Universidade Estadual de Londrina

TALITA PERDIGÃO DOMICIANO

A QUERCETINA INIBE A ATIVAÇÃO DOS INFLAMASSOMAS NLRP3 E AIM2 POR IMPEDIR A OLIGOMERIZAÇÃO DO ASC E PREVINE A VASCULITE MEDIADA PELA ATIVAÇÃO DA CASPASE-1 EM CAMUNDONGOS

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Tese apresentada à banca do Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina como requisito para obtenção do título de Doutora em Ciências da Saúde.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Jr.

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Londrina, 29 de fevereiro de 2016.

Dedico este trabalho aos meus pais que,
nunca mediram esforços para que
eu realizasse meus sonhos.

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A certeza de que estamos começando...
A certeza de que é preciso continuar...
A certeza de que podemos ser interrompidos antes de terminar...
Façamos da interrupção um caminho novo...
Da queda, um passo de dança...
Do medo, uma escada...
Do sonho, uma ponte...
Da procura, um encontro!

Fernando Sabino

DOMICIANO, Talita Perdigão. **A quercetina inibe a ativaçao do inflamassoma por impedir a oligomerização do asc e previne a vasculite mediada por Interleucina (IL-1) em camundongos.** 2016**.** 58 f. Tese (Doutorado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

A interleucina IL-1β (IL-1β) é uma citocina altamente inflamatória que contribui significativamente para o desenvolvimento de doenças inflamatórias tanto agudas quanto crônicas. A secreção de IL-1β depende principalmente de uma protease, a caspase-1, a qual é ativada por plataformas proteicas conhecidas como inflamassomas. Entre outros fatores, cada vez mais evidências indicam que as espécies reativas de oxigênio (EROS) desempenham um papel chave no processo de sinalização que leva à ativação do inflamassoma em diversas doenças inflamatórias. Os flavonoides são moléculas fenólicas que ocorrem naturalmente como metabolitos secundários de várias plantas e possuem uma ampla variedade de atividades biológicas tais como anti-inflamatórias, antidiabéticas, antimicrobiana e anticâncer bem como efeito antioxidante. Neste estudo, investigamos o efeito de três flavonoides, quercetina (QUC), naringenina (NRG) e silymarina (SIL) sobre a inibição da ativação dos inflamassomas. Encontramos que a QUC inibiu a secreção de IL-1β pelos inflamassomas NLRP3 e AIM2 de forma dose dependente, porém, não inibiu a ativação do inflamassoma NLRC4. O mesmo efeito não foi observado com os flavonoides NRG e SIL, uma vez que estes não inibiram significativamente nenhum do inflamassomas avaliados. A inibição do inflamassoma pela QUC também foi observada em macrófagos *knockout* para *Atg16l1*, indicando que o efeito da QUC não é dependente do processo de autofagia. Uma vez que a QUC inibiu tanto o inflamassoma NLRP3 quanto o AIM2, mas não o inflamassoma NLRC4, avaliamos então a formação de partículas de ASC. Neste aspecto, a QUC reduziu tanto a formação de partículas de ASC quanto sua oligomerização quando comparado com os controles. Finalmente, observamos que o tratamento com a QUC reduziu significativamente a vasculite induzida pelo extrato de parede celular bacteriana em um modelo murino de arterite coronariana aguda e aneurismas de aorta. Em conclusão, a QUC inibe os inflamassomas NLRP3 e AIM2 por prevenir a oligomerização do ASC e mostra-se como um candidato em potencial para o tratamento da vasculite da Doença de Kawasaki bem como outras doenças inflamatórias mediadas pela IL-1.

Palavras-chave: Quercetina. Inflamassoma. NLRP3. ASC. Vasculite. Arterite coronariana. Doença de Kawasaki.

DOMICIANO, Talita Perdigão. **Quercetin inhibits inflammasome activation by interfering with asc oligomerization and prevents Interleukin-1 (IL-1) mediated mouse vasculitis.** 2016. 58 p. Thesis (Doctoral degree in Health Sciences). – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

IL-1β is a highly inflammatory cytokine and significantly contributes to both acute and chronic inflammatory diseases. The secretion of IL-1β requires a unique protease caspase-1, which is activated by various protein platforms called inflammasomes. Accumulating evidence indicate a key role for mitochondrial reactive oxygen species (ROS) signaling for inflammasome activation during numerous inflammatory diseases. Flavonoids constitute a group of naturally occurring polyphenolic molecules that have been attributed with many biological activities including antioxidant effects. In this study, we investigated the effect of three flavonoids, quercetin (QUC), naringenin (NRG) and silymarim (SIL) on inflammasome activation. We found that QUC inhibits IL-1β secretion by both the NLRP3 and AIM2 inflammasome in a dose dependent manner, but not the NLRC4 inflammasome. A similar effect was not observed with the flavonoids NRG and SIL, once they did not significantly inhibited any of the evaluated inflammasomes. QUC inhibition of the inflammasome was still observed in *Atg16l1* knockout macrophages, indicating that QUC’s effect was autophagy independent. Since QUC inhibited both NLRP3 and AIM2 inflammasomes but did not NLRC4, we assessed ASC speck formation. QUC reduced ASC speck formation and ASC oligomerization compared with controls. Finally, we observed that QUC significantly reduced a bacterial cell wall extract induced-vasculitis in a mouse model of acute coronary arteritis and aorta aneurysms. In conclusion, QUC inhibits both the NLRP3 and AIM2 inflammasome by preventing ASC oligomerization and may be a further potential therapeutic candidate for Kawasaki disease vasculitis and other IL-1 mediated inflammatory diseases.

Key-words: Quercetin. Inflammasome. nlrp3. ASC. Vasculitis. Coronary arteritis. Kawasaki disease.

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LISTA DE ABREVIATURAS

BMDM	Macrófagos derivados da medula óssea
CAP	Síndrome periódica associada à criopirina
DAMP	Padrões moleculares associados a danos
DAPI	4',6-diamidino-2-fenilindol
DMSO	Dimetil sulfoxido
FLICA	Fluorocromo inibidor de caspases
GSH	Glutationa
IL-1β	Interlceucina-1β
IL-6	Interleucina-6
IL-17	Interleucina-17
IVIG	Imunoglobulina intravenosa
KD	Doença de Kawasaki
LOX	Lipoxigenase
LPS	Lipopolissacarídeo
LCWE	Extrato de parece celular de *Lactobacilos casei*
QUC	Quercetina
NF-κB	Fator nuclear kappa B
NLR	Receptor do tipo nod
NRG	Naringenina
MAPK	proteína quinase ativada por mitógeno
MMP-9	Matriz metalloproteinase 9
PAMP	Padrões moleculares associados a agentes patogênicos
PRR	receptores de reconhecimento de padrões
ROS	Espécies reativas de oxigênio
SIL	Silimarina
TLR	Receptor do tipo *toll*
TNF-α	Fator de necrose tumoral-α

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1 INTRODUÇÃO

1.1 A ativação do inflamassoma e a doença de Kawasaki

A inflamação é uma importante resposta celular com vários passos através dos quais os tecidos vasculares respondem a estímulos nocivos tais como patógenos infecciosos, toxinas, trauma ou calor. Neste cenário, o principal papel do sistema imunológico é manter a função do tecido afetado em homeostase (1). Normalmente a resposta inflamatória beneficia o organismo, no entanto, se esta resposta acontecer de forma intensa ou desregulada pode causar prejuízos permanentes levando ao aparecimento de doenças inflamatórias agudas ou crônicas (2).

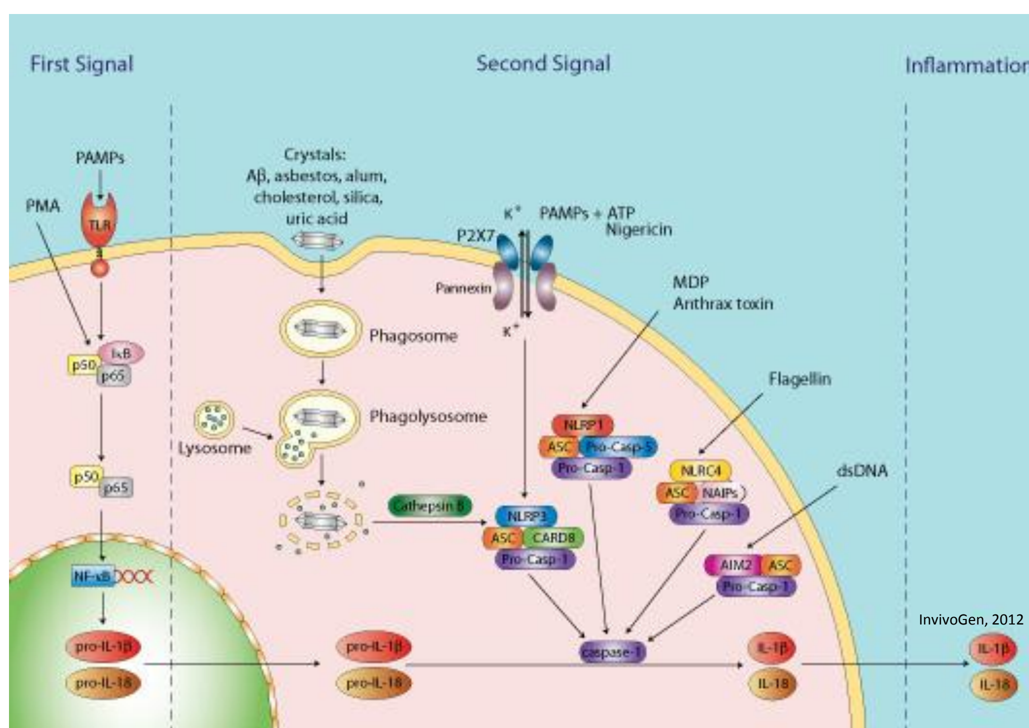
O desenvolvimento do processo inflamatório geralmente se inicia através da detecção de padrões moleculares associados a agentes patogênicos (PAMPs) ou padrões moleculares associados a danos (DAMPs) por receptores de reconhecimento de padrões (PRRs) em células imunes inatas ou células epiteliais. Os PRRs abrangem várias famílias de receptores entre estes estão os receptores do tipo *toll* (TLRs) e os receptores do tipo NOD (NLRs) que podem ser expressos tanto na superfície celular como no ambiente intracelular (3). Após a ligação, os PRRs dão início a eventos intracelulares que levam à produção de citocinas pró-inflamatórias, quimocinas e outros mediadores inflamatórios. A sinalização de diferentes eventos induzidos pelos PRRs leva à ativação da via do NF-κB, que medeia a transcrição de várias citocinas pró-inflamatórias como TNF-α, IL-1 e IL-6 bem como vários outros mediadores de processos inflamatórios (2,3). Entre estes, a IL-1β possui um papel central na imunidade contra várias classes de patógenos e atuando como peça chave em doenças inflamatórias. Esta citocina é produzida por macrófagos ativados em sua pró-forma e posteriormente ativada pelos inflamassomas (4).

O inflamassoma é um complexo proteico multimérico composto pela proteína adaptadora ASC e pela capsase-1 (5) que tem a sua ligação induzidas pela ativação de PPRs resultando na liberação de citocinas altamente pró-inflamatórias como IL-1β e IL-18 (6). O ASC, codificado pelo gene *Pycard*, é uma proteína citosólica que controla a ativação da capsase-1, atuando como uma ponte entre os componentes de inflamassomas como NLRP3 e AIM2, que se ligam entre si formando estruturas oligoméricas semelhantes a fibras, tais como partículas de ASC. Ainda não está bem definido se o ASC também é necessário para a ativação do inflamassoma NLRC4 uma vez que este possui um domínio CARD que pode interagir diretamente com o domínio CARD da pro-caspase-1 promovendo sua ativação (3).

Em suma, ativaçao do inflamassoma depende de dois sinais. O primeiro através da ativação do NF-κB que leva a produção de pró-IL-1β e pró-IL-18. E um segundo sinal responsável pela ativação do complexo proteico do inflamassoma que resultará na ativação da IL-1β e IL-18 conforme ilustrado na Figura 1.

O aumento da presença de IL-1β, de forma local ou sistêmica, tem sido relacionada com várias doenças tanto hereditárias como adquiridas, e inibidores de IL-1β ou antagonistas de seu receptor tem se mostrado como tratamentos eficazes em várias doenças tais como a síndrome periódica associada à criopirina (CAPS), gota, diabetes tipo II, aterosclerose, hipertensão e doença de Kawasaki (KD) (7-10).

Figura 1. Vias de ativação do inflamassoma.



A doença de Kawasaki é uma vasculite aguda multissistêmica que afeta principalmente crianças em países desenvolvidos, sendo a causa mais comum para doenças cardiovasculares infantis adquiridas (11). Os sintomas desta doença autoimune são febre persistente, eritema dos lábios e da mucosa oral, erupções cutâneas envolvendo o tronco e extremidades, conjuntivite bilateral e linfadenopatia unilateral (12). Enquanto as sequelas da inflamação arterial da fase aguda da doença de Kawasaki são geralmente autolimitadas e bem descritas, seus efeitos tardios, tais como aneurismas da artéria coronária, infarto do miocárdio ou insuficiência cardíaca podem levar à morte (13).

Recentemente, um grupo de pesquisadores propôs uma importante ligação entre a arterite coronariana e a subsequente aceleração do processo de aterosclerose, propondo

que a ocorrência da doença de Kawasaki na infância pode causar uma potencial predisposição para aterosclerose prematura na fase adulta (14) e relacionando ainda o envolvimento da IL-1β e ativação do inflamassoma no modelo animal da doença de Kawasaki (8).

Embora os mecanismos relacionados com o desenvolvimento da doença de Kawasaki e seu tratamento ainda não sejam completamente compreendidos a imunoglobulina intravenosa (IVIG) é atualmente a primeira linha terapêutica para a fase aguda desta doença. Além desta, a Associação Americana do Coração recomenda a combinação do tratamento com aspirina embora a evidência de seus benefícios sejam escassas (12). Apesar dos esforços para reduzir a inflamação, entre 11% a 23% dos pacientes podem apresentar resistência ao tratamento com IVIG, tornando-os uma população de alto risco para o desenvolvimento de problemas cardíacos. Neste sentido existe uma carência de novas opções terapêuticas para o tratamento da doença de Kawasaki.

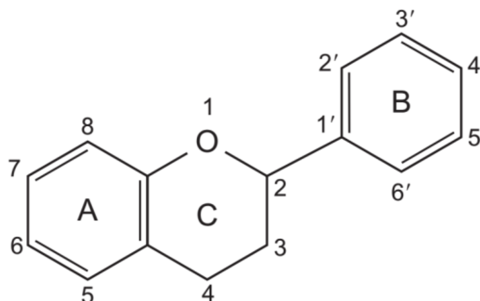
1.2 Flavonoides

Os flavonoides constituem um grupo de compostos fenólicos naturais que estão amplamente distribuídos, no reino vegetal, em várias classes de acordo com suas características estruturais. Estas classes são as flavonas, flavononas, isoflavonas, flavonóis, flavolonolois flavan-3-ois, antocianidinas, chalconas e auronas (15). As diferenças principais entre as classes de flavonoides estão relacionadas ao nível de oxidação e de substituição do anel C, enquanto os compostos individuais dentro de uma mesma classe diferem no padrão de substituição dos anéis A e B, conforme representados na Figura 2 (16). Devido a sua marcante presença nos produtos naturais, os flavonoides são um componente importante da dieta humana (16,17). Mais de 10 000 diferentes moléculas de flavonoides já foram identificadas, no entanto, poucas entre estas foram investigadas detalhadamente (17).

De modo geral, os flavonoides atuam como varredores de radicais livres e várias de suas funções biológicas são atribuídas a seus efeitos antioxidantes relacionados com a neutralização de espécies reativas de oxigênio celulares e mitocondriais (16,18). Existem evidências mostrando que radicais livres são capazes de ativar fatores de transcrição pró-inflamatórios sensíveis ao estresse oxidativo tais como o NF-κB, podendo ser este um dos mecanismos responsáveis pela ação anti-inflamatória dos flavonoides. No entanto, um estudo que investigou os mecanismos de ação da baicalina, demonstrou que este flavonoide age de forma independente do NF-κB, ligando-se seletivamente a quimiocinas tais como IL-8, MIP-1β e MCP-2 e limitando sua atividade biológica (19). Desta forma, devido à grande variação estrutural e por suas atividades biológicas variadas mesmo entre formas estruturais

semelhantes, cada flavonoide precisa ser avaliado separadamente em diferentes estudos *in vitro* e *in vivo* (16).

Figura 2. Estrutura de núcleo central de flavonóides



1.2.1 Quercetina

A quercetina é um flavonoide presente na dieta que pode ser encontrado em frutas e vegetais, como maçã, morango, tomate e cebola (20) e ainda em castanhas (21) geralmente em sua forma glicosilada. De acordo com diferentes estudos, entre os polifenóis, a quercetina é o que possui maior capacidade antioxidante (16). A quercetina apresenta várias propriedades terapêuticas com benefício em potencial para a saúde humana. Entre suas atividades biológicas descritas estão efeito gastroprotetor, antidiabético, cardioprotetor, antimicrobiano, anti-inflamatório e anticâncer (22-25). Tem sido demostrado que a quercetina inibe enzimas geradoras de espécies oxidativas como a xantina oxidase, LOX e nicotinamida adenina dinucleotídeo fosfato oxidase. A quercetina é um potente agente anticâncer, que apresenta atividades tais como regulação do ciclo celular, interação com sítios de ligação de estrógeno tipo II e inibição da tirosina quinase (26,27). Enquanto alguns parâmetros como controle da pressão arterial já foram investigados clinicamente, estudos de outros aspectos como a atividade anticâncer permanece no campo pré-clínico (23). Estudos recentes também têm apontado para um possível efeito da quercetina com respeito à inibição do inflamassoma NLRP3 em modelos de inflamação, diabetes e aterosclerose (25,28-32), no entanto, se este efeito inibitório está relacionado à inibição do sinal 2 de ativação do inflamassoma ou mesmo o mecanismo de inibição permanecem desconhecidos.

1.2.2 Naringenina

A naringenina, flavonoide pertencente ao grupo das flavanonas (33), é encontrada em frutas cítricas como laranja, limão e toranja (*grapefruit*) *(15).* A naringenina possui várias atividades biológicas incluindo antioxidante, antialérgica, antimicrobiana, anti-inflamatória e anticâncer (33). Estudos recentes mostram que os efeitos antioxidantes da naringenina estão relacionados com inibição da hipercolesterolemia e diabetes tipo II (34-36). Em

camundongos alimentados com dieta rica em colesterol, o tratamento com naringenina reduziu os níveis plasmáticos de colesterol por inibir a síntese e esterificação do colesterol hepático (37). Em um modelo de inflamação induzida por radiação ultravioleta (UV) a naringenina inibiu a formação de edema, recrutamento de neutrófilos, atividade de MMP-9 e várias citocinas pró-inflamatórias entre elas IL-1β, IL-6, IL-17 e TNF-α (38).

1.2.3 Silimarina

A Silimarina é um complexo de flavonóis extraídos da fruta do cardo de leite (*Silybum marianum*) e é um antioxidante eficaz, capaz de conservar a glutationa (GSH) em células hepáticas enquanto estabiliza as membranas celulares hepáticas contra o ataque oxidativo (39). A silimarina possui ainda atividade antiviral contra a vírus chikungunya reduzindo a eficiência da replicação viral (40). Também possui uma potente capacidade de controle sobre processos fisiopatológicos envolvidos na resposta inflamatória através da inibição das vias da MAPKs e NF-κB (41). Como recentemente demostrado por Abdel-Moneim e colaboradores (42), o tratamento de animais com intolerância glicose em animais alimentados com dieta rica em lipídeos com silimarina, restaurou parâmetros como ganho de peso, intolerância à glicose e resistência à insulina. Ainda restaurou a atividade enzimática e reduziu níveis plasmáticos de IL-1β e TNFα. Outras atividades como hepatoprotetora, imunomoduladoras, anti-inflamatória, antidiabético, anticâncer e antifibrótica também foram descritas para a silimarina (43-45).

Embora os três flavonoides escolhidos apresentem atividades biológicas potencialmente relacionadas com a inibição ou regulação do inflamassoma já descritas, quer pela inibição do NF-κB, inibição de IL-1β ou da expressão de NLRP3, o mecanismo molecular envolvido na atividade anti-inflamatória da quercetina, naringenina e silimarina ainda não está completamente compreendido. Desta forma, o foco deste estudo foi o mecanismo imune-farmacológico destes três flavonoides *in vitro* em macrófagos ativados por LPS e *in vivo* na arterite coronariana e aneurisma da aorta abdominal induzidos por extrato de parede celular bacteriana.

2 OBJETIVOS

2.1 Objetivo geral

Determinar o efeito dos flavonoides: quercetina, naringenina e silimarina sobre a ativação do inflamassoma.

2.2 Objetivos específicos

Avaliar o efeito dos flavonoides sobre diferentes estímulos/vias de ativação do inflamassoma;

Avaliar o efeito dos flavonoides sobre o processo de autofagia;

Avaliar o efeito dos flavonoides relacionados à ativação do inflamassoma auto reativo;

Investigar o mecanismo de ação dos flavonoides sobre a inibição da ativação do inflamassoma;

Avaliar o efeito do tratamento com a quercetina sobre a incidência de vasculite e sobre a inibição do inflamassoma no modelo de Doença de Kawasaki em camundongos;

3 MATERIAL E MÉTODOS

Animais.

Os camundongos C57BL/6 foram obtidos dos laboratórios Jackson (Bar Harbor, ME USA). Os camundongos *Nlrp3*$^{-/-}$ foram gentilmente cedidos pelo Dr. Fitzgerald KA (Univ. Massachusetts Medical School, MA USA). Os camundongos *Nlrp3*$^{A350V/350V}$ foram gentilmente cedidos pelo Dr. Hoffman HM (UC San Diego, CA USA). Os camundongos *Atg16l1*$^{fl/fl}$ foram gentilmente cedidos pelo Dr. Shih DQ (Cedars-Sinai Medical Center, CA USA) e cruzados com camundongos LysM^{Cre} (Laboratórios Jackson). Todos os camundongos utilizados eram machos e tinham entre 8 a 12 semanas de idade. Todos os camundongos foram mantidos em ambiente livre de patógenos no biotério do Cedar-Sinai Medical Center, Los Angeles, Califórnia, EUA. Os experimentos foram realizados de acordo com protocolos aprovados pelo Comitê Institucional de Usos e Cuidados animais.

Drogas e tratamentos

Os flavonoides quercetina, naringenina e silimarina foram adquiridos da Sigma. Para os experimentos *in vitro* foi preparado uma solução estoque de cada flavonoide dissolvido em 100% de sulfóxido de dimetil (DMSO) na concentração de 200 mg/ml e posteriormente diluído em meio de cultura antes da administração nas culturas de células. Para os experimentos *in vivo,* a quercetina na dose de 100 mg/kg foi primeiramente dissolvida em 100% DMSO e então diluída com PBS até o volume final de 100 µl/10 g de peso corporal.

Dosagem de citocinas

Os macrófagos derivados da medula óssea (BMDM) foram preparados como descrito previamente (47). Estes macrófagos foram estimulados com 500 ng/ml *E. coli* LPS (Invivogen, CA USA) e após 3 horas de incubação estimulados novamente com 5 mM ATP (Sigma, MO USA), 10 µM de nigericina (Enzo Life Science, INC., NY USA) ou 130 µg/ml de hidróxido de alumínio (Sigma) para ativação do inflamassoma NLRP3; poly(dA:dT) (Invivogen) para ativação do inflamassoma AIM2; e *Salmonella Typhimurium WT e ΔfljB/fliC* (MOI 5) para ativação de inflamassoma NLRC4 (48). Os BMDMs foram tratados com quercetina, naringenina ou silimarina nas doses de 20 e 100 µM, 30 minutos antes do estimulo com sinal-2. Os BMDMs obtidos de camundongos *Nlrp3*$^{A350/A350}$ foram estimulados com LPS (500 ng/ml) e tratados com quercetina 1 hora após o estimulo. Os sobrenadantes das culturas de BMDMs estimulados e tratados foram coletados e avaliados quanto a concentração das citocinas IL-1β e TNF-α através do método de ELISA de acordo com as orientações do fabricante (eBiosciences).

Ensaio de Immunoblot

Os BMDMs foram estimulados como descrito acima, os sobrenadantes foram coletados e as proteínas precipitadas por extração com metanol e clorofórmio. Os *pellets* obtidos foram ressuspendidos em tampão de lise. A análise do *imunoblot* foi realizada utilizando os seguintes anticorpos primários: anti-mouse IL-1β (AF-401-NA; R&D System, MN USA), anti-GAPDH (6C5; Santa Cruz Biotecnology). Para o ensaio de detecção de ASC, a suspensão de células foi lavada com PBS e incubada com 2 mM disuccinnimidyl suberate (DSS, No-Weigh™ Format, Pierce Protein Biology) durante 30 minutos em temperatura ambiente. Após lavagem com PBS gelado os precipitados foram suspendidos em tampão de lise. A análise por *immunoblot* foi realizada usando rabbit anti-mouse ASC (N15; Santa Cruz Biotecnology).

Coloração de imunofluorêscencia

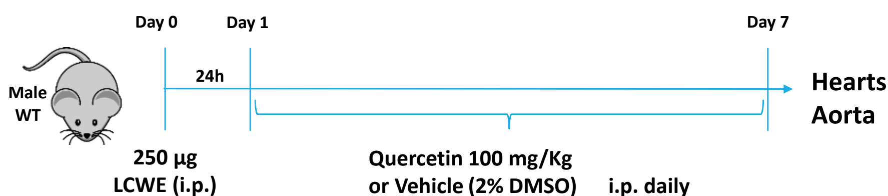
Os BMDMs foram plaqueados na densidade de 1 x 10^5 células/poço em uma placa de 24 poços com lamínulas redondas pré-revestidas com 0,2% de gelatina e estimuladas como descrito anteriormente. Uma hora após o estimulo as células foram lavadas com PBS bem gelado, fixadas com 1% de formalina e permeabilizadas com 0,1% de TritonX. Após o bloqueio, as células foram coradas utilizando como anticorpo primário rabbit anti-ASC (Santa Cruz Biotecnology), anticorpo secundário Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen) e em seguida montadas com DAPI (Life Technologies). Todas as imagens foram obtidas utilizando microscópio de fluorescência Keyence BZ-9000 (Keyence).

Preparação do extrato de parede celular de *Lactobacillus casei* e modelo animal da doença de Kawasaki.

O extrato de parede celular de *L. casei* (ATCC 11578) (LCWE) foi preparado como previamente descrito (46). Os camundongos machos com idade entre 4 a 5 semanas foram estimulados com injeção intraperitoneal de 250 µg LCWE ou PBS. O tratamento com quercetina 100mg/kg (grupo tratado) ou veiculo 2% DMSO (grupo controle) foi administrado diariamente, por via intraperitoneal, 24 horas após a injeção de LCWE (Figura 3). Após 7 dias da injeção de LCWE os camundongos foram sacrificados e tiveram os corações removidos e embutidos em *Tissue Tek OCT*, como descrito previamente (8). Os cortes de coronárias e aorta congelados foram analisados por imunofluorescência quanto a presença de macrófagos e atividade de caspase-1 utilizando anti-mouse F4/80 (eBioscience, CA USA) e FLICA (ImmunoChemistry Technologies LLC, MN USA) respectivamente, e em seguida montado com DAPI 4,6-diamidino-2 phenylindole; Life Technologies, USA). O anticorpo IgG2a foi usado como controle de isotipo (AbD Serotec, OX UK). Todas as imagens foram

obtidas usando um microscópio de fluorescência Keyence BZ-9000 (Keyence Corporation of America, IL USA).

Figura 3. Protocolo experimental da doença de Kawasaki.



Analise estatística

Todos os dados foram analisados com o programa estatístico Prisma 5.0 (Graphpad software, Inc.). Foi utilizado *one-way* ANOVA seguido do teste *post hoc* de Tukey. O valor de P menor do que 0,05 foi considerado estatisticamente significativo.

4 RESULTADOS E DISCUSSÃO – ARTIGO CIENTÍFICO

4.1 Artigo: **Quercetin Inhibits Inflammasome Activation by Interfering with ASC Oligomerization and Prevents Interleukin-1 (IL-1) Mediated Mouse Vasculitis.**

Running Title: Quercetin inhibits inflammasome via ASC oligomerization

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ABSTRACT

IL-1β is a highly inflammatory cytokine and significantly contributes to both acute and chronic inflammatory diseases. The secretion of IL-1β requires a unique protease caspase-1, which is activated by various protein platforms called inflammasomes. Accumulating evidence indicate a key role for mitochondrial reactive oxygen species (ROS) signaling for inflammasome activation during numerous inflammatory diseases. Flavonoids constitute a group of naturally occurring polyphenolic molecules that have been attributed with many biological activities including antioxidant effects. In this study, we investigated the effect of three flavonoids, quercetin (QUC), naringenin (NRG) and silymarim (SIL) on inflammasome activation. We found that QUC inhibits IL-1β secretion by both the NLRP3 and AIM2 inflammasome in a dose dependent manner, but not the NLRC4 inflammasome. QUC inhibition of the inflammasome was still observed in *Atg16l1* knockout macrophages, indicating that QUC's effect was autophagy independent. Since QUC inhibited both NLRP3 and AIM2 inflammasomes but did not NLRC4, we assessed ASC speck formation. QUC reduced ASC speck formation and ASC oligomerization compared with controls. Finally, we observed that QUC significantly reduced a bacterial cell wall extract induced-vasculitis in a mouse model of acute coronary arteritis and aorta aneurysms. In conclusion, QUC inhibits both the NLRP3 and AIM2 inflammasome by preventing ASC oligomerization and may be a further potential therapeutic candidate for Kawasaki disease vasculitis and other IL-1 mediated inflammatory diseases.

Keywords: Quercetin, inflammasome, nlrp3, ASC, vasculitis, coronary arteritis, Kawasaki disease.

INTRODUCTION

Inflammation is a fundamental multi-step cellular response to harmful stimuli such as pathogens, toxins, trauma, or heat injury. Thus, it can be considered that a major role of the immune system is to maintain homeostatic tissue function. However, if inflammation goes on unchecked, sustained, immune responses can lead to serious host inflammatory injury and various diseases. Increased IL-1β, locally or systemic, has been linked to a number of human hereditary or acquired diseases, and antagonists of IL-1β or its receptor are increasingly being used successfully for treatments for a number of these inflammatory diseases such as cryopyrin-associated periodic syndromes (CAPS), gout, atherosclerosis, type II diabetes and even in Kawasaki disease vasculitis (KD) (7,8). The inflammasomes are multimeric protein complexes that consist of a sensor molecule, the adaptor protein ASC and caspase-1 via Caspase activation and recruitment domains (CARD)-CARD interactions (5), which are induced by the activation of pattern recognition receptors (PPRs) resulting in the release of highly pro-inflammatory cytokines interleukin 1β (IL-1β) and IL-18 (6). Apoptosis-associated speck like protein containing a CARD (ASC), encoded by *Pycard*, is a cytosolic protein and can control the activation of caspase-1, bridging NLRP3 and AIM2 inflammasomes by self-assembly into fiber-like structures such as ASC specks (6). Whether ASC is also required to NLRC4 inflammasome activation is less clear (49). NLR family, pyrin domain containing 3 (NLRP3) and Absent in Melanoma 2 (AIM2) contain a Pyrin domain (PYD) and do not interact with caspase-1 directly. Instead, the PYD of these inflammasome receptors interacts with the PYD of ASC. The CARD domain of ASC then binds the CARD of caspase-1 via CARD-CARD interaction serving as a bridge between pro-caspase-1 and pyrin-containing inflammasomes such as NLRP3 and AIM2 (50). NLR family, CARD domain containing 4 (NLRC4) has its own PYD domain, thus ASC is mostly dispensable in NLRC4 inflammasome activation (50).

Kawasaki disease (KD) is a multisystem acute vasculitis that primarily affects young children and is the most common acquired cardiovascular disease among children in

developed countries (11). Without treatment, 25% of KD patient develop heart disease involving coronary aneurysms and dilatations (8,12,51). While sequelae of arterial inflammation in the acute phase of KD are generally self-limiting and well documented, its late effects, such as cardiovascular complications, can be life-threatening (13). A mouse model of Kawasaki Diseases vasculitis and coronary arteritis is available that closely mimics the important histological features of the coronary artery lesions seen in patients with KD (52). Lehman et al. reported in 1985 that a single i.p. injection of a cell wall extract from *Lactobacillus casei* (LCWE), reproducibly induces aortitis and proximal coronary arteritis that are histopathologically very similar to the coronary arteritis (CA) observed in human KD (52). Our group has recently shown that NLRP3 inflammasome activation and IL-1β are critically important in the development of coronary arteritis and abdominal aorta aneurysms (AAA) and dilatations seen in an experimental Kawasaki disease vasculitis mouse model (unpublished) (8,53).

Flavonoids constitute a large group of polyphenolic compounds broadly distributed in the plant kingdom and are divided into various classes according to their structural characteristics. These classes are flavones, flavanones, isoflavones, flavonols, flavanonols, flavan-3-ols, anthocyanidins, chalcones and aurones (16). Because of its presence in natural products, flavonoids are an important component of human diet (17). Flavonoids are free radical scavengers and many of the biological functions of flavonoids are attributed to their antioxidant effects related to the neutralization of cellular ROS as well as mitochondrial ROS (16,18). Because the biological activities of flavonoids vary due to the differences in the various structures, every flavonoid must be analyzed separately in both in vitro and in vivo assays (16).

Quercetin is a dietary flavonol widely found in fruits, vegetables, and nuts. Among polyphenols quercetin is one of the most potent anti-oxidants as demonstrated in different studies. Quercetin inhibits oxidative species generating enzymes such as xanthine oxidase, LOX, and nicotinamide adenine dinucleotide phosphate oxidase (NADPH) (54-56). It is a potent anti-cancer agent, exhibiting different activities such as cell cycle regulation,

interaction with type II estrogen binding sites, and tyrosine kinase inhibition (21). Silymarin, a flavonol complex extracted from the seeds of the milk thistle plant (*Silybum marianum*), an effective antioxidant, increases glutathione (GSH) in liver cells and provides important protective activities against oxidative stress (39). Silymarin has proven to possess potent anti-inflammatory activity by inhibition of MAPKs and NF-κB pathways (41). Naringenin, another flavonoid belonging to the flavanones group, has various impressive pharmacological activities including antioxidant, antimicrobial, anti-inflammatory, and anticancer activity (33).

The molecular mechanism underlying the anti-inflammatory activity of quercetin, silymarin and naringenin is not completely understood. Therefore, in this study we focused on the immuno-pharmacologic mechanism of these flavonoids on inflammasome activated macrophages in vitro, as well as and a bacterial cell wall extract induced coronary arteritis and abdominal aorta aneurysm. Here we report that quercetin inhibited inflammasome activity through inhibition of ASC oligomerization *in vitro* and quercetin treatment was also beneficial in preventing vascular inflammation in the KD vasculitis mouse model, which is IL-1-dependent experimental model.

MATERIALS AND METHODS

Mice.

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME USA). *Nlrp3*$^{-/-}$ mice were kindly provided by Dr. Fitzgerald (Univ. Massachusetts Medical School, MA USA). *Nlrp3*$^{A350V/350V}$ mice were kindly provided by Dr. Hoffman (UC San Diego, CA USA). *Atg16l1*$^{fl/fl}$ mice were kindly provided by Dr. Shih (Cedars-Sinai Medical Center, CA USA) and bred with LysM^{Cre} mice (Jackson Laboratories). All mice used were males 8-12 weeks of age. All animals were housed under specific pathogen-free conditions at the animal center of the Cedars-Sinai Medical Center. Experiments were conducted under approved Institutional Animal Care and Use Committee protocols.

Preparation of *Lactobacillus* cell wall extract and Kawasaki Disease mouse model.

L. casei (ATCC 11578) cell wall extract (LCWE) was prepared as we previously described (46). Four to five weeks aged male mice were injected intraperitoneally with 250 µg LCWE or PBS. Quercetin 100 mg/kg (treatment group) or 2% DMSO vehicle (control group) was administered daily i.p., 24 hours after LCWE injection. Mice were euthanized and hearts were removed at day 7 and embedded in optimal cutting temperature compound for histological examination as previously described (8). Frozen abdominal aorta sections were immunohistochemically analyzed for macrophage, and caspase-1 activity using anti-mouse F4/80 (eBioscience, CA USA) and FLICA (ImmunoChemistry Technologies LLC, MN USA), then mounted with DAPI (4,6-diamidino-2 phenylindole; Life Technologies, USA). IgG2a was used as the isotype control (AbD Serotec, OX UK). All images were obtained using a Keyence BZ-9000 fluorescent microscope (Keyence Corporation of America, IL USA).

Cytokine Measurement.

Bone marrow derived macrophages (BMDM) were prepared as previously described (57). BMDM were stimulated with 500 ng/ml *E. coli* LPS (Invivogen, CA USA) and 3 h later treated with either 5 mM ATP (Sigma, MO USA), 10 µM nigericin (Enzo Life Science, INC.,

NY USA), or 130 µg/mL alum (Sigma) stimulation for NLRP3 inflammasome, 400 ng/ml poly(dA:dT) (Invivogen) for AIM2 inflammasome, and *Salmonella Typhimurium* IR715 *and* Δ*fljB/fliC* (MOI 5) for NLRC4 inflammasome activation (58). BMDM were treated with quercetin, naringenin, or silymarin at the doses of 20 and 100 µM, 30 minutes before the signal-2 (ATP, nigericin etc.) stimulation. *Nlrp3*$^{A350/A350}$ BMDM were primed with LPS (500 ng/mL) and treated with quercetin 1 hour after priming. Supernatants were collected and assessed for IL-1β and TNF-α concentration by ELISA (eBiosciences).

Quercetin, Naringenin, and Silymarin were purchased from Sigma. For *in vitro* experiments, a stock solution of the flavonoids dissolved in 100% dimethyl sulphoxide (DMSO) at the concentration of 200 mg/ml was further diluted with culture medium prior to administration in cell cultures. For *in vivo* treatment, Quercetin (100 mg/kg) was first dissolved in 100% DMSO and then diluted with PBS to a final volume of 100 µl/10 g of body weight.

Immunoblot assay.

BMDM were stimulated as described above, supernatants were collected, and proteins were precipitated by methanol-chloroform extraction. Cell pellets were suspended in lysis buffer. Immunoblot analysis was performed using following primary antibodies: anti-mouse IL-1β (AF-401-NA; R&D System, MN USA), anti-GAPDH (6C5; Santa Cruz Biotechnology). For ASC oligomerization assay, cells suspension was washed with PBS and incubated with 2 mM disuccinnimidyl suberate (DSS, No-Weigh™ Format, Pierce Protein Biology) for 30 minutes in room temperature. After washing with ice cold PBS, precipitates were suspended in lysis buffer. Immunoblot analysis was performed using rabbit anti-mouse ASC (N15; Santa Cruz Biotechnology).

Immunofluorescence staining.

BMDM were plated at the density of 1 x 10^5 cells/well in 24-well plate with a 0.2% gelatin pre-coated cover slip and stimulated as described above. One hour after stimulation cells were washed with ice-cold PBS, fixed in 1% formalin and permeabilized with 0.1% Triton X-100. After blocking with serum free Protein Block (Dako), cells were stained with

primary rabbit anti-ASC (Santa Cruz Biotechnology), secondary Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen), then mounted with DAPI (Life Technologies). All images were obtained using a Keyence BZ-9000 fluorescent microscope (Keyence).

Statistical analysis.

All data were analyzed using Prism 5.0 statistical program (GraphPad software, Inc.). We used one-way ANOVA with Tukey's *post hoc* test for analysis with three or greater groups. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Quercetin inhibits NLRP3 and AIM2 inflammasome activation.

Inflammasome activation depends on 2 signals. The first, via NF-κB activation that leads to pro-IL-1β and pro-caspase-1 synthesis. The second signal is required for assembling the inflammasome complex, which recruits pro-caspase-1. The oligomerization of pro-caspase-1 triggers self-proteolysis to active caspase-1, which cleaves and releases mature IL-1β from the cell. While it is already known that flavonoids can potentially inhibit NF-κB activation (59) thereby preventing pro-IL-1β and pro-caspase-1 synthesis, it is not known whether flavonoids could inhibit inflammasome activation by interfering with signal 2. To investigate this, BMDM were primed with LPS followed by stimulation with ATP, nigericin, or alum (NLRP3 activators), or stimulated with dsDNA for AIM2 inflammasome activation. To distinguish from signal 2 from signal 1, we first primed the BMDM with LPS, incubated for 3 h to allow pro-IL-1β production, and then followed with flavonoid treatment before secondary stimulation. We found that treatment with quercetin inhibited IL-1β secretion by NLRP3 and AIM2 inflammasomes in a dose dependent manner (Fig. 1A-D). Interestingly, naringenin and silymarin only inhibited the Alum induced NLRP3 inflammasome (Fig. 1A-D), suggesting that these flavonoids might be associated with lysosomal destabilization (60). Importantly, under these conditions, quercetin treatment did not affect TNF-α production (Fig. 1E), suggesting that quercetin can inhibit IL-1β secretion by interfering with signal-2. Corroborating our secretion data, we observed more pro-IL-1β in the quercetin treated cell lysate compared with control and a reduced amount mature IL-1β in the quercetin treated cells. These data also indicate that quercetin interfered with activation inflammasome and not IL-1β secretion (Fig. 1F).

Quercetin does not inhibit NLRC4 inflammasome activation.

Since quercetin was able to inhibit both the NLRP3 and AIM2 inflammasomes, we investigated if this flavonoid could also inhibit the NLRC4 inflammasome. Flagellate intracellular bacteria, such as *Salmonella typhimurium*, activate this pathway. However,

unlike NLRP3 and AIM2, NLRC4 contains its own CARD domain, which can interact directly with capase-1, thus making ASC dispensable for NLRC4-dependent caspase-1 activation (7). Furthermore, ASC phosphorylation is not required in NLRC4 inflammasome while NLRP3 and AIM2 inflammasomes require ASC phosphorylation (61). Despite of these evidences, the possibility remains that ASC may still be required for full NLRC4 activation in different conditions (4). *S. typhinurium* induces both NLRP3 as well as NLRC4 inflammasomes (4,49). To address if quercetin also inhibits NLRC4 inflammasome, we used *Nlrp3*$^{-/-}$ BMDM. Additionally, we used type *S. typhinurium (St)* and mutant *St* (*St* ΔfljB/ΔfliC; non-flagellated) that does not induce NLRC4 inflammasome activation (49). We found that quercetin does not inhibit NLRC4 inflammasome activation (Fig. 2A). To assure whether the lack of inhibition was not due to a possible antibiotic action we incubated both *St* strains with quercetin and observed its growth after 8 hours of incubation. The growth levels did not change with quercetin confirming there was no antibiotic effect (Fig. 2B).

NLRP3 inflammasome inhibition by quercetin is independent of autophagy.

It has been reported that quercetin can induce autophagy in gastric cancer cells and HeLa cells (62,63). Autophagy is the process by which cellular components can be recycled, either as a normal process or to remove damaged organelles (64). Autophagy can also compensate for cellular stress and inhibit NLRP3 inflammasome activation (65,66). We next assessed whether quercetin affects pro-IL1β stability in BMDM in comparison to autophagy inducers. LPS-induced intracellular pro-IL-1β amounts did not change during quercetin treatment while both autophagy inducers, tamoxifen treatment and starvation, reduced intracellular pro-IL-1β levels (Fig. 3A), indicating that quercetin does not promote pro-IL-1β degradation (Fig. 3A). We also used *Atg16l1*$^{-/-}$ BMDM to observe inflammasome activation. LPS-primed BMDM secreted IL-1β in response to ATP or nigericin stimulation in both wild type and autophagy deficient BMDM. However, quercetin still inhibited IL-1β secretion in both WT and *Atg16l1*$^{-/-}$ BMDM (Fig. 3B, C) suggesting that inflammasome inhibition by quercetin does not involve autophagy.

Quercetin inhibits constitutively active NLRP3 inflammasome.

Cryopyrin-associated periodic syndrome (CAPS) is auto inflammatory disorder and associated with *Nlrp3* mutations (67). Brydges et al. (68) found that a specific mutations in BMDC resulted in NLRP3 activation and IL-1β secretion in response to only LPS; not requiring secondary stimulation such as ATP. Thus these gain of function mutations are considered to result in a constitutively active inflammasome. Using NLRP3 mutant mice expressing Muckle-Wells syndrome (MWS) mutation at A350V we addressed if quercetin still could inhibit IL-1β secretion. As expected, WT BMDM stimulated with LPS were unable to secrete IL-1β (Fig. 4). However, $NLRP3^{A350V}$ BMDM did secrete IL-1β in response to LPS alone (Fig. 4). Quercetin was also able to inhibit IL-1β secretion by $NLRP3^{A350V}$ BMDM treated with LPS (Fig. 4). These data suggest that quercetin inhibits the inflammasome activation downstream of the initial signal activating events.

Quercetin inhibits ASC-speck formation.

Since quercetin inhibited both the NLRP3 and AIM2 inflammasome, but not the NLRC4 inflammasome, we hypothesized that quercetin inhibition might be through the adaptor protein ASC. To investigate this possibility, we next visualized NLRP3 inflammasome complexes as ASC specks by immunofluorescence staining (69). As previously reported (61), after stimulation with nigericin, we also observed that the number of cells containing ASC specks increased (Figure 5A, B). However, we also observed that quercetin treated cells showed a significantly reduced number of ASC specks (Fig. 5A, B). In order to confirm this inhibition of ASC speck formation by quercetin, we assessed for ASC oligomerization (dimer and monomer forms) in the lysate by immunoblot. ASC dimers were detected in LPS + Nigericin stimulation, but the quercetin-treated cells had less ASC dimerization compared with controls (Figure 5C). Taken together, our data suggest that quercetin inhibits inflammasome activation by inhibiting ASC oligomerization.

Quercetin prevents mice from LCWE-Induced coronary arteritis and aortic aneurysms in experimental Kawasaki disease vasculitis model.

Since we have demonstrated that the cardiovascular lesion development in the KD vasculitis mouse model is IL-1-dependent (8,53), we then investigated whether quercetin could be beneficial for this KD vasculitis mouse model *in vivo*. We administered quercetin i.p. (daily) in LCWE-injected mice. At 7 days following LCWE injection, the hearts were collected and analyzed for coronary artery inflammation as described previously (8). Quercetin treatment significantly reduced the incidence of the coronary arteritis compared to control treated group (Fig. 6A, B). In addition to blocking coronary arteritis, quercetin treatment also led to reduced AAA formation as measured by maximal abdominal aorta diameter and inflammatory histology (Fig. 6C-E). Quercetin treatment prevented the significant intimal proliferation and massive myofibroblastic proliferation observed in the LCWE-induced vasculitis mice (Fig. 6D). We next determined if inflammasome was activated locally in the vascular tissues in the LCWE-induced vasculitis mice. For this purpose, we visualized Caspse-1 activity in the vascular lesions using the fluorescent inhibitor of caspases (FLICA) assay. Active caspase-1 has previously been identified in macrophages in the coronary and AAA lesions observed in this LCWE-induced vasculitis model (unpublished data) (53). As expected, FLICA-positive macrophages were observed in the abdominal aorta aneurysm lesions of the LCWE-injected mice (Fig. 6F). Importantly, quercetin completely inhibited this local caspase-1 activity in the LCWE-induced vascular lesions (Fig. 6F). Altogether, these results suggest that quercetin treatment may be beneficial to prevent vascular inflammation and vasculitis in this KD mouse model or other inflammasome/IL-1-mediated inflammatory diseases.

DISCUSSION

The inflammasome is a multiprotein oligomer consisting of caspase-1, ASC, and NLRs that regulates maturation of IL-1β and IL-18 (5). Inflammasome activation is required for many inflammatory processes and its activation generally requires two separate signals. NF-κB activation (signal 1), resulting from signaling such as TLRs, induces pro-IL-1β and pro-IL-18 production. The second signal is often a danger signal or a form of cellular stress. To date, the mitochondria and mitochondrial ROS has emerged as a central hub for NLRP3 inflammasome activation (58). Additionally, lysosomal damage and cytosolic K^+ efflux have been implicated in NLRP3 activation (48). In addition to their ability to inhibit NF-κB activation, flavonoids can scavenge ROS, thus we evaluated quercetin, naringenin, and silymarin in their ability to inhibit the second signal and inflammasome activation. We found that both ATP and nigericin (inducers of mitochondrial dysfunction and K^+ efflux), as well as alum (lysosomal damage) induced NLRP3 inflammasome activation were inhibited by quercetin. Similarly, quercetin also inhibited cholesterol crystal induced NLRP3 inflammasome activation. A previous study suggests that due to similar structures with allopurinol, quercetin and rutin were efficacious in reversing fructose-induced renal NLRP3 inflammasome activation in rats (31). Their proposed mechanism was by inhibiting signal 1. However, our data suggests that in addition to inhibiting signal 1, quercetin can also directly inhibit the inflammasome component ASC assembly in macrophages as evidenced by the inhibition by of a constitutively active NLRP3 inflammasome. We also observed that quercetin inhibited dsDNA-induced AIM2 inflammasome, but not flagellin-induced NLRC4 inflammasome. Our data suggest that the signal 2 inhibition by quercetin may act only on ASC dependent inflammasomes.

During inflammasome activation, autophagy is induced in parallel (70). Activation of autophagy itself inhibits the activation of NLRP3 inflammasome, likely through the prevention of apoptosis (48) and or mitochondrial dysfunction (58). Class III PI3K induces autophagy in a complex with Beclin 1, and thus class III PI3K inhibitors, such as wortmannin inhibits

autophagy (71). Walker et al. demonstrated that quercetin and wortmannin, a steroid metabolite of the fungi *Penicillium funiculosum*, possess important structure similarities and thus acts as inhibitors of phosphoinositide 3-kinases (PI3Ks) (72). Although, quercetin may potentially be able to block autophagy, we observed that quercetin inhibition of IL-1β secretion occurred normally in ATG16L1 deficient BMDM, suggesting the inflammasome inhibition by quercetin occurs in an autophagy independent manner.

Upon activation of NLRP3, ASC proteins assemble into large fiber-like structures that amplifies caspase-1 activation. Baroja-Mazo et al. (73) recently showed ASC-speck oligomerization 30 minutes after NLPR3 inflammasome activation. They also found that both ASC and the structural, gain-of-function, CAPS-associated NLRP3 mutant pD303N oligomerized into active particles detected in the serum of patients with CAPS. Previous studies show that NLRP3 mutant patients correlated with the aggregation of NLRP3 into particles with pro-inflammatory extracellular activity that induced the release of ASC specks (74). In addition, another study found that during active disease, patients with CAPS have enhanced serum concentrations of ASC oligomers (73). Indeed, in the current study, we discovered a novel mechanism by which quercetin blocks IL-1 production, and we demonstrated that quercetin leads to inhibition of ASC-speck formation in BMDM directly blocking the activation of the NLRP3 inflammasome. Thus in addition to inhibiting signal 1, quercetin directly inhibits inflammasome activation by preventing ASC oligomerization. Additional studies are required to further understand the exact molecular mechanisms of how quercetin blocks ASC oligomerization.

Quercetin is already known to exert immune and inflammation modulating activity in several biological and experimental murine models and as well as an inverse association between quercetin intake and coronary heart disease (23). Lara-Guzman et al. demonstrated that dyslipidemic *Apoe*$^{-/-}$ mice treated with quercetin had significant reduction in atherosclerosis (75). The ability of quercetin to inhibit signal 1 and prevent ASC oligomerization, directly inhibiting NLRP3 inflammasome activation make this molecule a potential therapeutic agent in inflammasome-mediated disorders. Using a mouse model of

LCWE-induced vasculitis, which is dependent on IL-1β and inflammasome activation, we observed that quercetin significantly inhibited the cardiovascular lesions in the LCWE-induced vasculitis mouse model. Current treatment options for KD include aspirin plus intravenous immunoglobulin (IVIG) therapy. However, the 20% of patients who do not respond to IVIG are at even increased risk of developing coronary artery aneurysms and cardiac sequelae (12). IL-1R antagonist (Anakinra) is currently in clinical trials for IVIG-resistant KD patients, as human data also suggests that IL-1 plays an important role in KD (76,77). Thus, quercetin may provide an alternative approach to prevent unwanted cardiovascular sequelae of KD that maybe due to over exuberant IL-1 signaling. Additionally, quercetin may also be a potential therapeutic candidate for CAPS and other inflammasome associated inflammatory diseases.

Author contributions: All authors discussed the results and implications and commented on the manuscript at all stages. Talita P. Domiciano: Experimental design and execution, data analyzing and manuscript writing. Daiko Wakita: *In vivo* experiments execution. Heather D. Jones: Experimental design. Timothy R. Crother: Experimental design and manuscript writing. Waldiceu Ap. Verri Jr: Experimental design and manuscript writing. Moshe Arditi: Supervised the project, contributed to experimental design and manuscript writing. Kenichi Shimada: Experimental design, data analyzing and manuscript writing.

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FIGURE LEGENDS

FIGURE 1. Quercetin inhibits NLRP3 and AIM2 Inflammasomes. LPS (500 ng/ml)-primed BMDM were treated with the indicated flavonoids and concentrations or vehicle (DMSO 0.01%), then stimulated with (A) 5 mM ATP (B) 10 μM Nigericin (C) 130 μg/ml Alum or (D) 400 ng/ml Poly (dA:dT) 30 minutes after treatment. (A-D) IL-1β and (E) TNF-α concentration in the culture supernatant were measured by ELISA. (B, F) LPS-primed BMDM were stimulated with Nigericin treated with quercetin 30 minutes before secondary stimulation. Supernatants and Lysate of BMDM were analyzed by immunoblotting. Data shown are representative of two or more independent experiments (means ± SD) *p<0.05, **p<0.01, ***p<0.001.

FIGURE 2. Quercetin does not inhibit NLRC4 inflammasome activation. (A) IL-1β concentrations (ELISA) in *Nlrp3*$^{-/-}$ BMDM culture supernatants primed with LPS (500 ng/ml) for 2h and stimulated with WT *Salmonella* or non-flagellated *Salmonella* Mutant (Δ*fljB/fliC)* (MOI 5) for 90 minutes and then treated with quercetin or vehicle (DMSO 0.01%) followed by 5 h incubation. (B) *Salmonella* growth in the presence of quercetin. O.D were measured after 8 h incubation. Data shown are representative of two or more experiments (means ± SD) ***p<0.001.

FIGURE 3. Autophagy is dispensable for NLRP3 inflammasome inhibition by Quercetin. (A) Intracellular pro-IL-1β concentrations (ELISA) in BMDM lysates. BMDM primed with LPS (500 ng/ml) were treated with the quercetin, tamoxifen or serum-free medium. (B, C) Wild type and ATG16L1$^{-/flox}$ BMDM primed with LPS (500 ng/ml) were stimulated with (B) ATP (5 mM) or (C) Nigericin (10 μM) and treated with quercetin 30 minutes before secondary stimulation. Data shown are representative of two or more independent experiments (means ± SD) *p<0.05, ***p<0.001.

FIGURE 4. Quercetin inhibits auto-reactive NLRP3 inflammasome. IL-1β concentrations in *Nlrp3*$^{A350V/A350V}$ and WT BMDM culture supernatants primed with LPS (500 ng/ml) and treated with quercetin 1 h after LPS. Data shown are representative of two or more experiments (means ± SD) ***p<0.001

FIGURE 5. Quercetin inhibits ASC speck formation and oligomerization. (A-C) LPS-primed BMDM were treated quercetin 30 minutes before stimulation with Nigericin (10 µM) and analyzed by (A) Immunostaining. (B) Percentage of cells containing ASC speck. (C) Cross-linked lysate of BMDM were analyzed with anti-ASC immunoblotting. Scale bar represents 10µm. Data shown are representative of two or more experiments (means ± SD) ***p<0.001.

FIGURE 6. Quercetin prevents mice from LCWE-Induced coronary and aortic lesions. Wild type mice were administered 250 µg of LCWE i.p., treated daily with quercetin 100 mg/kg or vehicle (control), i.p. and their hearts and aorta were harvested on day 7. A) Representative hematoxylin and eosin–stained heart sections (10X - Scale bar 500 µm). B) Heart lesions incidence C) Representative abdominal aorta. D) Representative hematoxylin and eosin–stained aorta sections (10X - Scale bar 200 µm). E) Average aortic diameter. F) Representative aorta section immunostaining (10X – Scale bar 100 µm; 40X - Scale bar 50 µm). Data shown are representative of two or more experiments (mean ± S.E.M.) and were compared by use of the Tukey test (B and E). **p<0.005 ***p<0.001

FIGURE 1.

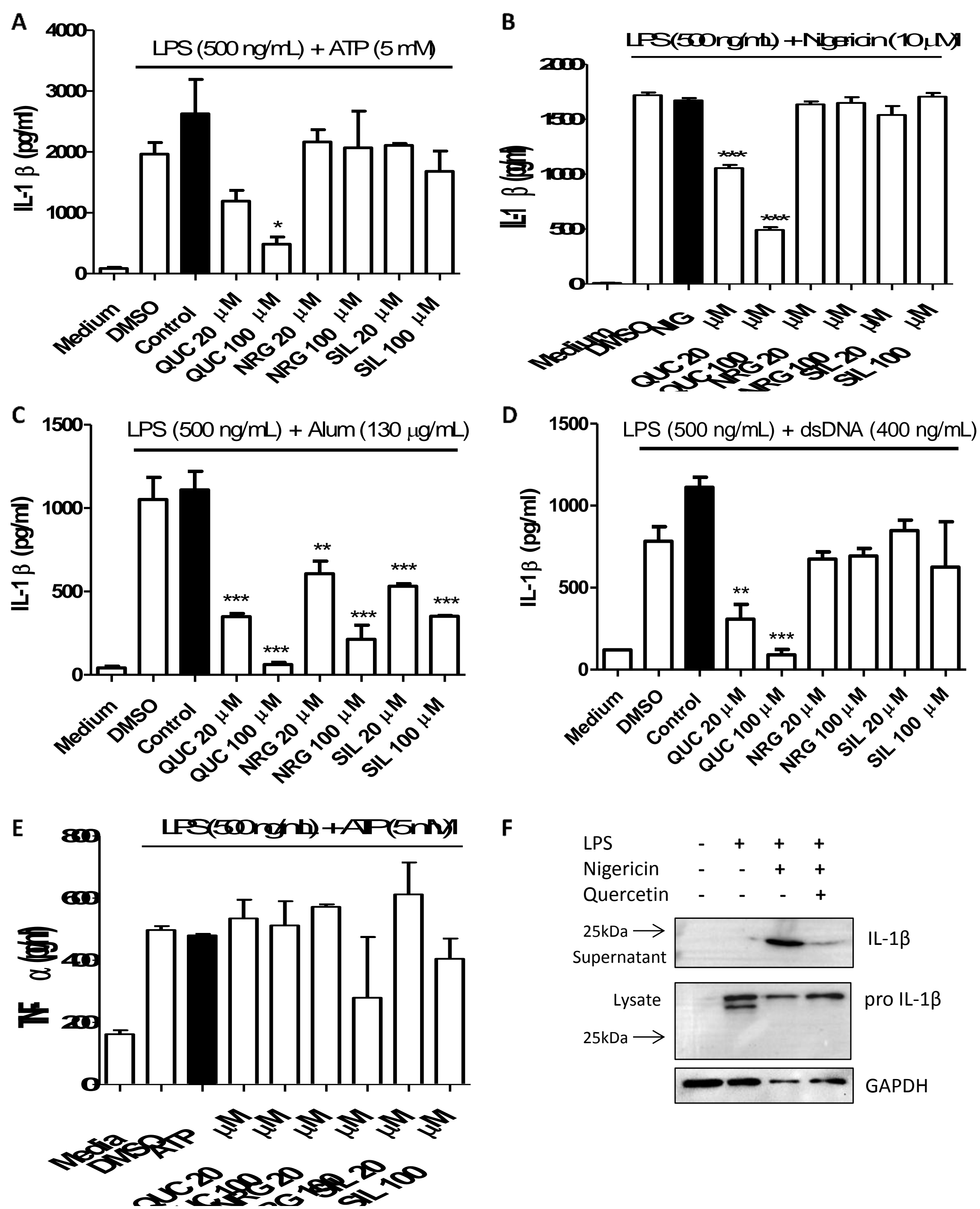


FIGURE 2.

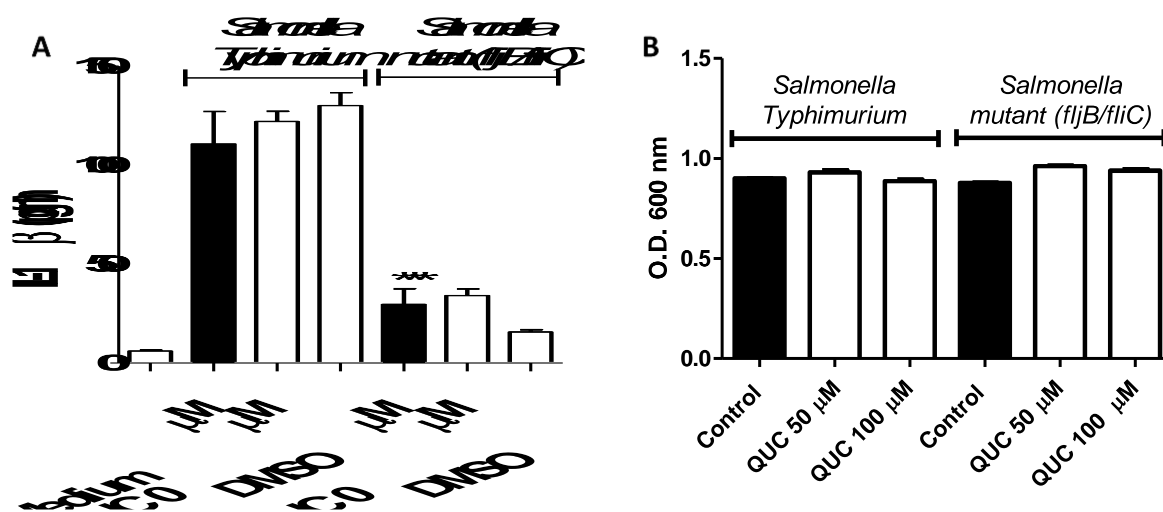


FIGURE 3.

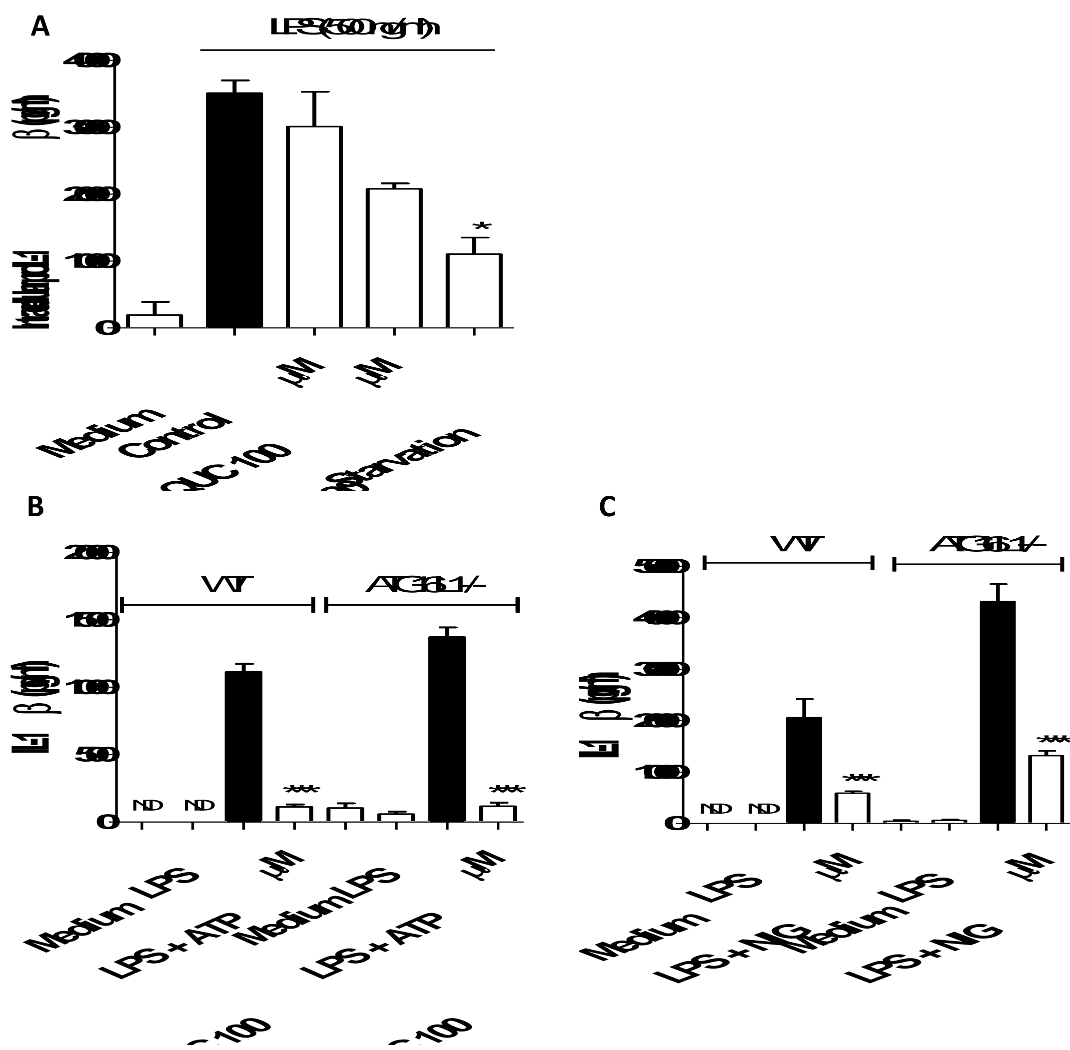


FIGURE 4.

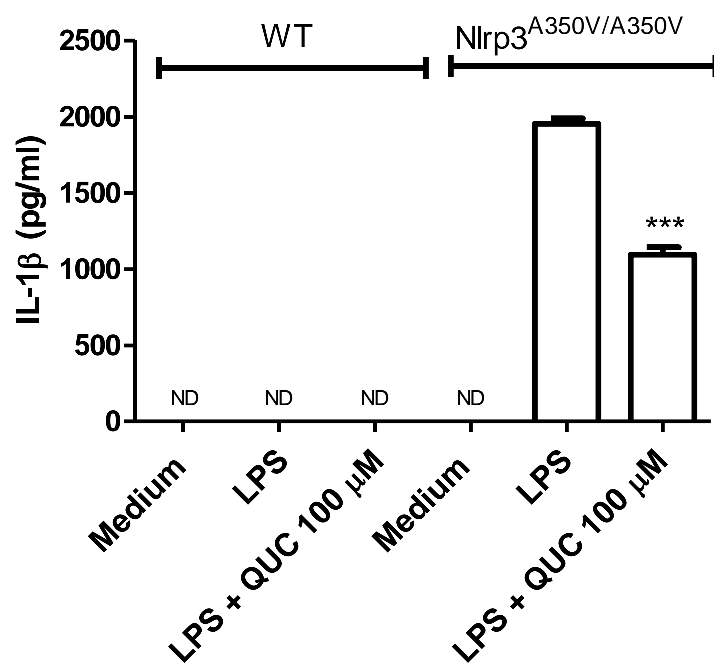
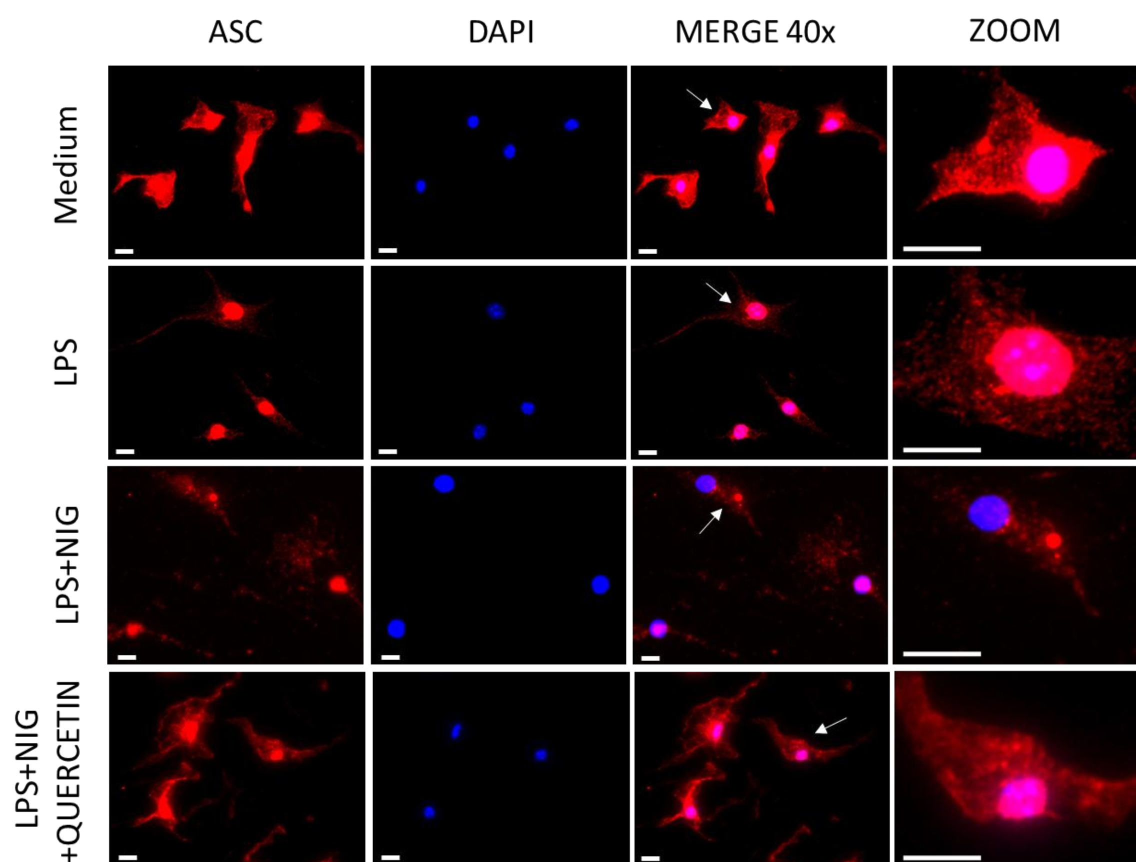
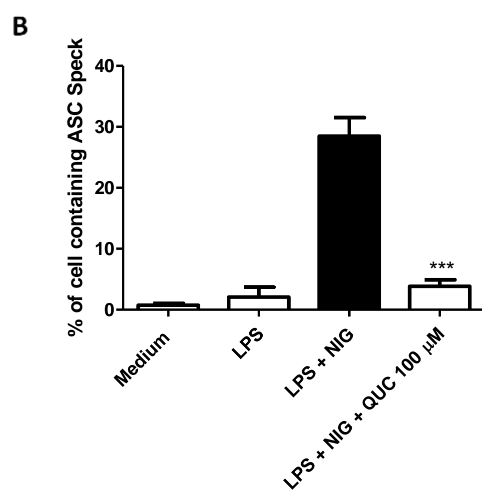


FIGURE 5.





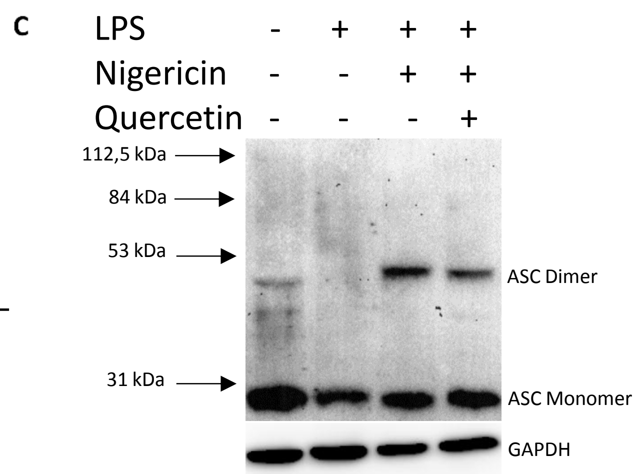
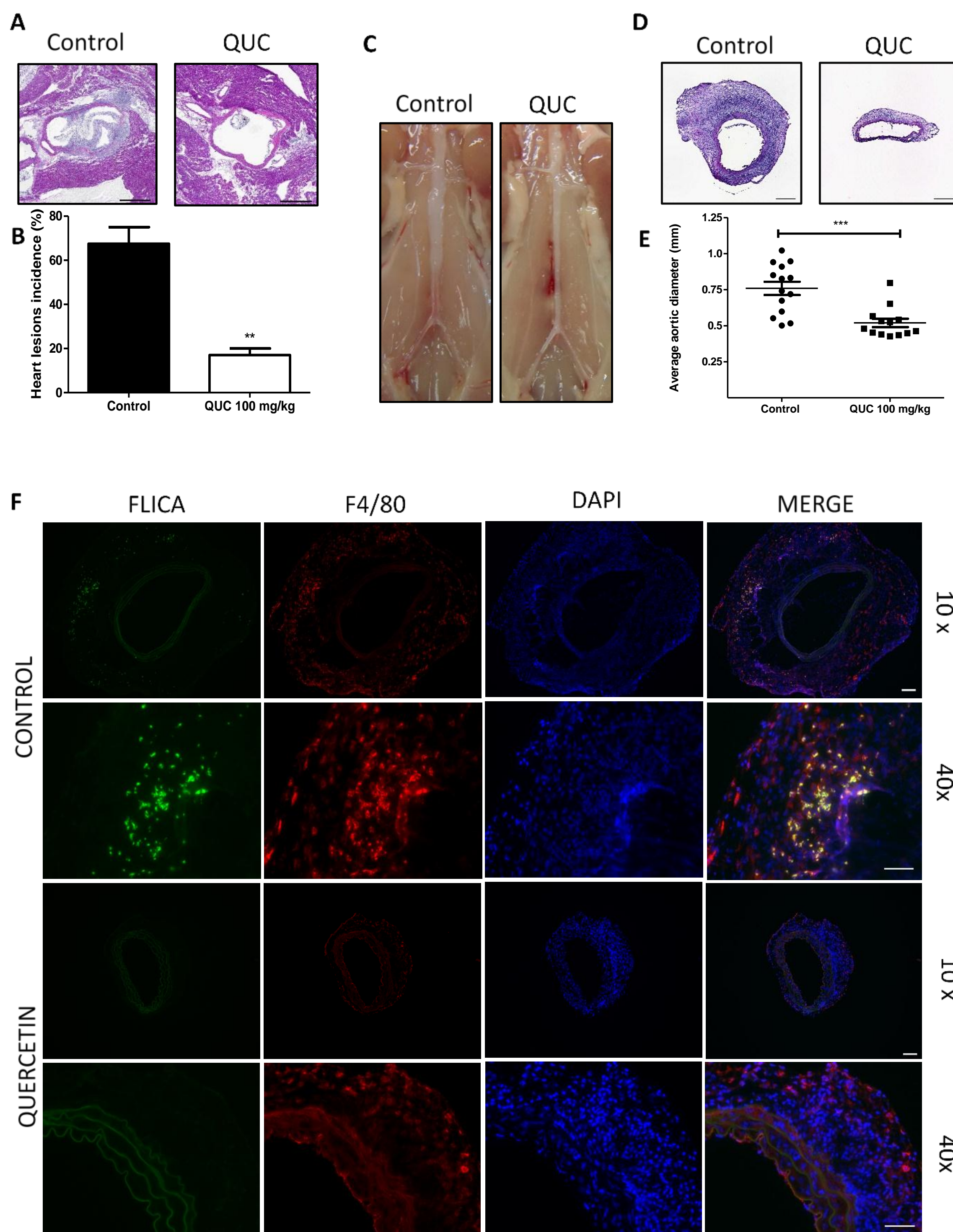


FIGURE 6.



5 CONCLUSÃO

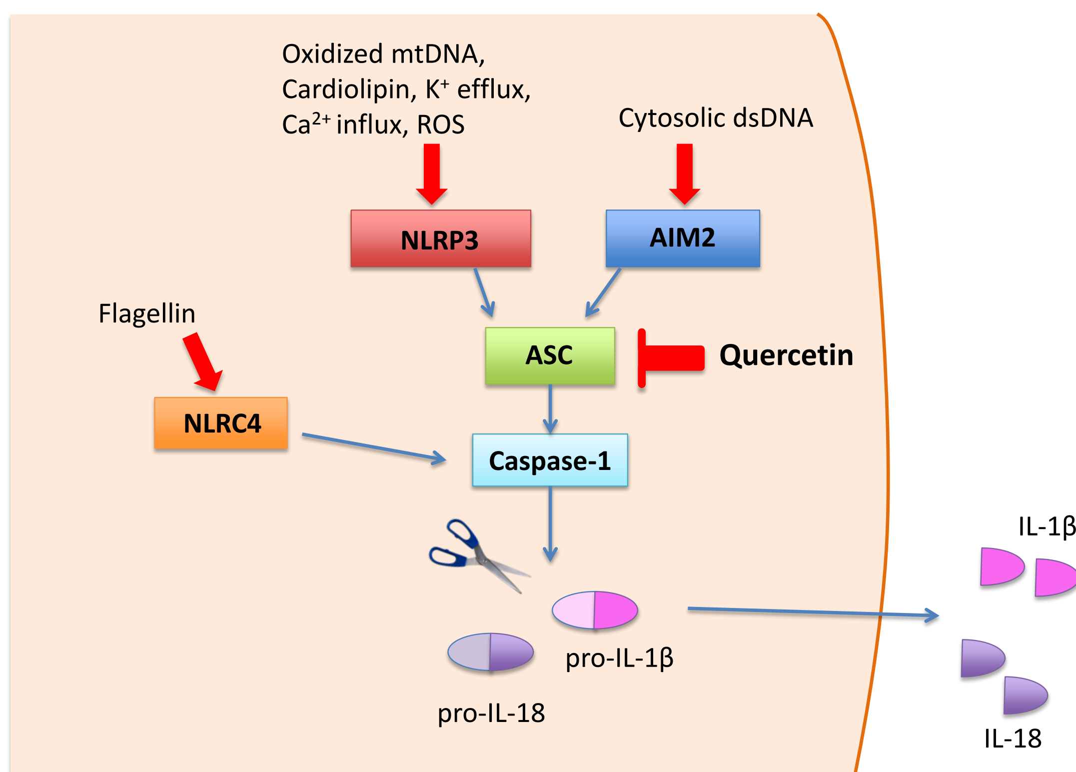
Diante dos resultados apresentados podemos observar que apesar dos três flavonoides estudados possuírem efeitos anti-inflamatórios descritos pela literatura, apenas a quercetina inibiu a ativação do inflamassoma e produção de IL-1β *in vitro*.

Neste trabalho demonstramos que a quercetina possui efeito inibitório sobra a ativação dos inflamassoma NLRP3 e AIM2, mas não possui efeito significativo sobre o inflamassoma NLRC4. Ainda, o efeito inibitório observado não é dependente da ativação do processo de autofagia. Através dos resultados obtidos propomos que o possível mecanismo de inibição do inflamassoma pela quercetina é através da inibição da oligomerização do ASC (Figura 4).

Além disso, o tratamento com quercetina no modelo de doença de Kawasaki *in vivo* previne a formação de lesões na aorta e coronária, reduz a incidência das lesões na aorta e inibe a ativação do inflamassoma nas lesões da aorta.

Desta forma, concluímos que o flavonoide quercetina inibe a ativação do inflamassoma e mostra-se como um potencial agente terapêutico para o tratamento da doença de Kawasaki e de outras doenças inflamatórias associadas ao inflamassoma.

Figura 4. Proposta de mecanismo de inibição do inflamassoma pela quercetina.



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