: Borges, Moret, Alves, Batista, Penido.

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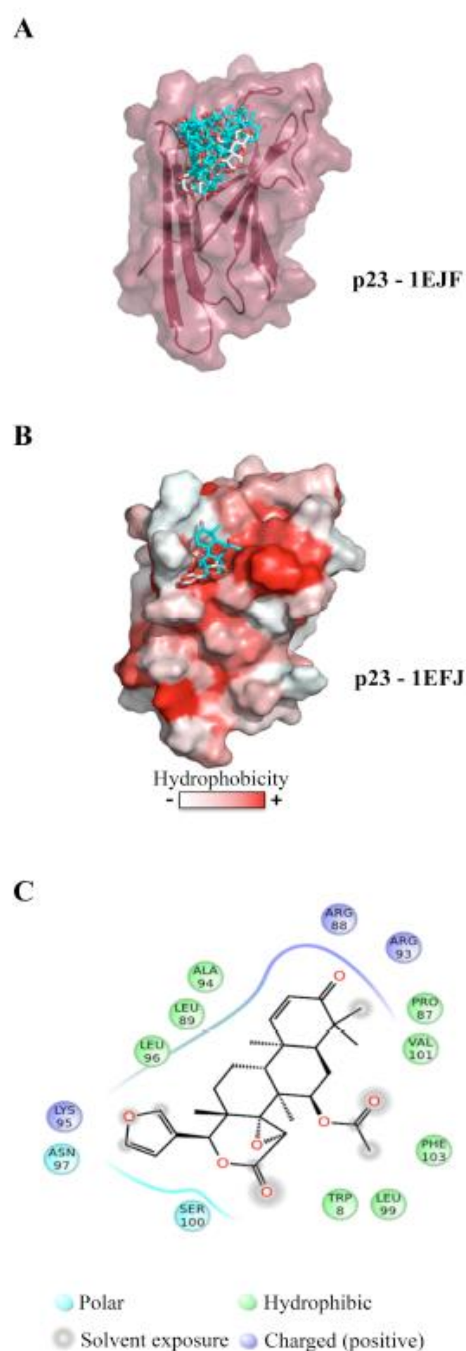


Figure S1. *In silico* molecular docking confirms the binding mode of gedunin on the crystal structure of the human co-chaperone p23 (PDB ID 1EJF). (A) 16-top high score conformations of gedunin within the hydrophobic pocket. Cartoon representation + molecular surface of human MD-2. (B) best-score predicted pose of gedunin on the molecular surface of the p23, colored accordingly the Eisenberg normalized consensus hydrophobicity scale (Eisenberg et al., 1984). (C) the 2D-Ligand Interactions Diagram, generated with Maestro program (2012). Polar, hydrophobic and positively charged aminoacids are illustrated in blue, green and purple circles.

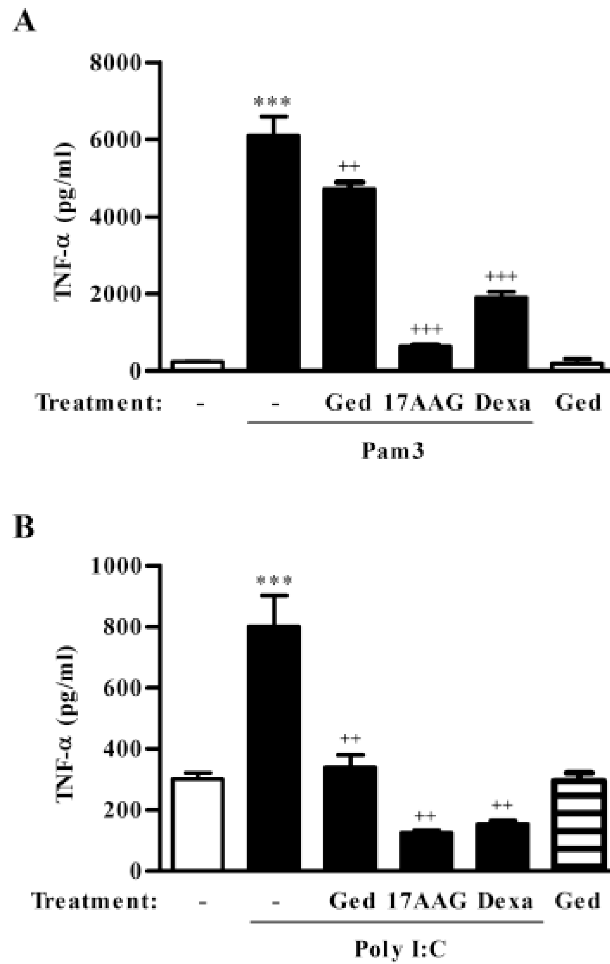
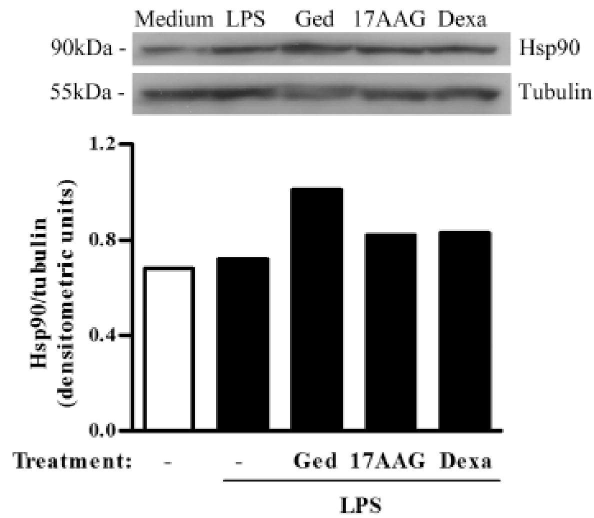


Figure S2. Gedunin impairs TLR2- and TLR3-dependent TNF-α production. WT immortalized macrophages (10^6 cells/well) were pretreated for 1h with gedunin (Ged, 10µM), 17-AAG (1µM) or dexamethasone (Dexa, 100nM) and stimulated with (A) Pam3 (palmitoyl-3-Cys-Ser-(Lys)4, Sigma Aldrich, 1µg/ml) or Poly I:C (Polyinosinic-polycytidylic acid, Sigma Aldrich, 10µg/ml,) for 24h. TNF-α levels were evaluated in the supernatants, as described in methods. Results are represented as the mean ± SEM for quadruplicate samples per group. Statistical analysis was performed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by *** (p<0.001), whereas $^{++}$ (p<0.01) and $^{+++}$ (p<0.001) represent differences between treated and non-treated groups.



Supplemental Figure 3

Figure S3. Gedunin induces Hsp90 expression in macrophages. WT immortalized macrophages (10^6 cells/well) were pretreated for 1h with gedunin (Ged, 10μM), 17-AAG (1μM) or dexamethasone (Dexa, 100nM) and stimulated with LPS (50ng/ml) for 24h. Hsp90 expression in whole cell extracts evaluated by western blot, as described in methods. Mouse monoclonal anti-mouse/human/rat Hsp90 (1:5000) was obtained from Santa Cruz Biotechnologies (Santa Cruz, USA). Representative western blot of one experiment is shown in the top panel.

3.2 Artigo 2

Protective effect of gedunin on TLR-mediated inflammation by modulation of inflammasome activation and cytokine production: evidence of a multitarget compound

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Pharmacological Research (16)30472-8, 2016

Demonstramos previamente que a gedunina apresenta um efeito modulador sobre a ativação das vias de sinalização aguda (dependente de MAL/MyD88) e tardia (dependente de TRAM/TRIF) de TLR4 em macrófagos *in vitro,* e também que a gedunina é capaz de se ligar ao MD-2 no mesmo sítio de ligação do LPS, em adição à sua ligação às co-chaperonas p23 e Cdc37, conforme demonstrado na literatura (109, 112). No presente trabalho, nós investigamos se a gedunina também poderia modular a ativação de outros TLRs, como TLR2 e TLR3, uma vez que a gedunina é uma substância multi-alvos e que a atividade da chaperona de Hsp90 é importante para a estabilização de diversas proteínas clientes envolvidas na sinalização destes receptores.

Neste estudo, nós observamos que, de fato, a gedunina modula a resposta inflamatória induzida por agonistas de TLR2 e TLR3, inibindo a produção de mediadores pró-inflamatórios e a ativação de inflamassoma NLRP3. Ainda, demonstramos que este limonóide induz a produção de fatores anti-inflamatórios por macrófagos estimulados por PAM3CSK4 e POLY I:C, o que reforça a sua atividade anti-inflamatória. Em adição, demonstramos através de ensaios *in silico* – que a gedunina apresenta alta probabilidade de ligação ao TLR2, ao TLR3 e à caspase-1, enzima ativada após formação do inflamassoma NLRP3. Considerando o efeito da gedunina sobre macrófagos estimulados com LPS *in vitro*, avaliamos neste estudo o efeito da gedunina sobre a resposta inflamatória *in vivo* desencadeada pelo LPS no modelo murino de pleurisia. Nós observamos que tanto o pré- quanto o pós-tratamento *in vivo* com a gedunina inibiram a ativação do inflamassoma NLRP3, a produção de mediadores pró-inflamatórios e o acúmulo de leucócitos no sítio inflamatório.

Em conjunto, nossos resultados demonstram que a gedunina suprime a resposta induzida por LPS em macrófagos murinos em modelo *in vitro* e no modelo de pleurisia, inibindo diferentes parâmetros da resposta inflamatória e a ativação de inflamassoma. Ainda, demonstramos que o mecanismo de ação da gedunina não se restringe à via de sinalização de TLR4, e sugerimos um que este tetranortriterpenóide se ligue a múltiplos alvos, apresentando alto potencial de ligação a TLR2, TLR3 e caspase-1.

Pharmacological Research 115 (2017) 65–77

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Protective effect of gedunin on TLR-mediated inflammation by modulation of inflammasome activation and cytokine production: Evidence of a multitarget compound

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ARTICLE INFO

Article history:
Received 19 May 2016
Received in revised form 9 August 2016
Accepted 14 September 2016
Available online 15 September 2016

Keywords:
Toll-like receptor
LPS
Gedunin
Macrophage
Inflammasome
Cytokines

ABSTRACT

Activation of toll-like receptors (TLRs) by pathogen-associated molecular patterns (PAMPs) triggers an innate immune response, *via* cytokine production and inflammasome activation. Herein, we have investigated the modulatory effect of the natural limonoid gedunin on TLR activation *in vitro* and *in vivo*. Intraperitoneal (i.p.) pre- and post-treatments of C57BL/6 mouse with gedunin impaired the influx of mononuclear cells, eosinophils and neutrophils, as well as the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and nitric oxide (NO), triggered by lipopolysaccharide (LPS) in mouse pleura. Accordingly, *in vitro* post-treatment of immortalized murine macrophages with gedunin also impaired LPS-induced production of such mediators. Gedunin diminished LPS-induced expression of the nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) on pleural leukocytes *in vivo* and in immortalized macrophages *in vitro*. In line with this, gedunin inhibited LPS-induced caspase-1 activation and the production of IL-1β *in vivo* and *in vitro*. In addition, gedunin treatment triggered the generation of the anti-inflammatory factors IL-10 and heme oxigenase-1 (HO-1) at resting conditions or upon stimulation. We also demonstrate that gedunin effect is not restricted to TLR4-mediated response, since this compound diminished TNF-α, IL-6, NO, NLRP3 and IL-1β, as well as enhanced IL-10 and HO-1, by macrophages stimulated with the TLR2 and TLR3 agonists, palmitoyl-3-Cys-Ser-(Lys)4 (PAM3) and polyriboinosinic:polyribocytidylic acid (POLY I:C), *in vitro*. *In silico* modeling studies revealed that gedunin efficiently docked into caspase-1, TLR2, TLR3 and to the myeloid differentiation protein-2 (MD-2) component of TLR4. Overall, our data demonstrate that gedunin modulates TLR4, TLR3 and TLR2-mediated responses and reveal new molecular targets for this compound.

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Abbreviations: ASC, apoptosis-associated speck-like protein containing caspase activation and recruitment domain; ATP, adenosine triphosphate; CARD, caspase activation and recruitment domain; DMEM, dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; HO-1, heme oxigenase-1; Hsp, heat shock protein; IL, interleukin; i.pl., intrapleural; LPS, lipopolysaccharide; MD-2, myeloid differentiation protein-2; NLRP3, nucleotide-binding domain and leucine-rich repeat protein-3; NO, nitric oxide; PAM3, palmitoyl-3-Cys-Ser-(Lys)4; PAMP, pathogen-associated molecular pattern; PDB, protein data bank; POLY I:C, polyriboinosinic:polyribocytidylic acid; PRR, pattern recognition receptors; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate (SDS); TLR, toll-like receptor; TNF-α, tumor necrosis factor-α.

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http://dx.doi.org/10.1016/j.phrs.2016.09.015
1043-6618/© 2016 Published by Elsevier Ltd.

1. Introduction

The innate immune system represents the first line of defense against invading microbial pathogens, by recognizing distinct conserved structures on pathogens denominated pathogen-associated molecular patterns (PAMPs), *via* pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). TLR specificity to basic molecular patterns affords host protection, and includes the recognition of bacterial lipopolysaccharide (LPS) by the TLR4/myeloid differentiation factor-2 (MD-2) complex, mostly expressed by macrophages [1–4]. TLR4 signaling pathways rely on the temporally distinct recruitment of the adaptor proteins MAL/MyD88 (MyD88-adapter-like/Myeloid differentiation primary response 88) and TRAM/TRIF (TRIF-related adaptor molecule/TIR-domain-containing adapter-inducing interferon-β) [5–7], which subsequently leads to the activation of nuclear factor (NF)kB and the production of inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and nitric oxide (NO) [8–12]. Other than LPS, a multiplicity of microbial products is recognized by macrophages, *via* the expression of other membrane bound and cytosolic PRRs. Among these PRRs, TLR3 is known to primarily recognize double-stranded RNA species produced during viral infections and to signal *via* TRAM/TRIF to ultimately induce type I interferons (IFNs) and other cytokines, *via* IFN regulatory transcription factor 3 (IRF3) and NFκB [13]. On the other hand, TLR2 recognizes a large variety of PAMPS from yeast, Gram-positive and Gram-negative bacteria, and requires MAL/MyD88 adaptor proteins, to finally trigger the transcription of inflammation-related genes, *via* NFκB and activator protein-1 (AP-1) [14,15].

PRR-induced responses can also be mediated by inflammasomes, a group of cytosolic multimeric protein complexes that can be distinctly formed in response to different stimuli [16,17]. The nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasome (also known as NALP3) can be triggered by a vast array of molecules originated from bacteria, yeast and viruses, and encompasses the NLRP3 protein, the adapter protein apoptosis-associated speck-like protein containing caspase activation and recruitment domain (CARD) (ASC) and pro-caspase-1 [18,19]. Activation of caspase-1 triggers the proteolysis of pro-IL-1β and pro-IL-18 and the subsequent release of IL-1β and IL-18 [16,17]. Inflammation is, undoubtedly, critical for effective host defense. However, new insights into pathogen–host interactions, inflammatory pathways and effects of immunomodulatory compounds during infection are paving the way for the application of host-directed therapies. The identification of immunomodulatory agents that can be used for improving antimicrobial treatment outcomes is a promising approach for the treatment of a range of infectious diseases [20–23].

Gedunin is a secondary metabolite of the Meliaceae species *Azadirachta indica* (Neem) and *Carapa guianensis* (andiroba). It is chemically classified as a limonoid, and has attracted substantial attention in view of its marked biological effects that include anti-tumor, anti-allergic and anti-inflammatory activities [for review, see [24]. More recently, our group has demonstrated that gedunin also modulates inflammatory responses triggered by TLR agonists [25,26]. Such broad spectrum of action of gedunin is partially explained by its molecular mechanism of action that relies on the inhibition of heat shock protein (Hsp)90 activity, an ubiquitously expressed chaperone that presents a vast repertoire of client proteins associated with membrane, cytoplasm and endoplasmic reticulum, being consequently involved in innumerous cellular processes [27–30]. In addition to the modulation of Hsp90 (*via* binding to its co-chaperones p23 and Cdc37) [31,32], our group has recently demonstrated that gedunin also binds to MD-2 and impairs TLR4/MD-2/CD14-mediated response in murine macrophages *in vitro* [26]. Considering the above, we hypothesize that gedunin modulates different parameters triggered by TLRs and that it binds to multiple molecular targets. In this paper we show that gedunin is a multitarget compound that efficiently docks to TLR2, TLR3 and caspase-1, and negatively modulates TLR2-, TLR3- and TLR4-mediated responses, by impairing inflammasome activation and cytokine production, as well as by inducing anti-inflammatory factors in macrophages.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (18 to 20 g, 5–7 weeks) were provided by the Oswaldo Cruz Foundation (Fiocruz, Brazil) Breeding Unit (Rio de Janeiro, Brazil). Animals were housed with free access to food and water in a room with temperatures ranging from 22 to 24 °C under a 12 h light/dark cycle at Farmanguinhos Experimental Animal Facility (Fiocruz). All experimental procedures were performed according to guidelines provided by the Committee on Ethical Use of Laboratory Animals of Oswaldo Cruz Foundation (#LW-62/12).

2.2. LPS-induced pleurisy

Pleurisy was induced by an intrapleural (i.pl.) injection of LPS (250 ng/cavity, from *E. coli* O111:B4, Sigma Aldrich, St. Louis, MO, USA) diluted in sterile saline solution to a final volume of 100 μl in non-anesthetized mice. Control animals received an equal volume of vehicle. Mice were euthanized with pentobarbital (250 mg/kg, 200 μl, i.p.). Leukocytes were recovered from pleural cavities after washing with 500 μl of PBS containing ethylenediamine tetraacetic acid (EDTA) (10 mM, pH 7.4) (Sigma-Aldrich), 24 h after LPS stimulation. Volumes of recovered pleural washes were determined with a pipette (Gilson Pipetman P1000) and used to normalize leukocyte counts. Total leukocyte counts were determined with an automated particle counter (Beckman Coulter, Inc., Fullerton, CA, USA). Differential leukocyte counts were made by using cytospins (Cytospin 3, Thermo Scientific, Waltham, MA, USA) stained by the May-Grunwald-Giemsa method, under light microscopy (100x lens objective; Olympus Optical do Brasil, Ltda, São Paulo, Brazil). Cell-free supernatants and cell lysates were kept at −20 °C until used.

2.3. *In vivo* treatments

One hour before or after i.pl. injection of LPS, mice received an intraperitoneal (i.p.) injection of gedunin (PubChem CID: 12004512) (0.5 mg/kg), after dilution in sterile saline solution containing 0.05% dimethyl sulfoxide (DMSO) to a final volume of 100 μl, as described [28]. Dexamethasone (1 mg/kg, 100 μl), was i.p. administered, as reference inhibitor. The same volume of vehicle plus DMSO was administered to control mice.

2.4. Cell culture, stimulus and treatment

Immortalized bone-marrow-derived macrophage cell lines generated from wild-type C57BL/6 mice (a kind gift from Douglas Golenbock; University of Massachusetts Medical School, [33]) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) supplemented with fetal bovine serum (FBS, 10%, Gibco/Life Technologies, Carlsbad, USA), sodium pyruvate (1 mM, Sigma Aldrich, St. Louis, USA), and ciprofloxacin (10 μg/ml, Fresoflox®, Fresenius Kabi, Barueri, SP, Brazil) until confluence. Cells were used at the forth passage for all *in vitro* experiments described in this report.

Macrophages (10^6 cells/well) were then cultured in 6- or 24-well plates in DMEM (supplemented with 2% FBS, 1 mM

sodium pyruvate, and 10 μg/ml ciprofloxacin) for 24 h. After confluence, cells were stimulated with LPS (50 ng/ml, from *E. coli* O111:B4, Sigma Aldrich) re-purified by a repeated phenolchloroform extraction [26,34], with palmitoyl-3-Cys-Ser-(Lys)4 (PAM3, 1 μg/ml, InvivoGen, San Diego, CA, USA) [35] or with polyriboinosinic:polyribocytidylic acid (POLY I:C, 1 μg/ml, InvivoGen) [36]. One hour before or after the stimulus, macrophages were treated with gedunin (0.01–100 μM; Gaya Chemical Corporation, New Milford, CT, USA) for 24 h and the cell-free supernatants recovered for analysis. In one set of experiments, cells were stimulated in the presence of IFN-γ (200 IU/ml), for nitrite determination. In another set of experiments, cells were stimulated with 1 μg/ml of LPS and pulsed with adenosine triphosphate (ATP) (2 mM) to activate NLRP3 inflammasome, for 20 min, 4 h after LPS stimulation [37]. The Hsp90 inhibitor 17-AAG (PubChem CID: 6440175) (1 μM) and dexamethasone (PubChem CID: 24893536) (100 nM) were used as reference inhibitors (Sigma Aldrich).

2.5. Cytokine analysis

Cytokine levels were evaluated in cell free pleural washes and in the supernatants of immortalized macrophages by enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs from R&D Systems (Quantikine, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.6. Nitrite determination

Nitrite, a stable metabolite of NO in aqueous solutions, was determined in cell-free pleural washes and in the supernatant of immortalized macrophages by Griess reaction in 96 well plates, after the addition of Griess reagent (1:1) (Sigma Aldrich) to the wells for 15 min at room temperature. Absorbance was read at 562 nm using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Western blot

Total protein content in cytoplasmatic extracts was determined by the Bradford reagent (Sigma Aldrich). Cell lysates were denatured in Laemmliís sample buffer (50 mM Tris-HCl, pH 6.8; 1% sodium dodecyl sulfate (SDS); 5% 2-mercaptoethanol; 10% glycerol and 0.001% bromophenol blue) and heated at 95 °C for 5 min. Aliquots containing 30 μg of protein were re-suspended in SDS-PAGE loading buffer, resolved on 15% SDS acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) Hybond™ membranes (Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% non-fat dry milk/Tris-buffered saline containing 0.1% Tween-20 for 2 h at room temperature, the membranes were probed with the following primary antibodies: rabbit polyclonal anti-mouse HO-1 (1:5000, Enzo Life Sciences, Farmingdale, NY, USA, #ADI-SPA-895-F), rat monoclonal anti-mouse NLRP3 (2 μg/ml, R&D systems, #MAB7578), rabbit monoclonal anti-mouse ASC (1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA, USA, #sc-22514-R), mouse monoclonal anti-mouse tubulin (1:1000, Santa Cruz Biotechnology, #sc5286) and mouse monoclonal anti-mouse β-actin (1:5000, Biolegend, San Diego, CA, USA, #643802), overnight at 4 °C. Membranes were then washed and incubated with horseradish peroxidase (HRP)-labeled goat polyclonal anti-mouse IgG (1:2500, Enzo Life Sciences, #ADI-SAB-101-J), rabbit anti-rat IgG (1:5000, Enzo Life Sciences, Farmingdale, USA, #ADI-SAB-200-J) and goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnologies, #sc-2004) antibodies for 1 h. PVDF membranes were developed using ECL®-plus reagent (Enhanced Chemiluminescence, Amersham Biosciences) and visualized on Hyperfilm (Amersham Biosciences). The bands were quantified by densitometry, using Image J software program (public domain).

2.8. Caspase-1 activation assay (FLICA)

Caspase-1 activity was evaluated in immortalized macrophage suspensions by FAM-FLICA Caspase-1 Assay Kit (Immunochemistry Technologies) in a FACScalibur flow cytometer (Becton and Dickinson), according to the manufacturer's instructions. Gating strategy and propidium iodide (PI) staining is shown in Supplemental Fig. 1.

2.9. Immunofluorescence staining

Immortalized macrophages seeded on glass coverslips in 24-well plates were washed three times with PBS after stimulation and treatments, and fixed for 30 min at 37 °C with 4% (vol/vol) paraformaldehyde in PBS. Cells were washed twice with PBS, incubated with blocking buffer (1% BSA, 0.2% gelatin and 0.05% tween 20 in PBS) for 1 h and subsequently incubated with primary goat anti-mouse NLRP3 antibody (1:20, Santa Cruz Biotechnologies, #sc-34411) in blocking buffer for 16 h at 4 °C. Cells were washed with PBS containing 1% BSA and 0.05% tween 20 and incubated with Alexa fluor 546 donkey anti-goat (1:1000, Invitrogen, Carlsbad, CA, USA, # A-11056) for 1 h. Cells were washed twice with PBS containing 1% BSA and 0.05% tween 20, twice with PBS, stained with Fluoroshield™ with DAPI histology mounting medium (Sigma Aldrich) and imaged with fluorescent microscope (40x lens objective, Olympus BX60 microscope; Olympus DP72 camera, Olympus Optical do Brasil, Ltda).

2.10. *In silico docking simulations*

The drug design softwares used for *in silico* assays were MGL Tools 1.5.4, Autodock Vina 1.5.6 [38] and Discovery Studio 4.1 client [39]. The protein structures were taken from the protein data bank (PDB), as follows: human caspase-1 (code 1RWX [40]), TLR2 (code 1O77 [41]) and TLR3 (1ZIW [42]). The protein caspase-1 (1RWX) has non-homologous chains A and B, TLR2 protein (1O77) has five homologous chains, and protein TLR3 (1ZIW) presents one chain. The water and ligand structures were excluded in all the proteins by Discovery studio [39]. In case of TLR2, only one chain was retained, while other chains were excluded. All the proteins were further processed using MGL Tools 1.5.4 [43] by adding polar hydrogens and creating grid box followed by energy minimization. The 3D-structures of gedunin, dexamethasone and 17-AAG were drawn using ChemDraw 8.0 and energy minimized by computing Gasteiger and Marsili atomic charge method with MGL Tools 1.5.4 [43]. The prepared proteins and the ligands were then subjected to molecular docking using the Autodock Vina 1.5.6 [38], in a blind docking procedure, which consisted of searching the entire protein surface in order to determine the potential binding pocket(s). This was achieved by aligning the grid center to the center of each protein and by using a grid size big enough to cover the entire protein surface. Once the grid box was created, the docking was performed using the following parameters: energy range = 10, num modes = 20 and exhaustiveness = 800. Protein ligand interactions were analyzed using Discovery Studio 4.1 and Pymol [44].

2.11. Statistical analysis

Data are reported as the mean ± standard error of the mean (SEM), and were analyzed by means of ANOVA followed by Student Newman-Keuls test. Values of $P < 0.05$ were regarded as significant. Outliers, if any, were identified and removed. In this sense, data points ± 3 standard deviations from calculated means were not included in statistical analysis.

3. Results

3.1. Gedunin impairs LPS-induced leukocyte infiltration and production of inflammatory mediators

LPS intrapleural (i.pl.) injection triggers an intense inflammatory response in mouse pleura, characterized by the production of inflammatory mediators and the accumulation of different cell populations 24 h after stimulation [45–48]. Here we show that the pre-treatment of mice with gedunin, at a dose previously established by our group [25,49,50] (0.5 mg/kg, i.p., 1 h before stimulus), impaired LPS (250 ng/cav, i.pl.)-induced leukocyte accumulation within 24 h, due to the inhibition of mononuclear cell, neutrophil and eosinophil migration, in the same extent as dexamethasone (1 mg/kg, i.p.), used as the reference antiinflammatory drug (Fig. 1A–D). Of note, gedunin pre-treatment also inhibited

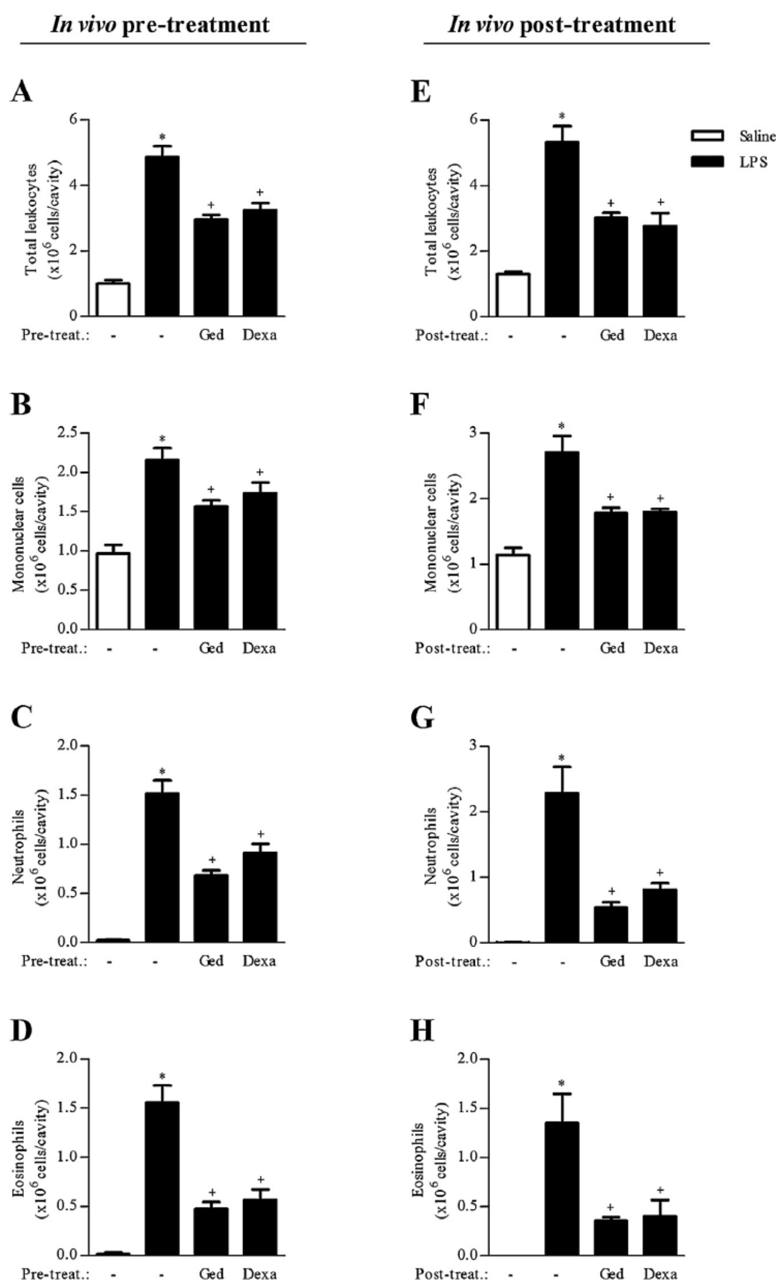

Fig. 1. Gedunin impairs LPS-induced leukocyte infiltration *in vivo*. Pleural leukocytes recovered 24 h after LPS (250 ng/cav, i.pl.) stimulation from C57BL/6 mice pretreated (A-D) or postreated (E-H) with gedunin (Ged, 0.5 mg/kg, i.p.) or dexamethasone (Dexa, 1 mg/kg). Analysis of total leukocytes (A, E), mononuclear cells (B, F), neutrophils (C, G) and eosinophils (D, H) are shown. Data are representative results (mean ± SEM) of one out of three independent experiments (n = 8 animals/group). *Indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates statistically significant differences between stimulated and treated groups.

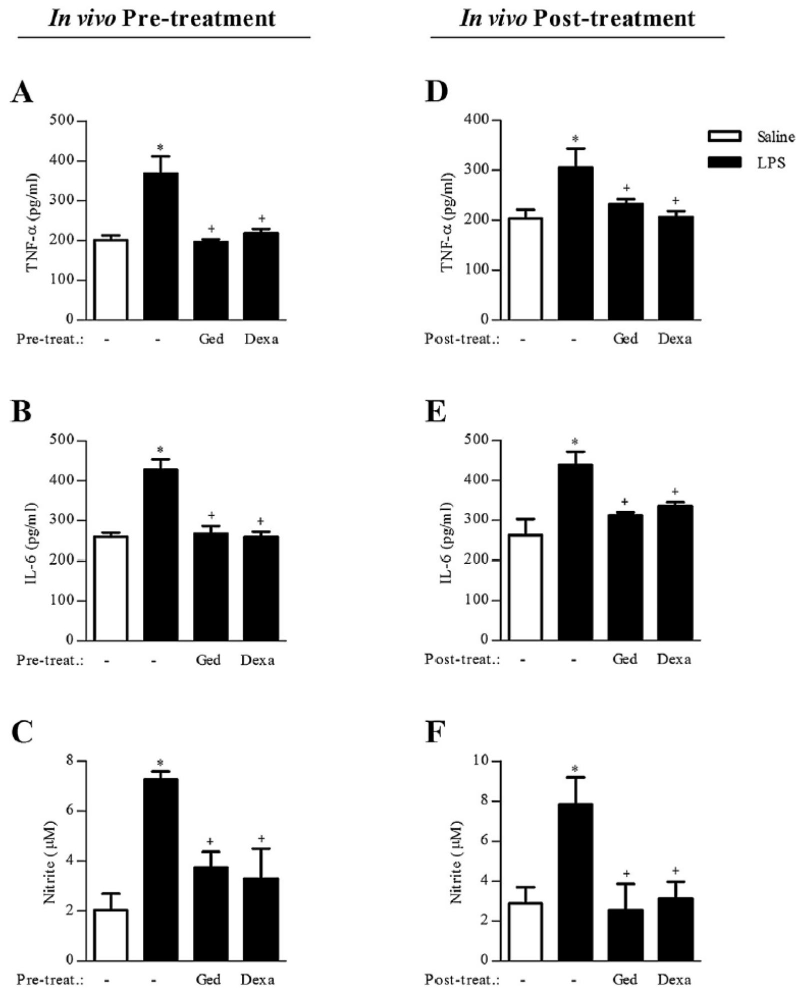

Fig. 2. Gedunin impairs LPS-induced inflammatory mediators *in vivo*. Effect of gedunin pre- and post-treatments on the production of TNF-α, IL-6 and NO by C57BL/6 mice triggered by LPS. Mice were treated with gedunin (Ged, 0.5 mg/kg, i.p.) or dexamethasone (Dexa, 1 mg/kg) 1 h before (A-C) or 1 h after (D-F) LPS (250 ng/cav, i.pl.) injection. Cytokine and nitrite levels were evaluated in cell-free pleural washes recovered 24 h after LPS stimulation. Data are expressed as the mean ± SEM for eight animals, from at least three independent experiments. *Indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates statistically significant differences between stimulated and treated groups.

LPS-induced total leukocyte and neutrophil influx 6 h after stimulation (data not shown). The post-treatment of mice with gedunin (0.5 mg/kg, i.p., 1 h after stimulus) also significantly reduced the numbers of all cell populations in mouse pleural cavities, similarly to dexamethasone (Fig. 1E–H).

Gedunin treatment also impaired *in vivo* production of TNF-α, IL-6 and NO induced by LPS within 24 h, when administered 1 h before (Fig. 2A–C) or 1 h after (Fig. 2D–F) LPS i.pl. stimulation. We have previously demonstrated that the *in vitro* pre-treatment of immortalized macrophages with gedunin inhibited LPS-induced production of inflammatory mediators in a concentration-dependent manner, 6 and 24 h after stimulation [26]. Similarly, here we show that gedunin pre-treatment (0.01–100 μM) diminished TNF-α and NO production by macrophages 24 h after LPS stimulation *in vitro*, a phenomenon also observed with dexamethasone (100 nM) (Fig. 3A,B). Based on these dose-response experiments and on previous studies from our group [25,26,49,50], we have chosen 10 μM of gedunin for further experiments. Fig. 3(C–E) shows that the 24 h-*in vitro* production of TNF-α, IL-6 and NO by macrophages, triggered by LPS, was also impaired by the post-treatment with gedunin and with the reference inhibitors 17-AAG (selective Hsp90 inhibitor) and dexamethasone. It is important to note that the incubation of macrophages with gedunin (≤10 μM) and LPS (≤500 ng/ml) for 24 h failed to induce cell death (Supplemental Table 1), as previously demonstrated by us [26].

3.2. Gedunin inhibits LPS-induced inflammasome activation

LPS binding to TLR4/MD-2 complex induces elevated expression of NLRP3 and leads to the assembly of NLRP3 inflammasome, which triggers caspase-1 activation and consequent maturation of IL-1β [19,51]. Our data show that neither gedunin nor dexamethasone treatments (i.p., 1 h after the stimulus) reduced LPS-induced expression of the adaptor protein ASC in leukocytes recovered from pleural cavity of LPS-stimulated mice (Fig. 4A). However, gedunin and dexamethasone inhibited NLRP3 expression *in vivo* (Fig. 4B),

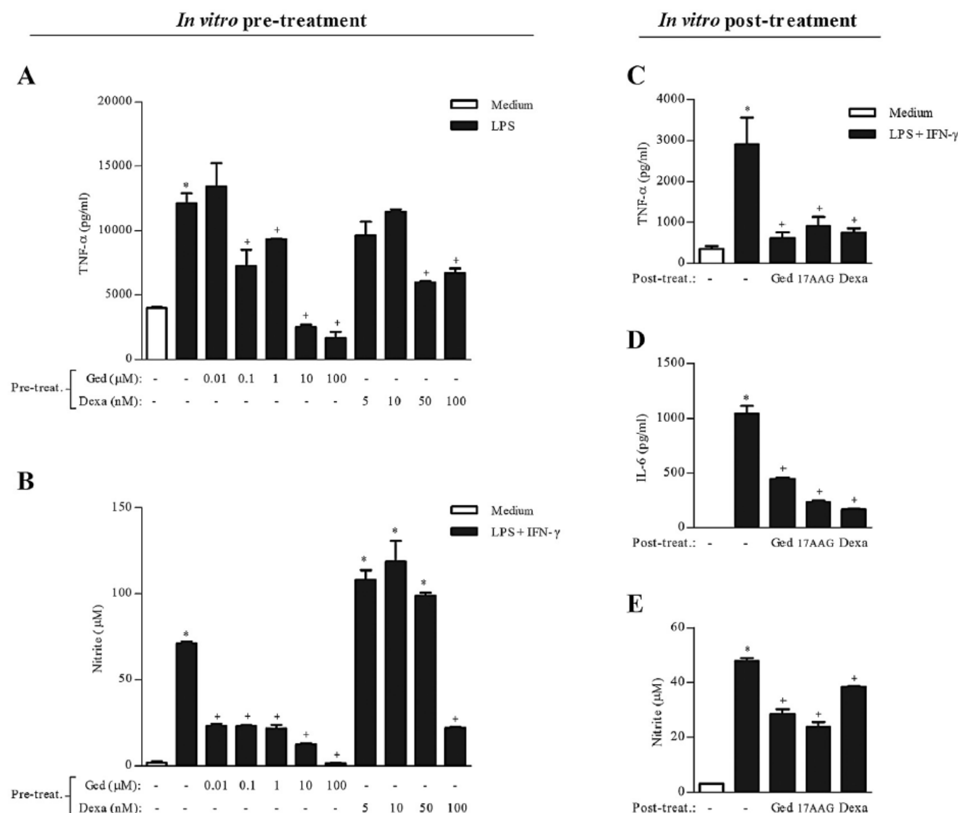

Fig. 3. Gedunin impairs LPS-induced inflammatory mediators *in vitro*. Immortalized macrophages (10^6 cells/well) were treated with gedunin (0.01–100 μM), 17-AAG (1 μM) or dexamethasone (5–100 nM) 1 h before (A, B) or after (C-E) LPS stimulation (50 ng/ml). For nitrite determination, cells were stimulated in the presence of IFN-γ (200 IU/ml). (C-E) Cells were treated with 10 μM of gedunin (Ged) or 100 nM of dexamethasone (Dexa). Cytokine and nitrite levels were evaluated in supernatants recovered 24 h after LPS stimulation. Data are expressed as the mean ± SEM for quadruplicate wells per group, from at least three independent experiments *indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates statistically significant differences between stimulated and treated groups.

a phenomenon also observed in LPS-stimulated macrophages *in vitro* by western blot and immunofluorescence staining (Fig. 4C–E), suggesting that this limonoid regulates inflammasome activation. Dexamethasone post-treatment also diminished NLRP3 expression *in vitro* and *in vivo*, (Fig. 4B–E). As expected, we also observed, by FLICA assay, that caspase-1 activation was inhibited in gedunin-treated macrophages *in vitro* (Fig. 5A,B). In line with these results, gedunin also impaired the production of the inflammasome-related cytokine IL-1β in mouse pleura and in macrophage supernatants, 24 h after LPS stimulation (Fig. 5C,D), as well as dexamethasone and 17-AAG.

We next aimed to investigate new molecular targets of this limonoid. *In silico* docking experiments revealed that gedunin efficiently docks to caspase-1 (PDB code: 1RWX), with a docking score of −8.1 kcal/mol, suggesting an additional putative mechanism by which gedunin impairs TLR4/MD-2 response (Fig. 5E). As described in the literature, the binding cavity of caspase-1 is in the shallow crevice on the terminal surface of the receptor protein [40,52–54], and our data revealed that the oxygen in the lactone ring of gedunin has hydrogen bond interaction with Arg383 at a distance of 2.9 Å, whereas the acetyl ketonic group of gedunin has hydrogen bond with Val348 at a distance of 3.0 Å. Moreover, hydrophobic interactions occurred between alkyl groups of gedunin and Val348, Arg383, Met345 and His342. Dexamethasone also binds in the same binding pocket, but with a higher binding energy score (-6.7 kcal/mol) compared to gedunin, indicating a low binding affinity (Supplemental Fig. 2). Worthy of note, we observed that gedunin also bound to NLRP3 (Pdb Code: 3QF2), however with a high docking score of −6.1 kcal/mol, indicating a low binding affinity.

3.3. *Gedunin enhances anti-inflammatory mediators during LPS-induced response* in vitro *and* in vivo

We have previously shown that gedunin induces antiinflammatory factors *in vitro*, indicating additional means by which such limonoid suppresses inflammation [26]. Here we show that gedunin post-treatment enhanced LPS-induced HO-1 expression (Fig. 6A,B) and IL-10 production (Fig. 6C,D) in pleural leukocytes *in vivo* and in immortalized macrophages *in vitro*. Of note, the incubation of non-stimulated macrophages with gedunin induced more prominent expression of HO-1, when compared to cells stimulated with LPS and post-treated with gedunin (Fig. 6B). Incubation of non-stimulated macrophages with gedunin also induced IL-10 production *in vitro* (Fig. 6D). According to previous data shown by us, dexamethasone post-treatment failed to enhance LPS-induced HO-1 expression (Fig. 6A,B), however increased LPS-induced IL-10 production *in vivo* (Fig. 6C). IL-10 levels in the supernatants of macrophages pre-treated with 17-AAG and dexamethasone were similar than the values obtained in the supernatant of LPS-stimulated group (Fig. 6D).

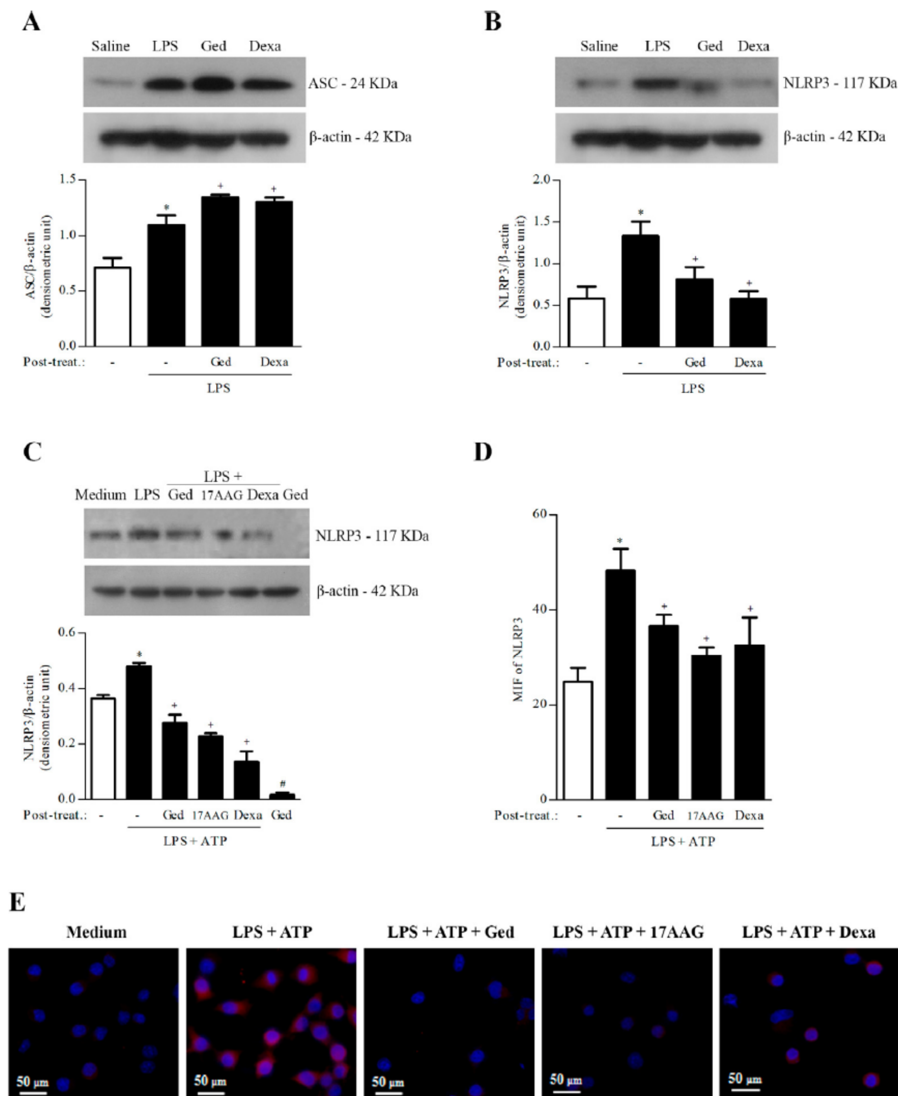

Fig. 4. Gedunin diminishes LPS-induced NLRP3 expression. Effect of gedunin post-treatment on LPS-induced ASC and NLRP3 and expression in whole extracts of pleural leukocytes (A–B) and immortalized macrophages (C-E), 24 h after LPS stimulation. C57BL/6 mice were treated with gedunin (Ged, 0.5 mg/kg, i.p.) or dexamethasone (Dexa, 1 mg/kg) 1 h after LPS (250 ng/cav, i.pl.) injection. Macrophages (10^6 cells/well) were treated with gedunin (10 μM), 17-AAG (1 μM) or dexamethasone (100 nM) 1 h after LPS stimulation (1 μg/ml) pulsed with ATP (2 mM). Representative western blots are shown on top, whereas densitometric analyses are shown in the graphs, normalized by β-actin expression. Western blot images were adjusted only for brightness and contrast using Adobe Photoshop CS5 software. (D) Mean fluorescence intensity (MFI) and (E) representative immunofluorescence staining of NLRP3. Data are expressed as the mean ± SEM for eight animals or quadruplicate wells per group from at least three independent experiments. *Indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates statistically significant differences between stimulated and treated groups. # indicates statistically significant differences between non-stimulated and treated groups.

3.4. Gedunin inhibits TLR2 and TLR3 activation

To examine whether gedunin modulates other TLR-dependent pathways in addition to TLR4, we have pre-treated immortalized macrophages *in vitro* and stimulated with PAM3 and with the synthetic analog of viral double stranded RNA POLY I:C, known TLR2 and TLR3 agonists, respectively. The pre-treatment of macrophages with gedunin impaired PAM3- and POLY I:C-induced TNF-α, IL-6 and NO production by macrophages within 24 h (Fig. 7A–C,H–J). Gedunin also impaired the expression of NLRP3 triggered by both PAM3 and POLY I:C, as well as inhibited the production of PAM3-induced IL-1β in macrophage supernatants, 24 h after PAM3 stimulation (Fig. 7D–E,K). Worthy of note, POLY I:C failed to induce IL-1β production by macrophages, even at high concentrations (10, 25, 50 and 100 μg/ml) (data not shown). On the other hand, gedunin enhanced HO-1 expression and IL-10 production in the presence of by PAM3 and POLY I:C (Fig. 7F–G,L–M). Similar to what was shown in Fig. 6, gedunin also triggered HO-1 and IL-10 (Fig. 7F,L) by non-stimulated macrophages, 24 h after incubation. Pre-treatment with 17-AAG was capable to impair PAM3-induced TNF-α, NLRP3, IL-1β, whereas POLY I:C-induced TNF-α, IL-6, NO and NLRP3 (Fig. 7A,D–E,H–K). Dexamethasone impaired the production of all pro-inflammatory mediators; however did not change NLRP3 expression. Dexamethasone and 17-AAG enhanced IL-10 production triggered by POLY I:C and PAM3 (Fig. 7G,M).

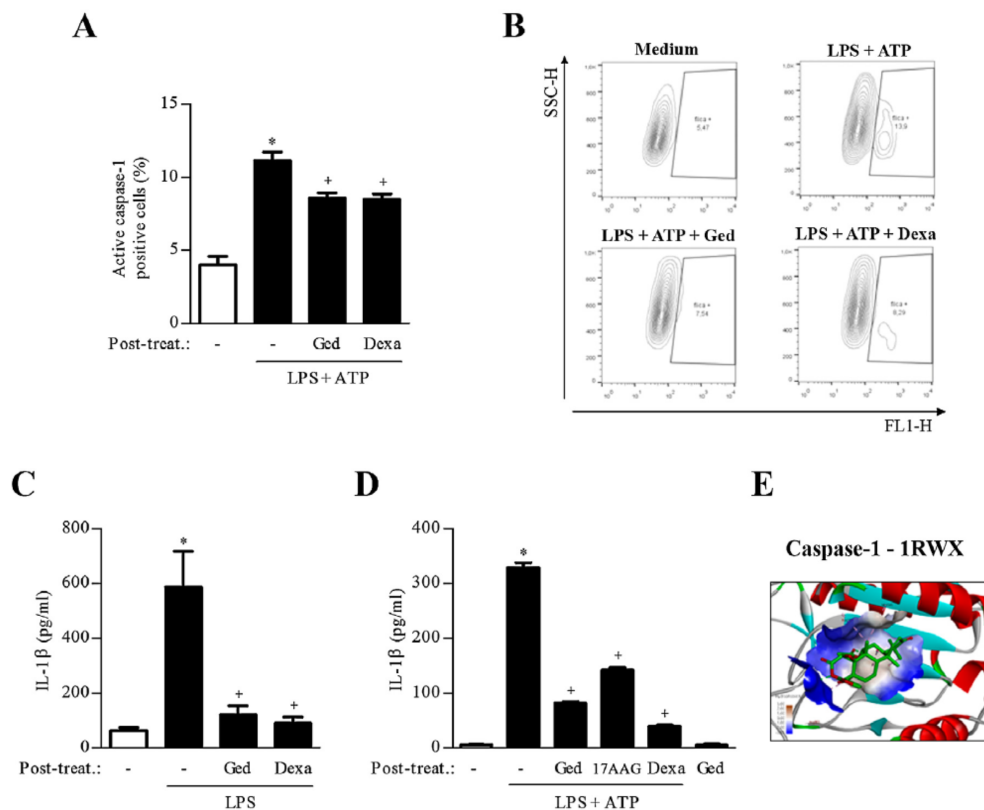

Fig. 5. Gedunin impairs caspase-1 activity and IL-1β production. (A-B) Analysis of active caspase-1 by intracellular FLICA staining of macrophages stimulated with LPS and post-treated with gedunin (Ged, 10 μM). Results shown are mean of FLICA-positive cells from three independent experiments (A) and representative FACS plots (B). IL-1β levels in cell-free pleural washes (C) and immortalized macrophage supernatants (D) evaluated by ELISA. Pleural washes were recovered 24 h after LPS (250 ng/cav) stimulation from mice treated with gedunin (Ged, 0.5 mg/kg, i.p.) or dexamethasone (Dexa, 1 mg/kg). Macrophages were stimulated with LPS (1 μg/ml), pulsed with ATP (2 mM) and treated with gedunin (10 μM), 17-AAG (1 μM) or dexamethasone (100 nM). Data are expressed as the mean ± SEM from eight animals or quadruplicate wells per group, from three independent experiments *indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates statistically significant differences between stimulated and treated groups. (E) Gedunin (green colored stick) interacting with polar and non-polar aminoacids of caspase-1 (PDB Code: 1RWX). Surface diagram represents hydrophobicity colored according to the Eisenberg normalized consensus hydrophobicity scale [55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Mapping gedunin binding sites in TLR2, TLR3 and MD-2

In the continuing search for new molecular targets of gedunin, we next performed *in silico* studies for TLR structures. Our *in silico* studies predicted that gedunin binds to the large hydrophobic site of MD-2 of TLR4 that overlaps to the LPS binding site (Supplemental Fig. 4), as previously published by our group [26]. In the present study, we have also investigated whether gedunin binds to TLR2 and TLR3.

TLR2 binding pocket is composed of hydrophobic residues and can accommodate hydrophobic ligands [56]. Gedunin, dexamethasone and 17-AAG was found to locate at the same TLR2 binding pocket (Fig. 8A and Supplemental Fig. 5). *In silico* analysis predicted that gedunin had better interactions with amino acids of the binding site of TLR2 compared to dexamethasone and 17-AAG. The binding energy (*i.e.* the summary of binding interactions with polar and non-polar amino acids) of gedunin, dexamethasone and 17-AAG to TLR2 was −7.9 kcal/mol, −6.5 kcal/mol and −5.7 kcal/mol, respectively (Fig. 8A and Supplemental Fig. 5B,C). Acetyl ketone of gedunin forms a hydrogen bond with Lys698 at a distance of 2.73 Å. Furan ring of gedunin forms π-σ interactions of alkyl protons of leu762. In addition, there were six σ-σ interactions of gedunin with amino acids of TLR2 (Ala732, Ala781, Ile733, Ph725 and leu734 (2)).

The horseshoe-shaped protein core of TLR3 provides a large surface area for ligand interactions and recognition, and can accommodate a wide diversity of ligand structures at different sites [57,58]. *In silico* molecular docking of gedunin to TLR3 predicted two different binding domains (Fig. 8B). One domain is located near the leucine-rich repeat (LRR) 9–12 insertion, whereas the other one is placed at the C-terminal domain. Interactions of gedunin at both the sites presented similar binding affinities. Our *in silico* results suggest that the binding of gedunin near LRR 9–12 insertion might be the most salient binding site, since this region is involved in dimerization of TLR3 and is the dsRNA binding site [58]. Gedunin interacts *in silico* to TLR3 at the binding site near the LRR 9–12 insertion with a docking energy of −7.6 kcal/mol near LRR 9–12. Asn257 of LRR 9 is found to have hydrogen bond with the ketonic oxygen of gedunin at a distance of 3.0 Å (Fig. 8C). The furan ring of and alkyl groups of gedunin presented π-π interactions had π-σ interactions with Tyr283 (LRR 10) and Tyr307 (LRR 11), respectively. In addition, there were intramolecular interactions, such as π-π stacked interactions between Tyr283 (LRR 10) and Tyr307 (LRR 11); and π-σ interactions of Tyr307 (LRR 11) with Lys330 (LRR 12)

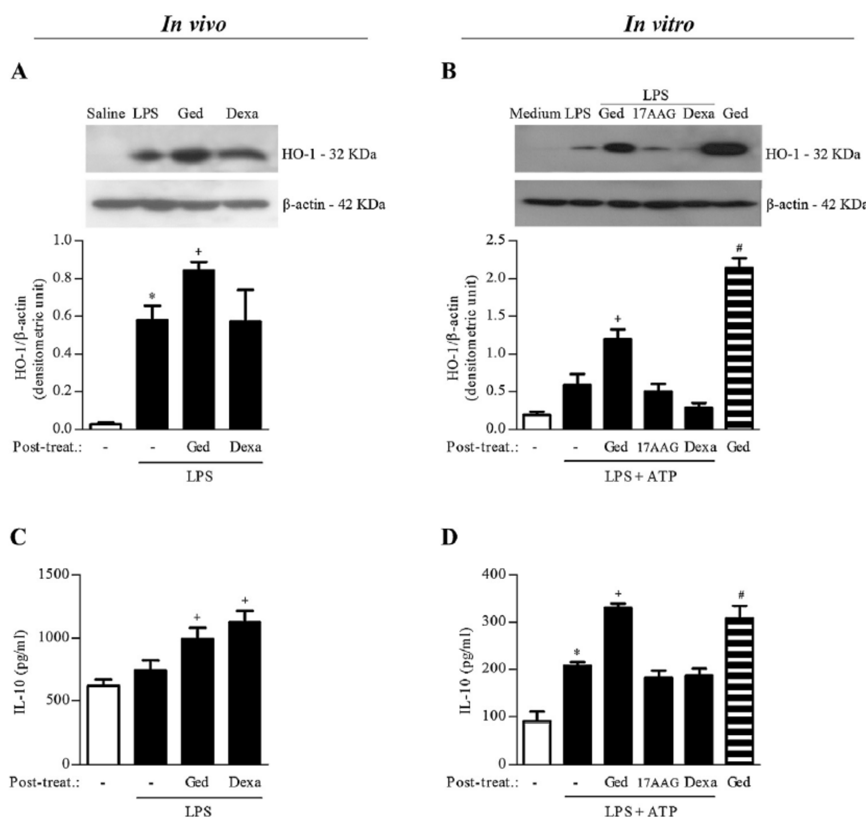

Fig. 6. Gedunin increases LPS-induced HO-1 expression and IL-10 production. (A, C) Mice were treated with gedunin (Ged, 0.5 mg/kg, i.p.) or dexamethasone (Dexa, 1 mg/kg) 1 h after LPS stimulation (250 ng/cavity, i.pl.). (B, D) Immortalized macrophages were stimulated with LPS (50 ng/ml) for 1 h and treated with gedunin (10 μM), 17-AAG (1 μM) or dexamethasone (Dexa, 100 nM). Analyses were performed 24 h (A-C) or 48 h (D) after LPS stimulation. Representative HO-1 western blots from three independent experiments are shown on top, whereas densitometric analyses are shown in the graphs, normalized by β-actin expression. Western blot images were adjusted only for brightness and contrast using Adobe Photoshop CS5 software. Data are expressed as the mean ± SEM for eight animals or quadruplicate wells per group, from at least three independent experiments. *Indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates differences between stimulated and treated groups. #Indicates differences between non-stimulated and gedunin-treated groups.

and Arg331 (LRR 12). Gedunin docks at the C-terminal domain of TLR3 with the binding energy of −7.5 kcal/mol. Ketonic oxygen of pyran-2-one of gedunin presented polar hydrogen bonding interactions with amino group of Cys651 at a distance of 2.23 Å (Fig. 8D). Gedunin has hydrophobic π-σ interactions with Ser653, Val658, Leu640, Trp660 and Phe647. Binding interactions of dexamethasone (binding energy: −6.5 kcal/mol) and 17-AAG (binding energy: −5.7 kcal/mol) to TLR3 are shown in Supplemental Fig. 6A–D.

Moreover, gedunin presents a log P of 3.1 (calculated by ChemDraw 8.0) and has excellent protein druggability, with ideal proportion of polar and hydrophobic groups required for binding. The polar groups of gedunin, from lactone and acetyl moiety, aid in hydrogen bonding, while the alkyl groups of gedunin make it feasible to have hydrophobic interactions. It is worthy of note that the binding energies of gedunin are superior then those of dexamethasone and of 17-AAG in all the above targets.

4. Discussion

Recognition of PAMPs by PRRs results in the activation of different intracellular signaling cascades that enables macrophages to rapidly mount a pro-inflammatory responses, through the expression of effector mediators involved in host defense [59]. However, exacerbated inflammatory response requires strict control to avoid tissue damage and host harm. In the present report, we demonstrate that gedunin is capable to modulate macrophage activation and to restrain the inflammatory response triggered by different TLR ligands, including bacterial LPS.

In accordance to our previous reports, we demonstrate here that LPS induces a marked increase in leukocyte numbers in pleural cavities of mice through an indirect mechanism that involves inflammatory mediators mainly synthesized by macrophages [45–48]. Indeed, resident macrophages comprise the majority of pleural leukocytes and are the major responders to LPS. TLR4 activation by LPS triggers the production of a wide variety of inflammatory mediators by macrophages, including chemokines and cytokines, such as TNF-α, IL-6 and IL-1β *in vivo* and *in vitro* [7]. While LPS-induced TLR4 activation has been consistently shown to be impaired by corticosteroids [60–63], our group has demonstrated that pre-treatment with gedunin also directly modulates LPS-induced macrophage activation, in a concentration-dependent manner [26]. Here, we demonstrate that gedunin also inhibits the inflammatory response triggered by LPS *in vivo* in a post-treatment regimen, impairing mononuclear cell, eosinophil and neutrophil migration, as well as the production of TNF-α, IL-6, IL-1β and NO *in vivo*.

Gedunin biological effects include antitumor, anti-arthritic and anti-alergic [24,25,49,50,64], and have been so far explained due to its ability to impair Hsp90 activity, by binding to its co-chaperone p23 [32,65,66]. Indeed, here we show that the treatment

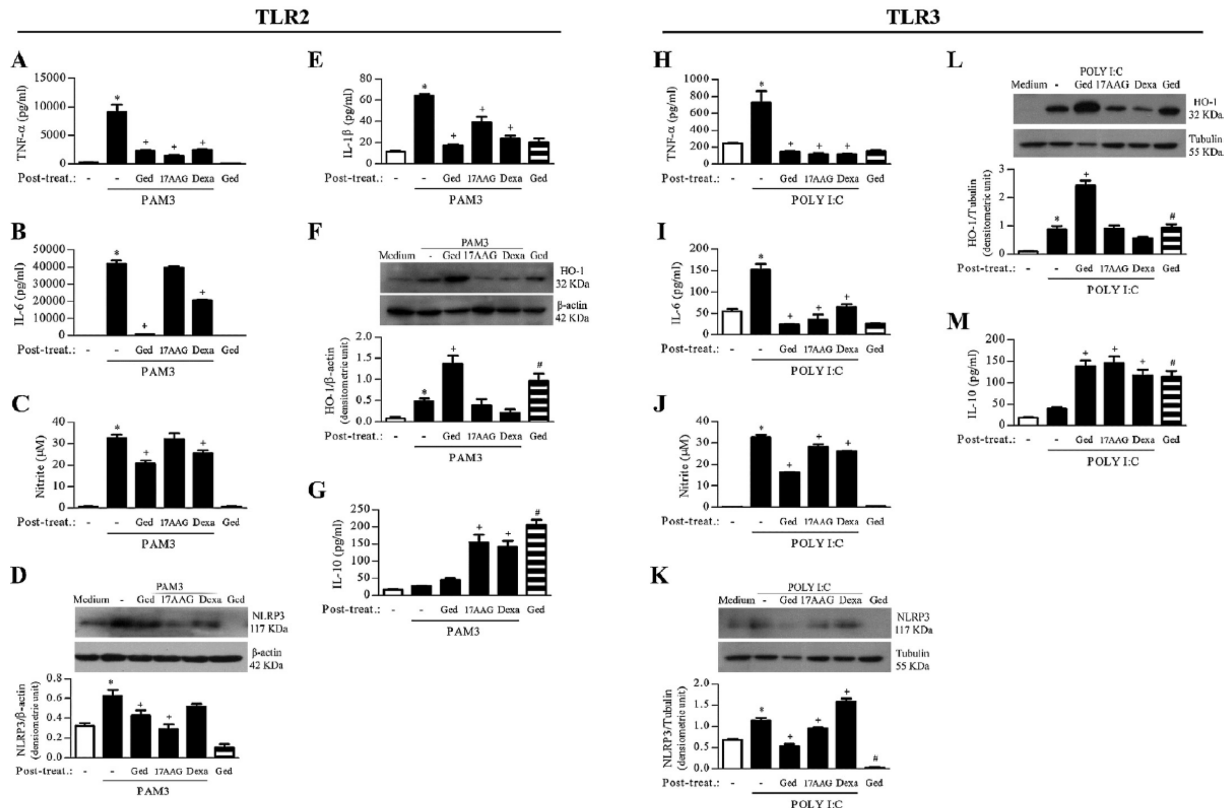

Fig. 7. Gedunin inhibits TLR2- and TLR3-induced responses. Effect of gedunin on *in vitro* production or expression of TNF-α, IL-6, NO, NLRP3, IL-1β, HO-1 and IL-10 triggered by PAM3 and POLY I:C. Macrophages (10^6 cells/well) were pre-treated with gedunin (10 μM), 17-AAG (1 μM) or dexamethasone (100 nM) and stimulated with PAM3 (1 μg/ml, left panel) or POLY I:C (1 μg/ml, right panel) 1 h after treatment. Cytokine levels were evaluated in the supernatants recovered 24 h after stimulation. Western blots were performed in whole cell extracts 24 h after stimulation, and Data are expressed as the mean ± SEM for quadruplicate wells per group, from at least three independent experiments. *Indicates statistically significant differences ($P \leq 0.05$) between non-stimulated and stimulated groups, whereas + indicates differences between stimulated and treated groups. #Indicates differences between non-stimulated and gedunin-treated groups. Whole uncropped images of western blots depicted in Figs. 4, 6 and 7 are shown in Supplemental Fig. 3.

of macrophages with 17-AAG, a semi-synthetic derivative of the specific Hsp90 inhibitor geldanamycin, also impaired the production of inflammatory mediators triggered by LPS. This data supports the notion that LPS-induced signaling depends on Hsp90 [66–69], since: *i*) Hsp90 has been reported to be part of the LPS receptor complex [70], *ii*) Hsp90 interacts with several mitogen-activated protein kinase (MAPK) involved in TLR4 signaling [65,71] and *iii*) Hsp90 is required for NFκB activation by different described mechanisms [72–74]. In accordance with this, geldanamycin treatment diminished NFκB nuclear translocation in murine macrophages [72], diminished the formation of NFκB/DNA complexes [73] and reduced the stability of TNF-α and IL-6 transcripts [74]. Recent data from our group has also demonstrated that gedunin impaired NFκB activation and the consequent production of inflammatory mediators in LPS-stimulated macrophages [26]. In this report we have shown that the modulation of Hsp90 activity might not be the only mechanism of action of gedunin, since this limonoid also binds to MD-2, as observed by *in silico* and surface plasmon resonance assays (SPR) and, therefore, it is likely impairing TLR4/MD-2 upstream signaling. Furthermore, SPR competition assays revealed that, even though gedunin binds more efficiently to MD-2 when added before LPS, such limonoid was capable to displace LPS and inhibited LPS/MD-2 complex formation [26].

The wide range of gedunin effects includes the modulation of NLRP3 inflammasome. The NLRP3 inflammasome can be triggered by a vast array of stimuli, including bacterial products, such as LPS [18,19,51]. Data presented here demonstrate for the first time that gedunin was capable to regulate LPS-induced NLRP3 inflammasome activation *in vivo* and *in vitro*, by controlling NLRP3 expression in pleural leukocytes and in immortalized macrophages. According to this, it has been proposed that Hsp90 activity is required for NLRP3 maturation, stabilization and activation, what is supported by the fact that Hsp90, in association with its co-chaperone SGT1 (suppressor of G2 allele of skp1), interacts with the leucine-rich repeat LRR and NACHT domains of NLRP3, forming a stable complex that is essential for inflammasome stability and activation [75–77]. Therefore, in addition to diminish NLRP3 expression, our data suggest that gedunin also inhibits NLRP3 assembly, what lines with the fact that diminished caspase-1 activation and IL-1β levels were detected in gedunin-treated mice or immortalized macrophages. Accordingly, caspase-1 activation and IL-1β production have been previously shown to be modulated by geldanamycin, in monosodium urate-stimulated THP-1 cells and primary monocytes [75].

In addition to the inhibition of pro-inflammatory mediators induced by LPS, we show that gedunin also triggered the antiinflammatory factors heme oxigenase-1 (HO-1) and IL-10 *in vitro*

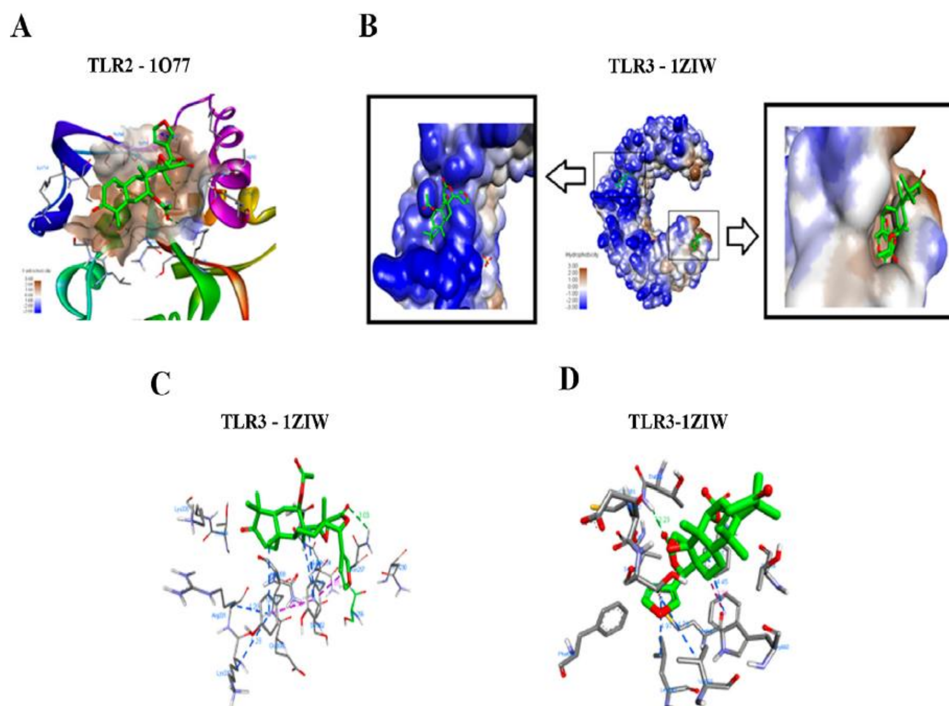

Fig. 8. *In silico* molecular docking of gedunin. (A) Polar and non-polar interactions of gedunin (green colored stick) with TLR2 (PDB code: 1O77). Binding site is represented as surface and protein (ribbon and non-polar surface). (B) Gedunin (green colored stick) interaction at two binding sites of TLR3 (surface representation, PDB code: 1Z1W). (C) Polar and non-polar interactions of gedunin (green colored stick) with amino acids of the LRR 9–12 region of TLR3 (PDB code: 1Z1W). (D) Polar and non-polar interactions of gedunin (green colored stick) with amino acids at C-terminal domain of TLR3 (PDB code: 1Z1W). Surface diagram represents hydrophobicity colored according to the Eisenberg normalized consensus hydrophobicity scale [55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and *in vivo*, which further expands the pharmacological effect of this molecule. Similar data has been previously demonstrated by our group *in vitro* [26], which is in accordance with previous reports that show that Hsp90 inhibitors, such as celastrol, induce the expression of HO-1, in addition to other anti-inflammatory factors, such as Hsp70 and heat shock factor (HSF)-1 [78–81]. It is well described that HO-1 and IL-10 are triggered by diverse stimuli, including LPS [82,83], and it was also demonstrated that HO-1 mediates the anti-inflammatory effect of IL-10 and suppresses TNF-α signaling [84]. Here we demonstrate that both IL-10 levels and HO-1 expression were increased by gedunin treatment in the presence or in the absence of TLR agonists, suggesting that gedunin anti-inflammatory effect is in part dependent on these factors. In agreement, the *in vivo* administration of gedunin led to increased levels of IL-10 in naïve mouse peritoneum (data not shown), reinforcing the hypothesis that IL-10 mediates the anti-inflammatory effect of this limonoid.

Our results also reveal that gedunin effect is not restricted to TLR4, and show that this compound also modulates TLR2- and TLR3-mediated responses. The mechanism by which gedunin impairs these responses is not yet clear; however, considering that TRAM/TRIF- and MAL/MyD88 dependent kinases, such as p38, depend on the chaperoning activity of Hsp90 [65,85–89], it is expected that TLR2, TLR3 and TLR4 signaling pathways are inhibited by Hsp90 modulators. This is further supported by the fact that gedunin impaired TNF-α production 6 h after LPS [26], PAM3 and POLY I:C stimulation (not shown). As stated above, Hsp90 is also necessary for NFkB activation that is one of the downstream events of TLR2, TLR3 and TLR4 activation, implying the requirement of Hsp90 for the induction of inflammatory cytokines triggered by all TLRs. However, our data (Fig. 7) shows that the selective Hsp90 inhibitor 17-AAG failed to modulate IL-6 and NO production during TLR2 activation, suggesting that this phenomenon might rely on Hsp90-independent pathways. In this regard, it is interesting to note that preliminary results (not shown) demonstrated that gedunin diminished luciferase activity on HEK cells transfected with TLR2-1 plasmid 4 h after PAM3 stimulation, giving further support that gedunin modulates TLR2 signaling, *via* both Hsp90 dependent and independent mechanisms. In addition, our *in silico* docking studies predicted that gedunin docks to TLR2, TLR3, MD-2 and caspase-1, suggesting gedunin as a multitarget ligand. However, further biochemical assays, such as SPR or drug affinity responsive target stability (DARTS) are required to confirm gedunin binding to these molecules and to determine its constant of affinity (*Keq*).

5. Conclusions

Overall, our data demonstrate that gedunin is a multitarget compound with antiinflammatory properties capable to modulate TLR-mediated responses, by impairing inflammasome activation, production of inflammatory mediators and leukocyte mobilization, as well as by triggering the production of antiinflamatory factors. The present work sheds light on the multifaceted pharmacological properties of gedunin, and implies that this limonoid may represent a candidate drug for the treatment of infectious inflammatory diseases.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

P.V.B., C.P. designed research. P.V.B., K.H.M., R.N.M., A.P.M., J.R.T., D.C.B.H. conceived and designed the experiments. P.V.B., K.H.M., R.N.M., T.E.M.C., A.P.M., A.B.C. performed the experiments and analyzed the data. M.G.H., D.C.B.H., P.P., A.P.M., A.B.C., provided essential reagents, experimental tools and advice. M.G.H., C.P. provided infrastructure and funding sources. P.V.B., R.N.M., C.P. wrote or contributed to the writing of the manuscript.

Acknowledgements

This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). P.V.B. and K.H.M. were supported by fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and CNPq, as students of the Graduate Program in Cellular and Molecular Biology from Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil. R.N.M. was supported by fellowship from CAPES/CDTS. The authors are grateful to Patricia Bozza, Thomas E. Krahe, Mariana Souza, Maria Fernanda Costa, Fátima Vergara and Clarissa Monteiro for suggestions and helpful comments on this manuscript. We also thank Thomas E. Krahe and Tatiana Pádua for providing help on graphical abstract preparation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.09.015.

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8. Abbreviations

ASC - apoptosis-associated speck-like protein containing caspase activation and recruitment domain

ATP - adenosine triphosphate

CARD - caspase activation and recruitment domain

DMEM - Dulbecco's modified Eagle medium

DMSO - dimethyl sulfoxide

HO-1 - heme oxigenase-1

Hsp - heat shock protein

IL - interleukin

i.pl. - intrapleural

LPS - lipopolysaccharide

MD-2 - myeloid differentiation protein-2

NLRP3 - nucleotide-binding domain and leucine-rich repeat protein-3

NO - nitric oxide

PAM3 - palmitoyl-3-Cys-Ser-(Lys)4

PAMP - pathogen-associated molecular pattern

PDB - protein data bank

POLY I:C - polyriboinosinic:polyribocytidylic acid

PRR - pattern recognition receptors

PVDF - polyvinylidene difluoride

SDS - sodium dodecyl sulfate (SDS)

TLR - toll-like receptor

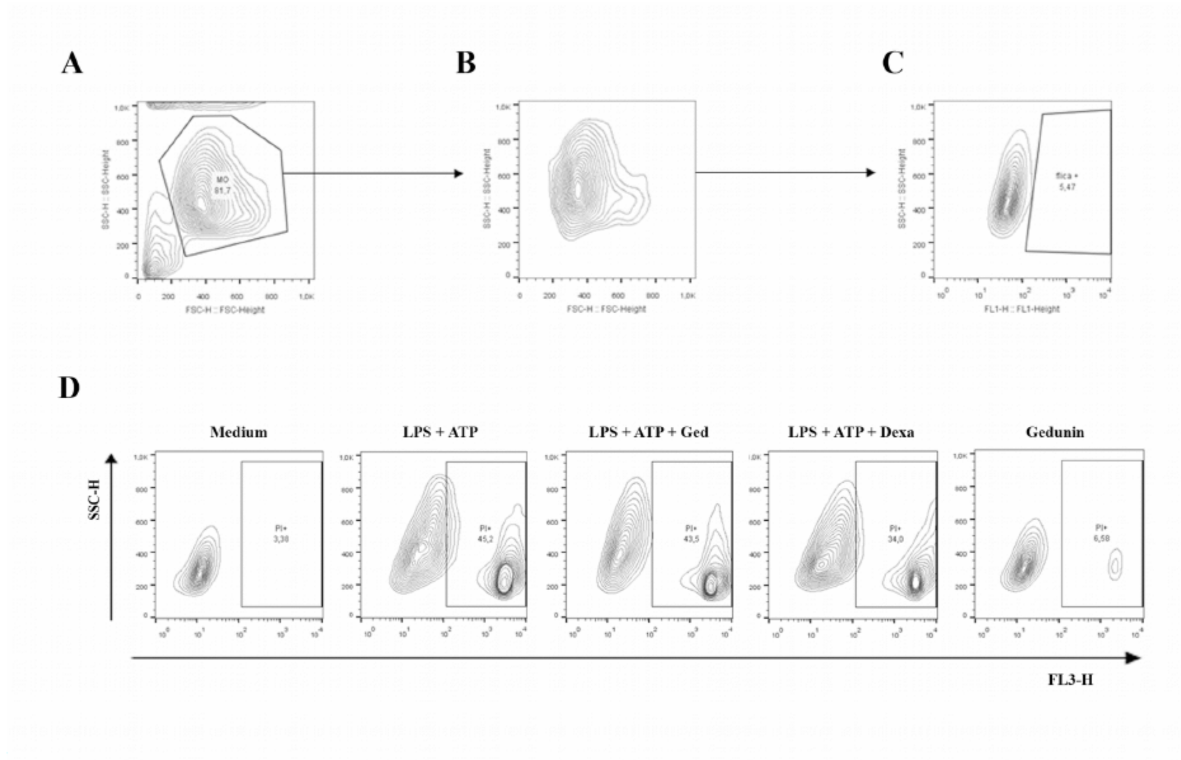
TNF-α - tumor necrosis factor-α

10. Supplemental Material

Table 1. Effect of gedunin and LPS on macrophage viability

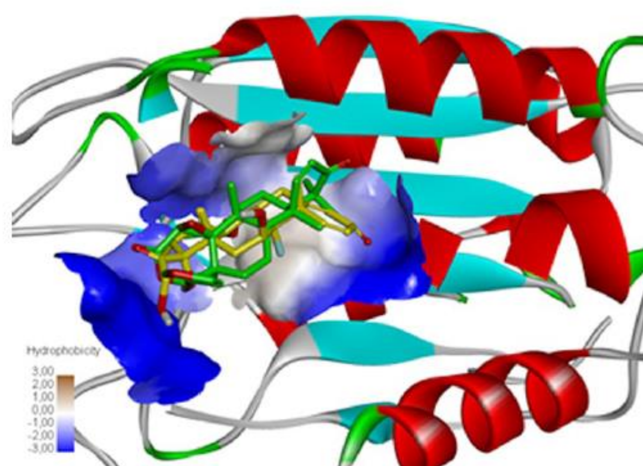
Medium	Tween 20 (3%)	Gedunin (µM)			
		1	10	100	1000
100	4.9	100	98.1	84.1	41.1
		LPS (ng/ml)			
		50	100	500	1000
100	3.6	100	100	100	94.6

Supplemental Table 1. Macrophage viability after exposure to gedunin and LPS. Immortalized macrophages (2 x 10^5 cell/well) were incubated with gedunin (1 – 100 µM) or LPS (50 – 1000 ng/ml) for 24h (37°C, 5% CO_2) and cell viability was determined by resazurin reduction method. The absorbance was read at 555/585nm ($\lambda_{ex}/\lambda_{em}$) using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, USA) and results are expressed as percentages (%) of viable cells from quadruplicate wells.

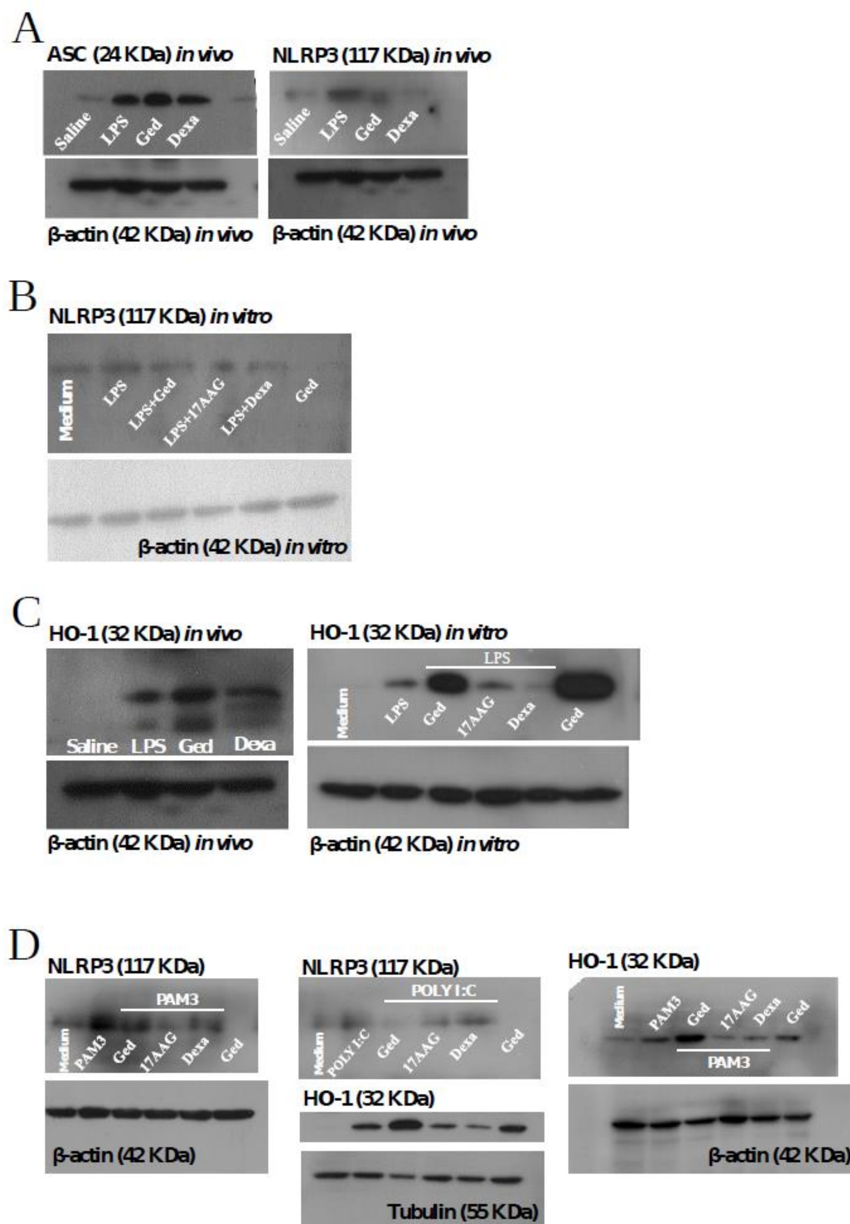


Supplemental Figure 1. Gating strategy for FLICA assay and propidium iodide (PI) staining. Macrophages were first gated using FSC-H/SSC-H parameters (A). Gated cells (B) were analyzed for caspase-1 expression (FL1) in non-stimulated cells (C). (D) FACS analyzes of PI staining of macrophages before and after LPS/ATP, gedunin or dexamethasone. Cells ($5x10^5$) were stained with 2 µl of PI for 5 min, washed and analyzed in FL3 channel (FACScalibur, Becton and Dickinson).

Caspase-1 - 1RWX

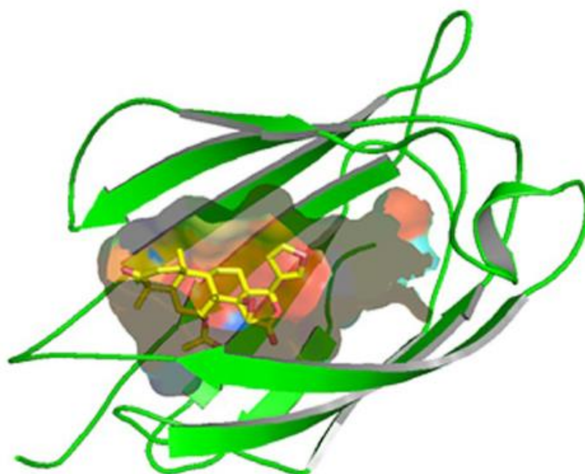


<u>Supplemental Figure 2</u>. *In silico* molecular docking of gedunin and dexamethasone to caspase-1. Gedunin (green colored stick) and dexamethasone (yellow colored stick) occupy the binding site (surface representation) of caspase-1 (cartoon shape) (PDB Code: 1RWX).

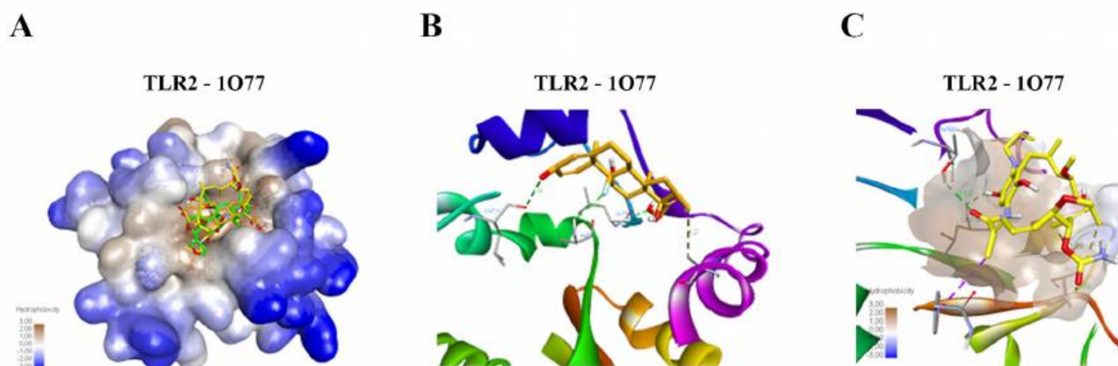


Supplemental Figure 3. Whole uncropped images of the original western blots depicted in Figures 4, 6 and 7. (A) Complete western blots of ASC, NLRP3 and β-actin in pleural leukocytes, shown in Figure 4 A-B. Protein levels were determined on the same blots after membrane stripping. (B) Complete western blots of NLRP3 and β-actin in immortalized macrophages shown in Figure 4 C. (C) Complete western blots of HO-1 and β-actin in pleural leukocytes (left) and in immortalized macrophages (right), shown in Figure 6 A-B. (D) **Complete western blots of NLRP3, HO-1, β-actin and tubulin in immortalized macrophages shown in Figure 7 (G, H, J, K)**. Molecular weight standards for all blots are shown in each panel.

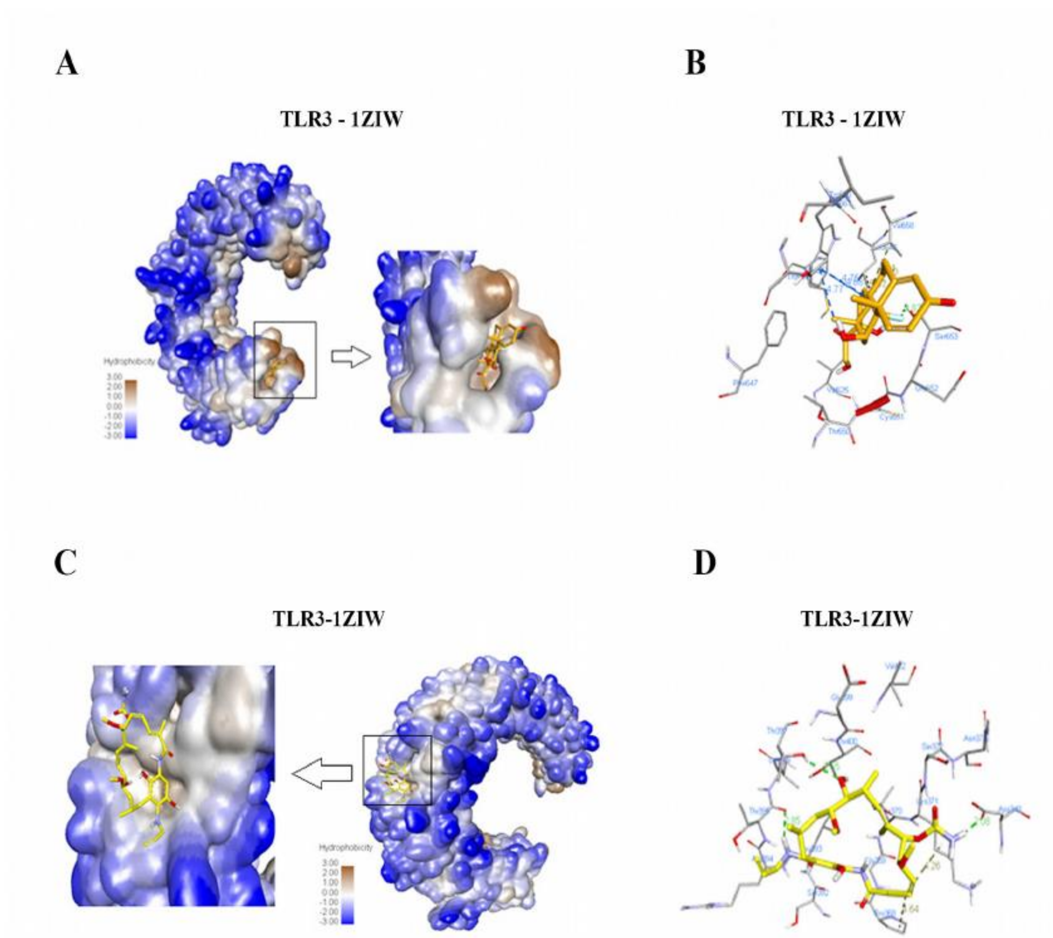
MD-2 - 2E56



Supplemental Figure 4. ***In silico* molecular docking of gedunin to MD-2.** Gedunin (yellow colored stick) and dexamethasone occupies the binding site (surface representation) of MD-2 (cartoon shape) (PDB Code: 2E56).



Supplemental Figure 5. ***In silico* molecular docking of dexamethasone and 17-AAG to TLR2.** (A) Superimposition of gedunin (green colored sticks), dexamethasone (orange colored sticks) and 17-AAG (yellow colored sticks) structures at the binding site of TLR2 (PDB code: 1O77, surface representation). (B) Polar interactions (Leu724, Ala732, Leu734) and non-polar interactions (Ala781) of dexamethasone (orange colored stick) with the binding site (ribbon representation) of TLR2. (C) Polar and non-polar interactions of 17-AAG (yellow colored stick) with the binding site (ribbon representation) of TLR2.



<u>Supplemental Figure 6</u>. *In silico* molecular docking of dexamethasone and 17-AAG to TLR3. (A) Dexamethasone (orange colored sticks) buried in the non-polar cavity at C-terminal domain of TLR3 (surface representation, PDB code: 1Z1W). (B) Hydrogen bonding and non-polar hydrophobic interactions of dexamethasone (orange colored sticks) with the amino acids of TLR3. (C) 17-AAG (yellow colored sticks) binding at LRR 12-14 region of TLR3. (D) Hydrogen bonding and hydrophobic interactions of 17-AAG (yellow colored sticks) with TLR3 amino acids at LRR 12-14 region.

4. DISCUSSÃO

Reconhecendo a importância dos produtos naturais como fonte de substâncias com atividades farmacológicas, investigamos neste trabalho o efeito anti-inflamatório da gedunina, um tetranortriterpenóide encontrado em espécies vegetais da família Meliaceae, sobre a resposta induzida por ligantes de TLR em macrófagos. Nós demonstramos que a gedunina modula negativamente a produção de mediadores inflamatórios e a ativação do inflamassoma NLRP3, assim como modula positivamente fatores anti-inflamatórios *in vitro* e *in vivo*. Ainda, identificamos possíveis alvos moleculares deste tetranortriterpenóide através de ensaios *in silico*.

Dentre os efeitos biológicos da gedunina já descritos, podemos destacar a sua atividade antitumoral, anti-alérgica e anti-inflamatória, em modelo de artrite reumatóide murina (128-130, 133, 172). Sabe-se que estes efeitos estão associados à sua capacidade de modular a atividade da chaperona Hsp90, que desempenha um importante papel na manutenção de diversas proteínas clientes. Foi descrito em 2013 que a gedunina modula a atividade da Hsp90 via ligação à sua co-chaperona p23, que é essencial à atividade desta chaperona (99-100, 112). Neste trabalho, nós demonstramos o efeito anti-inflamatório da gedunina na via de sinalização de TLR4, e sugerimos que este independe de proteínas adaptadoras da via aguda (MAL/MyD88) e tardia (TRAM/TRIF). Acreditamos que a modulação da gedunina ocorra *upstream,* por dois mecanismos: *i)* através da modulação da Hsp90 e *ii)* através da ligação a proteína MD-2, no mesmo sítio de interação de LPS. De fato, a ativação de TLR4 pode estar sendo modulada diretamente pela gedunina via inibição da Hsp90, tendo em vista que Triantofilou e colaboradores demonstraram por técnicas de FRET (*fluorescence ressonance energy transfer*) e FRAP (recuperação de fluorescência após a fotodegradação) que a Hsp90, em conjunto com a Hsp70, se associam ao complexo receptor TLR4/MD-2, constituindo os *lipid rafts* na superfície celular em resposta ao estímulo de LPS. Vale ressaltar que, além disso, estes autores demonstraram que a Hsp90 e a Hsp70 estão envolvidas no tráfego e internalização de TLR4 para o complexo de Golgi (100, 146-148). Em adição à modulação de Hsp90, nossos dados sugerem que a gedunina module negativamente a resposta de macrófagos ao LPS através da ligação à proteína MD-2 no mesmo sítio de interação de LPS, evitando, desta maneira, a formação do complexo TLR4/MD-2/LPS, inibindo a ativação da via de sinalização e, consequentemente, a produção de mediadores inflamatórios e o influxo de cálcio.

Sabe-se que o reconhecimento de LPS pelo complexo TLR4/MD-2/CD14 resulta na

produção de mediadores inflamatórios por ambas as vias de sinalização dependentes de MAL/MyD88 (via aguda) e TRAM/TRIF (via tardia) (139-140). Nós avaliamos o efeito da gedunina sobre estas vias, em macrófagos nocautes (KO) para as proteínas adaptadoras TRIF e MAL com LPS e observamos que o tratamento com a gedunina inibiu a produção de TNF-α, IL-6 e NO nas duas linhagens, de maneira semelhante à resposta observada em macrófagos WT. Acreditamos que, o fato de a gedunina inibir tanto a via aguda como a via tardia da sinalização de TLR4 possa ser decorrente da modulação da atividade da Hsp90 pela gedunina, conforme dito anteriormente (105, 112). Vale ainda ressaltar que, em adição à formação do complexo TLR4/MD-2, a Hsp90, em associação com Hsp70 (e outras chaperonas), mantém a conformação e a atividade de quinases dependentes de MAL/MyD88 e TRAM/TRIF tais como p38, ERK1/2 e JNK, que estão envolvidas na produção de TNF-α e IL-6 (14, 141-143). Isto pode ainda ser corroborado por dados da literatura, que demonstram que a inibição da Hsp90 pela geldanamicina reduz a estabilidade de transcritos de TNF-α e IL-6 em macrófagos induzidos por LPS, fenômeno este parcialmente dependente de p38 (99, 140, 144).

O fato de a gedunina modular o influxo de Ca^{2+} intracelular pode ser corroborado por trabalhos da literatura que demonstram que o estímulo com LPS induz o aumento transitório da concentração de Ca^{2+} intracelular via aumento de proteína quinase C (PKC) (138), que é cliente da Hsp90, envolvida na estabilização e maturação desta quinase. A modulação de PKC pela Hsp90 durante a ativação induzida por LPS também é importante, já que a ativação de NFκB, a produção de TNF-α e expressão de iNOS estão relacionadas com esta quinase e com o aumento de Ca^{2+} intracelular. Estes dados estão de acordo com os nossos resultados, uma vez que a inibição da Hsp90 pela gedunina modula estes mediadores via PKC. Somado a isto, a inibição da produção de NO pela gedunina pode ocorrer via modulação de Hsp90, tendo em vista que a síntese de NO é modulada por esta chaperona, através da interação Hsp90-iNOS (óxido nítrico sintase induzida) (35, 134, 136-138). Estes dados estão de acordo aos de um estudo que demonstra que o pré-tratamento com o análogo semi-sintético da geldanamicina (17-AAG) (inibidor específico de Hsp90) também inibiu a produção de NO em macrófagos induzidos por LPS (135).

Além da produção de NO e citocinas, a ativação da via de sinalização de TLR4 em macrófagos induz o aumento da expressão de COX-2 e da produção de PGE_2, principalmente através da via tardia dependente de TRIF, que requer a internalização de TLR4 (149). De acordo, nossos resultados *in vitro* demonstram que macrófagos WT estimulados com LPS

durante 24h produzem altas concentrações de PGE_2 quando comparados aos macrófagos estimulados por 6h, período de tempo no qual a via de sinalização mais atuante é a dependente de TRIF. Além disto, o pré-tratamento com gedunina foi capaz de inibir a produção de PGE_2 a níveis basais durante a resposta aguda e tardia de sinalização de TLR4. Vale ressaltar que várias proteínas clientes da Hsp90 estão envolvidas na regulação da liberação de ácido araquidônico mediada por fosfolipase A2, tais como a proteína quinase p54, que media a fosforilação e a consequente ativação de fosfolipase A2 (179). A Hsp90 desempenha um importante papel na regulação de outras proteínas envolvidas na sinalização de TLR, como as proteínas membros da família MAPKs em particular na estabilização da TAK-1, quinase que também está envolvida na ativação do fator de transcrição NFkB, que são proteínas chaves para a produção de citocinas e síntese de COX-2 (143, 150). De fato, foi demonstrado, em macrófagos estimulados com LPS *in vitro*, que a ativação do NFkB depende de Hsp90, uma vez que a geldanamicina foi capaz de inibir a translocação nuclear de NFkB (96). Além disto, outros autores demonstraram que a modulação de Hsp90 via geldanamicina se dá através da redução da formação do complexo DNA/NFkB (e não pelo impedimento da degradação de IkB ou a translocação nuclear de NFkB) e, desta forma, a ativação deste fator de transcrição é inibida (97).

De forma interessante, os resultados obtidos neste estudo demonstram um papel adicional da gedunina na via de sinalização de TLR4 que vai além da modulação de Hsp90, e pode estar relacionada ao bloqueio *upstream* da sinalização interferindo na ligação do LPS com o complexo receptor TLR4/MD-2. Isto porque, em adição às co-chaperonas da Hsp90 (p23 e Cdc37), o MD-2 aparece como mais um candidato a proteína alvo da gedunina. Considerando a similaridade estrutural do MD-2 à co-chaperona p23, comparamos as sequências e a estrutura cristalográfica desta proteína com as do MD-2, e observamos que ambas apresentam similaridades estruturais no enovelamento do sítio de ligação, representada por uma bolsa hidrofóbica bem definida (112). Em adição à semelhança estrutural do sítio de interação das proteínas p23 e MD-2 com seus ligantes, vale ressaltar que um dado da literatura demonstra que a sinalização de TLR4 pode ser também inibida pela ligação de um glicolipidio endógeno, o globotetraosilceramida (Gb4). Foi demonstrado que o Gb4 se liga ao MD-2, deslocando LPS do sítio de interação, atenuando a sua toxicidade (145). A hipótese de que a gedunina poderia também estar se ligando ao MD-2 como o Gb4 foi confirmada através de ensaios de modelagem *in silico* e de espectroscopia de ressonância plasmônica de superfície (SPR), que sugerem que

a gedunina pode modular a resposta ao LPS no início da cascata de sinalização do TLR4, se ligando no mesmo sítio de interação hidrofóbica ao MD-2 de forma espontânea. Ainda, foi demonstrado que mesmo variando a ordem de adição da gedunina e do LPS ao MD-2, este terpenóide foi capaz de deslocar o LPS, interferindo de maneira eficiente na formação do complexo de ligação entre o LPS/MD-2, e sugerindo que a gedunina atue como inibidor competitivo. Os resultados obtidos por SPR, mesmo que não reflitam o microambiente celular, corroboram os dados obtidos *in vitro* (pré- e pós-tratamento com a gedunina) e *in silico.* Vale ainda ressaltar que, em adição aos resultados de competição de SPR, nós observamos através de ensaio *in vitro* que o pré-tratamento de macrófagos estimulados com LPS com a gedunina pré-incubada com o MD-2 em diferentes concentrações não inibiu a produção de TNF-α de forma dependente (Anexo 1). Estes resultados sugerem que este limonóide forma um complexo em solução com o MD-2, impedindo que a gedunina module a resposta inflamatória em macrófagos induzidos por LPS, reforçando a premissa de que este tetranortriterpenóide é um inibidor competitivo ao LPS na ligação ao MD-2. Em conjunto, nossos dados nos indicam que a gedunina impede a formação do complexo TLR4/MD-2/LPS e a consequente sinalização desencadeada após o seu reconhecimento.

Partindo do fato de que *i)* a gedunina atua modulando a via de sinalização de TLR4 em macrófagos estimulados por LPS *in vitro* e que *ii)* dados prévios do nosso grupo que demonstram que o LPS induz um aumento acentuado no número de leucócitos na cavidade pleural de camundongos através de um mecanismo indireto que envolve mediadores inflamatórios produzidos principalmente por macrófagos residentes (164-167), avaliamos no presente estudo a capacidade da gedunina em inibir a resposta inflamatória desencadeada por LPS no modelo de pleurisia *in vivo.* Vale ressaltar que o nosso grupo só havia demonstrado a atividade anti-inflamatória e anti-alérgica da gedunina em respostas independentes de TLR4, que incluem pleurisia e inflamação pulmonar induzida por ovalbumina (OVA) e artrite induzida por zimosan (125, 126, 129, 130). Neste trabalho, demonstramos que a gedunina foi capaz de inibir a resposta inflamatória induzida por LPS *in vivo*, em um regime de pré- e pós-tratamento, reduzindo a migração de células mononucleares, eosinófilos e neutrófilos, como também a produção de TNF-α, IL-6, IL-1β e NO. Estes resultados obtidos *in vivo* corroboram os dados obtidos *in vitro* com macrófagos estimulados com LPS, considerando que os macrófagos residentes da pleura são as principais células que reconhecem o LPS e são células centrais nesta resposta. Em conjunto, podemos inferir que a resposta inflamatória induzida por LPS é

dependente de Hsp90 (100, 146-147, 173), tendo em vista que: *i*) Hsp90 faz parte do complexo receptor TLR4/MD-2 na ligação do LPS (174), *ii*) Hsp90 interage com diferentes proteínas quinases (MAPKs) envolvidas na sinalização de TLR4 (99, 175) e *iii*) Hsp90 é necessária para ativação de NFκB por diferentes mecanismos já descritos (96-97, 140).

Demonstramos que, dentre a grande variedade de efeitos da gedunina também se inclui a modulação do inflamassoma NLRP3. A ativação de NLRP3 ocorre em resposta a vários estímulos, que incluem produtos bacterianos como o LPS (79-80, 176). Dados apresentados aqui demonstram pela primeira vez que a gedunina foi capaz de regular a ativação do inflamassoma NLRP3 induzida por LPS *in vivo* e *in vitro*, reduzindo a expressão de NLRP3 nos leucócitos pleurais e em macrófagos murinos imortalizados. Neste contexto, foi demonstrado que a Hsp90 é necessária para maturação, estabilização e ativação de NLRP3, o que é corroborado pelo fato de que esta chaperona, em associação com sua co-chaperona SGT1 (supressor do alelo G2 de skp1), interage com os domínios NACHT e LRR (repetição rica em leucina) do NLRP3, formando um complexo estável essencial para estabilidade e ativação do inflamassoma (93, 106-107). Desta forma, nossos dados sugerem que a gedunina também inibe a formação do complexo do inflamassoma NLRP3 induzida por LPS, o que está de acordo com a redução da caspase-1 ativada e a produção de IL-1β obtidos *in vitro* e *in vivo.* Corroborando os resultados, dados prévios da literatura demonstraram que o tratamento de células THP-1 e de monócitos primários com geldanamicina reduziu a produção de IL-1β e caspase-1 ativada após estímulo com urato monossódico (106). Além disto, demonstramos o potencial de interação molecular da gedunina com a caspase-1, através da modelagem *in silico,* o que sugere um mecanismo de ação alternativo à modulação da Hsp90.

Reconhecendo os corticosteróides como uma importante classe de medicamentos anti-inflamatórios capazes de modular negativamente a via de sinalização de TLR e que estes apresentam similaridade estrutural com a gedunina (168-173), consideramos a possibilidade de que este tetranortriterpenóde poderia modular outras vias de sinalização, além do TLR4. Partimos então para a avaliação do efeito da gedunina na sinalização de TLR2 e TLR3, considerando que a sinalização de TLR2 depende de MyD88 e apresentaria semelhanças ao ensaio realizado com macrófagos KO de TRAM/TRIF estimulados com LPS, enquanto que TLR3 independe de MyD88 e se assemelharia ao ensaio realizado com macrófagos KO de MAL/MyD88 estimulados com LPS. Para tal, avaliamos o efeito da gedunina sobre a produção de mediadores pró-inflamatórios, fatores anti-inflamatórios, a formação do inflamassoma

NLRP3 e a ativação de caspase-1. Nossos resultados revelaram que o efeito da gedunina não é restrito a TLR4, demonstrando que este composto também modula a resposta mediada por TLR2 e TLR3, uma vez que este tetranortriterpenóide inibiu a produção dos mediadores inflamatórios TNF-α, IL-6, NO e IL-1β, assim como reduziu a expressão de inflamassoma NLRP3 após estímulo com PAM3 e POLY I:C. O mecanismo pelo qual a gedunina modula estas respostas ainda não está claro, entretanto, considerando que quinases dependentes da via MAL/MyD88 e TRAM/TRIF, como p38, ERK e JNK, são dependentes da atividade da chaperona Hsp90 (17, 99, 112, 141-143, 178), é esperado que as vias de sinalização de TLR2, TLR3 e TLR4 sejam inibidas por moduladores desta chaperona. Sabemos que a Hsp90 também é necessária para ativação de NFκB, que é um dos eventos comuns de ativação da via de sinalização de TLR2, TLR3 e TLR4, indicando que a inibição desta chaperona module negativamente a produção de citocinas pró-inflamatórias induzidas por todos estes agonistas de TLRs (96-97). Da mesma forma, seria esperado que a inibição da Hsp90, via co-chaperona SGT1, reduziria a formação de inflamassoma (106-107). De forma interessante, nossos dados demonstram que a produção de IL-6 e NO em resposta à ativação de TLR2 foi inibida pela gedunina, mas não pelo inibidor seletivo de Hsp90, a 17-AAG, sugerindo que, neste caso, o efeito deste limonóide possa estar ocorrendo de forma independente de sua modulação via Hsp90. Neste sentido, é interessante ressaltar que realizamos ensaios preliminares de luciferase em células HEK transfectadas com TLR2/1 estimuladas com PAM3 e observamos que o pré-tratamento com gedunina reduziu a atividade de luciferase nestas células (Anexo 2), contribuindo com os nossos dados que este tetranortriterpenóide modula negativamente a sinalização de TLR2. Em adição, nossos estudos de modelagem *in silico* prevêem que a gedunina se liga a TLR2 e TLR3, sugerindo que esta substância é um ligante multialvos. Entretanto, ensaios bioquímicos adicionais, como SPR ou DARTS (*drug affinity responsive target stability*) são necessários para a confirmação de ligação da gedunina a estas proteínas e para a determinação de sua constante de afinidade (K*eq*).

Demonstramos ainda que, além da capacidade da gedunina em inibir mediadores pró-inflamatórios induzidos por LPS, este tetranortriterpenóide também induz a expressão e a produção de fatores anti-inflamatórios, incluindo heme-oxigenase-1 (HO-1), IL-10 (*in vitro* e *in vivo)* e Hsp70, agregando mais valor ao efeito farmacológico desta molécula. Está bem descrito na literatura que a HO-1 e a IL-10 são induzidas por diversos estímulos, incluindo LPS (153, 177), como também foi demonstrado que a HO-1 modula o efeito anti-inflamatório da IL-

10 e inibe a sinalização de TNF-α (155). Demonstramos neste estudo que o tratamento da gedunina na presença ou ausência de agonistas de TLR (PAM3, POLY I:C e LPS), aumentou a expressão de HO-1 e a produção de IL-10, sugerindo que o efeito anti-inflamatório da gedunina se deve em parte por estes fatores. A indução de proteínas de choque térmico, incluindo HO-1 (Hsp32), pelo celastrol (inibidor de Hsp90) já foi demonstrado *in vitro* e *in vivo* (115, 151-152). A HO-1 é a isoforma induzida da enzima, responsável pela degradação do heme livre, e que é amplamente expressa durante estresse e inflamação, desencadeada por diversos estímulos, incluindo LPS e, mediadores inflamatórios, como TNF-α e IL-1β. Sabe-se que sua atividade é mediada pela produção de IL-10, de forma dependente da quinase p38 (153, 155). A atividade anti-inflamatória e imunomoduladora da HO-1 é mediada tanto pela degradação do grupo heme livre (pró-inflamatório), como também via produção de bilirrubina e monóxido de carbono (CO), que apresentam propriedades anti-inflamatórias (154). Além disto, foi demonstrado que o CO, um dos produtos de degradação do grupo heme da HO-1, atenua a atividade de Hsp90 e promove a dissociação de suas proteínas clientes (156). No presente estudo, nós demonstramos que a HO-1 induzida por estresse aumentou após incubação com gedunina (na ausência dos estímulos como PAM3, POLY I:C ou LPS), sugerindo que, em nosso modelo experimental, a gedunina apresente seus efeitos tanto de forma dependente quanto independente da resposta mediada por TLR, ou ainda, de Hsp90.

Vale ressaltar ainda que, observamos um aumento da expressão do fator anti-inflamatório Hsp70, em macrófagos estimulados com LPS pré-tratados com a gedunina. Sabe-se que a citoproteção durante o estresse oxidativo está relacionada ao aumento da expressão de Hsp70 e sua consequente liberação para o meio extracelular (116). Considerando que a gedunina apresenta seus efeitos via modulação de Hsp90 e que esta modulação induz a ativação de HSF-1 e o consequente aumento de expressão de Hsp70 e HO-1 (Hsp32), podemos entender o mecanismo pelo qual este tetranortriterpenóide induz o aumento da expressão de HO-1 e Hsp70 (157-158). Os mecanismos anti-inflamatórios da Hsp70 vêm sendo reportados na literatura, e, foi demonstrado que a indução de Hsp70 intracelular (por estresse ou por compostos indutores de Hsp70), leva à diminuição da translocação nuclear de NFκB, e consequente redução na produção de TNF-α, IL-6, IL-1β, NO, como também a expressão de iNOS (2, 150, 159-160). De forma interessante, dados da literatura demonstram que o tratamento *in vitro* de células dendríticas com Hsp70 antes do estímulo com LPS diminui a produção de TNF-α além de induzir a produção de IL-10 no líquido sinovial de pacientes com

artrite (161). Nosso estudo também demonstrou que a gedunina além de atuar como indutor de Hsp70 em macrófagos estimulados com LPS, aumenta a produção de IL-10 sob estímulo de PAM3 e POLY I:C. Ainda, reforçando a hipótese de que a IL-10 é um fator intermediário do efeito anti-inflamatório deste limonóide, observamos que a administração intra-peritoneal de gedunina em camundongos *naive* foi capaz de aumentar os níveis de IL-10 na cavidade pleural, 24h após a administração (Anexo 3). De fato, foi demonstrado na literatura que a IL-10 suprime a ativação de macrófago induzida por LPS por inibir a expressão de genes específicos pró-inflamatórios induzidos por TLR referidos como “genes contra-regulados por IL-10” (162-163). É importante salientar que a resposta anti-inflamatória mediada por IL-10 é também mediada pela expressão de HO-1 (155). Desta forma, demonstramos neste estudo, que a gedunina também atua de forma adicional à inibição de fatores pró-inflamatórios, via indução de mecanismos anti-inflamatórios e resolutivos.

5. CONCLUSÃO

Neste trabalho, demonstramos que a gedunina é uma substância multialvos, com propriedades anti-inflamatórias, capaz de modular a resposta mediada por TLR através da inibição da produção de mediadores inflamatórios, da ativação de inflamassoma NLRP3 e da migração de leucócitos. Além disto, demonstramos que a gedunina induz a produção de fatores anti-inflamatórios e protetores. Neste trabalho, evidenciamos ainda um novo mecanismo de ação deste terpenóide na via de sinalização de TLR4, atuando como um inibidor competitivo do sítio de interação do LPS ao MD-2. Nosso estudo aprofundou o conhecimento sobre as propriedades farmacológicas da gedunina e reconhece esta substância como um candidato a fármaco para o tratamento de doenças inflamatórias infecciosas (figura 5.1).

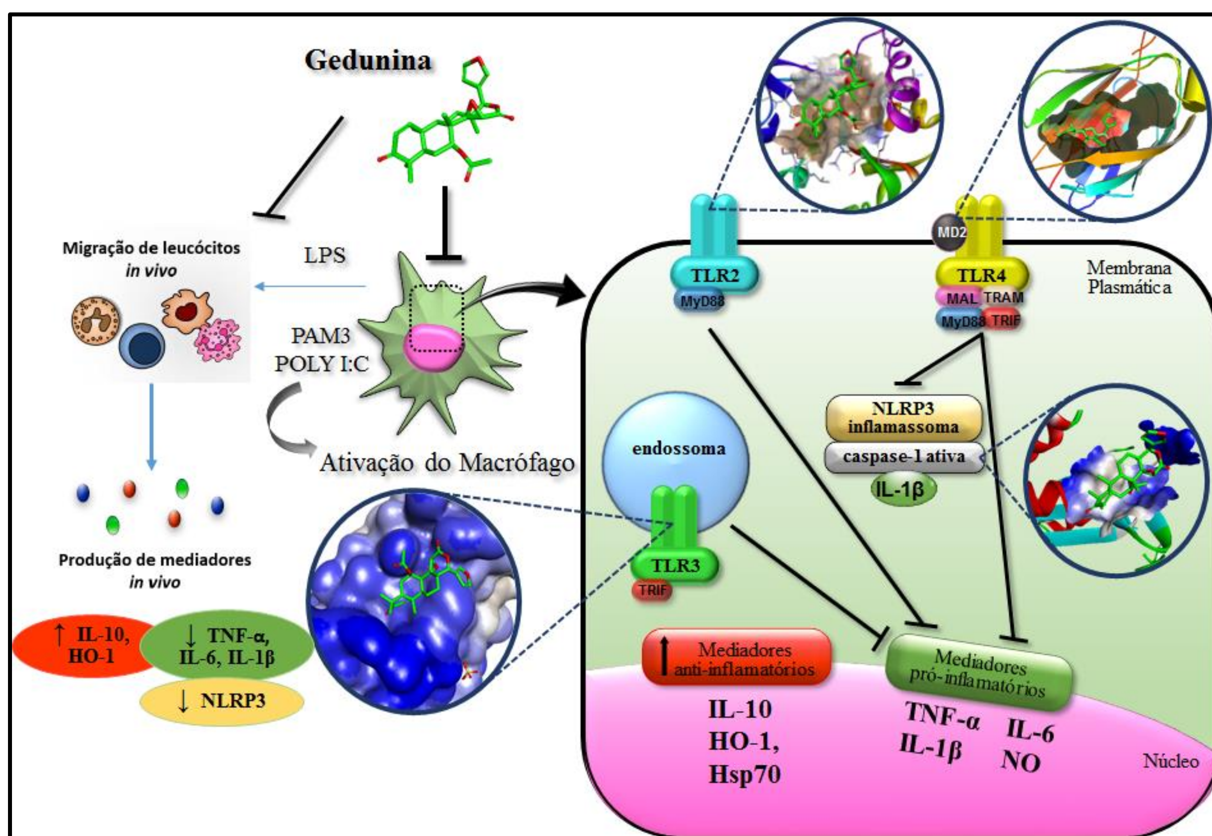


Figura 5.1. Esquema de conclusão. O tratamento com a gedunina inibe a ativação *in vitro* de macrófagos induzida por LPS, PAM3 e POLY I:C, impedindo a produção de mediadores inflamatórios (TNF-α, IL-6, NO e IL-1β), a ativação de inflamassoma (NLRP3, caspase-1) e induzindo a produção de mediadores anti-inflamatórios e resolutivos (IL-10, HO-1 e Hsp70). Em adição, a gedunina sob estímulo de LPS em macrófagos, *in vitro,* inibe o influxo de Ca^{+2} intracelular, a translocação de NFκB, a produção de citocinas e de PGE_2, e a expressão de COX-2, em ambas as vias aguda e tardia de TLR4, demonstrando que a gedunina além de modular a Hsp90, age se ligando ao MD-2/TLR4, impedindo a ligação de LPS e a cascata de sinalização. Ainda, demonstramos que este limonóide atua na resposta inflamatória induzida por LPS na cavidade pleural *in vivo,* impedindo a migração de leucócitos, inibindo a produção de mediadores inflamatórios (TNF-α, IL-6, NO e IL-1β), a ativação de inflamassoma NLRP3 e induzindo fatores anti-inflamatórios (IL-10 e HO-1). Ca^{+2} (cálcio); COX-2 (ciclo-oxigenase-2); HO-1 (heme-oxigenase-1); Hsp (proteína de choque térmico); IL (interleucina); LPS (lipopolissacarídeo); PAM3 (*palmitoyl-3-Cys-Ser-(Lys*)4) POLY I:C (*polyriboinosinic:polyribocytidylic acid*) MAL (*MyD88-adapter-like*); MD-2 (proteína mielóide de diferenciação-2); MyD88 (fator de diferenciação mielóide 88); NFκB (fator de transcrição nuclear κB); NO (óxido nítrico); PGE_2 (prostaglandina E_2); TLR (receptor do tipo Toll); TNF (fator de necrose tumoral); TRAM (molécula adaptadora relacionada à TRIF); TRIF (*Tir-domain-containing adapter-inducing interferon-beta*).

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7. ANEXOS

7.1 Anexo 1 – Efeito da resposta inibitória da gedunina sobre a produção de TNF-α, em macrófagos pré-incubados com MD-2 solúvel

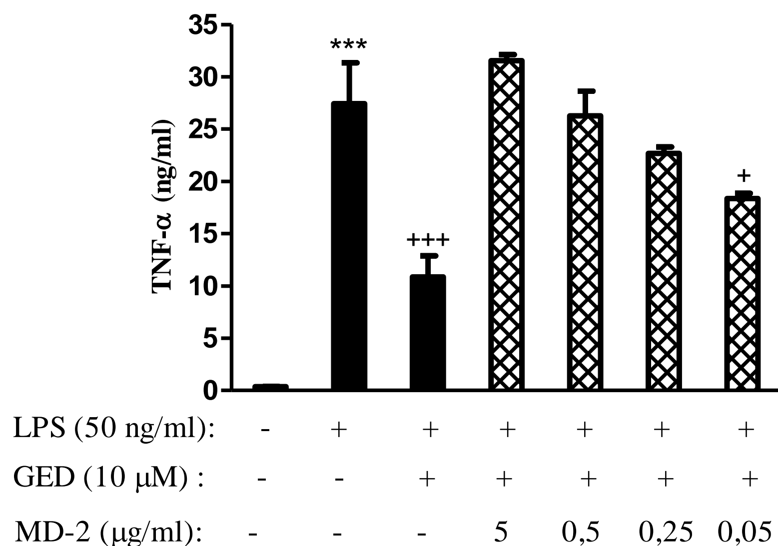

Figura Anexo 1 – Efeito da resposta inibitória da gedunina na produção de TNF-α, em macrófagos estimulados com LPS, quando pré-incubados com a proteína MD-2. Gedunina (10 µM) previamente incubada ou não por 1h, em meio DMEM, com diferentes concentrações de MD-2 (0,05, 0,25, 0,5 e 5 µg/ml) foi utilizada no pré-tratamento em macrófagos imortalizados que após 1h foram estimulados com LPS (50 ng/ml). Após 24h de estímulo as concentrações de TNF-α foram mensuradas no sobrenadante das células por ELISA. Os resultados estão expressos como média ± EPM em triplicata por grupo, de 2 experimentos independentes. As análises estatísticas foram realizadas pela análise de variância (ANOVA) seguido por *Newman Keuls*. As diferenças estatisticamente significativas ($p < 0,05$) entre o grupo estimulado e não estimulado estão indicadas por (*) e entre os grupos tratados e o estimulado por LPS ($^{+}$)

7.2. Anexo 2 – Efeito da gedunina sobre TLR2/1 em ensaio de luciferase

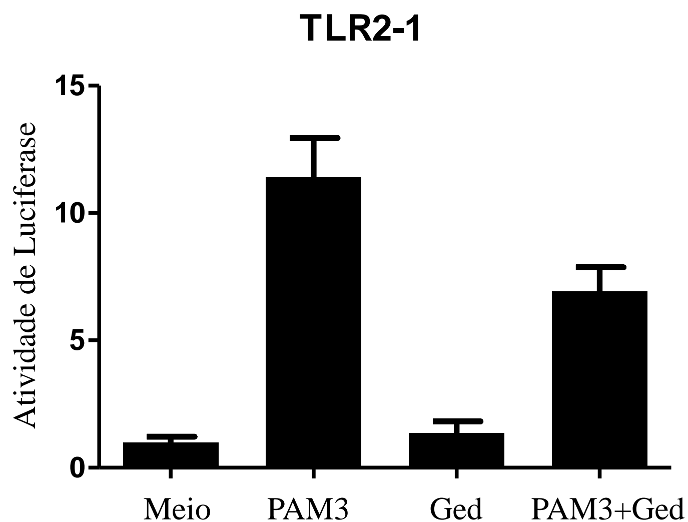

Figura Anexo 2. Efeito da gedunina sobre TLR2/1 em ensaio de luciferase. Células HEK293 foram cultivadas em placa de 96 poços ($3{,}5x10^4$ células/poço) em meio DMEM, suplementado com 10% de soro fetal bovino e pré-tratamento com a gedunina (10 µM), transfectadas com plasmídeo contendo o ectodomínio de TLR2/1 e estimuladas ou não com PAM3 (1 µg/ml) por 4h. As células foram então lisadas e a atividade luciferase foi determinada usando o kit Dual Luciferase Assay. O pré-tratamento da gedunina reduz a atividade de lucíferase após 4h de estímulo com PAM3 (1 µg/ml). Os resultados demonstrados no gráfico são representativos de simplicata dois experimentos independentes.

7.3 Anexo 3 - Efeito da gedunina sobre a indução de IL-10 na cavidade pleural de camundongos naive

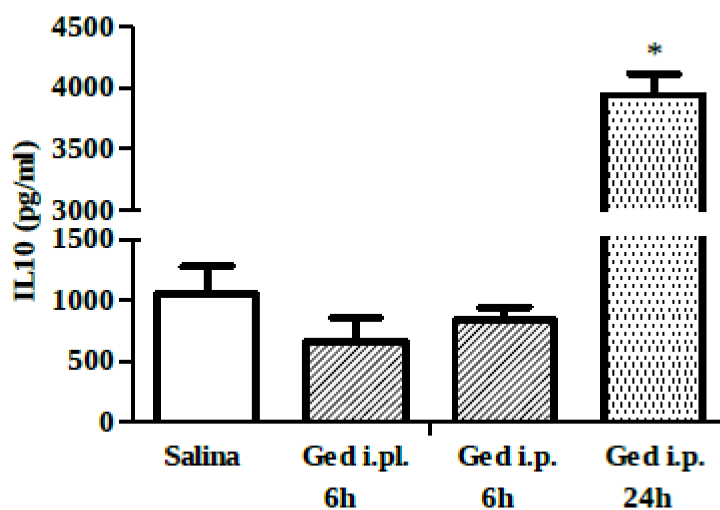

Figura Anexo 3. Efeito da gedunina sobre a indução de IL-10 na cavidade pleural em camundongos naive. Camundongos naive foram submetidos a administração intrapleural (i.pl.) e intraperitoneal (i.p.) de gedunina (0,5 mg/kg) e, após 6h e 24h do estímulo foram avaliados os níveis de IL-10 produzidos na cavidade pleural por ELISA. Os resultados estão expressos como média ± EPM em septupliclata por grupo, de 2 experimentos independentes. As análises estatísticas foram realizadas pela análise de variância (ANOVA) seguido por *Newman Keuls*. As diferenças estatisticamente significantivas entre os grupos estimulado e não estimulado estão indicadas por * ($p < 0,05$).