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Doutorado em Biologia Celular e Molecular

EFEITO ANTI-INFLAMATÓRIO DO GALATO DE METILA NA ARTRITE EXPERIMENTAL: ELUCIDAÇÃO DO MECANISMO DE AÇÃO

LUANA BARBOSA CORREA

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Ministério da Saúde

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

Orientador: Dra. Maria das Graças Müller de Oliveira Henriques
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ORIENTADOR: Dra. Maria das Graças Müller de Oliveira Henriques
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Rio de Janeiro, 20 de Agosto de 2019

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RESUMO

TESE DE DOUTORADO EM BIOLOGIA CELULAR E MOLECULAR

Luana Barbosa Correa

O galato de metila (GM) é um polifenol prevalente no reino vegetal e a sua presença em plantas medicinais pode estar relacionada com seus notáveis efeitos biológicos, tais como atividades antioxidantes, antitumorais e antimicrobianas. Embora seja amplamente descrito que os polifenóis tenham efeitos terapêuticos em doenças cardiovasculares, antitumorais e em reações inflamatórias, há poucos relatos científicos demonstrando que o GM possui ação anti-inflamatória em modelos experimentais *in vivo.* Neste estudo, foram utilizados modelos de artrite experimental como ferramenta para estudar o efeito farmacológico do GM sobre o influxo celular, a formação de edema e a produção de mediadores inflamatórios. Além disso, os estudos foram aprofundados buscando compreender e elucidar o mecanismo de ação deste derivado de polifenóis tão amplamente difundido na natureza, objetivando assim comprovar cientificamente o valor medicinal do GM e destacar a importância dessa substância como uma candidata com potencial efeito anti-inflamatório para o controle da artrite. Foi demonstrado que o GM (7 mg/kg) atenua a artrite experimental induzida por zimosan e antígeno, afetando a formação de edema, a migração de leucócitos, a produção de mediadores pró-inflamatórios (IL-1β, IL-6, TNF-α, IL-17, CXCL-1, LTB_4, e PGE_2), e a diferenciação e ativação de osteoclastos. O pré-tratamento com o GM inibiu a quimiotaxia de neutrófilos induzida por CXCL-1/KC, bem como a adesão dessas células a células endoteliais primadas com TNF-α *in vitro*. Além disso, o GM reduziu a produção de TNF-α, IL-6, CXCL-1/KC e NO, a expressão de enzimas como a COX-2 e iNOS, e a mobilização intracelular de cálcio em macrófagos ativados. Para investigar o mecanismo molecular do GM, foram utilizados macrófagos RAW 264.7 expressando de forma estável o gene repórter NF-κB-luciferase. O GM reduziu a atividade de NF-κB, quantificado indiretamente pela emissão de luminescência pelas células RAW 264.7 estimuladas com zimosan, $Pam3CSK_4$ e LPS, mas não com PMA. Assim, os resultados desse estudo demonstram que o GM possui um efeito anti-inflamatório promissor e sugere uma explicação do seu mecanismo de ação através da inibição da sinalização de NF-κB e da via das MAPKs.

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ANTI-INFLAMMATORY EFFECT OF METHYL GALLATE IN EXPERIMENTAL ARTHRITIS: MECHANISM OF ACTION ELUCIDATION

ABSTRACT

DOCTORAL THESIS IN CELLULAR AND MOLECULAR BIOLOGY

Luana Barbosa Correa

Methyl gallate (MG) is a prevalent polyphenol in the plant kingdom and its presence in medicinal plants may be related to its biological effects, such as antioxidant, antitumor and antimicrobial activities. Although it is widely reported that polyphenols have therapeutic effects on cardiovascular, antitumor, and inflammatory reactions, there are few scientific reports demonstrating that MG has anti-inflammatory action in experimental models *in vivo*. In this study, experimental arthritis models were used as a tool to study the pharmacological effect of MG on cell influx, edema formation and inflammatory mediators production. In addition, studies have been deepened in order to understand and elucidate the mechanism of action of this polyphenol derivative so widely diffused in nature, aiming to scientifically prove the medicinal value of MG and highlight the importance of this substance as a candidate with anti-inflammatory potential effects for arthritis control. It has been demonstrated that MG (7 mg/kg) attenuates experimental arthritis induced by zymosan and antigen, affecting edema formation, leukocyte migration, proinflammatory mediators production (IL-1β, IL-6, TNF-α, IL-17, CXCL-1, LTB_4, and PGE_2), and osteoclasts differentiation and activation. Pretreatment with MG inhibits CXCL-1/KC-induced neutrophil chemotaxis, as well as adhesion of these cells to TNF-α-primed endothelial cells *in vitro*. In addition, MG reduced the production of TNF-α, IL-6, CXCL-1/KC and NO, expression of enzymes such as COX-2 and iNOS, and the intracellular calcium mobilization in activated macrophages. To investigate the molecular mechanism of MG, RAW264.7 macrophages were stably expressing the NF-κB-luciferase reporter gene were used. MG reduced NF-κB activity, indirectly quantified by emission of luminescence by RAW 264.7 cells-stimulated with zymosan, Pam3CSK4 and LPS, but not with PMA. Thus, the results of this study demonstrate that MG has a promising anti-inflammatory effect and suggests an explanation of its mechanism of action through the inhibition of NF-κB signaling and the MAPK pathway.

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LISTA DE SIGLAS E ABREVIATURAS

ACPA	Anticorpos contra antígenos proteicos citrulinados
AIA	Artrite induzida por antígeno
AIE:	Anti-inflamatório esteroidal
AINE:	Anti-inflamatório não esteroidal
ALT	Alanina aminotransferase
AP-1:	do inglês, *activator protein-1*
AR:	Artrite reumatoide
AST	Aspartato aminotransferase
BAFF	Fator de ativação de células B
BMP	Proteína morfogenética óssea
BSA:	Albumina sérica bovina
CAM:	Molécula de adesão celular
CCL:	Ligante de quimiocinas CC
CD:	do inglês, *cluster of differentiation*
CIA	Artrite induzida por colágeno
CO_2:	Dióxido de carbono
COX:	Ciclo-oxigenase
CTR	Receptor de calcitocina
CXCL:	Ligante de quimiocinas CXC
Dexa:	Dexametasona
DMARD:	do inglês, *disease-modifying anti-rheumatic drug*
DNA:	Ácido desoxirribonucléico
EDTA:	Ácido etileno diamino tetra acético
EGCG	Epigalocatequina galato
EIA:	do inglês: *Enzyme Immunoassay*
ELISA:	do inglês, *Enzyme Linked Immuno Sorbent Assay*
EPM:	Erro padrão da média
ERK:	do inglês, *extracellular signal-regulated kinase*
F-actina:	Filamentos de actina
FR:	Fator reumatoide
GC	Glicocorticoide
G-CSF	Fator estimulador de colônias de granulócitos

GM: Galato de metila

GM-CSF: Fator estimulador de colônias de granulócitos e macrófagos

GPCR: Receptor acoplado à proteína G

H_2O_2: Peróxido de hidrogênio

HBSS: Solução salina tamponada de Hank's

HCl: Ácido clorídrico

HLA: Antígeno leucocitário humano

i.a.: Intra-articular

i.p.: Intraperitoneal

i.pl.: Intraplantar

IA: Índice de adesão

ICAM Molécula de adesão intracelular

IFN-γ: Interferon-γ

IgG: Imunoglobulina G

IL: Interleucina

iNOS: do inglês, *inducible nitric oxide synthase*

JAM: Molécula de adesão juncional

LDL Lipoproteína de baixa densidade

LFA-1: do inglês, *lymphocyte function-associated antigen-1*

LPS Lipopolissacarídeo

LT: Leucotrieno

MAC-1: do inglês, *macrophage-1 antigen*

MCP-1: do inglês, *monocyte chemoattractant protein-1*

MHC: Complexo de histocompatibilidade maior

MIP-1α: do inglês, *macrophage inflammatory protein-1α*

MMP: Metaloproteinase de matriz

MTX Metotrexato

NADPH-oxidase: do inglês, *nicotinamide adenine dinucleotide phosphate-oxidase*

NET Armadilha extracelular dos neutrófilos

NF-κB: Fator nuclear-κappa B

NO: Óxido nítrico

OA: Osteoartrite

OPG Osteoprotegerina

PAD do inglês, *peptidyl arginine deiminase*

PAF	Fator de agregação plaquetária
PBS:	Solução salina tamponada
PECAM-1	Molécula de adesão celular endotelial plaquetária
PG:	Prostaglandina
PI3K:	do inglês, *phosphatidylinositol-3kinase*
PLA:	do inglês, *phospholipase* A
PLC:	do inglês, *phospholipase* C
PMN:	Polimorfonuclear
RANKL	Ligante do receptor ativador nuclear kappa-B
RNA	Ácido ribonucleico
ROS:	Espécies reativas de oxigênio
TLR:	Receptor do tipo *Toll*
TNFR:	Receptor de TNF
TNF-α:	Fator de necrose tumoral-α
TRAP	Fosfatase ácida tartarato-resistente

1 INTRODUÇÃO

1.1 INFLAMAÇÃO ARTICULAR

Artrite é um termo usado de forma genérica para descrever doenças relacionadas a inflamação articular (CDC, 2016). Estima-se que existam mais de 100 tipos diferentes de artrite e doenças relacionadas, que afetam milhões de pessoas pelo mundo, só nos EUA 23% da população (aproximadamente 54 milhões de adultos) foram acometidos por algum tipo de artrite entre os anos de 2010 e 2012 (CDC, 2013). Os sintomas comuns da artrite incluem inchaço, dor, rigidez e diminuição da amplitude de movimento. Os sintomas podem ser intermitentes apresentando-se de forma leve, moderada ou grave. Eles podem permanecer os mesmos por anos, mas podem progredir ou piorar com o tempo levando a alterações articulares permanentes (Arthritis Foundation, 2018). Os tipos mais comuns de artrite são: a osteoartrite, a gota e a artrite reumatoide. Seus sintomas incluem rigidez e edema nas articulações ou ao seu redor, tendo como principais consequências a dor e a incapacidade funcional (Choi e Brahn, 2010; Niedermeier *et al.*, 2010).

A artrite reumatoide (AR) é a inflamação articular autoimune mais comum em adultos, com uma prevalência na população mundial entre 0,3-1 %, e afeta 3 vezes mais mulheres do que homens (Gabriel e Michaud, 2009). Além disso, em adultos idosos a AR está associada a alta morbidade e maior mortalidade (Naz e Symmons, 2007; Turesson, 2016). As taxas de mortalidade são duas vezes maiores em pacientes com AR do que na população em geral, devido principalmente ao aumento da incidência de doenças cardiovasculares (Gonzalez *et al.*, 2007; Crowson *et al.*, 2013; Radner *et al.*, 2017). Estima-se que gasto anual com o tratamento da AR é de aproximadamente dezesseis bilhões de dólares, incluindo os custos sócio econômicos diretos, como as despesas médicas, e custos indiretos, como a queda de produtividade e redução da qualidade de vida da população atingida (Dunlop *et al.*, 2003).

1.2 PATOGÊNESE DA ARTRITE REUMATOIDE

Na maioria dos pacientes, a doença AR começa anos antes que os sintomas clínicos sejam evidentes. O desenvolvimento da AR é determinado por predisposição genética, pois, mais de 80% dos pacientes com AR carregam o chamado epítopo compartilhado (um trecho de 5 aminoácidos na região responsável pela apresentação de antígenos aos linfócitos T) do cluster HLA-DRB1*04 (Raychaudhuri, 2010). Esse epítopo está associado a doença e pode apresentar peptídeos relacionados à artrite, levando a estimulação e expansão de células T específicas para autoantígenos nas articulações e linfonodos (Gregersen *et al.*, 1987; Smolen *et al.*, 2007).

Juntamente com a predisposição genética, fatores ambientais tais como tabagismo, composição da microbiota, alterações epigenéticas e inalação de sílica, influenciam no surgimento da resposta sinovial inflamatória e destrutiva (Smolen *et al.*, 2018). Porém, como esses fatores ambientais contribuem para a doença ainda não é completamente compreendido. Alguns estudos mostram que moléculas nocivas encontradas no ambiente, por exemplo, a fumaça do cigarro, pode atuar nas células de mucosas e promover a conversão pós-traducional do aminoácido arginina em citrulina. Isso pode ocorrer em uma variedade de proteínas, incluindo proteínas intracelulares (como as histonas) e proteínas de matriz (como fibronectina, colágeno, fibrinogênio, enolase e vimentina) via uma enzima denominada PAD (do inglês *peptidyl arginine deiminase*) em um processo chamado de citrulinação (Makrygiannakis *et al.*, 2008). Após a citrulinação, os peptídeos citrulinados são reconhecidos por APCs que apresentam estes antígenos via MHC aos linfócitos T, que por sua vez estimulam os linfócitos B a sintetizar uma gama de anticorpos que reconhecem proteínas próprias, incluindo o fator reumatoide (anticorpo contra a porção Fc da IgG) e anticorpos contra antígenos proteicos citrulinados (ACPAs) (Holers, 2013; Muller e Radic, 2015).

Os osteoclastos (células que compõem a matriz óssea) dependem de enzinas citrulinas para a sua maturação e exibem antígenos citrulinados em sua superfície celular em um estado de homeostase. Em humanos, a ligação de ACPAs aos osteoclastos no compartimento ósseo induz a secreção de interleucina (IL)-8 (Catrina *et al.*, 2017). A liberação da IL-8 contribui para o recrutamento dos neutrófilos, que desempenham papeis críticos na iniciação e manutenção dos processos inflamatórios articulares (Wipke e Allen, 2001; Németh e Mócsai, 2012; Wright *et al.*, 2014). Além disso, os neutrófilos também contribuem para a sinovite o influxo e/ou ativação local de células mononucleares (incluindo linfócitos T, linfócitos B, plasmócitos, células dendríticas, macrófagos e mastócitos) e a angiogênese.

Após a instauração do processo inflamatório, o revestimento sinovial torna-se hiperplásico, e a membrana sinovial se expande e forma vilosidades. A porção rica em osteoclastos da membrana sinovial destrói o osso, enquanto as enzimas secretadas pelos neutrófilos, sinoviócitos e condrócitos degradam a cartilagem, esse tecido invasivo é denominado de *pannus* sinovial (Smolen e Steiner, 2003). Outra característica importante da AR, é a excessiva produção de mediadores inflamatórios por células residentes e/ou infiltradas. Entre os principais mediadores envolvidos no dano articular estão os radicais livres, enzimas de degradação de matriz, citocinas pró-inflamatórias (incluindo IL-6, IL-17, IL-23, IL-1β e o fator de necrose tumoral (TNF)-α), assim como quimiocinas (como a IL-8), mediadores lipídicos (como o leucotrieno B_4) e endotelinas (Feldmann *et al.*, 1998; Maini e Taylor, 2000; Conte *et al.*, 2008; Karmakar *et al.*, 2010) (Figura 1.1).

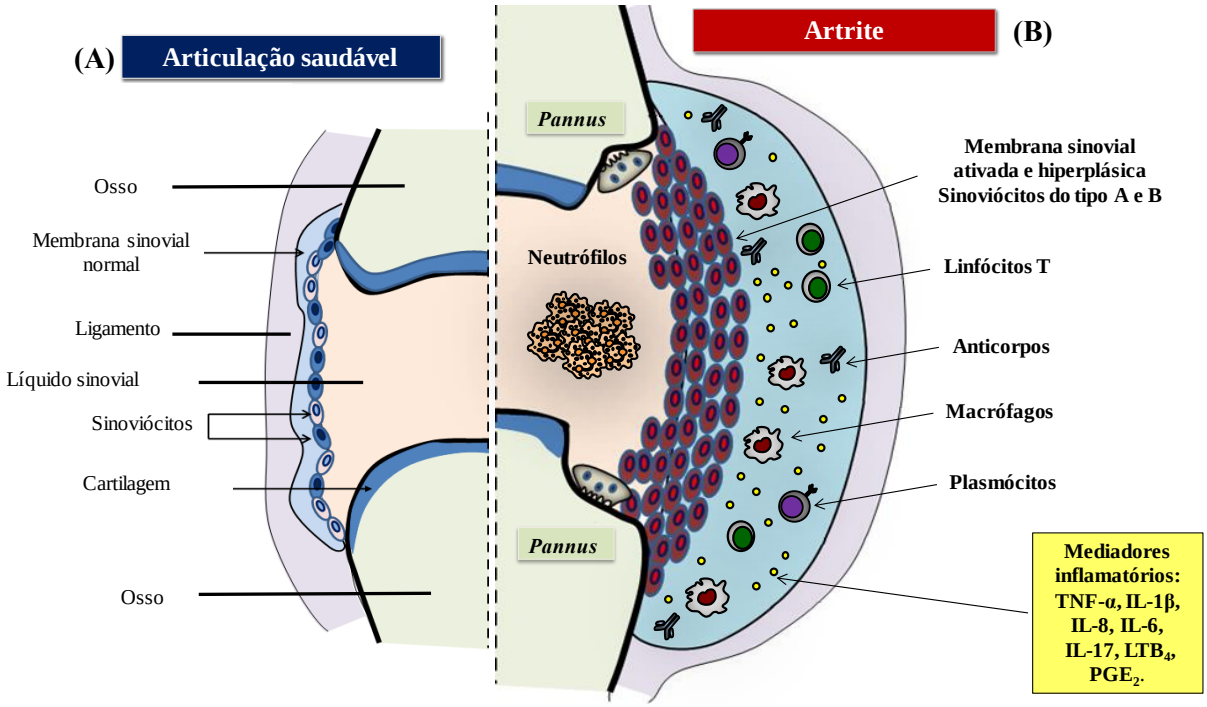

Figura 1.1: Estrutura da articulação sinovial de um indivíduo saudável e um indivíduo com artrite reumatoide. (A) Na articulação sinovial, as superfícies articulares dos ossos são protegidas por uma cartilagem fibrosa que forma uma bolsa, onde se encontra a articulação. A área dentro dessa cápsula articular é chamada de cavidade articular e está repleta de líquido sinovial que nutre a articulação e permite que as superfícies deslizem entre si. O líquido sinovial em condições fisiológicas é acelular. O revestimento sinovial é composto por dois tipos celulares que morfologicamente, fenotipicamente e funcionalmente podem ser subdivididos em sinoviócitos do tipo A (semelhantes a macrófagos) e do tipo B (semelhantes a fibroblastos). Os sinoviócitos servem como função de barreira e secretam ácido hialurônico e lubricina, sendo capazes de promover a lubrificação da articulação, reduzindo o atrito e facilitando o movimento (Kosinska *et al.*, 2015). (B) A membrana sinovial de pacientes com AR é ativada e hiperplásica com a proliferação local dos sinoviócitos. Simultaneamente, várias células do

sistema imune são recrutadas para a articulação inflamada. A membrana sinovial inflamada invade gradualmente as estruturas articulares como cartilagem e osso com a formação do *pannus* reumatoide. O aumento da celularidade da membrana sinovial requer uma oxigenação adequada que é suportada pelo aumento da angiogênese. Durante a AR ocorre a produção excessiva de mediadores inflamatórios e enzimas que degradam a cartilagem e desregula o metabolismo ósseo, o que eventualmente leva à destruição da articulação. Modificado de (Patakas, 2011).

1.2.1 PARTICIPAÇÃO DOS NEUTRÓFILOS NA AR

Entre as células circulantes, os neutrófilos são as primeiras a atingir a cavidade articular, e as mais abundantes no líquido sinovial (Wittkowski *et al.*, 2007). São também uma das principais responsáveis pelo surgimento e progressão da doença (Wipke e Allen, 2001; Tanaka *et al.*, 2006).

Agentes quimiotáticos como o fator estimulador de colônias de granulócitos (G-CSF) e a IL-8 estimulam a migração dos neutrófilos do sangue periférico para as articulações inflamadas na AR (Wipke e Allen, 2001; Eyles *et al.*, 2008). Em resposta à liberação de mediadores inflamatórios (como TNF-α e IL-17) o endotélio vascular adjacente às articulações inflamadas é ativado (Griffin *et al.*, 2012) e, proteínas da família das selectinas (E- e P-selectina) são expressas na superfície endotelial. A L-selectina, presente constitutivamente na superfície dos neutrófilos, medeia a captura inicial dessas células para a articulação (Hallmann *et al.*, 1991; Spertini *et al.*, 1991). Ao longo do endotélio vascular na proximidade da articulação inflamada, as quimiocinas estão concentradas por ligação a glicosaminoglicanos (GAGs), tais como heparina e heparan sulfato. Os GAGs permitem uma interação próxima entre as quimiocinas e os neutrófilos durante os processos de rolamento e adesão, potencializando a migração celular (Sanz e Kubes, 2012; Vestweber, 2015). A adesão firme é mediada por interações entre integrinas β2 (LFA-1, CD11a/CD18 e MAC-1, CD11b/CD18) e seu ligante ICAM-1. As integrinas estão geralmente em estado inativo nos neutrófilos e tornam-se funcionais após a ativação de receptores acoplados a proteínas G, como os receptores de quimiocinas (Tarrant e Patel, 2006). A ligação das integrinas aos seus ligantes e a ação das quimiocinas ativam vias de sinalização nos neutrófilos que estabilizam a adesão, iniciam a motilidade celular e regulam a polimerização da actina, responsável por controlar a direção do movimento da célula (Futosi *et al.*, 2013). O estágio final na cascata de adesão é a transmigração do neutrófilo do vaso sanguíneo para o tecido inflamado. A passagem através da camada de células endoteliais ocorre tanto paracelularmente (entre as células endotelias) usando ligantes de superfície incluindo ICAM-2, PECAM-1 e proteínas da família de moléculas de adesão juncional (JAM) (Woodfin *et al.*, 2009), como por uma via transcelular (através da célula

endotelial) sob condições de alta expressão e densidade de ICAM-1 (Yang *et al.*, 2005). Por fim, para finalmente alcançar a articulação inflamada, os neutrófilos devem passar pela membrana basal, que ocorre através da degradação de moléculas de matriz extracelular por proteases armazenadas no interior dessas células, como metaloproteinases de matriz (MMPs) e serino-proteases (Kolaczkowska e Kubes, 2013).

Uma vez ativados no foco inflamatório, os neutrófilos liberam altas concentrações de oxidantes e produtos citotóxicos como espécies reativas de oxigênio (ROS), citocinas (como o TNF-α) e grânulos contendo proteases, fosfolipases, defensinas e mieloperoxidase, no líquido sinovial ou diretamente na superfície da cavidade articular (Rollet-Labelle *et al.*, 2013; Milanova *et al.*, 2014).

Alguns estudos associam as funções dos neutrófilos às células Th17 na AR. Essas células são produtoras de IL-17, que é um potente mediador pró-inflamatório envolvido na indução da inflamação tecidual e por estimular o recrutamento de neutrófilos. Na AR, a IL-17 ativa sinoviócitos, macrófagos e osteoclastos (Assi *et al.*, 2007; Cua e Tato, 2010; Jaeger *et al.*, 2012). Assim, essas células ao serem ativadas na articulação produzem potentes quimioatraentes para os neutrófilos, como IL-8 e TNF-α que em combinação com a IL-17, estimulam as células endoteliais sinoviais a produzirem mais quimioatrativos neutrofílicos, formando um ciclo de *feedback* positivo que promove o recrutamento de mais neutrófilos e amplifica a resposta inflamatória aguda (Assi *et al.*, 2007; Cua e Tato, 2010; Jaeger *et al.*, 2012).

Neutrófilos recolhidos do líquido sinovial de pacientes com AR exacerbada expressam o ligante do receptor ativador nuclear kappa-B (RANKL), que ativa a osteoclastogênese (Sabroe *et al.*, 2005; Mócsai, 2013). Enquanto isso, os neutrófilos de sangue periférico expressam o fator de ativação de células B (BAFF), envolvido na regulação da autoimunidade dependente de células B (Chakravarti *et al.*, 2009). Ainda, na AR, ocorre a desregulação da apoptose dos neutrófilos, aumentando a sobrevida dessas células nos tecidos inflamados, prolongando a liberação de citocinas, quimiocinas e produtos citotóxicos o que leva a persistência da inflamação (Cross *et al.*, 2006; Cascão *et al.*, 2010).

Os neutrófilos presentes em pacientes com AR apresentam uma capacidade aumentada de liberar as armadilhas extracelulares de neutrófilos (NETs), através de uma forma de morte celular denominada de NETose (Khandpur *et al.*, 2013; Sur Chowdhury *et al.*, 2014), induzida por anticorpos, bem como por citocinas como a IL-17 e o TNF-α (Deane *et al.*, 2010). A NETose, consiste na liberação do conteúdo intracelular dos neutrófilos, incluindo redes fibrosas

compostas de componentes nucleares como DNA e histonas que são cobertas com enzimas antimicrobianas e componentes granulares, como mieloperoxidase, elastase, catepsina G e outros peptídeos microbicidas (Brinkmann *et al.*, 2004; Fuchs *et al.*, 2007). Durante a formação das NETs, ocorre a ativação intracelular da enzima PAD-4 e consequentemente proteínas externalizadas durante a NET tornam-se citrulinadas, e várias delas têm sido caracterizadas como importantes autoantígenos na AR (Khandpur *et al.*, 2013). Somado a isso, as NETs aumentam a resposta inflamatória dos fibroblastos sinoviais, estimulando a produção de IL-8, supermodulando a NETose (Lubberts *et al.*, 2002), a exposição aos autoantígenos citrulinados promovem o mecanismo amplificador da geração de anticorpos (Pratesi *et al.*, 2014). Em conclusão, as NETs exteriorizam várias moléculas imunoestimuladoras e antígenos autocitrulinados que, em indivíduos predispostos, podem ser responsáveis pela geração persistente dos ACPAs (Navegantes *et al.*, 2017). Na Figura 1.2 podemos ver uma representação esquemática destacando os principais papeis dos neutrófilos na artrite.

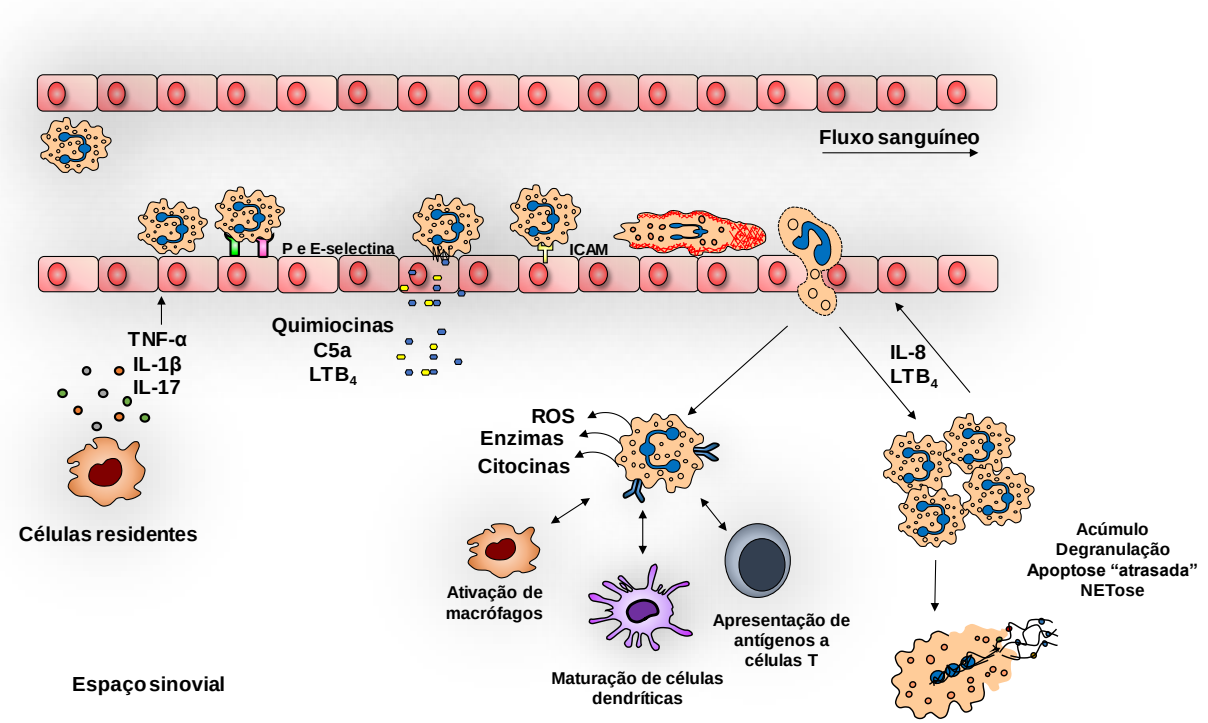

Figura 1.2: Participação dos neutrófilos na artrite. O rolamento dos neutrófilos ao longo do endotélio vascular envolve interações transientes entre as selectinas nas células endoteliais e nos neutrófilos. A expressão das selectinas nas células endoteliais é regulada positivamente por mediadores inflamatórios, como TNF-α e IL-17. Fatores quimiotáticos promovem a adesão firme dos neutrófilos as células endoteliais pelo aumento da expressão de integrinas. Durante a adesão firme os neutrófilos são ativados, ocorre a formação dos filamentos de actina seguidos da migração transendotelial em direção ao sítio inflamatório. Neutrófilos ativados produzem ROS e liberam enzimas responsáveis pela destruição da cartilagem. Além disso, comunicam-se com outras células do sistema imune através da secreção de citocinas e quimiocinas e pela apresentação de antígenos via MHC de classe II. Os neutrófilos podem sofrer uma forma especial de morte celular chamada NETose. Isso resulta na liberação de um complexo

de moléculas nucleares e granulares chamado NETs, que contribuem para o dano tecidual e para amplificação da geração de anticorpos ACPAs. Neutrófilos ativados também geram quimioatrativos, formando um ciclo de *feedback* positivo que promove o recrutamento de mais neutrófilos e amplifica a resposta inflamatória aguda. A apoptose efetiva dos neutrófilos é necessária para a resolução da inflamação. No entanto, na articulação inflamada ocorre a apoptose tardia dos neutrófilos, o que resulta em inflamação persistente e dano tecidual devido à liberação contínua de ROS, enzimas granulares e citocinas. Adaptado de (Rosas *et al.*, 2017).

1.2.2 A IMPORTÂNCIA DOS MACRÓFAGOS NA INICIAÇÃO E PROPAGAÇÃO DA AR

Os macrófagos são células residentes no tecido sinovial, juntamente com os fibroblastos (Kennedy *et al.*, 2011). No entanto, o número de macrófagos é maior na membrana sinovial inflamada da AR do que nas articulações normais e está bem correlacionado com danos radiológicos (Mulherin *et al.*, 1996), dor e inflamação articular (Tak *et al.*, 1997). Durante o processo inflamatório os macrófagos são recrutados para a cavidade sinovial principalmente pela ação do GM-CSF e do G-CSF que aumentam a maturação dessas células, seu efluxo da medula óssea e o tráfego para a sinóvia (Cornish *et al.*, 2009).

Macrófagos sinoviais são responsáveis pela produção das citocinas pró-inflamatórias TNF-α, IL-1β, IL-6, IL-23, peptídeos vasoativos, prostanoides e intermediários de oxigênio e nitrogênio que atuam na patogênese da AR (Kennedy *et al.*, 2011; Firestein e Mcinnes, 2017). Estas células podem estimular a angiogênese, o recrutamento de polimorfonucleares e linfócitos, a proliferação de fibroblastos, a secreção de proteases, além do processamento e apresentação de autoantígenos às células T, contribuindo para a destruição das articulações (Burmester *et al.*, 1997; Vallejo *et al.*, 2000; Takayanagi, 2007). Além disso, os monócitos/macrófagos também estão associados à erosão óssea patológica na AR, devido a essas células se diferenciarem em osteoclastos (células especializadas na reabsorção óssea) (Davignon *et al.*, 2013).

As quimiocinas liberadas pelos macrófagos, tais como a CXCL-1, MIP-1α e MCP-1 promovem o recrutamento de leucócitos para a articulação inflamada, que por sua vez, produzem mais mediadores pró-inflamatórios como IL-1β, TNF-α, IL-6 e metaloproteinases de matriz (Feldmann *et al.*, 1996; Kinne *et al.*, 2007). A grande concentração de citocinas e quimiocinas pró-inflamatórias produzida pelos macrófagos contribuem para a destruição da cartilagem e do osso e para a formação do *pannus* na AR. Além disso, ocorre uma regulação positiva da isoforma induzível da óxido nítrico sintase (iNOS) em macrófagos e sinoviócitos, o que resulta no aumento da formação de óxido nítrico (NO). Esse, mediador inibe a síntese de proteoglicanos e está elevado no líquido sinovial de pacientes com AR (Moilanen e Vapaatalo,

1995; Jang e Murrell, 1998). Macrófagos ativados também produzem a citocina IL-12 que induz uma alteração no equilíbrio Th1/Th2 em favor de uma atividade pró-inflamatória de Th1 que tem um papel pró-artritogênico (Germann *et al.*, 1995; Simon *et al.*, 2001).

Os macrófagos ativados atuam no espaço sinovial durante a AR, assim como, nos compartimentos extra-articulares, como por exemplo, no sangue periférico e no espaço subendotelial. O último é o local de formação de células espumosas e está relacionado ao desenvolvimento de placas ateroscleróticas na AR (Kinne *et al.*, 2007). Essa ativação destaca o caráter inflamatório sistêmico da AR e pode contribuir para a ocorrência de eventos cardiovasculares e o aumento da mortalidade (Sattar *et al.*, 2003; Monaco *et al.*, 2004).

1.2.2.1 TLRs NA AR

Assim como diversas células do sistema imune, os macrófagos expressam receptores do tipo Toll (TLRs) (por exemplo, TLR 2/6, 3, 4 e 8) e receptores do tipo NOD (NLRs) que reconhecem uma gama de padrões moleculares associados a patógenos e a danos teciduais (PAMPs e DAMPs), como por exemplo bactérias, vírus e ligantes endógenos (Seibl *et al.*, 2003). Os DAMPs são moléculas pró-inflamatórias endógenas geradas por lesão tecidual e incluem moléculas intracelulares liberadas por células necróticas, fragmentos de matriz extracelular ou moléculas de matriz extracelular reguladas sobre a lesão (Bianchi, 2007). Os TLRs são altamente expressos no tecido sinovial de indivíduos com AR (Radstake *et al.*, 2004; Sacre *et al.*, 2007), e camundongos com deleções direcionadas ou mutações de perda de função no TLR-4 são protegidos da artrite experimental (Choe *et al.*, 2003; Lee *et al.*, 2005). Ligantes endógenos são provavelmente liberados por células submetidas a estresse, dano ou morte necrótica e estão presentes na sinóvia inflamada. A maior rotatividade de células em condições de trauma ou inflamação, leva a níveis aumentados de ligantes de TLRs endógenos, portanto, é provável que inicie uma reação que leve a ativação celular mediada por TLRs em células inflamatórias na AR (Krieg, 2002; Radstake *et al.*, 2004). Uma vez ativados, os TLRs estimulam respostas imunes inatas e adaptativas, incluindo a indução de citocinas pró-inflamatórias e MMPs (Medzhitov e Janeway, 2002). Proteínas de choque térmico (HSPs), fragmentos de ácido hialurônico, fibronectina e tenascina-C (glicoproteína de matriz extracelular associada a lesão e reparo tecidual) são alguns dos ligantes endógenos reconhecidos pelos TLRs na AR (Ohashi *et al.*, 2000; Okamura *et al.*, 2001; Termeer *et al.*, 2002; Midwood *et al.*, 2009).

A sinalização dos TLRs é iniciada por hetero ou homodimerização induzida pelos ligantes dos receptores ou associação com proteínas acessórias (Wesche *et al.*, 1997). Todos os TLRs com exceção do TLR-3, compartilham uma via de sinalização comum que depende da molécula adaptadora MyD88 (fator de diferenciação mieloide 88) (Janssens e Beyaert, 2002). MyD88 contém um domínio amino terminal que é responsável pelo recrutamento *downstream* de mediadores de sinalização, incluindo a quinase associada ao receptor de IL-1 (IRAK)-1, IRAK-4 e o fator associado ao receptor de TNF (TRAF)-6 ao complexo do receptor (Burns *et al.*, 1998). Esse recrutamento leva à ativação de proteínas quinases ativadas por mitógenos (MAPKs), assim como o fator nuclear (NF)-κB (Takeda e Akira, 2015). A via das MAPKs e a via do NF-κB são duas importantes cascatas de sinalização que regulam a síntese e a ação de citocinas inflamatórias, como o TNF-α, e a produção de vários outros mediadores envolvidos na AR (Kumar *et al.*, 2001).

1.2.2.2 VIA DAS MAPKS

As MAPKs são divididas em três famílias, a quinase regulada por sinal extracelular (ERK), a quinase N-terminal c-Jun (JNK) e p38. Os mitógenos e fatores de crescimento ativam principalmente ERK1/2, enquanto as citocinas pró-inflamatórias como TNF-α e IL-1β e fatores indutores de estresse celular, como choque térmico, choque osmótico, radiação ultravioleta e radicais de oxigênio ativam principalmente JNK e p38 (Arthur e Ley, 2013). As três MAPKs controlam a ativação de muitos fatores de transcrição, incluindo AP-1 (homo ou heterodímero das proteínas c-Jun e c-Fos), NF-κB e C/EBP (Silvers *et al.*, 2003). Todas as MAPKs são expressas na membrana sinovial durante a AR, embora os locais de expressão sejam diferentes. A ativação de ERK ocorre principalmente nos microvasos, a ativação de JNK ocorre ao redor e dentro dos infiltrados de células mononucleares e a ativação da p38 ocorre dentro da camada de revestimento sinovial e das células endoteliais (Schett *et al.*, 2000). Em todos esses tipos celulares, as MAPKs são ativadas principalmente pelo TNF-α e IL-1β. A MAPK p38, mais notavelmente sua isoforma p38α, é ativada principalmente dentro das células envolvidas no processo inflamatório (Herlaar e Brown, 1999).

1.2.2.3 VIA DO NF-ΚB

Na artrite e na inflamação em geral, o NF-κB pode ser considerado um dos fatores de transcrição mais importantes, pois é utilizado por muitos complexos de ligante-receptor para modular a transcrição gênica (Kennedy *et al.*, 2011). Em modelos animais, a ativação de NF-

κB foi detectada antes do início clínico da artrite (Tsao *et al.*, 1997; Han *et al.*, 1998) e a via de NF-κB tem sido considerada um alvo para o tratamento da AR, confirmando seu papel essencial na patogênese da doença (Wakamatsu *et al.*, 2005). A ativação do NF-κB é regulada principalmente por IκB e IκB quinase (IKK). Na ausência de estímulo inflamatório, o NF-κB está acoplado à proteína inibidora IκB, o que impede sua translocação para o núcleo (Hayden e Ghosh, 2008). Uma vez ativado, o IKK induz a fosforilação de IκB, que então se separa do NF-κB. Uma vez que, o NF-κB se dissocia do IκB, ele migra para o núcleo, ligando-se ao DNA nas regiões promotoras e ativando a transcrição (Hayden e Ghosh, 2004; 2008). O NF-κB pode ser ativado por uma variedade de estímulos, incluindo citocinas pró-inflamatórias como TNF-α e IL-1β, componentes de microrganismos como o lipopolissacarídeo (LPS) e o zimosan, proteínas virais, radiação ultravioleta e estresse celular (Morel e Berenbaum, 2004). Moléculas de IκB fosforiladas se unem através da ubiquitinação antes de sofrerem degradação via proteassoma. Na Figura 1.3 estão representadas esquematicamente as principais vias ativadas pelos TLRs na patogênese da AR.

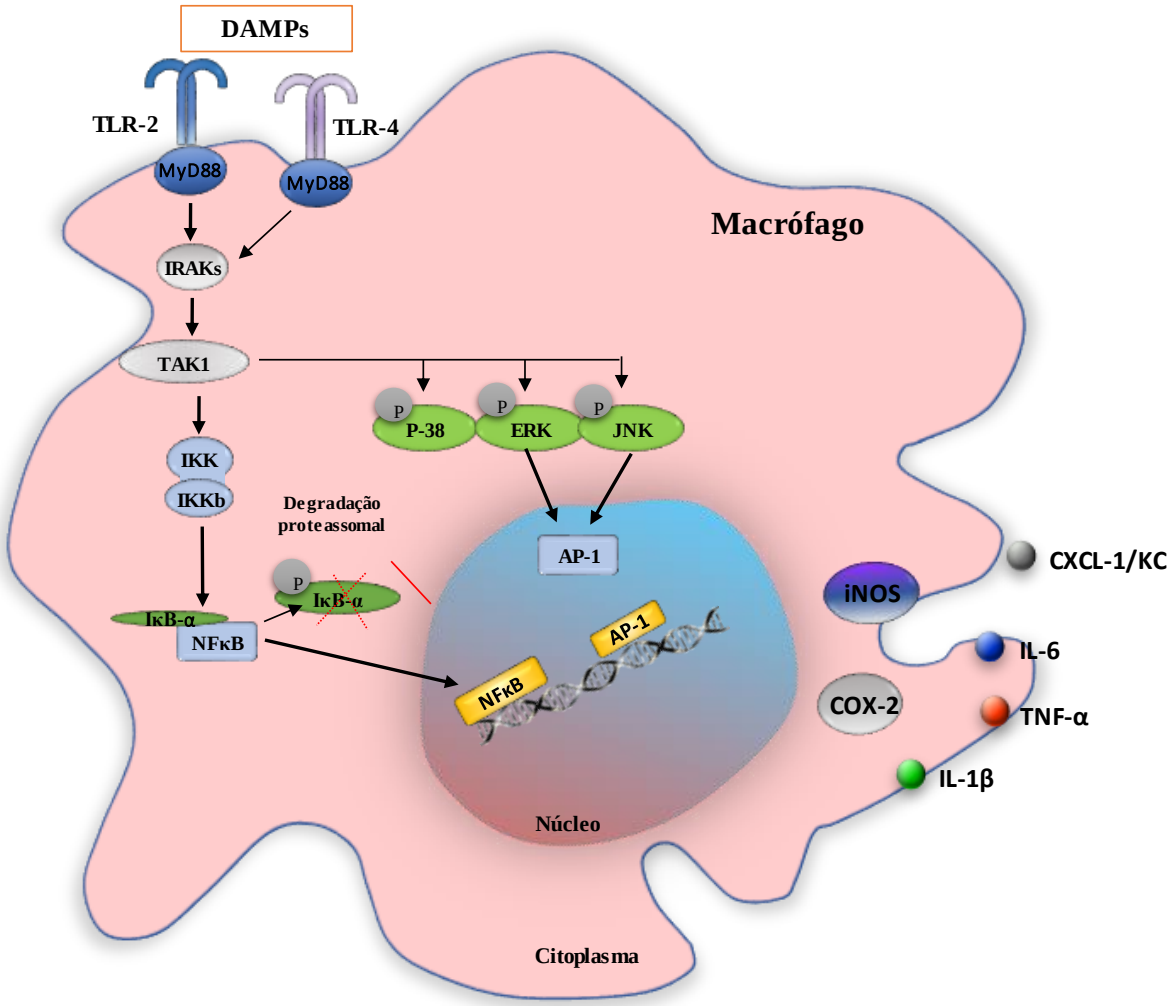

Figura 1.3: Sinalização de TLRs em macrófagos contribuem para a produção de mediadores inflamatórios envolvidos na patogênese da AR. Os TLRs são ativados por ligantes exógenos e endógenos. Após estimulação e dimerização, as vias de sinalização dos TLRs, com exceção de TLR-3, recrutam a molécula adaptadora MyD88 e induzem a ativação das IRAK-1, IRAK-4 e TRAF-6. Por sua vez, essas moléculas de sinalização induzem a ativação das vias IKK/NF-κB e da via das MAPKs, que culminam na translocação de fatores nucleares (como NF-κB e AP-1) responsáveis pela produção de mediadores, como TNF-α e IL-6, e pela expressão de iNOS e COX-2.

1.2.3 EROSÃO ÓSSEA NA AR

Além da inflamação sinovial, uma das principais manifestações clínicas da AR é a destruição progressiva das estruturas ósseas e cartilaginosas nas articulações dos pacientes, levando a características radiograficamente definidas (Choi *et al.*, 2009). As erosões ósseas aparecem precocemente no curso da AR, algumas vezes dentro de algumas semanas após o diagnóstico. Mais de 10% dos pacientes desenvolvem erosões ósseas dentro de 8 semanas após o início da doença, enquanto que 60% deles apresentam erosões após 1 ano (Machold *et al.*, 2007). As erosões ósseas progridem com o tempo e contribuem para o dano articular, levando ao comprometimento da capacidade funcional (Welsing *et al.*, 2001; Ødegård *et al.*, 2006).

Assim, a presença de erosões ósseas prediz um curso clínico mais severo, tornando-a um importante parâmetro de avaliação clínica na AR.

O mecanismo normal pelo qual os ossos são formados e reabsorvidos é mediado pela interação entre duas populações celulares, osteoblastos de formação óssea e osteoclastos de reabsorção óssea. Os osteoblastos diferenciam-se da linhagem celular mesenquimal sob o controle de sinais-chave, como o hormônio paratireoide, a via canônica Wnt-β catenina e a via da proteína morfogenética óssea (BMP) (Chen *et al.*, 2004; Kubota *et al.*, 2009). Essas vias de sinalização produzem produtos essenciais de matriz óssea que são subsequentemente mineralizados. Já os osteoclastos diferenciam-se dos precursores da linhagem mieloide sob o controle das principais vias envolvendo o fator estimulador de colônias de macrófagos (M-CSF) e o eixo RANKL-RANK, que atua nos estágios de diferenciação precoce e terminal, respectivamente (Suda *et al.*, 1999; Boyle *et al.*, 2003; Walsh *et al.*, 2006). Essas células degradam o osso através da expressão de moléculas efetoras, como catepsinas, MMPs e produção local de íons hidrogênio. Assim, estes dois tipos de células efetoras, derivadas de linhagens precursoras independentes e com funções opostas, atuam em conjunto para manter o metabolismo ósseo. A regulação cruzada desses tipos de células pode ocorrer, por exemplo, pelo receptor "isca" (*decoy receptor*) de RANKL, conhecido como osteoprotegerina (OPG), que é expresso por osteoblastos e age para reprimir o eixo osteoclástico através da regulação da sinalização de RANK (Choi *et al.*, 2009).

No caso da AR, o eixo osteoblastos-osteoclastos é severamente interrompido devido aos processos inflamatórios em curso, resultando em uma função aprimorada dos osteoclastos (Schett, 2007; Karmakar *et al.*, 2010). Citocinas pró-inflamatórias derivadas de macrófagos e células T (como TNF-α, IL-1β, IL-6 e IL-17) atuam em uma rede pleiotrópica para induzir a expressão de RANKL por fibroblastos sinoviais, osteoblastos e células estromais da medula óssea, que levam ao aumento da diferenciação dos osteoclastos (Kotake *et al.*, 1999; Gravallese *et al.*, 2000; Romas *et al.*, 2002; Dai *et al.*, 2004; Hashizume *et al.*, 2008). Além disso, o RANKL é expresso por células T $CD4^+$ ativadas, as quais estão infiltradas na sinóvia fornecendo outras fontes celulares dessa proteína geradora de osteoclastos sob condições inflamatórias (Kong *et al.*, 1999; Sato *et al.*, 2006; Hashizume *et al.*, 2008). A ligação de RANKL ao RANK ativa a sinalização de NF-κB em células precursoras de osteoclastos, que é essencial para a indução do fator nuclear de células T ativadas, proteína citoplasmática 1 (NFATc1). O NFATc1 induz a expressão de genes cruciais para a diferenciação e função de osteoclastos, como fosfatase ácida tartarato-resistente (TRAP), catepsina K, receptor de

calcitonina (CTR), MMPs e integrina β3 (Asagiri e Takayanagi, 2007). A importância do RANKL tem sido comprovada em ensaios *in vivo*, uma vez que, camundongos deficientes em RANKL são protegidos contra erosões ósseas em modelo experimental de artrite (Pettit *et al.*, 2001). Em estudo de fase II, a adição do denosumabe (anticorpo monoclonal anti-RANKL) ao tratamento com metotrexato inibiu o dano ósseo estrutural em pacientes com AR (Cohen *et al.*, 2008).

A segunda via possível para a perda óssea na AR envolve dois mecanismos autoimunes que atuam como um gatilho para danos ósseos estruturais. O primeiro mecanismo refere-se à formação de imunocomplexos e à diferenciação de osteoclastos mediada por receptores de Fc. O segundo é a formação de anticorpos anti-vimentina citrulinada, tornando os osteoclastos os alvos antigênicos ideais para os ACPAs (Harre *et al.*, 2012). A presença de proteínas citrulinas na superfície dos osteoclastos permite que os ACPAs se liguem aos osteoclastos, estimulando a liberação de IL-8, além do aumento autócrino da maturação e ativação dos osteoclastos (Catrina *et al.*, 2017). A ligação dos ACPAs aos precursores de osteoclastos induz a osteoclastogênese, a reabsorção e a perda óssea. Em 2012, foi demonstrado a primeira evidência *in vitro* de que os ACPAs poderiam se ligar especificamente aos osteoclastos e ativá-los (Harre *et al.*, 2012), sendo que o mesmo estudo também demonstrou que a administração de ACPAs isolados de pacientes com AR em camundongos ativa os osteoclastos gerando perda óssea (Harre *et al.*, 2012).

1.3 MEDICAMENTOS PARA O TRATAMENTO DA ARTRITE REUMATOIDE

O tratamento da AR evoluiu nos últimos 30 anos devido a disponibilidade de uma ampla gama de novas ferramentas terapêuticas, combinadas ao uso sistemático de abordagens não farmacológicas, com o diagnóstico precoce e o acompanhamento rigoroso. Apesar de ainda ser uma doença incurável, atualmente, o tratamento da AR visa alcançar remissão de longa duração ou pelo menos baixos níveis de atividade da doença (Cecchi *et al.*, 2018).

A terapia da AR inclui o uso cauteloso de glicocorticoides, fármacos anti-inflamatórios não esteroidais (AINEs) e fármacos anti-reumáticos modificadores da doença (DMARDs; do inglês *disease-modifying anti-rheumatic drugs*), incluindo DMARDs sintéticos como o metotrexato e biológicos como os inibidores de TNF-α (Kahlenberg e Fox, 2011; Crofford, 2013; Burmester e Pope, 2017).

Os AINEs são escolhas eficazes para aliviar a dor e melhorar a realização dos movimentos articulares nos pacientes com AR, mas não são capazes de prevenir danos estruturais da cartilagem e do osso (Crofford, 2013). Os AINEs diferem amplamente em sua classe química, mas compartilham a propriedade de bloquear a produção de prostaglandinas (PGs) (Crofford, 2013). Isto é conseguido através da inibição da atividade da enzima ciclo-oxigenase (COX). A COX ocorre em duas isoformas, conhecidas como COX-1 e COX-2, que diferem em sua distribuição e regulação tecidual. Essas isoformas possuem diferentes funções biológicas, tendo em vista que a COX-1 é expressa sob condições basais e está envolvida na biossíntese de PG que atende às funções homeostáticas, enquanto que a expressão de COX-2 é aumenta durante a inflamação e outras situações patológicas (Crofford *et al.*, 2000). A inibição de COX-2 pelos AINEs bloqueia a produção de PG em locais de inflamação, enquanto que a inibição da COX-1 em outros tecidos (principalmente em plaquetas e mucosa gastroduodenal) pode levar a efeitos adversos comuns dos AINEs como sangramento e ulceração gastrintestinal (Lanas, 2009). Os AINEs mais tradicionais inibem ambas as isoformas, embora com algumas diferenças na potência relativa para COX-1 e COX-2. Alguns AINEs não têm inibição da função plaquetária, que é a definição operacional dos AINEs seletivos para a COX-2 (Patrono *et al.*, 2001). No entanto, a utilização contínua de alguns inibidores específicos da COX-2 tem sido associada a efeitos adversos cardiovasculares e cerebrovasculares, particularmente em pacientes com um risco elevado de trombose. Esse risco aumentado pode ser devido à redução da síntese de prostaciclina (inibidor natural da ativação das plaquetas) mediada por COX-2 (Vonkeman e Van De Laar, 2010).

A descoberta das ações anti-inflamatórias esteroidais (glicocorticoides; GCs) foi um grande avanço para o tratamento de doenças inflamatórias e autoimunes (Rhen e Cidlowski, 2005). O tratamento da AR com GCs foi introduzido pela primeira vez na década de 40 (Hench *et al.*, 1949). Glicocorticoides a curto prazo reduzem a sinovite, porém, a longo prazo, diminuem o dano articular (Kirwan *et al.*, 2007), mas incidem em riscos adversos substanciais, como infecções e osteoporose, e sua relação risco/benefício em geral é considerada desfavorável (Ravindran *et al.*, 2009). Entretanto, os GCs podem ser especialmente úteis em dois contextos. Primeiro, o uso a curto prazo durante surtos da doença pode levar a uma melhora rápida e permitir que outros tratamentos - como os DMARDs, que tem um início de ação mais lento – sejam ajustados. O uso dos GCs dessa forma é de baixo risco. Segundo, os glicocorticoides intra-articulares são um tratamento local altamente eficaz para as articulações nos períodos de ativação da doença (Goossens *et al.*, 2000).

Os DMARD são definidos como medicamentos que interferem nos sinais e sintomas da AR, melhoram a função física e inibem a progressão do dano articular. Medicamentos que apenas melhoram os sintomas, como os AINEs ou analgésicos, não impedem a progressão do dano e a incapacidade irreversível (Aletaha e Smolen, 2018). Os DMARDs são classificados em agentes sintéticos (pequenas moléculas químicas administradas oralmente) e biológicos (proteínas administradas por via parenteral) (Smolen *et al.*, 2014). Os DMARDs sintéticos podem ser caracterizados como convencionais ou direcionados. Os DMARDs sintéticos convencionais entraram na prática clínica com base em observações empíricas, sendo utilizados por mais de 50 anos e tem alvos moleculares que ainda não foram identificados. Em contraste, os DMARDs sintéticos direcionados foram desenvolvidos para interferir com uma molécula específica, com base nos avanços da biologia molecular e estrutural (Aletaha e Smolen, 2018).

Entre os DMARDs sintéticos convencionais, o metotrexato (MTX) é considerado um dos pilares e a principal terapia inicial para a AR. Seu baixo custo, associado à boa eficácia a longo prazo e ao perfil de segurança, justificam a sua recomendação como o primeiro modificador da doença no tratamento da AR (Singh *et al.*, 2012; Smolen *et al.*, 2016). O mecanismo de ação preciso do MTX na AR ainda não está totalmente esclarecido, embora acredita-se que este fármaco previna a síntese *de novo* de pirimidina e purina necessária para a síntese de DNA e RNA e consequentemente inibe a proliferação celular de linfócitos envolvidos no processo inflamatório (Wessels *et al.*, 2008). O MTX é o principal medicamento para o tratamento da AR por vários motivos. Primeiro, uma grande proporção de pacientes (25-40%) melhora significativamente com a monoterapia com o MTX e, em combinação com glicocorticoides, quase metade dos pacientes pode atingir baixa atividade da doença ou remissão inicial, em uma taxa semelhante a obtida pela utilização dos DMARDs biológicos (Nam *et al.*, 2014; Emery *et al.*, 2017). Segundo, seus efeitos adversos são bem conhecidos e muitos, como náusea, perda de cabelo, estomatite e hepatotoxicidade, podem ser prevenidos pelo uso profilático de ácido fólico (Van Ede *et al.*, 2001). Terceiro, os DMARDs biológicos ou sintéticos, têm menos eficácia como monoterapias do que quando combinados com o MTX (Nam *et al.*, 2017). No entanto, apesar da sua eficácia uma porcentagem significativa de pacientes com AR é resistente ao tratamento com MTX, o que obriga o uso de outras estratégias terapêuticas (Peres *et al.*, 2015). Além disso, apesar de todos os benefícios conferidos ao tratamento com MTX o uso a longo prazo pode levar à fibrose hepática, que em alguns casos pode exigir um transplante de fígado para o seu tratamento (Carneiro *et al.*, 2008; Conway e Carey, 2017; Cheng e Rademaker, 2018). Marcadores de mau prognóstico, como a presença de

autoanticorpos, lesão articular precoce e alta atividade da doença, estão associados à uma possível falha terapêutica que pode ser interrompida ou retardada pela adição de um DMARD biológico ou outro DMARD sintético (Smolen *et al.*, 2006; Vastesaeger *et al.*, 2009). Outros DMARDs sintéticos convencionais incluem a sulfasalazina, a leflunomida e a hidroxicloroquina.

A última terapia aprovada para o tratamento da AR, denominados como DMARDs sintéticos direcionados, revolucionaram a clínica. O tofacitinibe é o primeiro de uma nova classe de fármacos orais que interferem especificamente nas vias de transdução de sinal, sendo a terceira classe dos DMARDs no tratamento da AR (Miller e Ranatunga, 2012; Kumar e Banik, 2013; Smolen *et al.*, 2016). O tofacitinibe inibe a Janus quinase (JAK), que é uma pequena enzima intracelular que modifica a função de outras proteínas, ligando-lhes grupos fosfato. JAKs medeiam a sinalização de citocinas e fatores de crescimento responsáveis pela hematopoiese e função imunológica. A sinalização mediada por JAK envolve o recrutamento de transdutores de sinal e ativadores de transcrição (STATs) para receptores de citocinas que levam à modulação da expressão gênica (Venkatesha *et al.*, 2014). Tofacitinibe foi recentemente aprovado pelo FDA para a AR moderada a grave refratária aos demais DMARDs com base em estudos de eficácia, com os benefícios associados ao tratamento precoce (Strand *et al.*, 2016).

Os medicamentos biológicos são proteínas purificadas, modificadas e/ou reconstruídas, derivadas de sequências genéticas de células vivas, que são usadas para modificar a resposta imunológica de um paciente. Na AR, eles são indicados para suprimir respostas inflamatórias ativas e destrutivas, visando especificamente mediadores-chave, como citocinas, células ou interações celulares (Meier *et al.*, 2013). Os medicamentos biológicos desenvolvidos para o tratamento da AR atualmente disponíveis incluem diferentes inibidores de citocinas tais como, TNF-α, IL-1β, IL-6 e inibidores de células como os linfócitos T e B (Miller e Ranatunga, 2012; Singh *et al.*, 2012; Meier *et al.*, 2013; Smolen *et al.*, 2016).

Cinco anti-TNF-α estão atualmente aprovados para o tratamento da AR, são eles: etanercepte, infliximabe, adalimumabe, golimumabe e certolizumabe. Todos eles são eficazes no controle da AR, mantendo a função e retardando a sua progressão (Miller e Ranatunga, 2012; Singh *et al.*, 2012; Kumar e Banik, 2013; Meier *et al.*, 2013; Smolen *et al.*, 2016). Quando combinados com MTX ou outros DMARDs, eles são mais eficazes do que quando usados em monoterapia (Meier *et al.*, 2013).

O abatacepte inibe a ativação da célula T pela ligação ao CD80 e ao CD86 em células apresentadoras de antígenos (APCs) e, assim, bloqueia a interação com CD28 necessária para a ativação das células T. É eficaz no tratamento de pacientes com AR ativa, que falharam em pelo menos um DMARD ou não responderam adequadamente à combinação de MTX com um anti-TNF-α. Pode ser administrado em monoterapia, embora sua eficácia seja aumentada quando administrado concomitantemente com outro DMARD (Alonso-Ruiz *et al.*, 2008; Meier *et al.*, 2013).

O rituximabe é atualmente a única terapia licenciada contra células B na AR. Tem como alvo o CD20 nos linfócitos B, resultando em depleção das células B e redução da produção dos autoanticorpos e interações entre células T/B, suprimindo potencialmente a autoimunidade humoral e celular (Miller e Ranatunga, 2012; Kumar e Banik, 2013; Meier *et al.*, 2013). A combinação do rituximabe e o MTX ou leflunomida resultam em uma boa resposta em pacientes com AR resistentes a monoterapia com DMARDs ou em combinação com anti-TNF-α. Recomenda-se como terapia de segunda linha, após falha do anti-TNF-α.

Anakinra é um antagonista recombinante do receptor da IL-1β humana. Nos últimos anos, demonstrou ser menos eficaz que a terapia anti-TNF-α e outros produtos biológicos. Assim, devido à incomoda administração diária por injeção subcutânea e a sua meia-vida muito curta, esse medicamento tem um papel menor no tratamento clínico de rotina dos pacientes com AR (Miller e Ranatunga, 2012; Kumar e Banik, 2013; Meier *et al.*, 2013).

O tocilizumabe é um anticorpo monoclonal humanizado que tem como alvo a IL-6 solúvel e ligada a membrana. A IL-6 é uma citocina pró-inflamatória produzida por linfócitos T e B, monócitos e fibroblastos, cujos efeitos incluem a indução de neovascularização sinovial, a ativação de osteoclastos e o aumento da expressão de MMPs e proteína C-reativa. Ela também promove a diferenciação de células Th17 (Miller e Ranatunga, 2012; Kumar e Banik, 2013; Meier *et al.*, 2013). O tocilizumabe reduz a sinovite e melhora as características sistêmicas da inflamação, incluindo anemia, anorexia, febre e fadiga. Ele foi aprovado para ser utilizado como terapia de primeira linha após resposta inadequada a um ou mais DMARDs, ou após falha terapêutica com anti-TNF-α, podendo ser utilizado em monoterapia ou em combinação com outros DMARDs (Miller e Ranatunga, 2012; Singh *et al.*, 2012; Kumar e Banik, 2013; Meier *et al.*, 2013; Smolen *et al.*, 2016). Na Figura 1.4, podemos observar o fluxograma esquemático empregado para o tratamento de pacientes com AR.

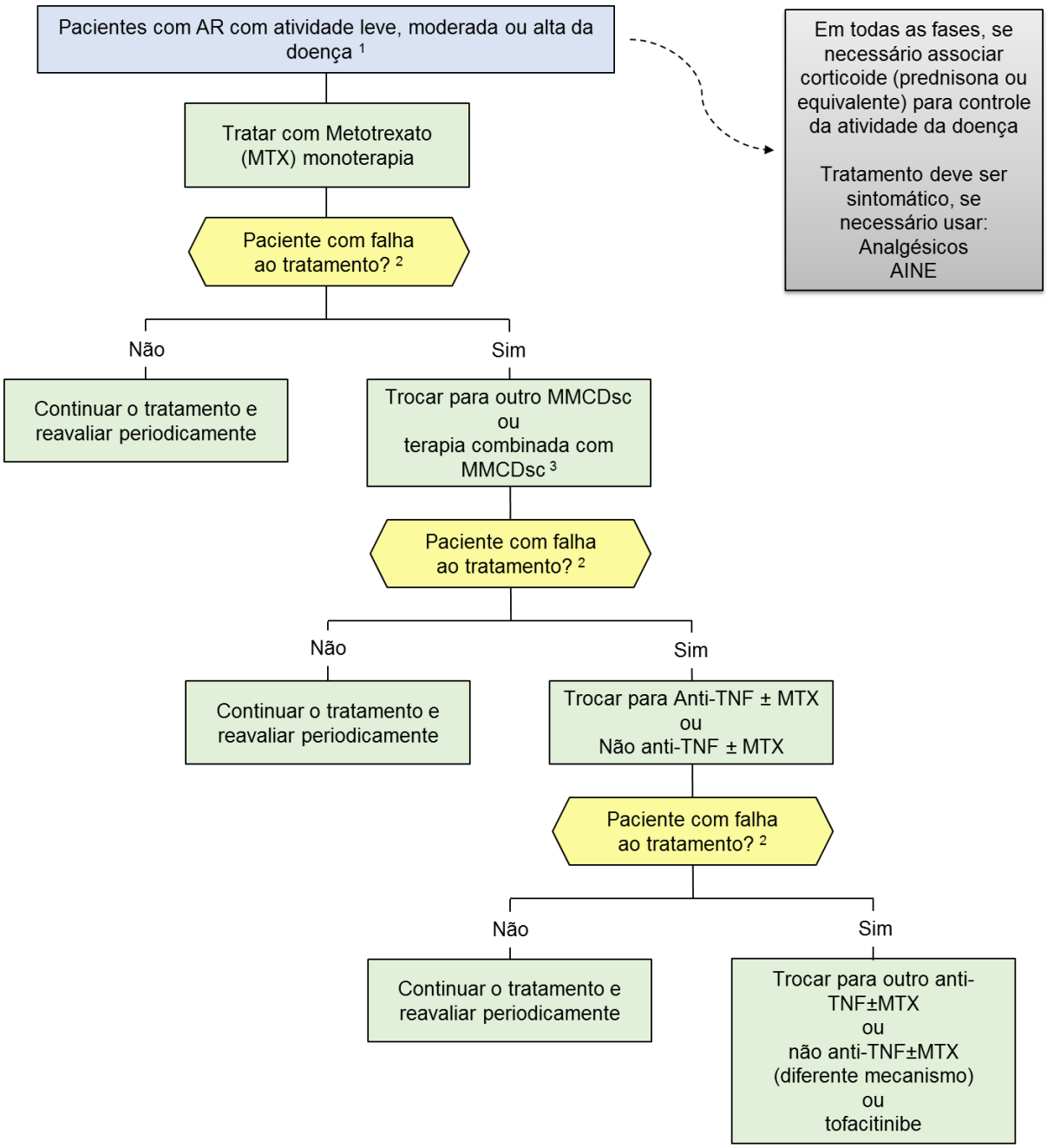

Figura 1.4: Fluxograma para o tratamento da AR. [1] Tratamento que tem como meta terapêutica a remissão ou baixa atividade da doença (deve ser reavaliado periodicamente). [2] A falha ao tratamento pode se dar por eventos adversos ou ausência de eficácia. Para avaliar a eficácia deve aguardar pelo menos 3 meses de tratamento com o esquema vigente, não devendo ser trocada de linha terapêutica em intervalo de tempo inferior. **MMCDsc:** medicamento modificador de curso da doença sintético convencional (Fonte: Ministério da Saúde, 2019).

Existem atualmente diversas terapias que fornecem o controle da inflamação em pacientes com AR. No entanto, várias dessas estratégias levam à imunossupressão secundária que aumentam o risco de infecção e o desenvolvimento de tumores. Além disso, dois terços dos pacientes com AR ainda não respondem ou são intolerantes ao tratamento atual disponível. Sem

contar, que o custo dessas medicações é uma questão muito importante para os pacientes, principalmente em países em desenvolvimento ou subdesenvolvidos (Yen, 2006). Como a maioria dos tratamentos existe há apenas uma década ou menos, ainda é preciso avaliar a segurança a longo prazo desses medicamentos. Assim, os esforços para desenvolver novas terapias ainda são necessários e a busca por novos alvos e abordagens deve ser levada em conta para esse crescente desafio. O uso de moléculas de fontes naturais representa um terreno promissor para o tratamento da AR, atuando como terapia primária ou pelo menos como adjuvantes terapêuticos para reduzir as doses diárias de drogas convencionais que os pacientes com AR recebem (Gelderman *et al.*, 2007; Khanna *et al.*, 2007; Natarajan *et al.*, 2015). As substâncias derivadas de plantas que podem modular sinais pró-inflamatórios têm claramente um potencial contra doenças inflamatórias, tais como a artrite (Khanna *et al.*, 2007).

1.4 MOLÉCULAS DERIVADAS DE PLANTAS COMO TERAPIAS EMERGENTES PARA ARTRITE

Desde os tempos antigos, as plantas são utilizadas pela população na cura e prevenção de diferentes doenças (Phillipson, 2001; Kong *et al.*, 2003). Um estudo realizado pela Organização Mundial de Saúde (OMS) relatou que cerca de 80% da população mundial depende da medicina tradicional (WHO, 2002). Terapias à base de plantas podem ser uma fonte alternativa para aliviar os sintomas em pacientes com AR. Produtos derivados de plantas, como polifenóis, flavonoides e tetranortriterpenóides, são metabolitos secundários com potencial atividade para reduzir a inflamação, podendo ser novos agentes terapêuticos com custo relativamente mais baixo (Choudhary *et al.*, 2015; Farzaei *et al.*, 2016; Dudics *et al.*, 2018). Estes produtos naturais atraíram considerável interesse na última década devido as suas múltiplas propriedades, como antioxidantes, anti-inflamatórios, antiproliferativos e imunomoduladores (Sung *et al.*, 2019).

Compostos fenólicos são metabólitos secundários de plantas, amplamente distribuídos por todo o reino vegetal. Estão disponíveis em diferentes tipos de frutas, vegetais ou ervas e atuam como micronutrientes (Pandey e Rizvi, 2009). Os compostos fenólicos são essenciais para o crescimento e a reprodução das plantas e são produzidos para defesa contra patógenos e lesões (Sung *et al.*, 2019). Os polifenóis compreendem uma extensa diversidade de moléculas que compartilham uma estrutura polifenólica básica similar (vários grupos hidroxila de anéis aromáticos), bem como moléculas com um único anel fenólico, incluindo ácidos fenólicos e álcoois fenólicos. A classificação dos polifenóis baseia-se no número de anéis fenólicos ou no

tipo de elementos estruturais que unem os anéis uns aos outros. Alguns dos principais grupos são: flavonoides, ácidos fenólicos, estilbenos e lignanos (D'archivio *et al.*, 2007). Esta classe de compostos fitoquímicos também tem um papel potencial em diferentes complicações induzidas pelo estresse oxidativo, como doenças cardiovasculares, câncer e doenças neurodegenerativas (Manach *et al.*, 2004). Foi constatado que uma dieta regular compreendendo a ingestão frequente de derivados polifenólicos reduz o risco de deposição de lipoproteína de baixa densidade (LDL) dificultando a aterosclerose (Marrugat *et al.*, 2004; Scalbert *et al.*, 2005; D'archivio *et al.*, 2007; Singla *et al.*, 2019).

O efeito imunomodulador dos polifenóis é suportado por diferentes estudos: alguns polifenóis afetam diferentes populações de células imunes, modulam a produção de citocinas e a expressão de genes pró-inflamatórios (John *et al.*, 2011; Karasawa *et al.*, 2011). A ação dos polifenóis tem sido investigada, em particular, sobre a secreção de MMPs, citocinas e fatores de crescimento e sobre os mecanismos que contribuem para a perda da homeostase normal na articulação sinovial e levam a erosão óssea (Funk *et al.*, 2006). Dentre os polifenóis estudados na AR pode-se destacar a quercetina, a curcumina e a epigalocatequina galato.

A quercetina é um dos bioflavonóides mais comuns na natureza e apresenta baixa toxicidade (Okamoto, 2005). Este flavonoide é encontrado em grandes quantidades em muitos alimentos vegetais, como maçãs, chás e cebola, e forma uma parte significativa da ingestão diária de polifenóis (Manach *et al.*, 2005). A quercetina apresenta propriedades antioxidantes proeminentes, incluindo eliminação de radicais de oxigênio, redução da peroxidação lipídica e quelação de íons metálicos (Kandaswami e Middleton, 1994; Formica e Regelson, 1995). Este polifenol inibe a artrite gotosa em camundongos pela redução do edema articular, hiperalgesia, infiltração de leucócitos, produção de IL-1β, PGE_2, óxido nítrico (NO) e a expressão da COX-2 (Huang *et al.*, 2012; Ruiz-Miyazawa *et al.*, 2017). Além disso, a quercetina inibe efetivamente a proliferação de sinoviócitos e a angiogênese em um processo inflamatório associado à artrite (Jackson *et al.*, 2006). Recentemente foi demonstrado que a quercetina diminui a gravidade dos sinais clínicos da doença na artrite induzida por colágeno (CIA) e protege a cartilagem e o osso da destruição (Haleagrahara *et al.*, 2017). Curiosamente, a quercetina demonstrou, no modelo de CIA, ser uma terapia anti-inflamatória, imunossupressora e protetora melhor do que o MTX (Haleagrahara *et al.*, 2017).

A curcumina é um polifenol que possui uma ampla variedade de propriedades anti-inflamatórias que a tornam uma potente molécula bioativa anti-artrítica. A curcumina é

derivada da cúrcuma (*Curcuma longa*), um produto vegetal que tem demonstrado atenuar a inflamação (Gupta *et al.*, 2013; Srivastava *et al.*, 2016). O potencial da curcumina em pacientes com AR foi relatado pela primeira vez em 1980, tendo apresentado redução do inchaço das articulações, rigidez matinal e melhora do tempo de caminhada (Deodhar *et al.*, 1980). A administração oral de curcumina inibiu a CIA em camundongos, reduzindo a infiltração celular, a hiperplasia sinovial a destruição da cartilagem e a erosão óssea (Mun *et al.*, 2009). Em ensaios clínicos em pacientes com AR foi demonstrado que a curcumina reduz a dor, a sensibilidade e inchaço da articulação (Chandran e Goel, 2012). Corroborando, Meriva® (um medicamento a base de curcumina) demonstrou eficácia em ensaios clínicos com pacientes com osteoartrite, onde diminuiu a dor relatada (escala VAS), a rigidez e os sinais físicos (teste em esteira), juntamente com os baixos níveis de IL-1β, IL-6 e sVCAM (Belcaro *et al.*, 2010; Di Pierro *et al.*, 2013).

Outro produto natural amplamente estudado em modelos de artrite é a epigalocatequina galato (EGCG), um dos principais polifenóis presentes no chá verde (Khan *et al.*, 2006). Em modelo experimental de AR, a administração profilática de polifenóis do chá verde melhorou a gravidade da artrite (Haqqi *et al.*, 1999). Estudos subsequentes mostraram que o EGCG possui potencial para prevenir doenças crônicas como a osteoartrite e a AR (Singh *et al.*, 2002; Ahmed *et al.*, 2004; Ahmed *et al.*, 2005; Ahmed *et al.*, 2008; Yun *et al.*, 2008). Os efeitos anti-inflamatórios e antiartríticos do EGCG são suportados por dados *in vitro* e *in vivo*, indicando que o EGCG pode regular a produção de citocinas, quimiocinas, mediadores lipídicos, MMPs, ROS, NO, bem como a expressão de COX-2 em diversos tipos celulares relevantes para a patogênese da AR (Haqqi *et al.*, 1999; Singh *et al.*, 2002; Ahmed *et al.*, 2004; Ahmed *et al.*, 2005; Ahmed *et al.*, 2008; Yun *et al.*, 2008). Em estudos *in vivo*, foi observado que o EGCG inibe a inflamação em modelos animais, afetando o funcionamento de células T e neutrófilos (Donà *et al.*, 2003; Aktas *et al.*, 2004).

Os ácidos fenólicos são responsáveis por cerca de um terço dos compostos polifenólicos na nossa dieta e são encontrados em todas as plantas, mas são particularmente abundantes em frutas com sabor ácido. Ácido caféico, ácido gálico e o ácido ferúlico são alguns ácidos fenólicos comuns. Os ácidos fenólicos também vêm sendo descritos como tendo efeitos anti-AR (Kwak *et al.*, 2013; Yoon *et al.*, 2013; Pašková *et al.*, 2016; Neog *et al.*, 2017). Macrófagos pré-incubados com ácido ferúlico, que é encontrado em grãos, vegetais, frutas e nozes, tiveram uma redução na expressão de NFATc1, c-Fos, NF-κB, TRAP, MMP-9 e na atividade de catepsina (Doss *et al.*, 2018).

Existem vários outros produtos naturais além dos descritos anteriormente que possuem atividade anti-inflamatória e são relatados como benéficos no tratamento da artrite e alguns outros distúrbios muscoloesqueléticos em modelos experimentais e pacientes (Goldbach-Mansky *et al.*, 2009; Choudhary *et al.*, 2015; Hoscheid e Cardoso, 2015; Farzaei *et al.*, 2016; Dudics *et al.*, 2018). Os produtos derivados de plantas são muito promissores, mas exigem ampla investigação em vários estudos pré-clínicos e clínicos para comprovarem sua utilidade. Apesar de limitados, os estudos em humanos sugerem que plantas medicinais tradicionais usadas para a AR têm menos efeitos adversos do que os fármacos convencionais (Farzaei *et al.*, 2016). Estudos bem planejados são necessários para avaliar os efeitos dos produtos naturais tradicionais em termos de resultados sintomáticos, funcionais e biológicos. Os agentes naturais atuais também podem ser testados como terapias adjuvantes em combinação com drogas convencionais para AR.

1.5 GALATO DE METILA

O galato de metila (GM) (Figura 1.5), um éster metílico do ácido gálico, é um composto fenólico com notável efeito antioxidante, sendo amplamente distribuído em plantas medicinais e alimentícias, como *Schinus terebinthifolius*, *Galla Rhois*, *Rosa rugosa* e *Givotia rottleriformis* Griff. (Cho *et al.*, 2004; Kang *et al.*, 2009; Kamatham *et al.*, 2015; Rosas *et al.*, 2015). Além da sua ação antioxidante, o GM tem uma série de efeitos biológicos descritos, tais como atividade antialérgica (Cavalher-Machado *et al.*, 2008), antitumoral (Lee *et al.*, 2010; Lee *et al.*, 2013), antimicrobiana (Choi *et al.*, 2014; Acharyya *et al.*, 2015) e anti-plaquetária (Hsieh *et al.*, 2004). Como antioxidante o GM protege o DNA do dano causado pelo estresse oxidativo e possui propriedades benéficas contra o dano induzido por peróxido de hidrogênio na viabilidade celular (Hsieh *et al.*, 2004; Crispo *et al.*, 2010).

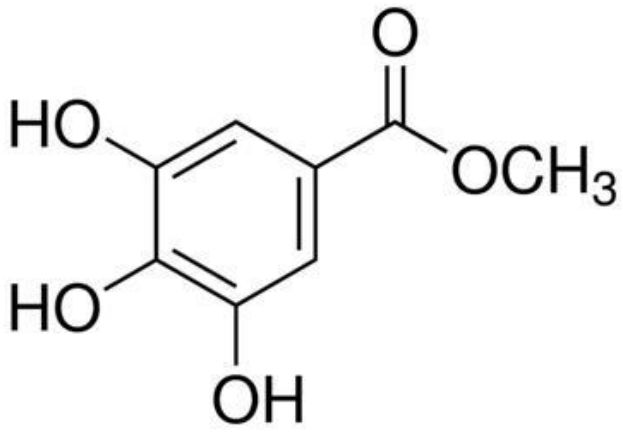

Figura 1.5: Estrutura química da substância galato de metila.

Alguns estudos sugerem que o GM possui efeito anti-inflamatório (Kim *et al.*, 2006; Chae *et al.*, 2010), dentre esses efeitos destacam-se a eliminação de radicais livres, e a inibição da expressão e da atividade de mediadores inflamatórios. Chae e colaboradores (2010) relataram que o GM extraído da espécie vegetal *Galla Rhois* foi capaz de inibir a produção de IL-6 e NO através da redução da fosforilação da proteína quinase regulada por sinal extracelular (ERK) em macrófagos. Além disso, foi demonstrado que o GM atua como um potente inibidor da atividade de COX-2 e 5-lipoxigenase (LOX) (Kim *et al.*, 2006; Kamatham *et al.*, 2015; C S *et al.*, 2018). Baek e colaboradores (Baek *et al.*, 2017), demonstraram recentemente que o GM atenua a diferenciação e maturação de osteoclastos dependente de RANKL, incluindo a estrutura da F-actina e a atividade de reabsorção óssea *in vitro*, sugerindo que o GM pode ser um candidato para o tratamento da osteoporose. Apesar dos efeitos descritos, não há relatos científicos que demonstrem que o GM tenha efeitos anti-inflamatórios em modelos *in vivo*.

Neste trabalho, foi investigado as propriedades anti-inflamatórias do GM em modelos de inflamação induzida por zimosan, dando ênfase ao modelo de artrite experimental. Foi avaliado também os efeitos dessa substância no modelo de artrite por antígeno que propicia a observação de respostas imunes mais próximas daquelas observadas na artrite humana. Além disso, buscou-se elucidar o mecanismo de ação desse derivado de polifenol tão amplamente difundido na natureza, objetivando assim comprovar cientificamente o valor medicinal do GM e destacar a importância dessa substância como uma candidata com potencial anti-inflamatório para o controle da artrite.

2 OBJETIVOS

2.1 Objetivo geral:

Estudar o efeito anti-inflamatório do galato de metila (GM) em modelos de artrite experimental em camundongos e elucidar o seu mecanismo de ação, estudando vias de sinalização em células envolvidas no processo inflamatório articular.

2.2 Objetivos específicos:

1. Estudar o efeito anti-inflamatório do GM na artrite experimental induzida por zimosan avaliando diversos parâmetros inflamatórios: a) formação de edema articular; b) influxo de células para a cavidade sinovial; c) avaliação histopatológica do tecido; d) produção de mediadores inflamatórios (citocinas, quimiocinas, leucotrienos e prostaglandinas);

2. Estudar o mecanismo de ação do GM, avaliando as vias de sinalização intracelular envolvidas na produção de mediadores inflamatórios;

3. Estudar o efeito do tratamento prolongado com o GM na artrite experimental induzida por antígeno avaliando diversos parâmetros inflamatórios e bioquímicos.

3 ARTIGOS

3.1 ARTIGO 1

Anti-inflammatory Effect of Methyl Gallate on Experimental Arthritis: Inhibition of Neutrophil Recruitment, Production of Inflammatory Mediators, and Activation of Macrophages

Autores: Luana Barbosa Correa, Tatiana Almeida Pádua, Leonardo Noboru Seito, Thadeu Estevam Moreira Maramaldo Costa, Magaiver Andrade Silva, André Luis Peixoto Candea, Elaine Cruz Rosas e Maria G. Henriques.

Journal of Natural Products, 79(6), 1554-66, 2016

Trabalhos prévios descrevem efeitos biológicos para o GM, tais como atividade antioxidante, antitumoral e antimicrobiana. No entanto, ainda não existiam evidências sobre o efeito anti-inflamatório do GM em modelos de inflamação *in vivo*. Nesse trabalho, foi avaliado o efeito anti-inflamatório do GM nos modelos de edema de pata, pleurisia e artrite experimental induzida pelo zimosan. Foi demonstrado o efeito farmacológico do GM sobre o influxo celular, formação de edema e a produção de mediadores inflamatórios. Somado a isso, aprofundou-se no estudo sobre o mecanismo de ação celular do GM na migração de neutrófilos avaliando sua quimiotaxia e adesão, aferindo também diversos parâmetros de ativação de macrófagos, que são células cruciais para o desenvolvimento desse modelo. Os resultados obtidos nesse trabalho demonstraram que o GM possui um notável efeito anti-inflamatório, reduzindo significativamente a migração de neutrófilos, a produção de mediadores inflamatórios e a ativação de células. Esses dados destacam a importância de um estudo mais aprofundado sobre

o mecanismo de ação do GM, reforçando a importância dos produtos naturais como fontes terapêuticas para a modulação do processo inflamatório.

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Anti-inflammatory Effect of Methyl Gallate on Experimental Arthritis: Inhibition of Neutrophil Recruitment, Production of Inflammatory Mediators, and Activation of Macrophages

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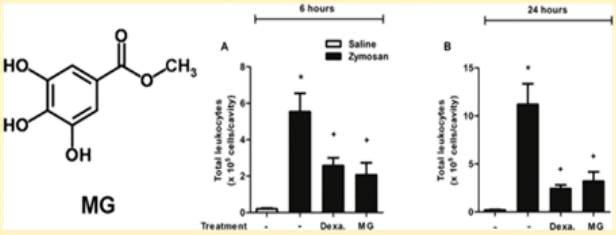
Supporting Information

ABSTRACT: Methyl gallate (MG) is a prevalent phenolic acid in the plant kingdom, and its presence in herbal medicines might be related to its remarkable biological effects, such as its antioxidant, antitumor, and antimicrobial activities. Although some indirect evidence suggests anti-inflammatory activity for MG, there are no studies demonstrating this effect in animal models. Herein, we demonstrated that MG (0.7−70 mg/kg) inhibited zymosan-induced experimental arthritis in a dose-dependent manner. The oral administration of MG (7 mg/kg) attenuates arthritis induced by zymosan, affecting edema formation, leukocyte migration, and the production of inflammatory mediators (IL-1β, IL-6, TNF-α, CXCL-1, LTB_4, and PGE_2). Pretreatment with MG inhibited *in vitro* neutrophil chemotaxis elicited by CXCL-1, as well as the adhesion of these cells to TNF-α-primed endothelial cells. MG also impaired zymosan-stimulated macrophages by inhibiting IL-6 and NO production, COX-2 and iNOS expression, and intracellular calcium mobilization. Thus, MG is likely to present an anti-inflammatory effect by targeting multiple cellular events such as the production of various inflammatory mediators, as well as leukocyte activation and migration.



Arthritis is joint inflammation that can cause edema, pain, and loss of function in the joints. The most common types of arthritis are osteoarthritis, gout, and rheumatoid arthritis (RA).[1,2] The inflammatory process in RA results from the dysregulation of pro-inflammatory cytokines. Tumor necrosis factor (TNF-α), interleukin (IL)-1β, cyclooxygenase-2 (COX-2)-derived pro-inflammatory prostaglandins (PG), and the leukotrienes (LT) produced by lipoxygenases, together with the infiltration of the polymorphonuclear (PMN) and mononuclear leukocytes, are all important elements that highlight the crucial role of the immune system in the pathogenesis of RA.[3,4]

Experimental models of arthritis provide an important approach for evaluating potential anti-inflammatory molecules.[5,6] Zymosan-induced articular inflammation is an arthritis experimental model associated with increases in vascular permeability, neutrophil and mononuclear cell migration, hyperalgesia, and the production of inflammatory mediators, such as TNF-α, IL-1β, IL-6, CXCL-1, PGE_2, and LTB_4.[7−9]

Methyl gallate (MG) is a polyphenol found in various plants and natural products including *Schinus terebinthifolius*, *Rosa rugosa*, and Galla rhois.[10−12] It has been extensively studied because of its antioxidant, antitumor, and antimicrobial activities.[13−15] Regarding antioxidant effects, it has been demonstrated that MG is a free radical scavenger that inhibits lipid peroxidation and shows protective effects against DNA damage resulting from oxidative stress.[16,17] Crispo and co-workers (2010)[18] demonstrated that MG exhibits defensive effects against mitochondrial depolarization, caspase-9 cleavage, and DNA fragmentation in H_2O_2-induced apoptotic conditions. MG has also been shown to delay tumor progression in tumor-bearing animals, displaying an anticancer effect.[19] Indirect evidence suggests an anti-inflammatory activity of MG. Chae and co-workers (2010)[20] showed that MG from Galla rhois inhibited lipopolysaccharide (LPS)-induced IL-6 secretion, nitric oxide (NO) production, and decreasing ERK1/2 phosphorylation in a murine macrophage cell line. However, until now, there have been no descriptions in the literature concerning the *in vivo* anti-inflammatory effect of MG.

Herein, we have demonstrated that oral pretreatment with MG suppresses articular inflammation in the experimental model of zymosan-induced arthritis (ZIA) and elucidated the cellular mechanism of action of this polyphenol.

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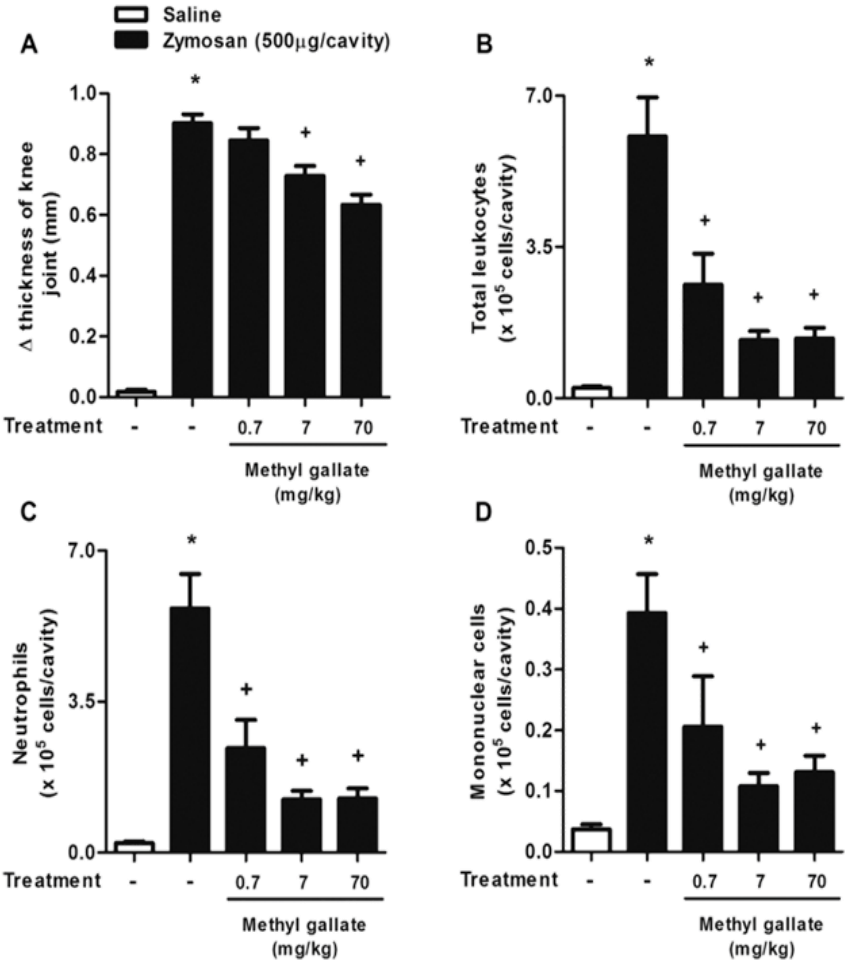

Figure 1. Dose−response analysis for methyl gallate on experimental arthritis induced by zymosan. (A−D) Mice were treated with MG (0.7−70 mg/kg) or vehicle (saline) orally 1 h before i.a. injection of zymosan (500 μg per cavity in 25 μL of sterile saline). The control group was injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper 6 h after zymosan stimulation (panel A). The numbers of total leukocytes (B), neutrophils (C), and mononuclear cells (D) in the synovial cavity were assessed 6 h after zymosan stimulation. The results are presented as the means ± SEM of seven mice per group per experiment and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group; $^{+}p \leq 0.05$ compared to the zymosan group (one-way ANOVA followed by Student−Newman−Keuls test)].

RESULTS AND DISCUSSION

Dose−Response Analysis of Methyl Gallate on Cellular Influx and Edema Induced by Zymosan in Mice. The intra-articular injection of zymosan produces a severe and erosive synovitis associated with acute increases in vascular permeability and cell migration, followed by a progressive synovitis.[7,21] In this study, we evaluated the time course of zymosan-induced arthritis in C57BL/6 mice (Figure S1, Supporting Information). In agreement with previous reports,[22,23] the intra-articular (i.a.) injection of zymosan (500 μg/cavity) induced increased knee joint thickness within 6 and 24 h. Here, we also observed that total leukocyte accumulation with a predominance of neutrophils increases within 6 h and remains high until 24 h. Pretreatment with MG (0.7−70 mg/kg, p.o.) was able to inhibit zymosan-induced edema formation at 6 h, whereas no effect was observed for pretreatment with MG at 0.7 mg/kg (Figure 1A). As shown in Figure 1B−D, the leukocyte recruitment was significantly inhibited by the three doses of MG tested, and the same inhibitory effect was observed with doses of 7 and 70 mg/kg. Considering these results, further analyses were performed within 6 and 24 h after stimulation using a dose of 7 mg/kg. Reinforcing the anti-inflammatory effect, the treatment with MG was also able to inhibit two additional models of inflammation, pleurisy and paw edema (Figure S2, Supporting Information). Figure S2A shows that pretreatment with MG (from 1 to 50 mg/kg, p.o.) was able to significantly inhibit paw edema formation (4 h) at doses of 10 and 50 mg/kg. Pretreatment with MG also reduced protein extravasation and neutrophil influx in a pleurisy model at the same doses observed in the paw edema model (10 and 50 mg/kg) (Figure S2B,C). It is likely that MG anti-inflammatory activity involves inhibition of the events that lead to edema formation, plasma exudation, and leukocyte recruitment.

Methyl Gallate Attenuates Articular Inflammatory Response Induced by Zymosan. We investigated the effect of MG at both of the time points discussed above, and we compared its action with that of dexamethasone, an anti-inflammatory reference drug. Oral pretreatment with 7 mg/kg of MG significantly inhibited edema formation at 6 h (24%) and 24 h (49%) after zymosan injection in the knee joint (Figure 2A,B). The reference drug dexamethasone (10 mg/kg, i.p.) also inhibited edema formation at both times (42% and 48%, respectively; Figure 2A,B). The joint swelling in response to zymosan is the result of cascading events that include complement system activation, mast cell degranulation, the generation of 5-lipoxygenase and cyclooxygenase metabolites, and NO production.[8,24−27] It was previously demonstrated that MG inhibited the NO production by LPS-stimulated macro-

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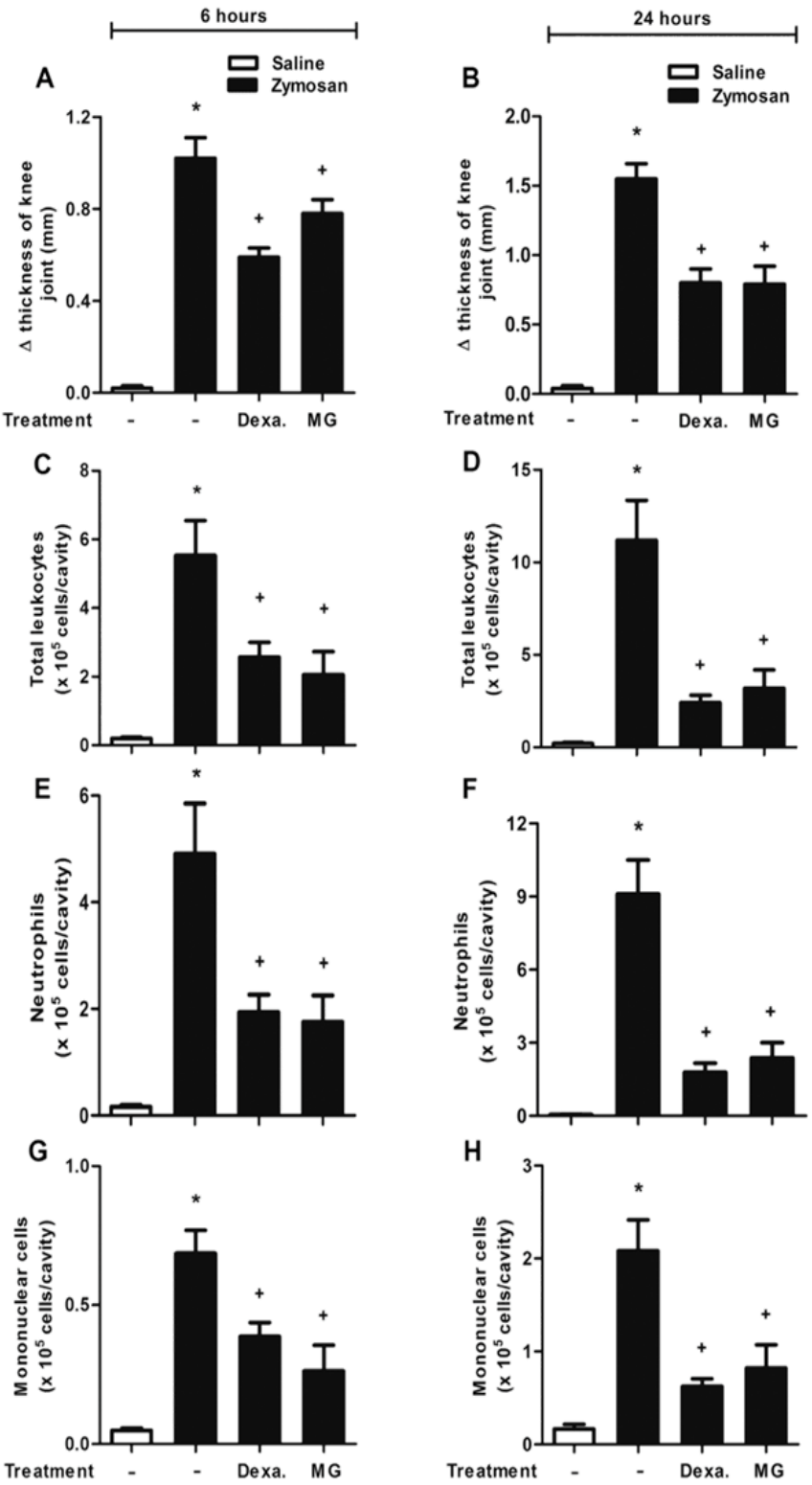

Figure 2. Methyl gallate inhibits edema and leukocyte influx into the articular cavity induced by zymosan. (A–H) Mice were treated with saline, dexamethasone (Dexa, i.p., 10 mg/kg), or MG (p.o., 7 mg/kg) 1 h before i.a. injection of zymosan (500 μg per cavity in 25 μL of sterile saline). The control group was injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper 6 and 24 h after zymosan stimulation (A and B). The numbers of total leukocytes (C and D), neutrophils (E and F), and mononuclear cells (G and H) are plotted as number of cells × 10^5. Knee synovial cells were recovered 6 and 24 h after zymosan stimulation. The results are presented as the means ± SEM of seven mice per group per experiment and are representative of three separate experiments [$^*p \leq 0.05$ compared to the saline group; $^+p \leq 0.05$ compared to the zymosan group (one-way ANOVA followed by Student–Newman–Keuls)].

phages.[20] Moreover, MG reduced histamine release by mast cells stimulated with C48/80.[28] In light of these findings, the antiedematogenic effect of MG might be exerted through the inhibition of NO and histamine release during joint swelling.

Pretreatment with MG (7 mg/kg) reduced total leukocyte accumulation in the joint cavity 6 and 24 h after stimulation (Figure 2C,D), particularly by the reduction of neutrophil migration at both times studied (64% and 74%, respectively)

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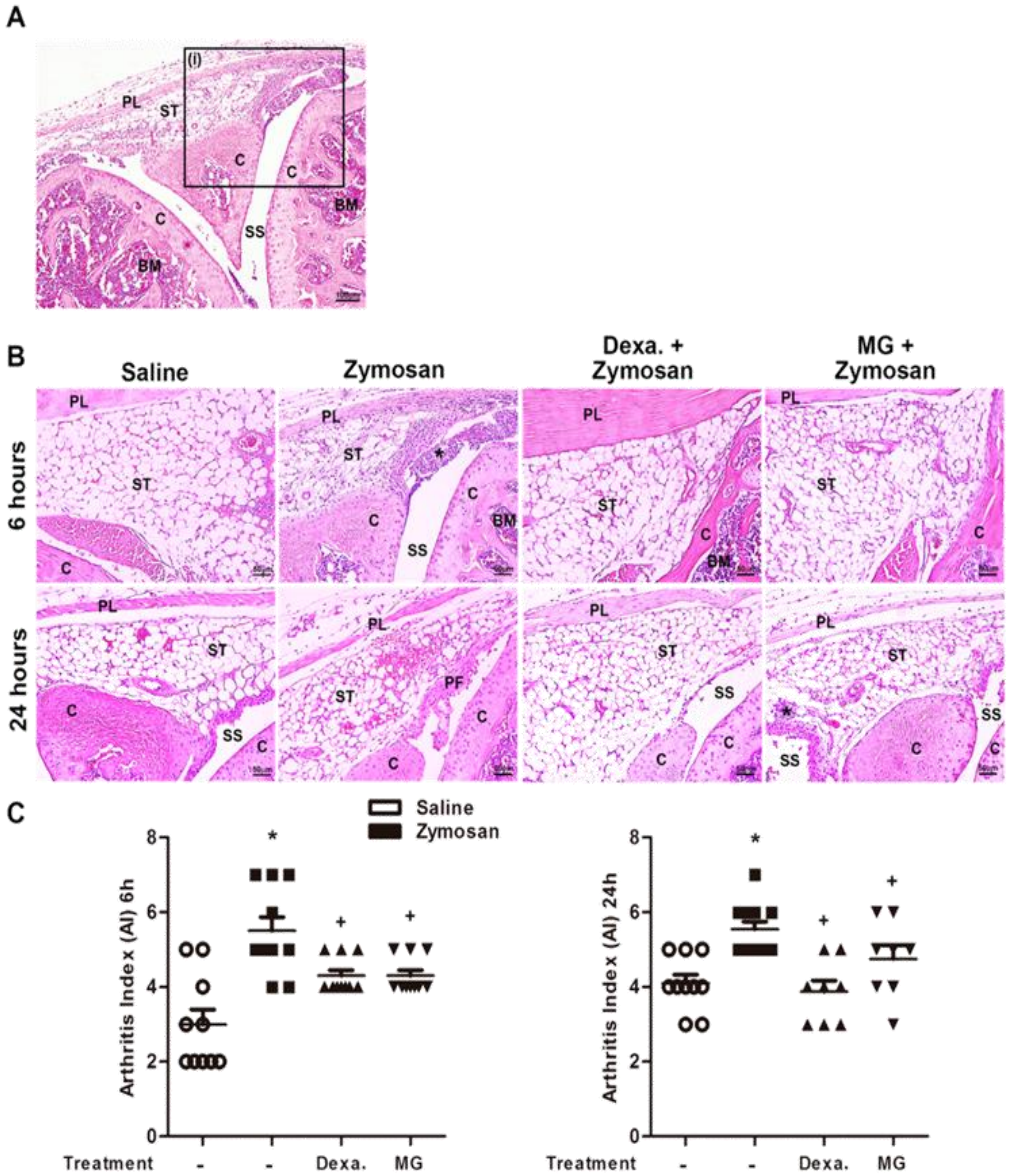

Figure 3. H&E-stained representative histological images from different knee joints injected with saline or zymosan collected 6 or 24 h after stimulation. (A) Image representative of at least six different animals stimulated with zymosan, (i) detached area containing the structures that were evaluated in panel B at higher magnification (10×; bars = 100 μm). (B) Representative images demonstrating histopathology after 6 and 24 h of zymosan stimulus (20×; bars = 50 μm). Saline controls show normal articular cartilage surface and synovial membrane, with absence of inflammatory cells. Non-zymosan-treated group shows the intense infiltration of inflammatory cells (*) and bleeding scattered diffusely in the synovial tissue. A significant reduction in the infiltration of inflammatory cells and local bleeding can be observed in mice pretreated with dexamethasone or MG at both assessed times. (C) Arthritis index (AI) analysis after ZIA onset. The results are presented as the means ± SEM from six mice per group and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group; $^{+}p \leq 0.05$ compared to the zymosan group (one-way ANOVA followed by Student−Newman−Keuls test)]. C: cartilage; SS: synovial space; PL: patellar ligament; ST: synovial tissue; BM: bone marrow; PF: pannus formation.

(Figure 2E,F). In addition, MG decreased the number of mononuclear cells within 6 and 24 h after stimulus (Figure 2G,H). The positive control, dexamethasone, similarly inhibited zymosan-induced migration of total leukocytes, neutrophils, and mononuclear cells. To confirm these findings, we evaluated the effect of MG on histological joint sections of knee joints (Figure 3). Figure 3A shows representative images of at least six different animals. The highlighted region (i) is shown at higher magnification (20×) in Figure 3B, and it includes part of the synovial tissue, patellar ligament, cartilage, and synovial space. Histopathological analysis of the femorotibial joints of zymosan-stimulated (6 and 24 h) mice revealed intense infiltration of polymorphonuclear cells into the synovium and the periarticular tissues (Figure 3B). The zymosan-stimulated mice showed an increase in the overall arthritis score in comparison to control mice (Figure 3C). Histologic examination of serial joint sections showed that synovial hyperplasia, inflammation, and bleeding were significantly lower in mice treated with MG and dexamethasone, as could be seen in the representative figures for each group (Figure 3B) and their arthritis index scores (Figure 3C).

Neutrophils are the most abundant leukocytes in inflamed joints, and some reports have demonstrated the importance of these cells in the initiation and perpetuation of human RA, as well as in murine models.[29,30] Potential neutrophil effector mechanisms include the release of granules containing

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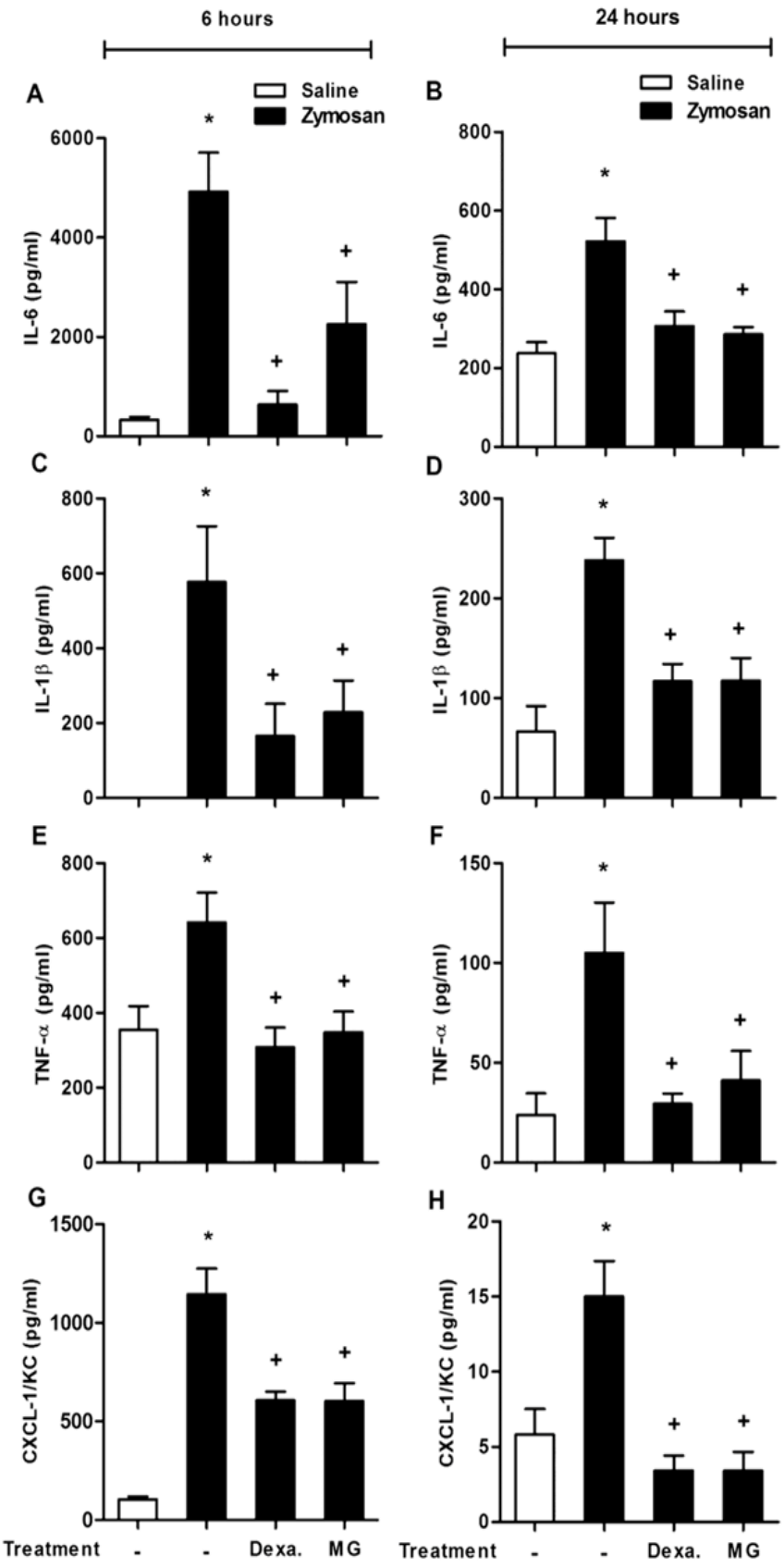

Figure 4. Methyl gallate inhibits inflammatory mediators induced by zymosan. (A–H) Mice were treated with saline, dexamethasone (Dexa, i.p., 10 mg/kg), or MG (p.o., 7 mg/kg) 1 h before i.a. injection of zymosan (500 μg per cavity in 25 μL of sterile saline). The control group was injected with the same volume of sterile saline. Protein levels of IL-6 (A and B), IL-1β (C and D), TNF-α (E and F), and CXCL-1/KC (G and H) were determined in cell-free supernatant of knee-joint synovial washes by ELISA. Analysis was performed 6 and 24 h after zymosan injection. The results are presented as the means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group; $^{+}p \leq 0.05$ compared to the zymosan group (one-way ANOVA followed by Student–Newman–Keuls test)].

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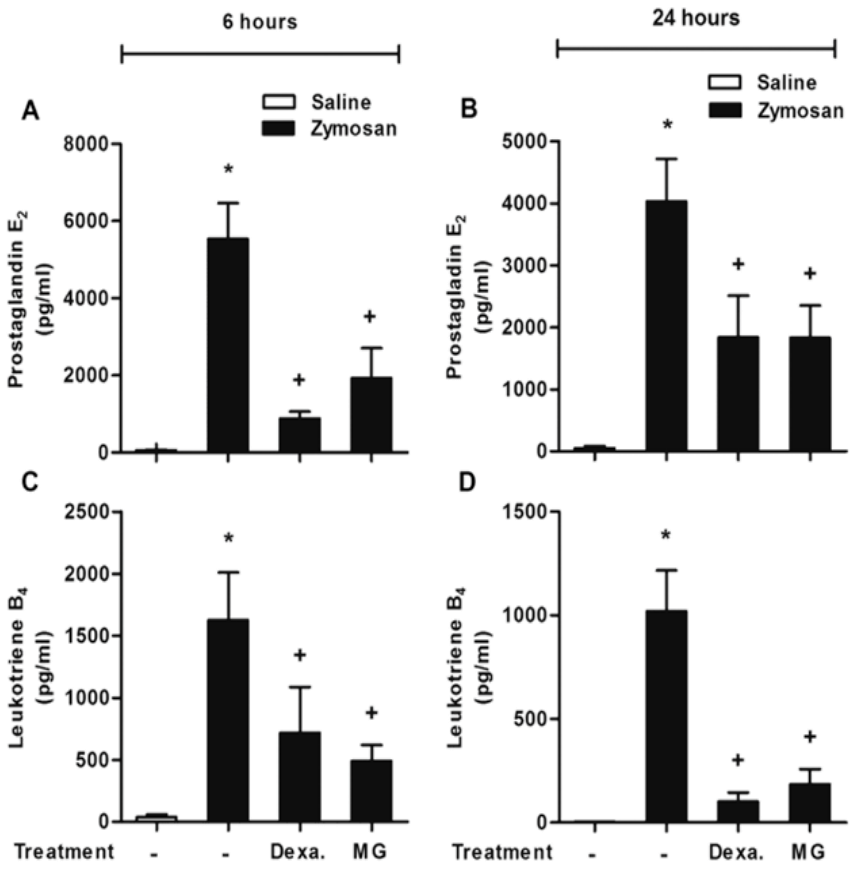

Figure 5. Methyl gallate inhibits lipid mediators induced by zymosan. (A–D) Mice were treated with saline, dexamethasone (Dexa, i.p., 10 mg/kg), or MG (p.o., 7 mg/kg) 1 h before i.a. injection of zymosan (500 μg per cavity in 25 μL of sterile saline). The control group was injected with the same volume of sterile saline. Levels of prostaglandin E_2 (A and B) and leukotriene B_4 (C and D) were determined in cell-free supernatant of knee-joint synovial washes by EIA. Analysis was performed 6 and 24 h after zymosan injection. The results are presented as the means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group; $^{+}p \leq 0.05$ compared to the zymosan group (one-way ANOVA followed by Student–Newman–Keuls test)].

degradative enzymes such as myeloperoxidase, elastase, matrix metalloproteinases (MMPs), and collagenase that can cause further damage to the tissue and amplify the neutrophil response.[31,32] Activated neutrophils release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 that potentially affect the activities of both neutrophils and other cell types, such as resident mononuclear cells and chondrocytes.[33,34] Therefore, neutrophils play an essential role in joint inflammation, and the modulation of neutrophil functions is considered a potential target for pharmacological intervention in arthritis.[35−37] Our findings suggest that treatment with MG is a plausible approach to control neutrophil recruitment during inflammation, preventing neutrophil-mediated tissue injury.

Methyl Gallate Reduces the Production of Inflammatory Mediators in Experimental Arthritis. Inflammatory signaling pathways in response to zymosan lead to the expression of many pro-inflammatory cytokines, chemokines, and lipid mediators that are detected at early time points in murine models of inflammation.[38,39] In this study, we observed an increase in the production of cytokines, chemokines, and lipid mediators in the mouse joint cavity at 6 and 24 h after zymosan stimulus (Figures 4 and 5). Pretreatment with MG (7 mg/kg) significantly inhibited the zymosan-induced production of IL-6, TNF-α, IL-1β, and CXCL-1/KC, as well as the lipid mediators PGE_2 and LTB_4, at 6 and 24 h, as observed with the reference drug dexamethasone (10 mg/kg, i.p.) (Figures 4 and 5).

Therapeutic strategies that block or antagonize pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been used for the treatment of RA and found to be beneficial in preventing the progression of the disease in patients.[40−42] Experimentally, these cytokines play a critical role in inflammatory hypernociception, drive the production of a neutrophil chemoattractant factor, and increase cell adhesion molecule expression in arthritis experimental models.[43−46] The inhibition of TNF-α and IL-1β receptors (TNFR1 and IL-1R1, respectively) reduced neutrophil migration in the model of antigen-induced arthritis (AIA).[47] Moreover, Sachs and co-workers (2011)[47] showed that the blockade of neutrophil influx with fucoidan decreases TNF-α and IL-1β production. Thus, in AIA, TNF-α and IL-1β are necessary for adequate neutrophil influx, but neutrophils are also essential for the full production of these cytokines. Here, we show that pretreatment with MG was able to reduce the levels of these cytokines at 6 and 24 h after zymosan injection (Figure 4C–F). However, it is necessary to clarify whether MG inhibits the early production of these mediators or whether the reduction in neutrophil accumulation was the reason for the TNF-α and IL-1β decrease observed in our results.

Multiple chemoattractants can directly induce neutrophil migration. Chou and co-workers (2010)[48] demonstrated in the K/BxN murine model of arthritis that LTB_4 was critical for initial neutrophil infiltration. A significant release of LTB_4 1 h after zymosan injection was demonstrated in zymosan-induced

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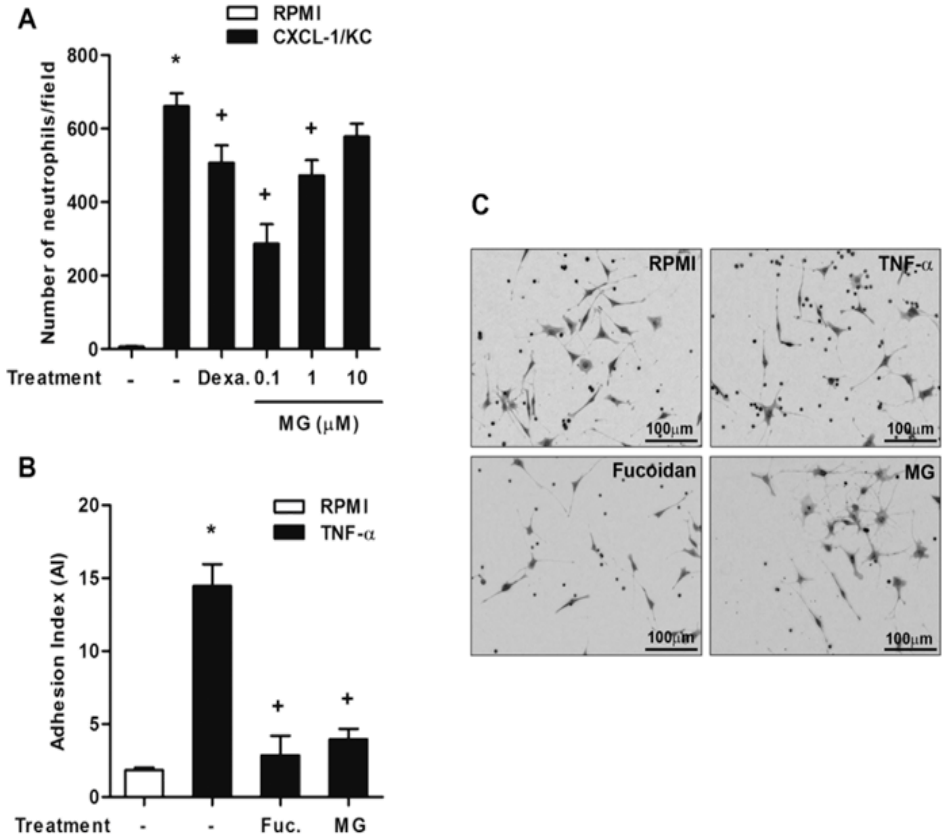

Figure 6. Methyl gallate inhibits murine neutrophil chemotaxis and adhesion. (A) Neutrophils recovered from mouse bone marrow were preincubated for 1 h with medium (RPMI), dexamethasone (Dexa, 50 nM), or MG (0.1, 1, or 10 μM) and allowed to migrate toward CXCL-1/KC (250 nM) for 1 h in a chemotaxis chamber. The migrated cells were counted using light microscopy. The results are expressed as numbers of neutrophils per field per well. (B) Neutrophils were preincubated for 1 h with medium, fucoidan (25 μg/mL), or MG (0.1 μM) and allowed to adhere to previously stimulated tEnd.1 (50 neutrophils/tEnd.1) for 1 h. Fucoidan was used as the positive control. Adhesion was quantified by an association index. (C) Photomicrographs of adherent neutrophils on stimulated tEnd.1 (original magnification 60×; bars = 100 μm). The results are presented as the means ± SEM of quadruplicate wells per group per experiment and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group; $^{+}p \leq 0.05$ compared to the stimulated group (one-way ANOVA followed by Student−Newman−Keuls test)].

arthritis.[49] Because the neutrophils' influx into the joints in ZIA starts at least after 2 h, it is clear that the LTB_4 production precedes the influx of these cells. However, the treatment with fucoidan or antineutrophil antibody inhibited the zymosan-induced LTB_4 production in the joint 7 h after zymosan stimulation.[9] These data show that although resident cells, such as synoviocytes, might account for the immediate LTB_4 release, neutrophils are responsible for further production of LTB_4.

These LTB_4-recruited neutrophils produce cytokines such as IL-1β, which then act on resident cells, inducing the expression of chemokine ligands for CXCR-1 and CXCR-2 (including CXCL-1/KC).[8,50] These data show the existence of a positive-feedback loop involving lipid−cytokine−chemokine cascades that drive neutrophil recruitment and the development of arthritis.[51] In this study, we demonstrated that MG pretreatment reduces the increases in LTB_4, CXCL-1/KC, and IL-1β in knee joint fluids after zymosan stimulus (Figures 4C−H and 5B,C), as well as the zymosan-induced massive neutrophil migration. It is very likely that MG modulates lipid−cytokine−chemokine cascades and impairs neutrophil influx into the inflammatory site.

We also observed an inhibition of PGE_2 production by MG pretreatment at 6 and 24 h (Figure 5A,B). This result is in agreement with the findings of Kim and co-workers (2006),[52] who showed that mast cells failed in PGD_2 generation due to the inhibition of COX-2-enzyme activity by MG. PGE_2 produced by synoviocytes and macrophages in synovial space induces increased vascular permeability and is one of the main mediators responsible for edema formation, including in ZIA,[24] and it is involved centrally in fever and pain production.[53] Studies *in vitro* and in animal models have demonstrated that PGE_2 induces bone resorption by osteoclasts[54] and regulates type II collagen synthesis and degradation and that a high concentration of PGE_2 stimulates the release of cartilage-degrading MMPs.[55] The reduction in the levels of PGE_2 in the joint after pretreatment with MG might be related to the reduction in paw edema formation and tissue damage as observed in the above results.

Methyl Gallate Inhibits the Chemotactic Activity and Adhesion of Neutrophils *in Vitro*. We have previously shown that MG can reduce the *in vivo* recruitment of neutrophils, as well as the production of pro-inflammatory cytokines and chemokines. In this part of the study, we evaluated whether MG could directly inhibit neutrophil migration upon chemotactic stimulus. The effect of MG (0.01−100 μM) on neutrophil viability was initially assessed using the lactate dehydrogenase assay, which showed that none of the tested concentrations were cytotoxic (≥92% viability at 100 μM) (Supplemental Table 1). Next, we tested the *in vitro* effect of MG on neutrophil chemotaxis. As shown in Figure 6A, CXCL-1/KC induced a significant increase in the number of migrant neutrophils compared to basal migration in the control

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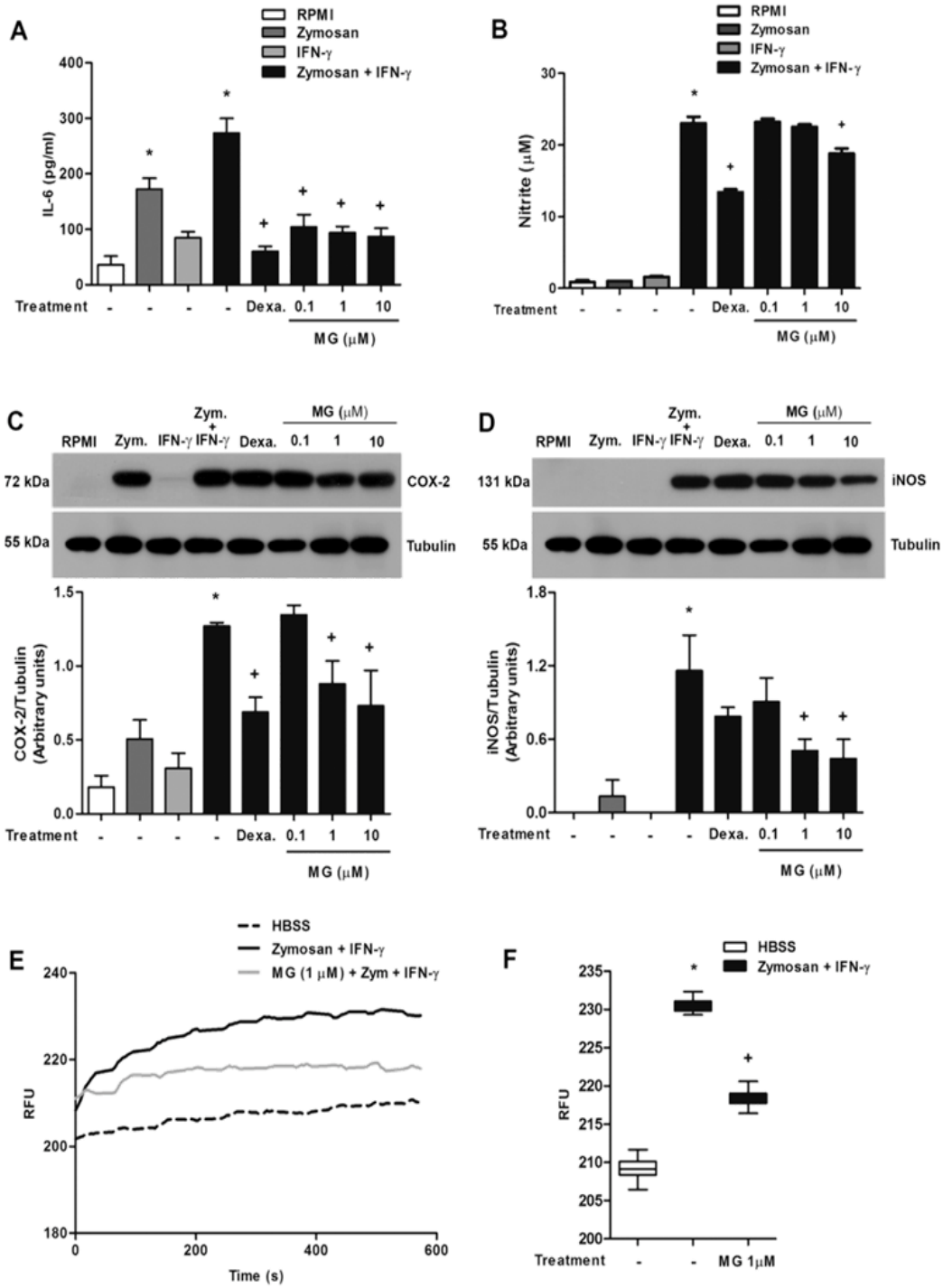

Figure 7. Methyl gallate inhibits macrophage activation. (A–D) J774A.1 macrophages were preincubated for 1 h with medium, dexamethasone (Dexa, 1 μM), or MG (0.1, 1, or 10 μM) and subjected to stimulation with zymosan (10 μg per 10^5 cells), IFN-γ (25 U/mL), or zymosan plus IFN-γ for 24 h. Levels of IL-6 (A) and NO (B) were determined in cell-free supernatant by ELISA and the colorimetric Griess test, respectively. (C and D) COX-2 and iNOS expression were quantified from cellular extracts by Western blot. (E and F) J774A.1 macrophages were preincubated for 1 h with HBSS or MG (1 μM) and stimulated with zymosan plus IFN-γ. (E) Kinetics of calcium influx of zymosan-stimulated macrophages over 600 s as measured using the FLIPR Calcium Plus assay kit and (F) means of values obtained at 360–600 s for each group after the stimulus. The data were analyzed using SOFTmax Pro. The results are presented as the means ± SEM of quadruplicate wells per group per experiment and are representative of two separate experiments [$^*p \leq 0.05$ compared to the saline group; $^+p \leq 0.05$ compared to the stimulated group (one-way ANOVA followed by Student–Newman–Keuls test)].

wells. Interestingly, MG pretreatment (0.1–10 μM) 1 h prior to CXCL-1/KC stimulation reduced neutrophil migration only at the lower concentrations (0.1 and 1 μM). Recently, Eler and co-workers (2013)[56] showed that MG has lipophilicity, which would facilitate its access to various intracellular environments. Herein, we observed that MG in the two greatest concentrations was not able to inhibit the neutrophil chemotaxis, and this unexpected effect may be due to MG binding to nonspecific sites within the cell and triggering various signaling pathways. It is evident that MG has a potent inhibitory effect on neutrophil migration; however, it seems likely that MG at higher concentrations interacts with different

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molecular targets, modifying the effects observed for lower concentrations. Dexamethasone (50 nM) also impaired neutrophil migration.

CXCL-1/KC initiates neutrophil chemotaxis through binding to G protein-coupled receptors (CXCR-1 and CXCR-2) located on the surfaces of these cells.[57] The activation of those receptors then leads to the activation of protein kinase B (PKB/Akt) and of small GTPases. This signaling regulates F-actin polymerization, which controls the direction of neutrophil migration.[58,59] A recent study showed that MG significantly reduced glioma cell migration by inhibiting the phosphorylation of Akt and the formation of focal adhesions.[15] Because MG inhibited the neutrophil chemotaxis elicited by CXCL-1, possible mechanisms involving G protein-dependent pathways should be considered in further studies.

Another important event associated with neutrophil migration is the interaction of circulating leukocytes with the vascular endothelial cells. To understand the mechanisms involved in the MG-mediated inhibition of neutrophil accumulation, we performed an *in vitro* adhesion assay. The binding of chemokines to their receptors on neutrophils almost instantaneously triggers the activation of surface integrin, which participates in the adhesion cascade of neutrophils.[60] Accordingly, neutrophils in contact with TNF-α-primed endothelial cells exhibited higher adhesion than the control group in this assay. The pretreatment of neutrophils with MG (0.1 μM, 1 h before stimulus) impaired their adhesion to endothelial cells. A similar result was obtained when neutrophils were pretreated with fucoidan, a carbohydrate that inhibits leucocyte migration by binding to L- and P-selectins, consequently inhibiting leukocyte rolling and subsequent adhesion (Figure 6B,C). This result supports the hypothesis that MG might be interfering in the signaling pathway of the chemotactic receptor, thereby avoiding neutrophil activation and the expression of adhesion molecules and consequently preventing the entry of neutrophils into sites of inflammation.

Methyl Gallate Impairs Macrophage Activation. The interaction of zymosan with resident macrophages (type A synovial lining cells) is the first step in the initiation of ZIA.[61,62] To assess the effect of MG on macrophage activation, we evaluated the production of IL-6 and NO, the expression of COX-2 and inducible nitric oxide synthase (iNOS), and the mobilization of intracellular calcium. Thus, macrophages were incubated with different concentrations of MG (0.1−10 μM) or dexamethasone (1 μM) for 1 h. The cells were stimulated with zymosan and/or IFN-γ. The cytotoxic effect of MG in macrophages was examined. As shown in Supplemental Table 2, the incubation of macrophages with MG for 24 h was not cytotoxic at any of the concentrations analyzed (≥95% viability at 0.01−100 μM). Zymosan is recognized through Toll-like receptor 2 (TLR-2) and dectin-1 receptor, resulting in activation of nuclear factor-κB (NF-κB), thereby triggering the production of inflammatory cytokines and chemokines.[63,64] Classically, macrophage activation induced by the combination of a Toll ligand with IFN-γ leads to the production of cytokines such as TNF-α, IL-1β, IL-6, and IL-12 in large quantities.[65,66] In addition, activated macrophages produce reactive nitrogen species, such as the NO produced by iNOS.[67]

IL-6 is a pleiotropic cytokine that regulates the immune response, inflammation, hematopoiesis, and bone metabolism.[68] An increase in IL-6 levels was observed in both the serum and synovial fluid in patients with RA, and serum IL-6 levels were correlated with disease activity and radiographic joint damage.[69,70] Macrophages are the cells that produce inflammatory mediators, including IL-6, in the joint in large quantities.[71] Figure 7A shows that stimulation of macrophages (J774A.1) with zymosan plus IFN-γ significantly increased IL-6 production. Pretreatment with MG (0.1−10 μM) reduced the levels of IL-6 at all concentrations used. In the same way, dexamethasone pretreatment reduced IL-6 production. Unlike in neutrophils, MG at high concentrations was able to inhibit macrophage activity. One explanation for this effect is that the macrophages used are a cell line, whereas the neutrophils are primary cells. Moreover, these cells were stimulated by zymosan plus IFN-γ, which triggers different signaling pathways from those observed in the neutrophils, which were stimulated with CXCL-1/KC. Finally, we evaluated different activities in the two cell types. Thus, MG might be interacting with different molecular targets as well as modulating diverse cellular events to reduce the leukocyte infiltration in the knee joint cavity.

PGE_2 is a lipid mediator produced by COX-2, which is an inducible enzyme expressed in inflammatory conditions such as RA.[54] As previously demonstrated, MG was able to reduce PGE_2 production in ZIA. We then investigated whether this reduction resulted from the interference of MG in the expression of COX-2. Macrophages were pretreated with MG (0.1−10 μM) or with dexamethasone (1 μM) and then stimulated with zymosan and/or IFN-γ for 24 h. As shown in Figure 7C, only stimulation with zymosan plus IFN-γ significantly increased COX-2 expression. Pretreatment with MG at concentrations of 1 and 10 μM reduced the expression of COX-2, and dexamethasone inhibited the expression of this enzyme. We suggest that the reduction of *in vivo* PGE_2 production is due to decreased COX-2 expression in macrophages.

In Figure 7B and D, it shown that only stimulation with zymosan plus IFN-γ induced increases in NO production and iNOS expression. Pretreatment with MG significantly reduced NO production only at the highest concentration tested (10 μM). Moreover, MG at both 1 and 10 μM significantly inhibited iNOS expression, whereas in cells pretreated with dexamethasone there was a significant reduction only in NO production and not in enzyme expression (Figure 7B and D). The overproduction of NO induces the generation of reactive nitrogen species, which has been associated with autoimmune disease severity.[72] In the experimental model of ZIA in rats, it was observed that the production of NO occurred in association with the severe migration of neutrophils into the synovial cavity.[25] Increased iNOS expression in inflamed synovia, particularly in synovial cells and neutrophils, contributes to NO release.[73] Our results showed that MG inhibits *in vitro* NO production by macrophages (type A synoviocytes), which suggests that in ZIA macrophages might be one of the cell types involved in NO production during the exacerbation of the inflammatory process.

Dectin-1 is a β-glucan receptor expressed on the surfaces of monocytes and macrophages and is involved in zymosan recognition and internalization. Dectin-1 signaling stimulates intracellular calcium mobilization after the activation of phospholipase Cγ (PLC-γ) and the release of inositol triphosphate in the cytoplasm.[74] In macrophages, the combined stimulus of zymosan and IFN-γ was accompanied by a prolonged increase in the cytoplasmic calcium concentration, which was inhibited by pretreatment with MG (1 μM) (Figure 7E). For statistical analysis of this assay, the means of values

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obtained between 360 and 600 s for each group were plotted (Figure 7F). Calcium is a versatile intracellular messenger in eukaryotic cells, regulating many cellular processes including the cell cycle, the transport system, mobility, gene expression, and cell metabolism.[75] The presence of calcium is essential for the action of the enzyme phospholipase A_2 (PLA_2).[76] Cytosolic free calcium binds to the catalytic site of PLA_2, inducing the release of arachidonic acid from membrane phospholipids for the biosynthesis of eicosanoids.[77] The MG-induced reduction in intracellular calcium mobilization may reduce the activity of PLA_2, thereby decreasing the release of arachidonic acid. This process might partially explain the decrease in the observed PGE_2 and LTB_4 production in the synovial fluid of mice treated with MG. Indeed, we found that MG pretreatment reduced both migration and adhesion of neutrophils, as well as the production of PLA_2-dependent lipid mediators from macrophages. It is likely that MG might interfere in the signaling pathways of chemotactic receptors, consequently impairing neutrophil migration. Moreover, the inhibition of calcium influx by MG demonstrates a direct effect of MG on the modulation of macrophage functions.

The present study demonstrates for the first time the anti-inflammatory effect of MG in animal models. According to the results shown in this work, we can suggest that MG acts on signaling cascades and that its actions are not exclusively dependent on its antioxidant properties. It is possible that MG interacts with multiple molecular targets, as has been observed for numerous natural products,[78] since the activation of macrophages by zymosan triggers different signaling pathways than those observed downstream of G protein-coupled receptors. On the other hand, there is evidence that the PI3K signaling pathway is activated in response to numerous TLR stimuli and subsequently induces Akt phosphorylation.[79] Moreover, it has been demonstrated that $G\beta\gamma$ signaling contributes to macrophage membrane dynamics and zymosan phagocytosis.[80] Thus, MG may be acting on the same target via different signaling pathways, or it may have pleiotropic cellular functions. Indeed, future experiments are necessary to clarify the mechanism of action of MG.

Taken together, we demonstrated in this study that MG, a phenolic acid derivative found in various plant species, exerts an important anti-inflammatory effect on zymosan-induced inflammation, particularly in experimental arthritis. Our results showed that MG significantly reduces neutrophil migration, the production of inflammatory mediators, and the activation of cells crucial to the development of this disease. In addition, these data highlight the importance of further study on the mechanism of action of MG, reinforcing the importance of natural products as sources of therapeutics for the modulation of the inflammatory process and the control of arthritis.

■ EXPERIMENTAL SECTION

Chemicals and Reagents. Methyl gallate (98% purity) was purchased from Fluka (Sigma-Aldrich). Zymosan A, phosphate-buffered saline (PBS) tablets, perborate buffer, Tween-20, Hank's balanced salt solution (HBSS), probenecid, fucoidan, *o*-phenylenediamine dihydrochloride, Percoll, Bradford reagent, bovine serum albumin, ethylenediaminetetraacetic acid sodium salt (EDTA), phenylmethylsulfonyl fluoride (PMSF), protease inhibitors cocktail, and RPMI 1640 were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). May-Grünwald and Giemsa stains were purchased from Merck (Darmstadt, Hessen, Germany). Fetal bovine serum (FBS) and gentamicin were obtained from Gibco (Grand Island, NY, USA). Polyvinylidene difluoride membrane was purchased from GE Healthcare (Chicago, IL, USA). Purified anti-murine TNF-α, CXCL1/KC, IL-1β, and IL-6 mAbs, biotinylated anti-TNF-α, CXCL-1/KC, IL-1β, and IL-6 mAbs, and recombinant TNF-α, CXCL1/KC, IL-1β, and IL-6 were obtained from R&D Systems (Minneapolis, MN, USA). LTB_4 and PGE_2 immunoassay kits were obtained from Cayman Co. (Ann Arbor, MI, USA). Goat polyclonal purified anti-COX-2 and biotin-conjugated anti-goat IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Dexamethasone (Decadron; Aché, SP, Brazil) and sodium pentobarbital 3% (Hypnol; Syntec, SP, Brazil) were commercially obtained.

Animals. Male C57BL/6 mice (18−22 g) from the Oswaldo Cruz Foundation Breeding Unit (Fiocruz, Rio de Janeiro, Brazil) were kept caged with free access to food and fresh water in a room with the temperature ranging from 22 to 24 °C and a 12 h light/dark cycle. The animals were housed at the Farmanguinhos experimental animal facility unit until use. All animal care and experimental procedures performed were approved by the institution's Committee of Ethics in Animal Care and Use (CEUA) (registered under the number CEUA LW-43/14) and were performed according to the ethical guidelines of the International Association for the Study of Pain.[81]

Treatments. Animals were fasted overnight and then received MG in doses ranging from 0.7 to 70 mg/kg orally (p.o.) diluted in filtered water in a final volume of 200 μL, 1 h before zymosan stimulation. Dexamethasone (10 mg/kg, 100 μL) was administered intraperitoneally (i.p.) 1 h prior to stimulation and used as a reference inhibitor. The same volume of vehicle (200 μL) was administered orally to the control groups.

Paw Edema. To evaluate the effect of MG on paw edema induced by zymosan, C57BL/6 mice were pretreated orally with various doses of MG (1−50 mg/kg) and subjected to zymosan (i.pl., 100 μg/paw) diluted in sterile saline to a final volume of 50 μL.[82] The contralateral paw received an i.pl. injection of equal volume of sterile saline and was used as a control. Four hours after stimulus, paw edema was evaluated by plethysmography (plethysmometer 7140, Ugo Basile, Comerio, Italy). Paw edema values are expressed in microliters (μL), and the difference (Δ) between the right and left paws was taken as the edema volume.

Induction of Pleurisy. One hour after the respective treatments, pleurisy was induced in C57BL/6 mice by an i.t. injection of zymosan (100 μg/cavity) diluted in sterile saline to a final volume of 100 μL, according to the technique of Spector[83] as modified for mice by Henriques.[84] The control group received an i.t. injection of an equal volume of vehicle. The mice were euthanized 4 h after stimulus using a lethal dose of pentobarbital sodium 3% (Hypnol), and their thoracic cavities were washed with 1 mL of PBS containing EDTA (10 mM). Total leukocyte counts were performed in an automatic particle counter (Coulter Z2, Beckman Coulter Inc., Brea, CA, USA). Differential counts were performed under a light microscope (100×) using Cytospin smears (Cytospin 3, Shandon Inc., Pittsburgh, PA, USA) stained using the May-Grumwald-Giemsa method. The counts are reported as the number of cells ($\times 10^6$) per cavity. Pleural washes were centrifuged at 400*g* for 10 min to obtain the supernatant for total protein quantification by the Bradford method according to the manufacturer's instructions.

Experimental Arthritis Model. Joint inflammation was induced by the i.a. injection of zymosan (500 μg per cavity in 25 μL of sterile saline) by inserting a 27.5 G needle through the suprapatellar ligament into the left knee joint cavity, as previously described.[5,12] The contralateral knee was injected with the same volume of the vehicle and used as control. Knee joint swelling was evaluated by measurement of the transverse diameters of each knee joint with a digital caliper (Digmatic Caliper, Mitsutoyo Corporation, Japan) at different time points after stimulation. Values of knee joint thickness are expressed as the difference (Δ) between the diameters measured before (basal) and after the induction of articular inflammation in millimeters (mm). After induction of joint inflammation, mice were euthanized by a lethal dose of pentobarbital sodium 3% (Hypnol) at various time points. Knee synovial cavities were washed twice with 150 μL of PBS containing EDTA (10 mM) by the insertion of a 21 G needle into mouse knee joints, and the synovial washes were recovered

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by aspiration. The volume was inserted in stages (50 μL each time, per needle insertion). Total leukocyte counts were performed in an automatic particle counter (Coulter Z2, Beckman Coulter Inc.). Differential counts of leucocytes were performed using May-Grunwald-Giemsa-stained cytospin smears (Cytospin 3, Shandon Inc.), and the values are reported as the number of cells per cavity ($\times 10^5$). After cellular counts, the synovial washes were centrifuged at 400*g* for 10 min at 4 °C, and the supernatant was stored at −80 °C for further analysis.

Histology. Whole knee joints were obtained from six different C57BL/6 mice at 6 and 24 h after i.a. administration of zymosan or saline. The knee joints were removed, dissected, and fixed in 10% formalin for 48 h. After that, the knees were kept in 10% EDTA in PBS solution for 1−2 weeks for decalcification. Subsequently, the specimens were processed for paraffin embedding. Tissue sections (5 μm) were stained with H&E, mounted on permanent glass slides, and analyzed under an optical microscope (Olympus BX41, Olympus, Japan). Images were taken at lower magnification (10×) to allow viewing of the entire area of the synovial cavity and were graded subjectively for the following parameters: synovial hyperplasia (pannus formation; from 0 = no hyperplasia, to 3 = most severe hyperplasia); synovial infiltrate (from 0 = no inflammation, to 3 = most severe inflammation), and bleeding (from 0 = no bleeding, to 3 = most severe bleeding).[85] To eliminate potential bias, the slides were scored by two independent observers who were blinded to the experimental procedure. Scores for all parameters were subsequently summed to give an arthritis index (AI; expressed as the mean ± SEM).

Cytokine and Chemokine Measurements (ELISA). The concentrations of TNF-α, IL-1β, IL-6, and CXCL-1/KC in the knee joint washes 6 and 24 h after stimulation with zymosan were evaluated by sandwich ELISA using matched antibody pairs (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The results are expressed as picograms of each cytokine or chemokine per milliliter (pg/mL).

Enzymatic Immunoassay for LTB_4 and PGE_2. LTB_4 and PGE_2 levels were evaluated in cell-free synovial washes recovered from zymosan-stimulated C57BL/6 mice 6 and 24 h after zymosan (500 μg per cavity) injection. LTB_4 and PGE_2 were assayed by immunosorbent assay (EIA) according to the manufacturer's protocol (Cayman Chemical). The results are expressed as picograms of LTB_4 or PGE_2 per milliliter (pg/mL).

Preparation of Murine Bone Marrow PMN Cells. C57BL/6 mice were euthanized, and the femurs from both hind legs were dissected and removed, free of soft tissue attachments. The extreme distal tip of the bone was cut out, and each femur was washed with 2 mL of Ca^{2+}-/Mg^{2+}-free HBSS−EDTA solution. The cell suspension was then centrifuged at 400*g* for 15 min at 20 °C and resuspended in 2 mL of Ca^{2+}-/Mg^{2+}-free HBSS−EDTA. The cells were purified using Percoll discontinuous gradients (65% and 72% diluted in Ca^{2+}-/Mg^{2+}-free HBSS−EDTA). For that purpose, the cells were centrifuged at 1200*g* for 35 min at room temperature without braking, and neutrophils were recovered from the 65%/72% interface. Next, neutrophils were counted in a Neubauer chamber and identified by cytospin centrifugation followed by Wright-Giemsa coloration (~85−90% final purity) according to the manufacturer's instructions.

Neutrophil Viability. Neutrophil viability in the presence and absence of MG was determined using the lactate dehydrogenase-based *in vitro* toxicology assay kit (Sigma). After isolation, neutrophils were plated into 96-well culture plates at a density of 1×10^5 cells/well. Then, the cells received fresh medium with or without Tween 20 (3%) or MG (0.01−100 μM) in a quadruplicate assay. After 2 h of incubation, the plate was centrifuged at 250*g* for 4 min to pellet the cells. Aliquots (100 μL) were transferred to a clean flat-bottom plate, and the lactate dehydrogenase assay mixture was added to each sample (200 μL per well) and incubated at room temperature for 20−30 min. The reaction was terminated by the addition of 1 N HCL to each well. The absorbance was read using a SpectraMax 190/Molecular Devices at a wavelength of 490 nm. The background absorbance was read at 690 nm, and this value was subtracted from the primary wavelength measurement (490 nm).

Chemotaxis Assay. Neutrophil chemotaxis was assayed in a 48-well Boyden chamber (Neuroprobe Inc., Cabin John, MD, USA). The bottom wells of the chamber were filled with 29 μL of a chemoattractant stimulus, CXCL-1/KC (250 nM) or RPMI 1640 (control), while the upper wells were filled with neutrophils (10^5 cells, 50 μL) that had been preincubated with MG (0.1, 1, and 10 μM), dexamethasone (50 nM), or medium for 1 h. The lower and upper cells were separated using a 5 μm polycarbonate filter (Nuclepore, Sigma-Aldrich). The chamber was incubated in humidified air with a 5% CO_2 atmosphere at 37 °C for 60 min, and the filter was fixed and stained using Wright-Giemsa coloration according to the manufacturer's instructions. Neutrophils that had migrated through the membrane were counted under light microscopy (100× magnification) in 15 random fields. The result is expressed as the mean of migrated neutrophils per field.

Cell Adhesion Assay. The cell adhesion assay used the murine thymic endothelioma cell line tEnd.1 cultured in RPMI 1640 medium supplemented with 10% FBS containing 0.1% gentamicin. These cells were incubated at 37 °C in a humidified incubator containing 5% CO_2 and plated onto four-well culture chambers (10^4 cells/well) (Lab-Tek chambers, Nunc, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 24 h. Before each experiment, the tEnd.1 cells were stimulated with recombinant mouse TNF-α (10 ng/mL) for 4 h. Neutrophils recovered from naïve C57BL/6 mice were pretreated with fucoidan (25 μg/mL) or MG (0.1 μM) for 1 h and then allowed to adhere to the tEnd.1 cultures (50 neutrophils/tEnd.1) for 1 h at 37 °C with shaking. Nonadherent cells were gently washed away with PBS, and the remaining cells were subsequently stained with Giemsa and visualized by light microscopy (100× magnification). The number of adhered neutrophils per tEnd.1 cell was determined by direct counting. The data are expressed as an index of adhesion (IA), calculated as follows: IA = (tEnd.1 cells with bound neutrophils)/(total number of tEnd.1 cells) × (neutrophils bound to tEnd.1 cells)/(total number of tEnd.1 cells) × 100, as previously described.[86]

Macrophage Viability. Cellular viability in the presence and absence of MG was determined using an Alamar Blue assay (Invitrogen, Carlsbad, CA, USA). Cells of the mouse cell line J774A.1 were plated into a black flat-bottomed 96-well plate at a density of 2.5×10^5 cells/well. After 24 h of incubation in a controlled atmosphere (5% CO_2, 37 °C), the cells received fresh medium with or without Tween 20 (3%) or MG (0.01−100 μM) in a quadruplicate assay. After 20 h of further incubation, 20 μL of Alamar Blue solution was added to each well, and after 4 h, the fluorescence was read using a SpectraMax M5/M5e microplate reader (Molecular Devices; λ_{exc} = 555 nm, λ_{em} = 585 nm).

Macrophage Activation and IL-6 and Nitric Oxide Production. Mouse macrophages J774A.1 (1×10^5 cells/well in RPMI with 10% FBS and gentamicin) were plated into 96-well culture plates and allowed to adhere for 24 h in a controlled atmosphere. The macrophages were incubated with dexamethasone (1 μM) or various concentrations of MG (0.1−10 μM) for 1 h. Then, the cells were stimulated with zymosan (10 μg per 10^5 cells) and/or IFN-γ (25 U/mL) for 24 h. After 24 h, the amount of IL-6 was evaluated by sandwich ELISA as described above. The amount of nitrite, a metabolite of nitric oxide, was assessed in the supernatant from the macrophages using the Griess method.[87] The absorbance was measured at 540 nm in a plate reader (Softmax Pro 190-Molecular Devices).

Western Blot. J774A.1 cells were plated into six-well plates and allowed to adhere for 24 h. The cells were then treated with MG (0.1−10 μM) or dexamethasone for 1 h before stimulation with zymosan and/or IFN-γ as described above. Whole cell lysates were obtained with lysis buffer (20 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF, and protease inhibitor cocktail), and the total protein concentration of each sample was determined according to the Bradford method. Cell lysates were denatured in Laemmli buffer (50 mM Tris-HCL, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) at 90 °C for 5 min. Protein samples (30 μg of protein) were resolved by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed

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with antibodies against COX-2, iNOS, and α-tubulin (Santa Cruz Biotechnology). Antibodies were diluted in Tris-buffered saline containing 0.1% Tween 20 and 5% skimmed milk and incubated overnight at 4 °C. On the following day, the membranes were incubated with anti-goat, anti-rat, or anti-mouse peroxidase-conjugated secondary IgG antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed with the use of a chemiluminescent substrate (ECL Western blotting analysis system; Amersham Biosciences Buckinghamshire, UK), and images were acquired for densitometric analysis using ImageJ software.

Intracellular Calcium Measurement. The intracellular calcium was assayed using the FLIPR calcium assay kit (Molecular Devices, Sunnyvale, CA, USA), according to the manufacturer's instructions. Mouse cell line J774A.1 was plated into a black flat-bottomed 96-well plate at a density of 5×10^4 cells/well. After 24 h of incubation in a controlled atmosphere, the macrophages were incubated with probenecid (inhibitor for the anion-exchange protein) at 2.5 mM in a final volume of 100 μL. Then, MG (1 μM) was added for 1 h at 37 °C in loading buffer at a final volume of 100 μL, according to the manufacturer's protocol. Following incubation, the macrophages were either unstimulated or stimulated with zymosan (10 μg per 10^5 cells) and IFN-γ (25 U/mL). Intracellular Ca^{2+} spikes in macrophages were monitored for 10 min. The data were analyzed using SOFTmax Pro (Softmax Pro190-Molecular Devices).

Statistical Analysis. The data are reported as the mean ± SEM and analyzed statistically using analysis of variance (ANOVA) followed by the Newman-Keuls-Student test or Student's *t*-test. Values of $p \leq 0.05$ were regarded as significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01115.

Additional figures (PDF)

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Notes

The authors declare no competing financial interest.

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Anti-Inflammatory Effect of Methyl Gallate on Experimental Arthritis: Inhibition of Neutrophil Recruitment, Production of Inflammatory Mediators, and Activation of Macrophage.

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Supporting information

Figure S1.

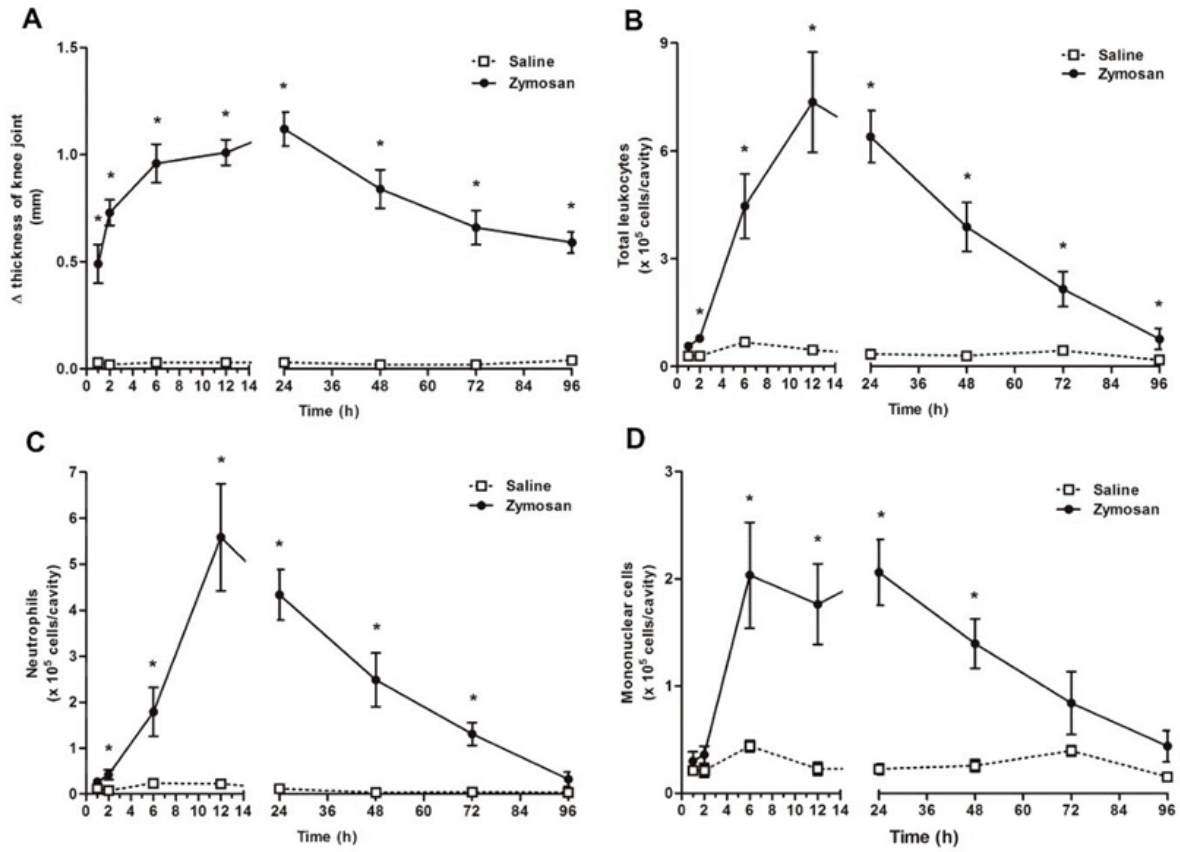

Figure S1. Time-course of zymosan-induced articular inflammation. Panels A-D: Evaluation of the knee joint diameter (A) and the number of total leukocytes (B), neutrophils (C) and mononuclear cells (D) in the synovial cavity after zymosan injection (500 µg per cavity in 25 µl sterile saline). The control group was injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper, and knee synovial cells were recovered from 1 h until 96 h after stimulation. The results are presented as the means ± SEM of seven mice per group per experiment and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group (Student's t-test)].

Figure S2.

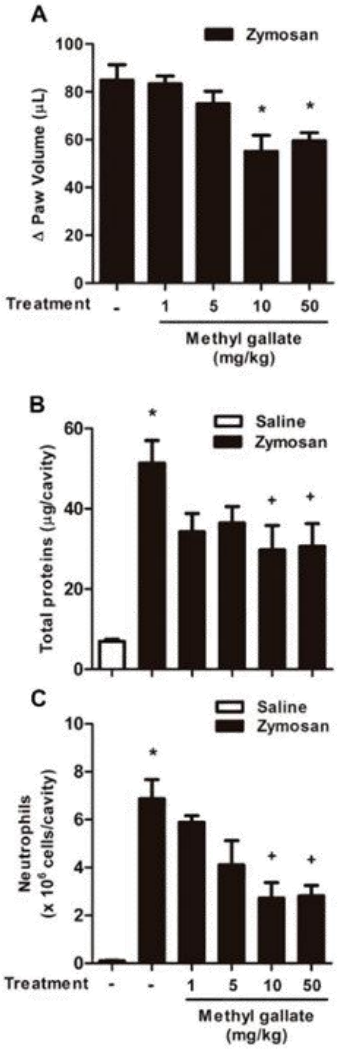

Figure S2. Methyl gallate inhibits the inflammatory response induced by zymosan. Panels A-C: Mice were treated with MG (1–50 mg/kg) or vehicle (saline) orally 1 hour before zymosan stimulus. Paw edema was evaluated by plethysmography 4 h after zymosan injection (i.pl., 100 µg/paw, panel A). Protein extravasation and neutrophil migration in the thoracic cavity were assessed 4 h after zymosan-induced pleurisy (i.t., 100 µg/cavity, panels B and C). Control animals received saline i.t. injection (100 µl/cavity). We calculated that effective dose of MG to decrease 50% of inflammation (ED_{50}) was also 7 mg/kg in the pleurisy model. To calculate the ED_{50}, we used GraphPad Prism software, adopting the median values from the neutrophil counts. This parameter was chosen because it provided the best result for evaluating the MG dose-response curve (data not shown). The results are presented as the means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p \leq 0.05$ compared to the saline group; $^{+}p \leq 0.05$ compared to the zymosan group (one-way ANOVA followed by Student-Newman-Keuls)].

Table S1: Cellular viability of MG in murine bone marrow neutrophils.

	Vehicle	0.01 µM	0.1 µM	1 µM	10 µM	100 µM
Neutrophils viability (%)	100	100	100	100	100	92

The results were represented as percentage of cellular viability.

Table S1. The results are expressed as the cell viability percentage (%) from quadruplicate wells (1 x 10^5 cells/well) after incubation of cells with MG for 2 h (37 °C, 5% CO_2). Cell viability was assessed using the Lactate Dehydrogenase-Based *In Vitro* Toxicology Assay Kit as described in the experimental section.

Table 2: Cellular viability of MG in J774A.1 macrophages.

	Vehicle	0.01 µM	0.1 µM	1 µM	10 µM	100 µM
Macrophages viability (%)	100	100	100	100	97	95

The results were represented as percentage of cellular viability.

Table S2. The results are expressed as the cell viability percentage (%) from quadruplicate wells (2.5 x 10^5 cells/well), after incubation of cells with MG for 20 h (37 °C, 5% CO_2). Cell viability was assessed using the Alamar Blue assay as described in the experimental section.

3.2 ARTIGO 2

Methyl gallate attenuates Toll-Like ligands-induced inflammation: effect on NF-κB and MAPK activation.

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Foi demonstrado previamente que o pré-tratamento oral com o GM reduz a formação de edema, a migração de leucócitos, principalmente de neutrófilos, e a produção de diversos mediadores inflamatórios tais como: TNF-α, IL-6, IL-1β, CXCL-1, LTB_4 e PGE_2 nos modelos de inflamação induzido por zimosan. Na busca do mecanismo de ação, avaliamos *in vitro* possíveis mecanismos celulares, pelos quais esta substância reduz a ativação de células cruciais para o desenvolvimento da artrite, como migração e adesão de neutrófilos e ativação de macrófagos. Apesar de tais evidências pouco se sabe sobre os mecanismos moleculares envolvidos na ação anti-inflamatória do GM. No presente trabalho, foram investigados os mecanismos moleculares responsáveis pelos efeitos anti-inflamatórios do GM.

Nesse estudo, para identificar o alvo molecular, foi avaliada a ação do GM na via de sinalização dos receptores do tipo Toll (TLRs), em macrófagos ativados *in vitro* com ligantes destes receptores. Para elucidar o mecanismo pelo qual o GM reduz a produção das citocinas pró-inflamatórias, investigou-se a ação deste polifenol nas vias de ativação do NF-κB e das MAPKs (ERK, JNK e p38). Estes eventos foram reduzidos, após a incubação dos macrófagos com GM e estimulados com os ligantes de TLRs. Somado a isso, foi avaliado se essa substância seria capaz de interferir na ativação do inflamossoma NLRP3. Observou-se uma inibição significativa da produção da citocina IL-1β e da expressão do NLRP3.

Em conjunto, os resultados demonstraram que o GM desempenha efeitos supressivos em vias envolvidas na ativação de macrófagos durante a inflamação. O GM inibiu a translocação nuclear dos fatores de transcrição NF-κB e AP-1, que desempenham papeis críticos na indução de genes inflamatórios. Esses resultados sugerem fortemente que enzimas *upstream* que regulam a ativação transcricional do NF-κB e AP-1 podem ser alvos moleculares do GM. De fato, nossos resultados demonstraram uma atividade inibitória do GM prejudicando a degradação do IκB-α e ativação das MAPKs ERK, JNK e p38 que estão envolvidas na modulação de NF-κB e AP-1. Com base nesses achados, fornecemos evidências para alguns possíveis mecanismos de ação do GM.

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Methyl gallate attenuates Toll-Like ligands-induced inflammation: effect on NF-κB and MAPK activation.

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Abstract:

Background: Polyphenols are secondary metabolites fully distributed troughout the Plant Kingdom. Methyl gallate (MG) is a phenolic compound widely distributed in medicinal and food plants and has been described as an antioxidant and anti-inflammatory molecule. Ligands for innate immune receptors such as the TLRs activate macrophages to produced pro-inflammatory cytokines by triggering complex intracellular signaling cascades as MAPK pathways and inflammation-regulatory transcription factors including NF-κB and AP-1. The aim of this study was to test the effect of MG on TLR agonists-induced cytokines production by macrophages and address the possible molecular mechanism involved.

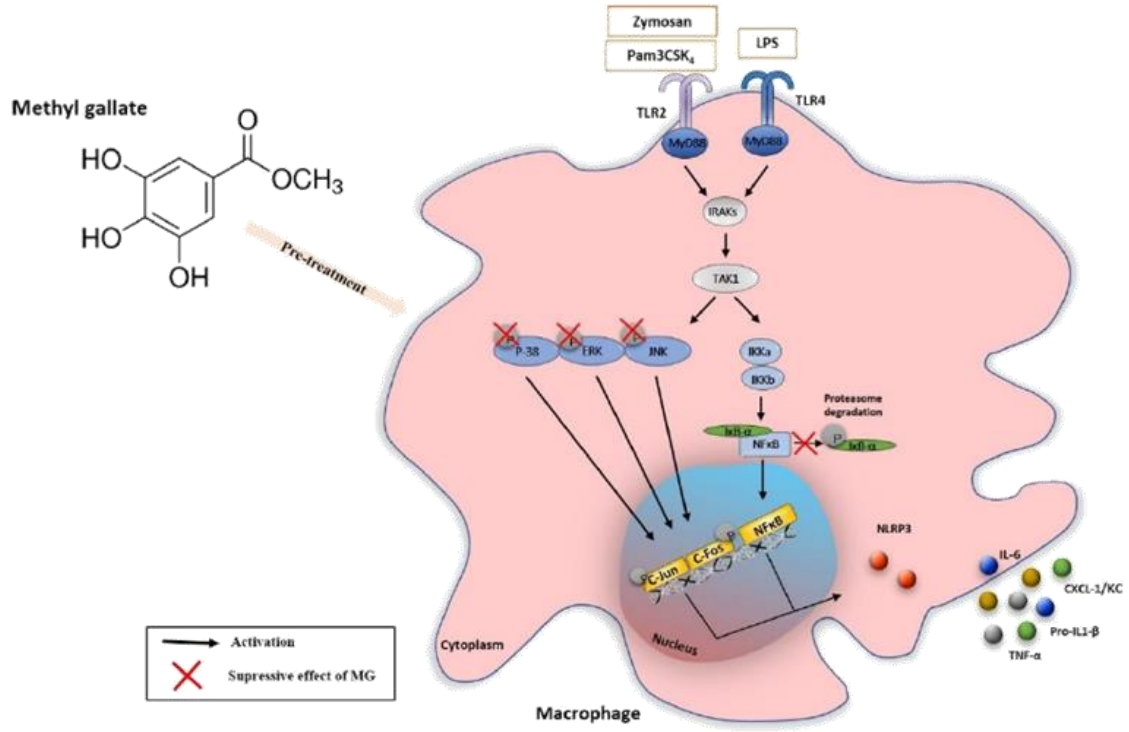
Methods: Macrophages were activated with TLR agonists as zymosan, LPS, and $Pam3CSK_4$, after previous incubation with MG.

Results: Pre-treatment with MG inhibited the production of cytokines in macrophages stimulated with zymosan, LPS and $Pam3CSK_4$. MG reduced the luminescence emission by RAW264.7 NF-κB-Luc stimulated by different TLR agonists but not by PMA. Moreover, pre-treatment with MG decreased IκB degradation and nuclear translocation of NF-κBp65 induced by $Pam3CSK_4$. Besides, MG also reduced ERK-1/2, p38 and JNK phosphorylation, and c-Jun and c-Fos nuclear translocation.

Conclusion: The results suggest that MG inhibits the activation of macrophages stimulated with TLRs agonists, by reducing cytokines production through reduction of NF-κB and c-jun and c-fos translocation, particularly affecting the ERK1/2, JNK and p38 MAPK phosphorylation as well as the IκB-α degradation.

Keywords: methyl gallate, polyphenols, TLRs, NF-κB signaling, MAPKs quinases.

Graphical abstract



1. Introduction

Toll-like receptor (TLR) play an essential role in the innate immune system through its ability to recognize specific molecules released by bacteria and viruses (PAMPs, pathogen-associated molecular patterns) and molecules released by injured cells and tissues (DAMPs, damage-associated molecules pattern)[1].

The mammalian Toll-like receptor (TLR) family consists of 13 members[2] that may bind various ligands, such as zymosan (TLR2/6), tri-acylated lipopeptide $Pam3CSK_4$ (TLR2/1), and lipopolysaccharide (LPS) (TLR4)[3]. TLR triggering leads to the induction of proinflammatory cytokines such as tumor necrosis factor (TNF)-α), hydrolytic enzymes, toxic molecules (nitric oxide (NO) reactive oxygen species (ROS)), and lipid mediators (prostaglandin E_2)[4]. The activation of TLRs stimulates different intracellular signaling cascades via two adaptor molecules (TRIF and MyD88). In MyD88-dependent pathway, there are the recruitment and activation of IRAK kinase family members, which leads the activation of TAK-1 protein kinase complex, triggering two different signaling pathways[5]. TAK1 coupling to the IKK complex leads to IkB phosphorylation and nuclear translocation of NF-κB whereas TAK1-dependent activation of mitogen-activated protein kinase (MAPK) promotes AP-1 transcription factor induction[6-9].

Polyphenols are secondary metabolites of plants with a broad range of therapeutic effects due to their potent antioxidant, immunemodulatory, and anti-inflammatory actions. This phytochemical class of compounds, including flavonoids, phenolic acids, phenolic alcohols, stilbenes, and lignans, has a potential role in different oxidative stress-induced complications, such as cardiovascular disease, cancer, and neurodegenerative diseases[10]. In the recent years, a considerable attention has been paid to the potential of polyphenols in regulating the signaling pathway of TLR and aftermath suppress the overexpression of inflammatory mediators and regulating several pathologies such as ischemia/reperfusion injury, neuropathic pain, neurodegenerative diseases, and cancer[11-13].

Methyl gallate (MG), is a ubiquitous phenolic acid, widely distributed in medicinal and food plants such as *Schinus terebinthifolius*, *Galla Rhois*, *Rosa rugosa*, and *Givotia rottleriformis*[14-17]. Several studies demonstrate that MG is related to the notable biological effects of those plants such as its antioxidant[18, 19], antitumor[20, 21], and antimicrobial activities[22, 23]. Besides, a potent anti-inflammatory effect of

MG was demonstrated on zymosan-induced experimental arthritis, by reducing neutrophil migration, decreasing the production of inflammatory mediators and inhibiting the activation of cells crucial for arthritis development[24]. However, the molecular mechanism behind the anti-inflammatory activity of MG is not thoroughly understood. In the present study, we investigated the effects of MG on the signaling pathway downstream of TLRs activation.

2. Results and discussion

2.1 Methyl gallate reduces the release of cytokines and NF-κB activity on macrophages induced by zymosan.

In our previous study, we described that MG impaired J774A.1 macrophage activation by inhibiting IL-6 and NO production, COX-2, and iNOS expression and intracellular calcium mobilization induced by zymosan and IFN-γ[24]

Zymosan particles are recognized simultaneously by dectin-1 and TLR-2. These receptors trigger NF-κB activation in a different way that leads to the production of inflammatory cytokines and ROS[25]. Therefore, we investigated the effect of MG on NF-κB activation. To assesses the effect of MG on cytokine production, RAW264.7 cells were pretreated for 1 h with different concentrations of MG (1-100 μM) or dexamethasone (1 μM) and stimulated with zymosan (30 μg/mL). After 6 h, the levels of TNF-α, IL-6, and CXCL-1 were measured in the culture medium. The cell viability (≥ 90%) was evaluated by MTT assay to exclude a cytotoxicity effect of MG. (Supplemental Table 1). Pre-treatment with MG significantly inhibited the production of IL-6 and CXCL-1 induced by zymosan in a concentration-dependent manner, although it was not able to inhibit the production of TNF-α (Figure 1A-C). Dexamethasone was used as reference inhibitory drug and reduced the production of all cytokines evaluated (Figure 1A-C). We further examined whether the cytokine production inhibition occurred at the transcriptional level. We investigated the effect of MG on NF-kB activity using the RAW264.7 macrophages stably bearing the luciferase gene-containing vector controlled by an NF-κB-activated promoter RAW-NF-κB/Luc. Notably, the preincubation of macrophages with MG (1-100 μM) promoted a concentration-dependent reduction of luminescence emission induced by zymosan (Figure 1D). These findings suggest that the modulatory effect of MG on the production of pro-inflammatory mediators by macrophage is due to the inhibition on NF-κB activity.

Figure 1.

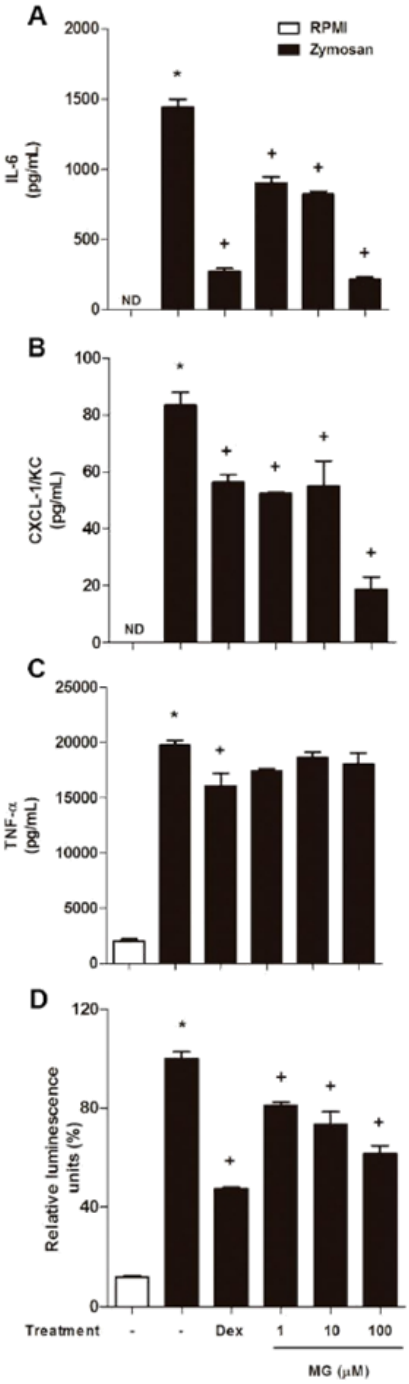


Figure 1. MG inhibits inflammatory cytokines production and NF-κB activity on zymosan-stimulated macrophages. (A-D) RAW264.7 macrophages were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with zymosan (30 μg/mL) for 6 h. (A-C) The supernatant was collected (6 h after stimulus) to evaluate IL-6, CXCL-1 and TNF-α production by ELISA. (D) NF-κB activity was quantified using RAW264.7 macrophages stably expressing the NF-κB pLUC gene. After 6 h of incubation, cells were lysed and incubated with luciferin. Luminescence units were measured by a luminometer. Results are expressed as the mean ± SEM for quadruplicate wells per group from at least two independent

experiments and statistically analyzed by means of the analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by $^{*}P \leq 0.05$. Whereas $^{+}P \leq 0.05$ represent significant differences between treated and stimulated groups. ND= not detectable.

2.2 Methyl gallate inhibits zymosan-induced IL-1β secretion and NLRP3 expression.

Inflammasome activation is a two-step process, which is initiated by priming macrophages with TLR agonists such as zymosan[26] that leads to NF-κB activation and induce the expression of NLRP3 and pro-IL-1β[27, 28]. Enhanced NLRP3 expression is required for responsiveness to the second stimulus, ATP, which signals through the $P2X_7$ receptor for assembling the inflammasome complex, which recruits procaspase-1[29]. The oligomerization of procaspase-1 triggers self-proteolysis to active caspase-1, which cleaves and releases mature IL-1β from the cell[30]. IL-1β is a potent pro-inflammatory mediator and induce further activation of transcription factor NF-κB[31] which in turn, is able to stimulate the expression of more pro-IL-1β as well as inflammasome components[27, 31].

Since MG inhibited NF-κB activation induced by zymosan, we analyzed whether it could interfere with NLRP3 expression. Bone marrow-derived macrophages (BMDM) stimulated with zymosan followed by ATP presented significant expression of NLRP3 and IL-1β release, which were inhibited by the pre-treatment with MG in a dose-dependent manner (Figure 2A-B). These data corroborate with our results showing that the inhibitory effect of MG on pro-inflammatory cytokine production could be due to down-modulation of the NF-κB signaling pathway.

Figure 2.

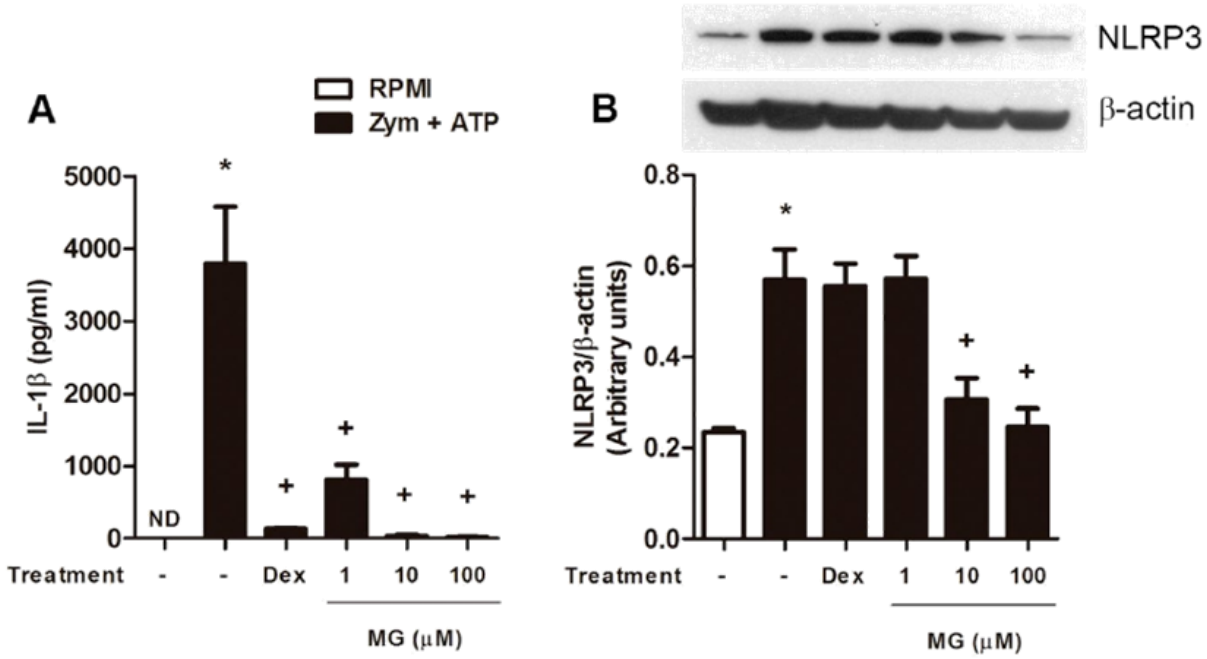


Figure 2. MG inhibits IL-1β secretion and NLRP3 expression. (A – D) BMDMs were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with zymosan (30 μg/mL) following by ATP (2 mM) stimulation for 4 h. (A) The supernatant was collected (4 h after stimulus) to evaluate IL-1β production by ELISA. (B) Total cell lysates were separated by SDS-PAGE and subjected to western blotting for NLRP3. Representative of three different western blots are shown in top, whereas densitometric analyses are shown in the graphs. Results are expressed as the mean ± SEM for quadruplicate wells per group from at least three independent experiments and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by $^{*}P \leq 0.05$. Whereas $^{+}P \leq 0.05$, represent significant differences between treated and stimulated groups. ND= not detectable.

2.3 Methyl gallate reduces the release of cytokines induced by Pam3CSK$_4$, a specific TLR-2 agonist.

Since MG reduced the NF-κB activation in macrophage stimulating by zymosan, we decided to investigate the effect of this substance on exclusive activation of the TLR-2 pathway. For this, we use a specific agonist for TLR-2, Pam3CSK$_4$. As shown in Figure 3A-C, RAW macrophages stimulated with Pam3CSK$_4$ produce TNF-α, IL-6 and CXCL-1. The pretreatment with MG was able to inhibit the cytokines production in all concentration tested. The cell viability (≥ 90%) among the different concentrations was accessed by MTT assay to exclude cytotoxicity effect (Supplemental Table 2). These results demonstrate a remarkable effect of MG on activation of TLR-2.

Figure 3.

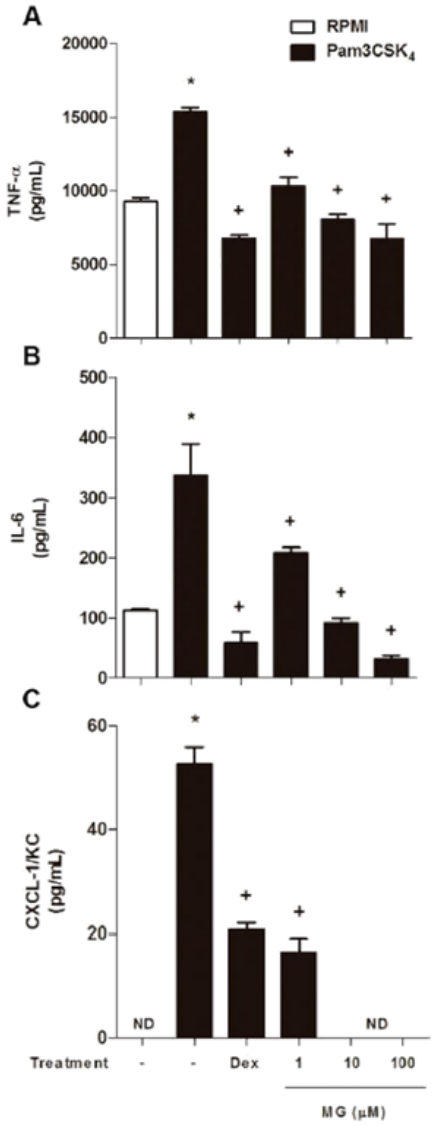


Figure 3. MG inhibits inflammatory cytokines production on Pam3CSK$_4$-stimulated macrophages. (A-C) RAW264.7 macrophages were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with Pam3CSK$_4$ (1 μg/mL) for 6 h. (A-C) The supernatant was collected (6 h after stimulus) to evaluate TNF-α, IL-6 and CXCL-1 production by ELISA. Results are expressed as the mean ± SEM for quadruplicate wells per group from at least two independent experiments and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by *P≤ 0.05. Whereas $^{+}$P≤ 0.05 represent significant differences between treated and stimulated groups. ND= not detectable.

2.4 Methyl gallate impairs NF-κB activity, translocation and IκB-α degradation on macrophage.

We further investigated the effect of MG in the NF-κB signaling pathway triggered $Pam3CSK_4$. The stimulation of TLR2 triggered different intracellular signaling cascades via adaptor molecules that induce NF-κB activation[1]. Once activated, NF-κB induces the transcription of genes encoding inflammatory mediators, such as chemokines (e.g., CXCL-1)[32], cytokines (TNF-α, IL-1β) and enzymes such as COX-2[33-35].

$Pam3CSK_4$ induce an augment in NF-κB activity in RAW264.7 macrophages, as evidenced by luminescence emission increased. The pre-treatment with MG was able to reduce the NF-κB activity in the same intensity of the pre-treatment with dexamethasone (Figure 4A).

NF-κB exists in the cytoplasm in an inactive complex bound to the NF-κB inhibitory protein (IκB)[36]. In response to an inflammatory stimulus, IκB is phosphorylated and then degraded by the proteasome which allows free NF-κB to translocate into the nucleus and priming transcription of pro-inflammatory mediators[36, 37].

Since MG inhibited NF-kB activation induced by $Pam3CSK_4$, we investigated whether this compound could inhibit IkB-α degradation or NF-κB translocation to the nucleus. The stimulation of BMDMs with $Pam3CSK_4$ (100 ng/mL) resulted in translocation of the cytosolic NF-κBp65 for the nucleus (Figure 4B). Besides, stimulation with $Pam3CSK_4$ triggered the overall degradation of IκB-α 30 min after the stimulus (Fig. 4C). Preincubation of cells with MG significantly reduced the nuclear translocation of NF-κB in all concentrations tested (Fig. 4B) and effectively suppressed IκB-α degradation within 30 min in a concentration-dependent manner (Figure 4C).

These results indicate that the suppression of IκB-α degradation by MG might inhibit pro-inflammatory gene expression due to reduce the translocation of NF-κBp65 to the nucleus and consequently, NF-κB activity.

Figure 4.

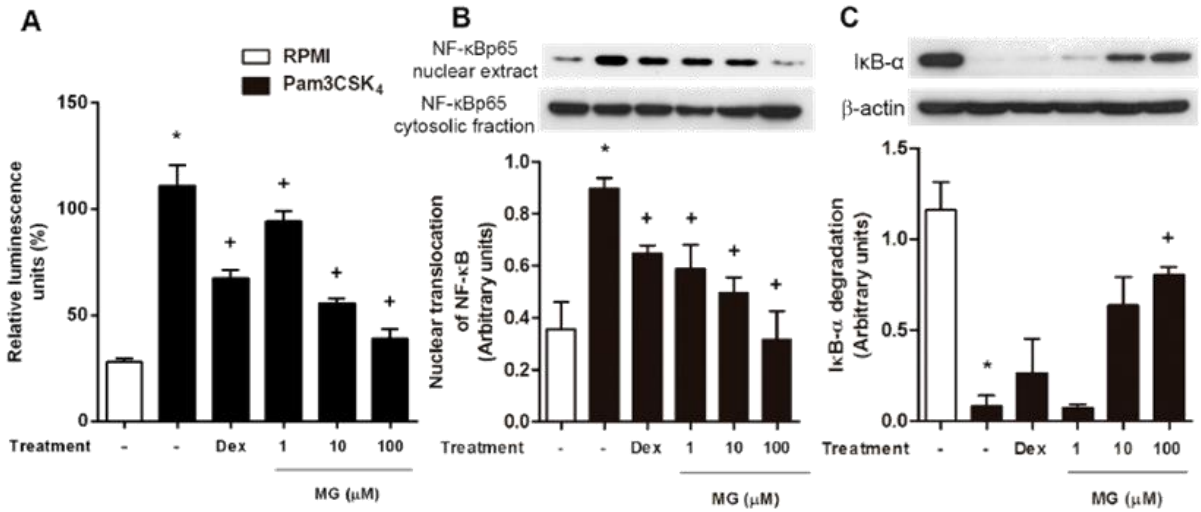


Figure. 4. MG inhibits NF-κB activity, nuclear translocation and IκB-α degradation on BMDMs. (A) RAW264.7 macrophages were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with $Pam3CSK_4$ (1 μg/mL) for 6 h. NF-κB activity was quantified using RAW264.7 macrophages stably expressing the NF-κB pLUC gene. After 6 h of incubation, cells were lysed and incubated with luciferin. Luminescence units were measured by a luminometer. (B–C) Bone marrow-derived macrophages were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with $Pam3CSK_4$ (100 ng/mL) for 1 h (for NF-κB) or 30 min (for IκB-α). Total cell lysates or nuclear and cytoplasm protein fraction were separated by SDS-PAGE and subjected to western blotting for NF-κBp65 or IκB-α. A representative of three different western blots is shown in the top, whereas densitometric analyses are shown in the graphs. Results were expressed as the mean ± SEM of three independent experiments and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by *P≤ 0.05. Whereas +P≤ 0.05 represent significant differences between treated and stimulated groups.

2.5 Methyl gallate inhibits cytokine production and NF-κB activity induced by TLR4 activation.

Toll-like receptor 4 (TLR4) is another member of the TLR family that plays an essential role in innate immunity through inflammatory cytokine induction[38]. It has been described that polyphenols, including flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans can target TLR4 signaling pathways in multiple ways[11].

We next evaluated the effect of MG on macrophages stimulated with LPS, a TLR4 agonist. As observed in Figure 5, the stimulation of RAW264.7 macrophages with LPS (100 ng/mL) induced the production of TNF-α, IL-6 and CXCL-1 and the activation of

NF-κB. Pre-treatment with MG did not affect TNF-α production (Figure 5A) whereas significantly inhibited IL-6 and CXCL-1 production in a concentration-dependent manner (Figure 5B-C). Notably, pre-treatment of RAW264.7 macrophages with MG (1-100 μM) promoted a concentration-dependent reduction of luminescence emission induced by LPS (Figure 5D). To verify whether MG inhibits NF-κB activity by signaling pathways different from TLRs, we use the specific activator of protein kinase C [PKC], PMA as the stimulus. As shown in the insert of Figure 5, the pre-treatment with MG (1-100 μM) was not able to inhibit NF-κB activation induced by PMA. This result suggests that MG modulates NF-κB activity through the signaling pathway activated by TLRs ligands.

Figure 5.

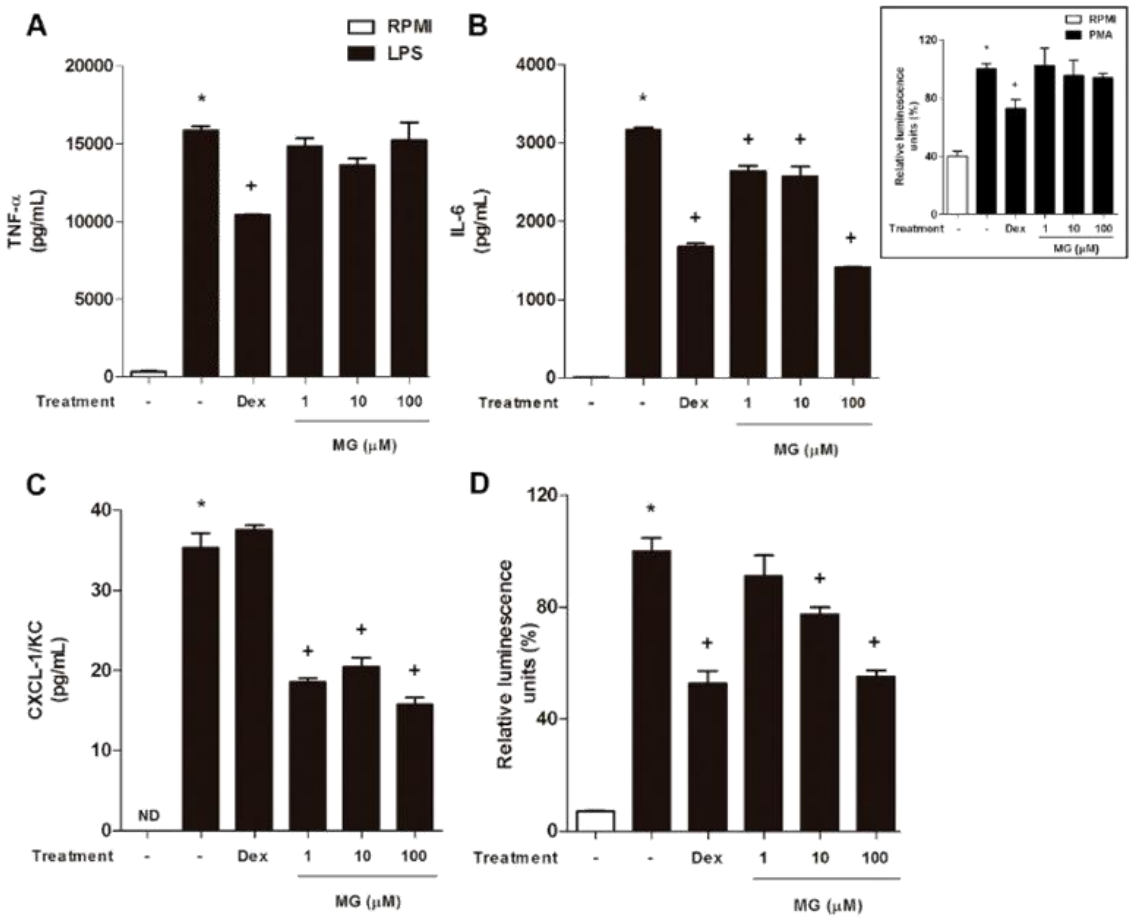


Figure. 5. MG inhibits inflammatory cytokines production and NF-κB activity in LPS-stimulated macrophages. (A-E) RAW264.7 macrophages were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with LPS (100 ng/mL) or PMA (100 ng/mL) for 6 h. (A-C) The supernatant was collected (6 h after stimulus) to evaluate TNF-α, IL-6 and CXCL-1 production by ELISA. (D-E) NF-κB activity was quantified using RAW264.7 macrophages stably expressing the NF-κB pLUC gene. After 6 h of incubation, cells were lysed and incubated with luciferin. Luminescence units were measured by a luminometer. Results are expressed as the mean ± SEM for quadruplicate wells per group from

at least two independent experiments and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by $^{*}P \leq 0.05$. Whereas $^{+}P \leq 0.05$ represent significant differences between treated and stimulated groups.

2.6 Methyl gallate decreases MAPK phosphorylation and nuclear translocation of c-Jun and c-Fos in stimulated-macrophage.

The TLRs activation via MyD88-dependent pathway can activate alternatively, the TAK1-dependent mitogen-activated protein kinase (MAPK) pathway. MAPK is a highly conserved family of serine/threonine protein kinases which plays a crucial role in many aspects of immune-mediated inflammatory responses. Among the subfamilies of MAPKs, the extracellular signal-regulated kinase (ERK), the c-Jun NH terminal kinase (JNK) and the p38 MAP kinase are extensively studied[39].

The effect of few polyphenols on MAPK pathway have been described at different levels of the signaling pathway. Luteolin reduces TNF-α production in LPS-stimulated-macrophages by blocking ERK1/2 and p38 phosphorylation[40]. Resveratrol downregulated the phosphorylation of p38 MAPK and c-Jun N-terminal kinase in IL-1β-stimulated RSC-364 cells[41]. In epithelial cells, luteolin, as well as chrysin and kaempferol inhibit ERK, JNK and P38 blocking ICAM-1 expression induced by TNF-α[40, 42]. Quercetin downregulates the phosphorylation of p38 MAPK in different cells[43, 44].

In this study, we investigated the effect of MG in activation MAPKs (ERK1/2, JNK and p38). As shown in Figure 6, the stimulation of BMDM with $Pam3CSK_4$ (100 ng/mL) for 30 min. triggered the phosphorylation of ERK1/2 (Figure 6A), JNK (Figure 6B), and p38 MAPK (Figure 6C). The pre-treatment with MG (1 – 100 μM) decreased the phosphorylation of ERK1/2, JNK and p38 MAPK mainly in the concentration of 100 μM.

Activation of JNK and ERK1/2 lead to phosphorylation of c-Jun and c-Fos, respectively, which constitute the transcription factor AP-1 (activator protein-1)[45]. AP-1 is an important regulatory protein involved in cell growth, differentiation, transformation, apoptosis, and also contribute to inflammatory response[46]. In the

inflammatory progression, AP-1 regulates the production of cytokines like TNF-α, IL-1, and IL-2[47].

In order, to determine whether MG regulates the translocation of c-jun and c-fos from the cytoplasm into the nucleus, BMDM were pre-treated with MG for 1 h prior the stimulation with $Pam3CSK_4$ for 1 h, and the nucleus fraction was prepared and subjected to western blot analysis. The pre-treatment with MG significantly reduced the translocation of c-jun and c-fos induced by $Pam3CSK_4$ in all concentration tested (Figure 6A-B).

These results suggest that the inhibition of MG in c-Jun and c-Fos translocation through inhibiting ERK1/2, JNK, and p38 phosphorylation could be involved in the modulation of cytokines production, observed after TLR2 activation.

Figure 6.

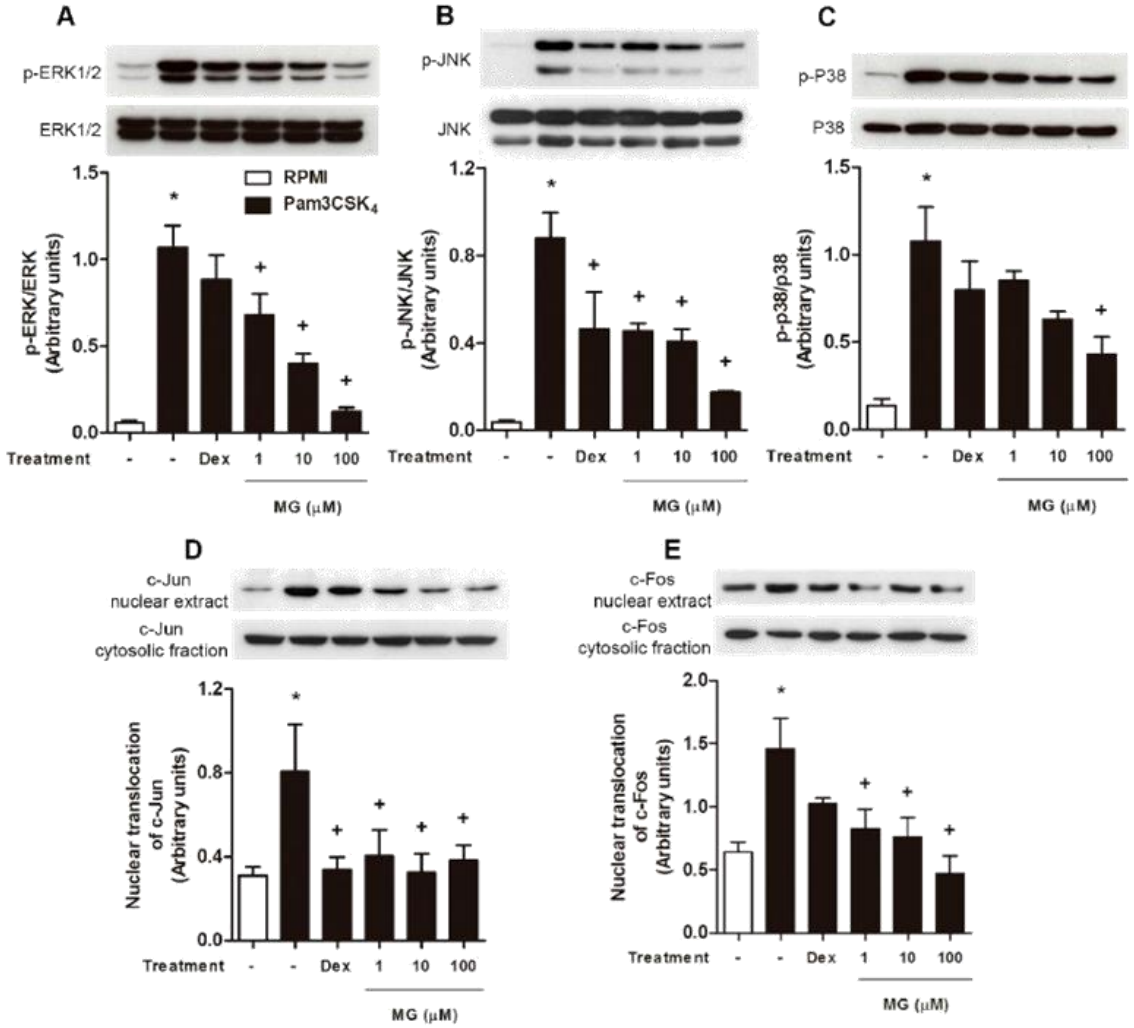


Figure. 6. MG inhibits activation of MAPKs and nuclear translocation of c-Jun and c-Fos on BMDMs. (A–E) Bone marrow-derived macrophages were treated with vehicle, dexamethasone (1 μM) or MG (1, 10 or 100 μM) for 1 hour and then incubated with Pam3CSK4 (100 ng/mL) for 30 min (for MAPKs) or 1 h (for c-Jun and c-Fos). Total cell lysates or nuclear and cytoplasm protein fraction were separated by SDS-PAGE and subjected to western blotting for ERK1/2, JNK, p38, c-Jun or c-Fos. The representative of three different western blots is shown in top, whereas densitometric analyses are shown in the graphs. Results were expressed as the mean ± SEM of three independent experiments and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by $^{*}P \leq 0.05$. Whereas $^{+}P \leq 0.05$ represent significant differences between treated and stimulated groups.

Herein, we demonstrate that MG plays an inhibitory effect in two pathways involved in macrophages stimulate by TLRs agonists.

MG inhibited the nuclear translocation of the transcription factor, NF-κB, and the MAPK pathway that leads to AP-1 activation. These results strongly suggest that the

upstream enzymes regulating the translational activation of NF-κB and AP-1 might be directly targeted by MG. In fact, our results showed a suppressive effect of MG to impairs IκB-α degradation and, ERK, JNK and p38 phosphorylation, which are involved in the modulation of NF-κB and AP-1. However, the association of TLRs and MyD88 stimulates the recruitment of members of the IRAK family that interact with members of the TRAF family. In sequence, TRAF activates TAK1, which in turn activates two downstream pathways the NF-κB complex and the MAPKs[48]. We cannot rule out the possibility that some of these proteins will be a molecular target of MG and therefore, this substance could be interfering with both pathways. However, more studies will be needed to clarify this hypothesis.

In the present study we provide molecular evidence for the anti-inflammatory mechanism of MG. We demonstrate that MG exerted its anti-inflammatory action via inhibition of cytokines production (TNF-α, IL-6, CXCL-1, and IL-1β) through reduction of NF-κB and c-Jun and c-Fos translocation, particularly affecting the ERK1/2, JNK and p38 MAPK phosphorylation as well as the IκB-α degradation. Moreover, MG may represent a potential phytochemical or template molecule that should be investigated in different types of inflammation that involves macrophages activation.

3. Materials and Methods

3.1 Antibodies e reagents

Methyl gallate (98% purity) was purchased from Fluka (Sigma-Aldrich). Zymosan A, dexamethasone, phosphate buffered saline (PBS) tablets, penicillin, perborate buffer, Tween-20, *o*-phenylenediamine dihydrochloride (OPD), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), protease inhibitors cocktail, lipopolysaccharide (LPS), and phorbol myristate (PMA) were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine and RPMI-1640 were obtained from Gibco (Grand Island, NY). $Pam3CSK_4$ was obtained from InvivoGen (San Diego, CA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from GE Healthcare (Chicago, IL, USA). Purified anti-murine TNF-α, CXCL-1/KC, IL-1β and IL-6 mAbs, biotinylated anti-TNF-α, IL-CXCL-1, IL-1β and IL-6 mAbs and recombinant TNF-α, CXCL-1/KC, IL-1β and IL-6 were all obtained from R&D Systems (Minneapolis, MN, USA). Rabbit polyclonal anti-phospho-ERK1/2 (cat. #4370S), anti-ERK1/2 (cat. #4695P), anti-phospho-JNK1/2 (cat. #4668S), anti-JNK1/2 (cat. #9258S), anti-phospho-p38 (cat. #9216S), anti-p38 (cat. #9212S), NLRP3 (cat. #15101) and β-actin (cat. #4967L) were obtained from Cell Signaling (Danvers, MA, USA). Polyclonal IκB-α (sc-371), anti-NF-κB (sc-372), c-jun (sc-45) and c-fos (sc-52) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

3.2 Macrophage culture, stimulus and treatment

RAW264.7 cell line murine macrophage was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere. Macrophages were detached from flasks by gentle scraping and plated in 24-well culture plates (1 x 10^5 cells/well/500µL) overnight prior stimulus. The macrophages were treated with dexamethasone (1 µM), MG (1, 10 or 100 µM) or vehicle (RPMI) one hour before challenge with zymosan (30 µg/mL), $Pam3CSK_4$ (1 µg/mL), PMA (100 ng/mL) or lipopolysaccharide (LPS; 100 ng/mL). After 6 h, the supernatants were recovered and centrifuged at 400 x g for 10 min

at 4°C. The cell-free supernatants were stored at -70°C for analysis of pro-inflammatory mediators.

3.3 Cell viability assay by MTT

RAW264.7 macrophage was cultured in 96-well plates (2 x 10^5 cells/well) at 37°C, 5% CO_2 overnight. Then, the cells were treated with dexamethasone (1 μM) or MG (1, 10 or 100 μM) and stimulated with zymosan (30 μg/mL), $Pam3CSK_4$ (1 μg/mL) or LPS (100 ng/mL). After the incubated period, supernatants were removed, and the cells were incubated with MTT solution (200 μL; 1 mg/mL) (tetrazolium salt; Sigma) in RMPI-1640 medium for 4 h. Thereafter, the solution was removed and intracellular reduced MTT (formazan) was dissolved in 200 μL of DMSO. The absorbance was read at 540 nm wavelength in a SpectraMax M5 (Molecular Devices), and the results are expressed as percentage reduction compared to the control group (vehicle).

3.4 Chemokine and cytokines quantification

The levels of TNF-α, IL-6, CXCL-1/KC and IL-1β were determined 4 or 6 h after stimulation with zymosan (30 μg/mL), $Pam3CSK_4$ (1 μg/mL) or LPS (100 ng/mL). Sandwich enzyme-linked immunosorbent assay (ELISA) was performed using protocols supplied by the manufacturers (R&D Systems, Minneapolis, MN, USA). Results are expressed as pg per milliliter (pg/mL).

3.5 Luciferase-Nuclear Factor Kappa B (NF-κB) reporter assay

RAW264.7 macrophages that stably bear the luciferase reporter gene controlled by an NF-κB-sensitive promoter (pNF-κB-Luc) were obtained from Dr. Thiago Mattar Cunha. For luciferase reporter assay, RAW264.7 macrophages (5 x 10^5 cells/well) were grown in 24-well plates overnight prior to stimulus. After culturing, cells were pretreated with MG in different concentrations (1, 10 or 100 μM) or dexamethasone (1 μM) 1 h prior to challenge with zymosan (30 μg/mL), $Pam3CSK_4$ (1 μg/mL), LPS (100 ng/mL) or PMA (100 ng/mL) for 6 h. For analysis, cells were lysed with 50 μL of TNT lysis buffer at 4°C for 20 min. Lysates (10 μL) were transferred to an opaque white plate and then incubated with 25 μL of Luciferase Assay Reagent containing luciferin (Promega, Madison, WI,

USA). The activation of the NF-κB transcription factor in these cells leads the transcription of the luciferase gene, by catalyzing the luciferin oxidation reaction, producing oxyluciferin and photons, and the activity of this enzyme can be quantified by the emission of photons. Luminescence emission was measured using a luminometer (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA).

3.6 Bone marrow-derived macrophages

To evaluated the effects of MG on intracellular signaling, murine bone marrow-derived macrophages (BMDMs) were obtained from femurs and tibias of 6-to-8-week-old mice after 7 days of differentiation in RPMI-1640 (Gibco) containing 20% FBS (v/v), penicillin (100 U/mL) and 20% of L929 cell culture supernatant (v/v) expressing mouse macrophage colony-stimulating factor (M-CSF), at 37ºC in a 5% CO_2 atmosphere. After differentiation, the cells were seeded at a density of 2 x 10^6 cells/well in 6-well plates and were treated with vehicle (RPMI), dexamethasone (1 μM) or different concentrations of MG (1, 10 or 100 μM). One hour after treatment, the cells were stimulated with $Pam3CSK_4$ (100 ng/mL) for 30 min to verify the MAPKs expression and IKB-α degradation. For analysis of NF-κB, c-Fos and c-J translocation, the stimulus remained for 1 h.In one set of experiments, BMDMs were stimulated with 30 μg/mL of zymosan for 4 hour and pulsed with adenosine triphosphate [ATP] (2 mM), for the last 30 minutes to activate NLRP3 inflammasome.

3.7 Western blot

Bone marrow-derived macrophages (BMDM) were plated in six-well plates (2 x 10^6 cell/well) and allowed to stabilize overnight. Then, cells were treated and stimulated as described earlier. For total cell extract, macrophages were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, and protease and phosphatase inhibitors). For cytoplasmic protein fractions, cells were lysed (Cytoplasmic Lysis Buffer; 10 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Triton X-100, 0.5 M Sucrose, 1 mM DTT, 1 mM PMSF, and protease inhibitors cocktail) and centrifuged at 2.000 rpm for 10 minutes. Supernatants containing cytoplasmic proteins were recovered and pellets with nuclei were lysed (Nuclear Lysis Buffer; 10 mM HEPES, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitors cocktail),

sonicated for 5 minutes, and centrifuged at 14.000 rpm for 10 minutes. Nuclear protein fractions as well as cytoplasmic fractions were recovered and stored at -80°C. The protein concentration of each sample was determined using the Lowry method (Sigma Chemical Co). Lysates were denatured in Laemmli buffer (50 mM Tris-HCL, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) at 90 ºC for 5 min. Protein samples (30 µg/well) were resolved by 10% acrylamide gels for SDS/PAGE. Thereafter, proteins were transferred onto polyvinylidene difluoride (PVDF) Hybond™ membranes (Amersham Biosciences, Buckinghamshire, UK), non-specific binding sites were blocked with 5% BSA or 5% milk in Tris-buffered saline-Tween (TBST; 20 mM Tris-HCl pH 7.6, 140 mM NaCl, 0.05% Tween 20) for 2 h at room temperature. The membranes were probe with rabbit polyclonal anti-phospho-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), anti-phospho-JNK1/2 (1:1000), anti-JNK1/2 (1:1000), anti-phospho-p38 (1:1000), anti-p38 (1:1000), anti-NF-κB (1:1000), IKB-α (1:2500), anti β-actin (1:1000), anti-c-fos (1:1000), anti-c-jun (1:1000) and anti-NLRP3 (1:100) in TBST with 5% BSA or 5% milk overnight. To detect proteins bands, the membranes were incubated with HRP-conjugated secondary antibodies in TBST 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.

3.8 Statistical Analysis

Results are reported as the mean ± SEM and were statistically analyzed by means of analysis of variance (ANOVA) followed by Newman-Keuls-Student test. All tests were performed in GraphPad Prism 5.00 (GraphPad Software, La Jolla, CA, USA). Values of $p \leq 0.05$ were regarded as significant.

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Conceptualization, Maria G. Henriques; Formal analysis, Elaine C. Rosas and Maria G. Henriques; Funding acquisition, Maria G. Henriques; Investigation, Luana B. Correa, Leonardo N. Seito and Elaine C. Rosas; Methodology, Luana B. Correa, Leonardo N. Seito, Thiago M. Cunha and Elaine C. Rosas; Project administration, Elaine C. Rosas and Maria G. Henriques; Supervision, Thiago M. Cunha, Elaine C. Rosas and Maria G. Henriques; Writing – original draft, Luana B. Correa and Elaine C. Rosas; Writing – review & editing, Thiago M. Cunha, Elaine C. Rosas and Maria G. Henriques.

All authors read and approved the final manuscript

Conflicts of interest

The authors declare no conflicts of interest.

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Methyl gallate attenuates Toll-Like ligands-induced inflammation: effect on NF-κB and MAPK activation.

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Supporting information

Table S1: Macrophage viability after treatment with MG

	Vehicle	Zymosan	Dex.	1 µM	10 µM	100 µM
Macrophage viability (%)	100	98%	100%	100%	100%	100%

Results were expressed as percentage of viability (%). Cells were plated, in quadruplicate (2 x 10^5 cell/well), pretreated with MG (1-100 µM) or dexamethasone (1 µM), and stimulated with zymosan (30 µg/mL) for 6 hours. Cell viability was assessed by MTT assay. Compound concentrations that induced ≥ 10% of cell death were considered cytotoxic.

Table S2: Macrophage viability after treatment with MG

	Vehicle	$Pam3CSK_4$	Dex.	1 µM	10 µM	100 µM
Macrophage viability (%)	100	91%	90%	91%	90%	90%

Results were expressed as percentage of viability (%). Cells were plated, in quadruplicate (2 x 10^5 cell/well), pretreated with MG (1-100 µM) or dexamethasone (1 µM), and stimulated with $Pam3CSK_4$ (1 µg/mL) for 6 hours. Cell viability was assessed by MTT assay. Compound concentrations that induced ≥ 10% of cell death were considered cytotoxic.

4 RESULTADOS COMPLEMENTARES

4.1 MATERIAL E MÉTODOS

4.1.1 ANIMAIS

Neste estudo foram utilizados camundongos machos da linhagem isogênica C57BL/6, com peso médio variando entre 18-25 g, oriundos do Centro de Criação de Animais de Laboratório (CECAL-FIOCRUZ), Rio de Janeiro, RJ, Brasil.

Os camundongos foram mantidos no biotério experimental do laboratório de Farmacologia Aplicada (Farmanguinhos/FIOCRUZ) com temperatura controlada de 23° C ± 2°C e ciclo claro-escuro constante de 12/12 horas com livre acesso à água e ração até o momento do uso.

Os experimentos envolvendo animais foram conduzidos de acordo com as normas éticas internacionais, sendo o projeto devidamente aprovado no Comitê de Ética em Uso de Animais (CEUA) da Fundação Oswaldo Cruz sob a licença de número LW-14/18 e de acordo com as recomendações da *International Association for the Study of Pain* (Zimmerman, 1983).

4.1.2 TRATAMENTOS

Após aproximadamente 12 horas de jejum, os animais receberam o galato de metila (GM) por via oral (v.o), diluído em água filtrada, na dose de 7 mg/kg em um volume final de 200 µL, 1 hora antes da indução do desafio intra-articular (i.a.) com o antígeno. A dexametasona foi utilizada como inibidor de referência e foi administrada por via intraperitoneal (i.p.) 1 hora antes do estímulo na dose de 10 mg/kg em um volume final de 100 µL. Os animais do grupo controle receberam 200 µL de água filtrada por via oral.

Em outro conjunto de experimentos, os camundongos foram tratados diariamente com o GM (7 mg/kg; v.o.) ou com a dexametasona (10 mg/kg; i.p.) durante sete dias consecutivos.

4.1.3 INDUÇÃO DA ARTRITE POR ANTÍGENO (AIA)

Camundongos C57BL/6 foram imunizados através de injeção subcutânea (s.c.) no dia 0 com 500 µg de albumina de soro bovino metilada (mBSA) em 0,2 mL de uma emulsão contendo: 0,1 mL de solução salina e 0,1 mL de adjuvante completo de Freund's. Os camundongos receberam reforço da mesma solução no dia 07. Camundongos *sham* (falso-imunizado) receberam injeções semelhantes, no dia 0 e no dia 07, mas sem o antígeno (mBSA). No vigésimo primeiro dia após a injeção inicial, a artrite foi induzida nos animais imunizados ou *sham* através de injeção intra-articular (i.a.) de mBSA (30 µg/cavidade; 25 µL) ou salina. As análises foram realizadas 7 h ou 24 h após o desafio i.a (Figura 4.1, esquema 1).

Em outro conjunto de experimentos, no vigésimo quinto dia foi realizado uma reinjeção do antígeno (30 µg/cavidade) e os camundongos foram submetidos a eutanásia no vigésimo oitavo dia (Figura 4.1, esquema 2)**.**

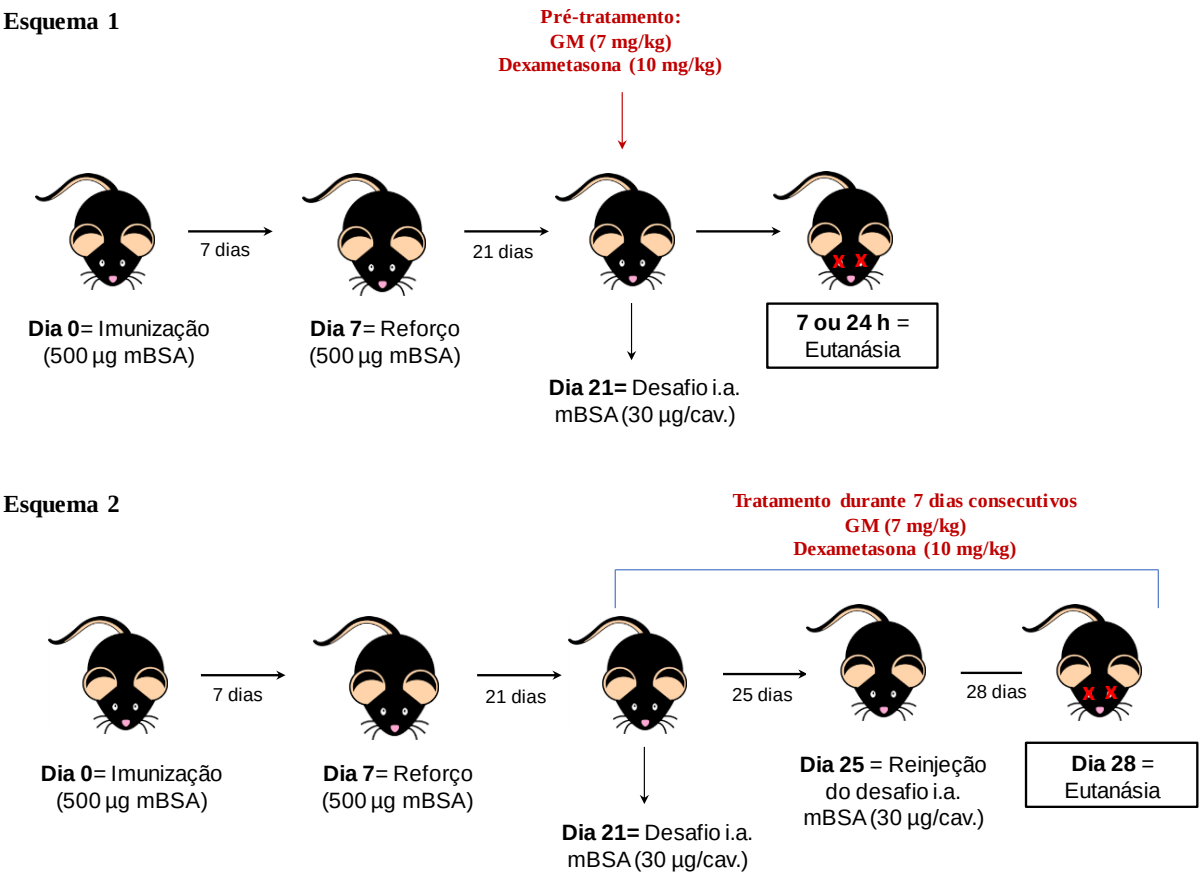


Figura 4.1: Representação esquemática do protocolo experimental de artrite induzida por antígeno.

4.1.3.1 AVALIAÇÃO DO EDEMA ARTICULAR

O edema da articulação do joelho foi avaliado pela medida do diâmetro transverso da articulação fêmuro-tibial, com o auxílio de um paquímetro digital (Digmatic caliper, Mitutoyo Corp. Kanagawa, Japão), antes e depois do desafio i.a. Os valores dos edemas foram expressos pela diferença (Δ) entre os diâmetros medidos antes (basal) e após a indução da inflamação articular e foram representados em milímetros (mm).

4.1.3.2 COLETA DO LÍQUIDO SINOVIAL

Os camundongos foram submetidos à eutanásia com excesso de anestésico (pentobarbital sódico 3% - Hypnol) 7 ou 24 horas após a injeção intra-articular de mBSA. A cavidade sinovial foi lavada com auxílio de uma seringa acoplada a uma agulha de 21G contendo 300 µL de PBS/EDTA (10 mM). A agulha foi introduzida através do ligamento suprapatelar do joelho de camundongos, permitindo o acesso, a lavagem e a aspiração do

líquido presente no interior da cavidade articular. Foi realizada a contagem total e diferencial dos leucócitos presentes no lavado sinovial. O lavado sinovial foi centrifugado a 400 x *g* por 10 minutos para recolhimento do sobrenadante para análises posteriores.

4.1.4 CONTAGEM DOS LEUCÓCITOS

4.1.4.1 CONTAGEM DE LEUCÓCITOS TOTAIS

A contagem do número total de leucócitos presentes no lavado sinovial foi realizada através da diluição de uma alíquota (50 µL) dos lavados em isoton (1:100), e a análise foi realizada através de um contador automático de micropartículas (Z1; Beckman-Coulter, EUA).

4.1.4.2 CONTAGEM DIFERENCIAL DE LEUCÓCITOS

Para a contagem diferencial, alíquotas das suspensões celulares foram centrifugadas em lâminas de vidro utilizando-se citocentrífuga (Cytospin 3, Shandon Inc., - EUA, 450 RPM, 5 min). A coloração foi realizada pelo método de May-Grunwald-Giemsa. De acordo com este método, as lâminas ficam imersas em May-Grunwald por 5 minutos, para fixação e coloração de grânulos celulares, 3 minutos em água para retirada do excesso de corante e 15 minutos em Giemsa, para corar o núcleo. A contagem das células foi feita em microscópio óptico com objetiva de imersão em óleo, com aumento de 100x.

4.1.5 HISTOLOGIA

As articulações fêmuro-tibial de camundongos C57BL/6, 24 horas ou 7 dias após o desafio i.a. com mBSA (30 µg/cavidade), foram removidas, dissecadas e fixadas por 48 horas em formalina a 10% em tubos contendo aproximadamente 50 mL. Logo após, a articulação foi descalcificada em solução tampão fosfato (0,1 M)/EDTA 10% durante aproximadamente 3 semanas, com trocas diárias do tampão, sendo posteriormente processada para histologia convencional, sofrendo inclusão em parafina. Com o auxílio de um micrótomo (Shandon), os blocos contendo as peças foram seccionados em uma espessura de 5 µm e colocados em uma lâmina de vidro para posterior coloração pela técnica de hematoxilina e eosina ou safranina. As lâminas foram montadas com lamínulas de vidro em meio próprio e analisadas em microscópio óptico (*Olympus* BX41, Japão). As seções de menor aumento (100x) foram utilizadas para permitir a visualização de toda a área da cavidade sinovial e foram classificadas subjetivamente,

avaliando os seguintes parâmetros: hiperplasia sinovial (formação de pannus; de 0 = sem hiperplasia, a 3 = hiperplasia severa); presença de infiltrado inflamatório (de 0 = sem inflamação, a 3 = inflamação severa), e sangramento (de 0 = sem sangramento, a 3 = sangramento severo). A coloração da safranina foi utilizada para avaliar a perda de proteoglicanos das cartilagens, sugerindo degradação da cartilagem e também foi incluída na avaliação (de 0 = sem degradação da cartilagem, a 3 = degradação da cartilagem severa). Todos os parâmetros foram subsequentemente somados para fornecer o índice de artrite (IA; expresso como a média ± EPM)(Williams *et al.*, 2007).

Em outro conjunto de experimentos, as articulações fêmuro-tibial foram coradas com kit TRAP 387A (Sigma-Aldrich, St. Louis, MO, USA) e as células positivas para TRAP ficam destacadas na cor vermelho-púrpura. As lâminas coradas foram analisadas e foi realizada a busca e contagem de células TRAP-positivas na área de epífise de crescimento da articulação (fêmur). O resultado foi expresso como número de células TRAP-positivas.

4.1.6 PREPARAÇÃO DO EXTRATO DA ARTICULAÇÃO FÊMURO-TIBIAL

O extrato da articulação fêmuro-tibial foi realizado como previamente descrito por Rosengren e colaboradores (Rosengren *et al.*, 2003) e adaptado para uso em camundongos. Os camundongos foram eutanasiados 7 horas após a injeção i.a. de mBSA (30 µg/cavidade; 25 µL) ou salina estéril, e quase a totalidade do tecido adjacente a articulação fêmuro-tibial, incluindo tendões, osso e tecido muscular, foram removidos resultando em um espécime triangular bem definido e padronizado da articulação em estudo, como descrito por Van Meurs (Van Meurs *et al.*, 1997). Após a remoção e dissecação do espécime, o tecido foi imediatamente congelado em nitrogênio líquido, durante 5 minutos. A articulação foi homogeneizada manualmente com auxílio de um pistilo de teflon em *potter* de vidro (Kontes Glass Company, New Jersey, EUA) contendo 1 mL de solução gelada de HBSS contendo 0,4% de Triton-X e 0,2% de inibidor de protease (Sigma Aldrich, EUA), em uma proporção indicada pelo fabricante. O homogenato foi então centrifugado (5000 x g por 10 min a 4ºC), o sobrenadante foi recolhido e armazenado a -70ºC até o momento do uso.

4.1.7 *ENZYME-LINKED IMMUNOSORBENT ASSAY* (ELISA)

As concentrações das citocinas TNF-α, IL-17 e das quimiocinas CCL-2 e CCL-3 presente nos sobrenadantes dos lavados ou nos extratos da articulação fêmuro-tibial foram

determinadas pelo método de ELISA sanduíche, utilizando pares de anticorpos específicos de acordo com as instruções do fabricante R&D Systems (Minneapolis, EUA). A absorbância foi determinada a 490 nm em espectrofotômetro (SpectraMax M5/M5e; Molecular Devices). Os resultados foram expressos como picograma de cada citocina ou quimiocina por mililitro (pg/mL).

4.1.8 MEDIÇÃO DO TÍTULO DE ANTICORPOS ANTI-MBSA

Os títulos séricos de anticorpos anti-mBSA no soro de camundongos C57BL/6 foram determinados por ELISA de captura 7 h, 24 h ou 7 dias após a indução da AIA. Em resumo, placas de 96 poços (Nunc, Roskilde, Dinamarca) foram revestidas com 50 µL de solução mBSA (10 µg/mL; 2 µg/poço, em tampão PBS) overnight a 4ºC. No dia seguinte, foi adicionado aos poços 200 µL de solução de PBS-*Milk* 3%, para bloqueio dos locais de ligação não específicos, durante 2 horas. Posteriormente, diluições seriadas dos soros foram adicionadas e incubadas overnight a 4ºC. Foi utilizado IgG anti-mouse conjugado a HRP como anticorpo secundário (1/800 diluição), e após 1 hora, as placas foram lavadas com PBS tween 0,5%, e o reagente OPD (0,5 mg/mL; 100 µL) foi adicionado. Após 10 min, a reação foi parada com H_2SO_4 1 M (100 µL). A absorbância foi lida a 490 nm e os valores comparados entre grupos dentro da mesma diluição.

4.1.9 QUANTIFICAÇÃO DE DNA EXTRACELULAR

Os líquidos sinoviais dos animais submetidos a AIA foram recolhidos 7 e 24 h após o desafio i.a. com mBSA. Os lavados foram centrifugados para remoção de células e 50 µL do sobrenadante foi adicionado a 50 µL de Quanti-it™PicoGreen® dsDNA (Invitrogen), e a leitura determinada a 485/538 nm emissão/excitação conforme Gardinassi et al (2017). Os resultados foram expressos em unidades arbitrárias (UA).

4.1.10 ANÁLISE DE PARÂMETROS BIOQUÍMICOS

Foi realizada a coleta de sangue por punção cardíaca, após a eutanásia dos animais com excesso de anestésico (pentobarbital sódico 3% - Hypnol) 7 dias após a injeção intra-articular de mBSA. As amostras foram acondicionadas em microtubos contendo anticoagulante heparina. O plasma foi separado por processo de centrifugação a 2.500 rpm/10 min, obtendo-

se o plasma livre de hemólise. A quantificação dos parâmetros bioquímicos contou com as dosagens de: uréia Bum, ácido úrico, aspartato aminotransferase (AST) e alanina aminotransferase (ALT). Os ensaios foram realizados através da metodologia de química seca pelo equipamento Vitros 250 (Ortho clinical; Jonhson & Jonhson).

4.1.11 CULTURA DE OSTEOCLASTOS E COLORAÇÃO DE TRAP

Camundongos C57BL/6 foram submetidos à eutanásia com excesso de anestésico (isoflurano 100%) em seguida, seus fêmures e tíbias de ambas as patas traseiras foram dissecados e removidos. A extremidade distal do osso foi removida e cada fêmur e cada tíbia foram lavados com 2 mL de PBS e centrifugados a 2000 x g por 5 min. As células obtidas foram ressuspensas em PBS e novamente centrifugadas a 600 x *g* durante 5 min e o sobrenadante foi descartado. As células foram ressuspendidas em meio α-MEM (Thermo Fisher Scientific, Waltham, MA, USA) completo com M-CSF (30 ng/mL; R&D Systems,Minneapolis, MN, USA), 10% de SFB e 1% de P/S (penicilina/estreptomicina; Sigma-Aldrich) e semeadas em placas de Petri (13 x 10^6 células/placa) por 72 h. Para o ensaio de coloração de TRAP, as células não aderentes foram removidas (após as 72 h), e as BMDMs (do inglês *bone marrow-derived macrophages*) foram semeadas de acordo com cada experimento e posteriormente cultivadas em meio α-MEM com M-CSF (30 ng/mL), 10% de SFB, 1 % de P/S e RANKL (10 ng/mL; R&D Systems).

Os pré-osteoclastos foram semeados em placa de 96 poços (2 x 10^4 células/poço) e tratados com diferentes concentrações de GM (3, 10, 30 e 100 μM) por mais 96 h, com uma troca de meio após 48 h. Em seguida, as células foram coradas com TRAP, como descrito anteriormente (Da Cunha *et al.*, 2017). Brevemente, as células foram fixadas e coradas com TRAP com um kit comercial específico, seguindo as instruções do fabricante. Células $TRAP^+$ contendo mais de três núcleos/célula foram considerados osteoclastos, e nenhuma distinção foi feita entre osteoclastos grandes e pequenos. Nove campos foram selecionados e contados para osteoclastos maduros e representados como células $TRAP^+$ multinucleadas/poço. Para determinar a área dos osteoclastos, as imagens foram analisadas usando o software *ImageJ*. Foram utilizados cinco poços por grupo em dois experimentos independentes.

4.1.12 ANÁLISE DA VIABILIDADE DOS OSTEOCLASTOS *IN VITRO*

Os pré-osteoclastos foram semeados em placas de 96 poços (2 x 10^4 células/poço) e cultivados com meio osteoclástico (meio α-MEM com M-CSF, 10% de SFB, 1 % de P/S e RANKL) e diferentes concentrações de GM (3, 10, 30 e 100 µM). No terceiro dia, 5 mg/mL da solução de MTT (22,5 µL) foram adicionados em cada poço, conforme descrito anteriormente (Oliveira *et al.*, 2011). Após 3 h, a placa foi centrifugada (200 x g/ 5 min), o meio foi removido e foi adicionado 150 µL DMSO para dissolver os cristais de formazan. A densidade óptica (DO) foi medida a 570 nm usando um leitor de microplacas ((Molecular Devices, Sunnyvale, CA, USA). A OD média do grupo controle (RANKL) foi estabelecido em 100%, e os grupos experimentais foram comparados a RANKL.

4.1.13 PCR QUANTITATIVO EM TEMPO REAL (RT-PCR)

Pré-osteoclastos foram semeados (2 x 10^5 células/poço) em placas de 24 poços em meio osteoclástico (M-CSF + RANKL) e tratados com GM (30 µM) por 48 h. O RNA total foi isolado a partir da cultura de células utilizando o kit *SV Total Isolation System* e foi então avaliado sua quantidade e pureza usando um espectrofotômetro (Nanodrop Technologies, Wilmington, DE, EUA). O DNA complementar foi sintetizado utilizando o kit *High Capacity*, que requer 500 ng de RNA total. O qPCR foi realizado utilizando o kit *TaqMan* PCR (PCR BioSystems, Londres, RU), de acordo com as instruções do fabricante em um sistema ABI *One Step Sequence Detection System* (Applied Biosystems, Foster City, CA, EUA), usando um conjunto de primers/sondas TaqMan pré-projetados. Os seguintes ciclos de reação foram ajustados para amplificação do cDNA sintetizado: 95ºC durante 2 min, seguidos de 40 ciclos de 60ºC por 30 s e 95ºC por 5 s. O NFATc1, Acp5 (TRAP), integrina β3 (Itgb3) e CTR foram avaliados utilizando o GAPDH (gliceraldeído-3-fosfato desidrogenase) como controle endógeno (gene de referência). Os gráficos mostram a expressão relativa do gene de interesse normalizado para a população controle (RANKL). O experimento foi feito em quadruplicada em dois experimentos independentes.

4.1.14 WESTERN BLOT

Os lisados celulares totais e as proteínas das amostras da articulação fêmuro-tibial de camundongos submetidos a AIA foram obtidos utilizando tampão RIPA (Sigma-Aldrich) com

um *cocktail* de inibidores de protease e fosfatase (Sigma-Aldrich). A concentração de proteínas foi determinada utilizando um kit comercial Pierce BCA (Thermo Fisher Scientific). Os *westerns blots* foram realizados como descrito anteriormente por Silva *et al.*, 2018. Brevemente, as amostras de proteína (20 µg/poço) foram resolvidas em gel de acrilamida 12% para SDS/PAGE. Posteriormente, as proteínas foram transferidas para membranas Hybond ™ de difluoreto de polivinilideno (PVDF) (Amersham Biosciences, Buckinghamshire, Reino Unido). As membranas foram bloqueadas com leite em pó desnatado (Molico) a 5% durante 1 h em temperatura ambiente e incubadas *overnight* com os seguintes anticorpos LC3 e β-actina (para os lisatos celulares) e NFATc1, MMP-9, TRAP, CTSK, β-actina e α-tubulina (para as proteínas obtidas das articulações fêmuro-tibial). Após 4 lavagens com TBS contendo Tween -20 (TBS-T) (para LC3, não foi utilizado Tween 20), as membranas foram incubadas com anticorpos secundários conjugados com HRP apropriado durante 4 h a temperatura ambiente. Após a incubação as membranas foram novamente lavadas 4 vezes com TBS-T, e incubadas usando o reagente ECL®-plus (Enhanced Chemiluminescence, Amersham Biosciences) para obtenção do sinal proteico. As imagens foram visualizadas e capturadas usando o sistema ChemiDoc-XRS (Life Science Research, Bio-Rad). A quantificação da densitometria das bandas foi realizada com o *software Image* J (domínio público) utilizando β-actina ou α-tubulina como controle basal.

4.1.15 ANÁLISE ESTATÍSTICA

Os resultados foram expressos como a média ± erro padrão da média (EPM) e analisado estatisticamente através do teste de análise da variância (ANOVA), seguido pelo teste de *Newman-Keuls-Student* ou *Tukey* para comparação entre mais de dois grupos. Para a comparação entre dois grupos experimentais foi utilizado o teste "t" de *Student*. Todos os testes foram realizados no GraphPad Prism 5.00 (GraphPad Software, La Jolla, Califórnia, EUA). Valores de *p* inferiores ou iguais (≤) a 0,05 foram considerados significativos (*;$^{+}$).

4.2 RESULTADOS

4.2.1 EFEITO DO GALATO DE METILA NA FORMAÇÃO DE EDEMA E ACÚMULO DE LEUCÓCITOS NA ARTRITE EXPERIMENTAL INDUZIDA POR ANTÍGENO EM CAMUNDONGOS

Após avaliar o efeito do GM na artrite induzida por zimosan, foi investigado se essa substância seria capaz de modular a artrite experimental induzida por antígeno (AIA) que é um modelo de artrite bem estabelecido dependente de células T $CD4^+$ com subsequente ativação de células B e produção de anticorpos específicos para o antígeno (Ferraccioli *et al.*, 2010).

Nesta etapa do trabalho, foi avaliada a ação da administração oral do GM na inflamação articular induzida por antígeno. Camundongos previamente imunizados com mBSA (500 µg) foram desafiados após 21 dias com uma injeção i.a. de mBSA na articulação fêmuro-tibial. O desafio induziu um significativo edema articular tanto em 7 quanto em 24 h em comparação ao grupo não-imunizado (*sham)* ou o imunizado e desafiado com veículo (salina) (Figura 4.2 A-B). A administração oral prévia do GM (7 mg/kg) reduziu significativamente o aumento do diâmetro articular nos dois tempos avaliados de forma semelhante ao fármaco de referência, dexametasona (10 mg/kg; i.p.) (Figura 4.2 A-B). Nos animais imunizados e desafiados com mBSA foi observado um aumento do número de leucócitos na cavidade sinovial, devido ao acúmulo de neutrófilos e células mononucleares 7 e 24 h após o estímulo. Observou-se ainda que a administração de mBSA em animais falso-imunizados (*sham*) não foi capaz de promover uma significativa migração de células para a cavidade articular (Figura 4.2 C-H). Em 7 e 24 h, o pré-tratamento oral com o GM reduziu o acúmulo de leucócitos na articulação fêmuro-tibial (Figura 4.2 C-D) referente à diminuição no número de neutrófilos e células mononucleares (Figura 4.2 E-H). A dexametasona apresentou efeitos similares ao GM em todos os parâmetros avaliados.

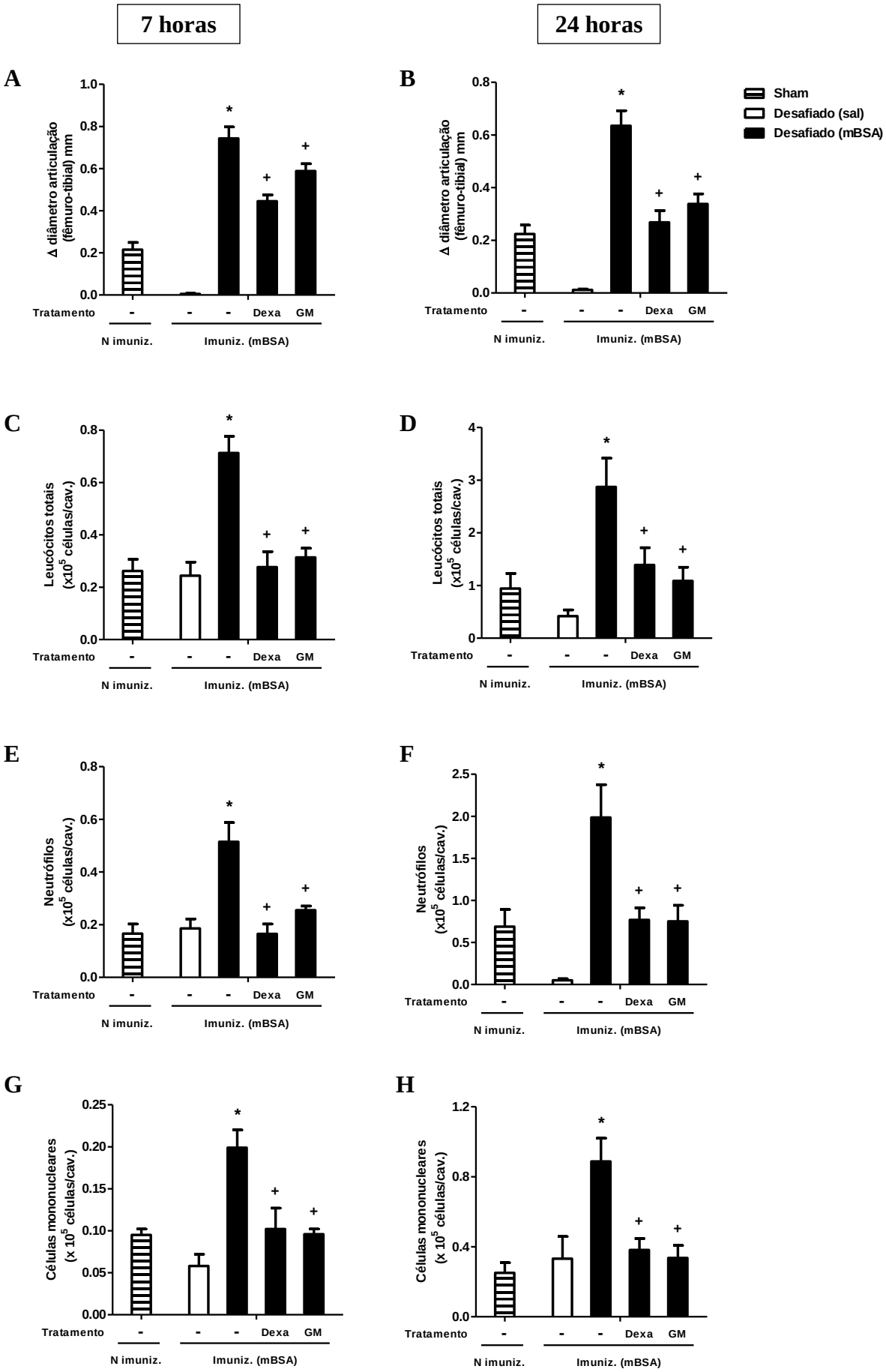

7 horas
24 horas
A
B
C
D
E
F
G
H
Sham
Desafiado (sal)
Desafiado (mBSA)
Δ diâmetro articulação (fêmuro-tibial) mm
Leucócitos totais (x10^5 células/cav.)
Neutrófilos (x10^5 células/cav.)
Células mononucleares (x 10^5 células/cav.)
Tratamento
- - - Dexa GM
N imuniz.
Imuniz. (mBSA)

Figura 4.2: Efeito do tratamento oral com o GM sobre a formação de edema e influxo de leucócitos na artrite induzida por antígeno. Os animais foram pré-tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) 1 h antes do desafio com mBSA (30 µg/cavidade). Os animais falso-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. O diâmetro articular foi avaliado 7 e 24 h após o desafio com o auxílio de um paquímetro digital (A-B). O número total de leucócitos (C-D), neutrófilos (E-F) e células mononucleares (G-H) na cavidade sinovial foram avaliados 7 e 24 h após a injeção i.a. de mBSA e foram apresentados como número de células $x10^5$. Os resultados foram expressos como média ± erro padrão da média (EPM) de 7 animais por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+). N imuniz.= não-imunizado; Imuniz. (mBSA) = imunizado.

4.2.2 HISTOPATOLOGIA DO EFEITO DO GM SOBRE A INFLAMAÇÃO ARTICULAR INDUZIDA POR ANTÍGENO

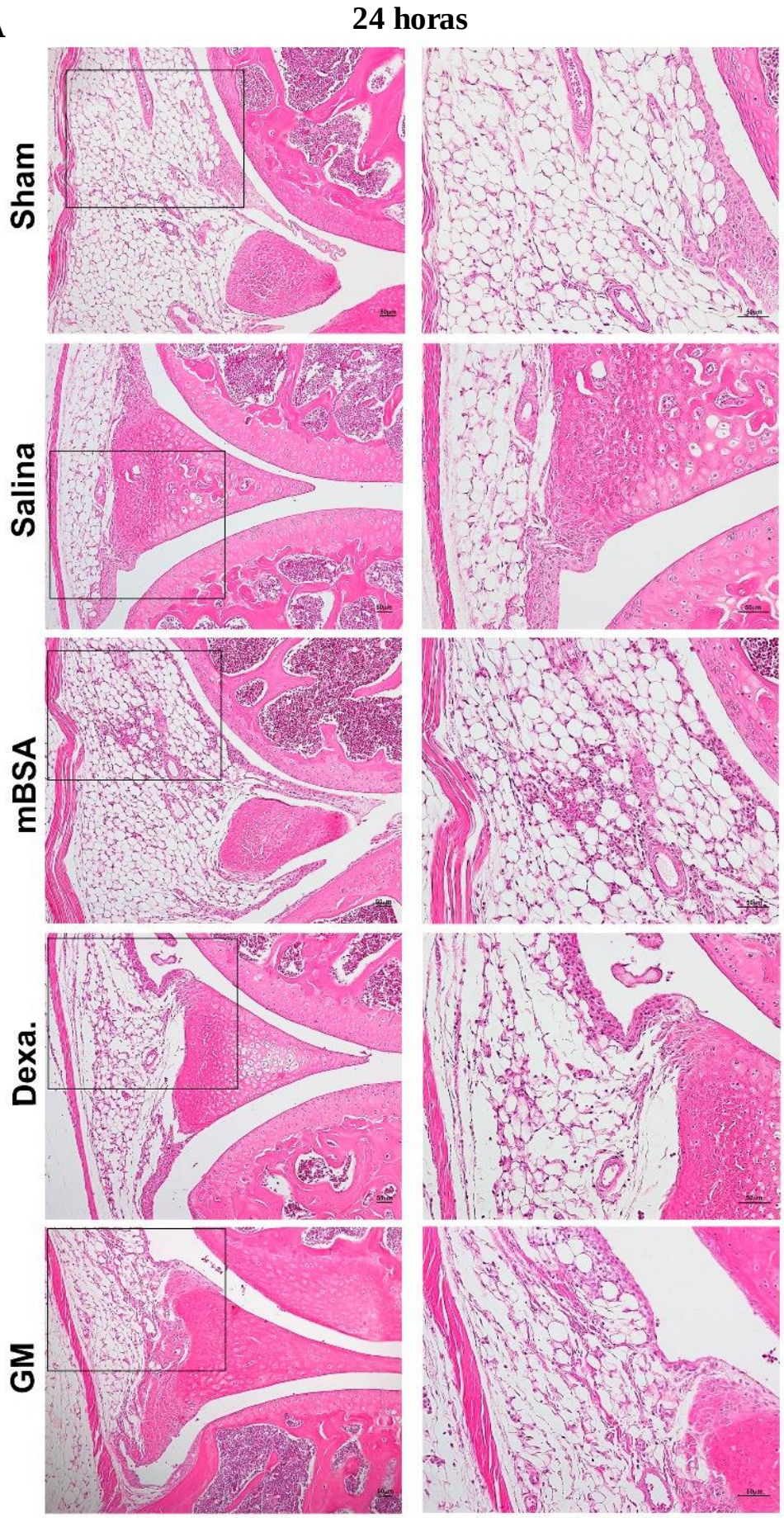
Os cortes histológicos da articulação fêmuro-tibial de camundongos foram obtidos 24 horas e 7 dias após o desafio i.a. com o antígeno (mBSA). A Figura 4.3 A-B e Figura 4.4 A-B mostra imagens representativas de pelo menos 6 animais diferentes. A região destacada está representada em maior amplificação (200x) nas imagens ao lado.

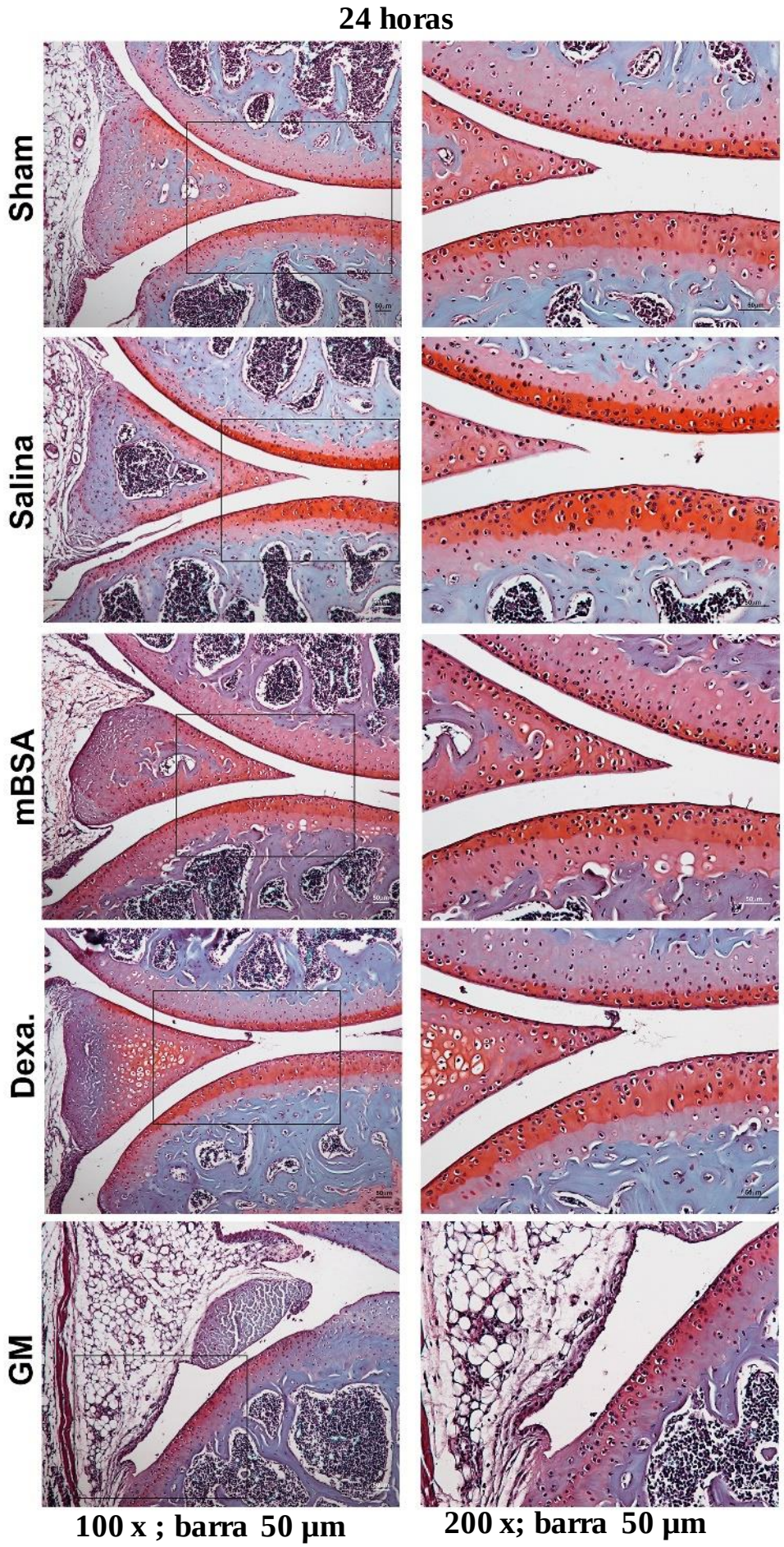
Na Figura 4.3 A observa-se, nas lâminas coradas por H&E, um aumento do infiltrado inflamatório nos animais imunizados e desafiados com mBSA em relação aos grupos controle *sham* e os animais desafiados i.a. com veículo. Além disso, também é possível observar uma perda de proteoglicanos na cartilagem dos animais desafiados com o antígeno em comparação aos grupos controle, observada através da coloração das lâminas com safranina (Figura 4.3 B). As articulações dos animais desafiados com mBSA apresentaram um aumento na pontuação geral do índice de artrite em comparação com os grupos controle. Entretanto, esse aumento não foi estatisticamente significativo (Figura 4.3 C). Apesar de se observar uma redução do infiltrado inflamatório nas articulações fêmuro-tibial dos animais que foram tratados com dexametasona ou com o GM (Figura 4.3 A) não foi notado uma proteção significativa em relação a perda de proteoglicanos das cartilagens após os tratamentos com Dexa ou GM (Figura 4.3 B). Além disso, não houve uma redução significativa do índice de artrite em comparação aos animais desafiados com mBSA (Figura 4.3 C).

A análise histopatológica das articulações fêmuro-tibiais de camundongos desafiados com mBSA 7 dias após a injeção i.a. do antígeno revelaram uma infiltração de células inflamatórias na sinóvia e intensa hiperplasia sinovial (Figura 4.4 A). Esta foi atenuada pelo tratamento dos animais com a dexametasona, mas não com o GM (Figura 4.4 A). Com relação a perda de proteoglicanos da cartilagem, observa-se que nem a dexametasona nem o GM foram

capazes de reduzir a degradação da cartilagem (Figura 4.4 B), bem como o índice de artrite (Figura 4.4 C).

A


24 horas
Sham
Salina
mBSA
Dexa.
GM
100 x ; barra 50 µm
200 x; barra 50 µm


B
24 horas
Sham
Salina
mBSA
Dexa.
GM
100 x ; barra 50 µm
200 x; barra 50 µm

C

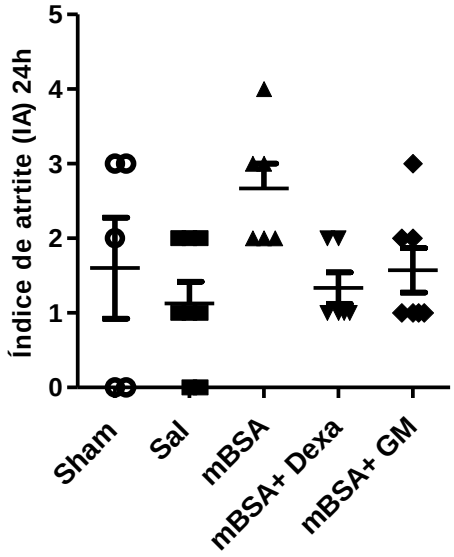
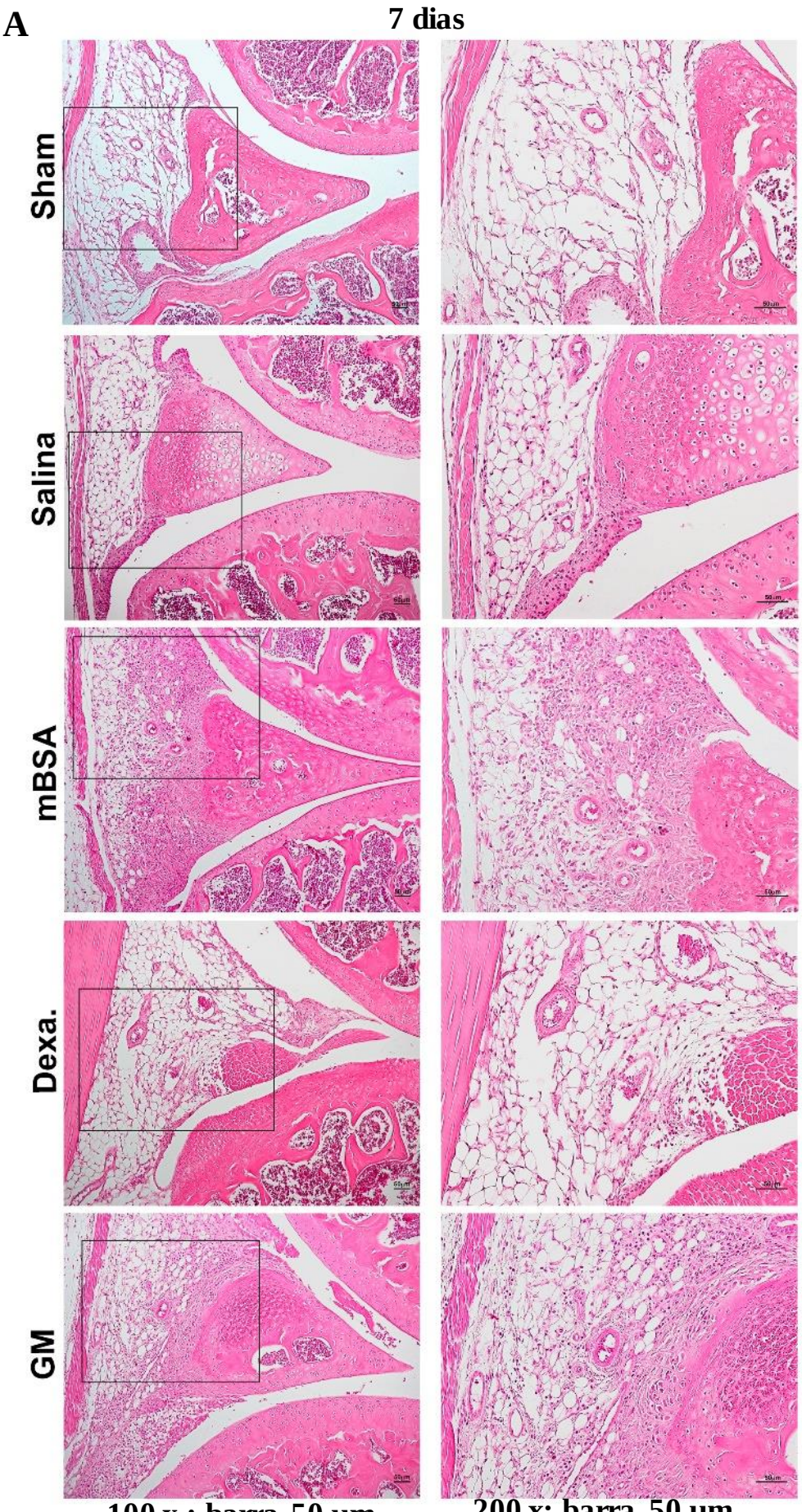
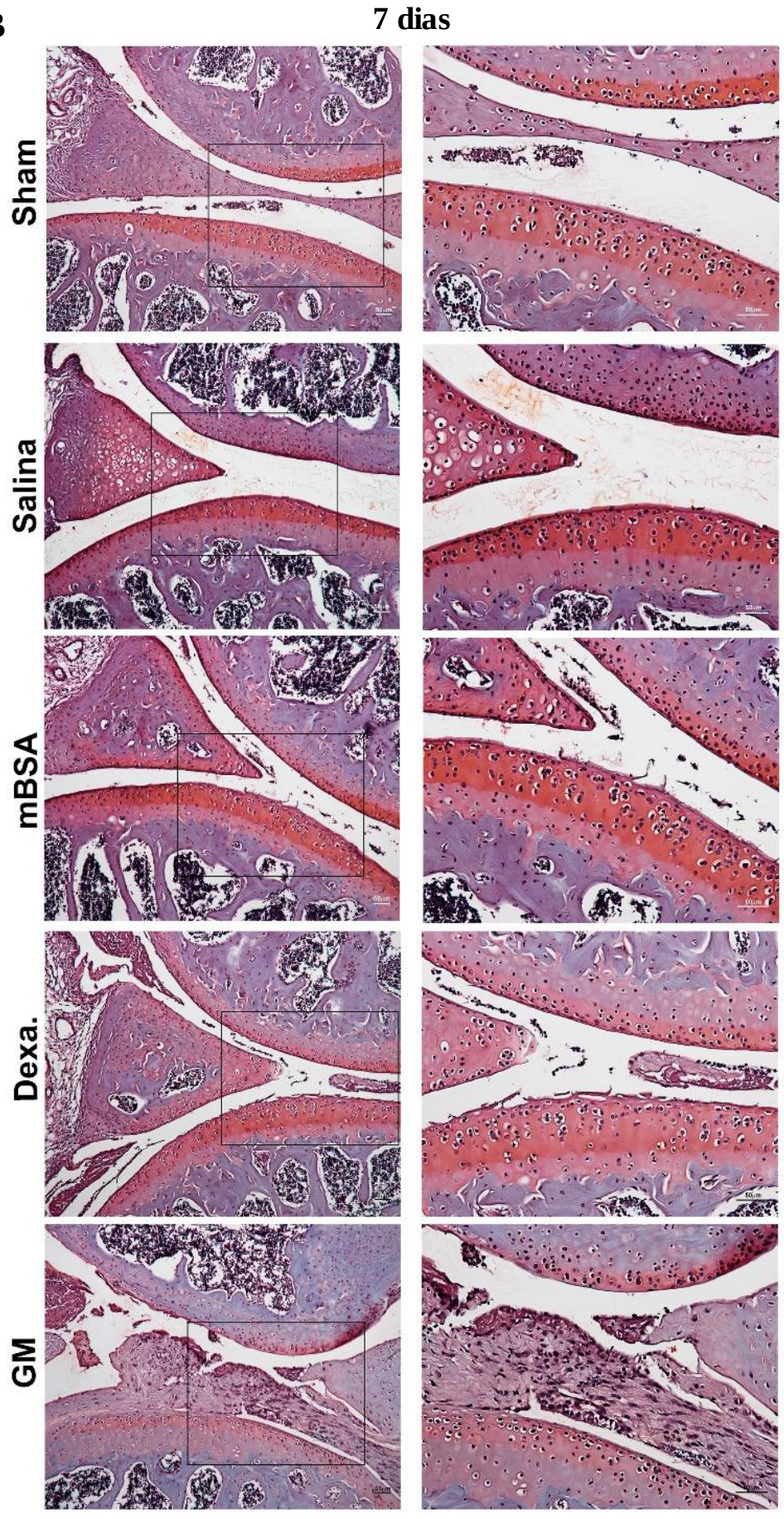


Figura 4.3: Histopatologia qualitativa e quantitativa do efeito do GM sobre a inflamação articular induzida por antígeno 24 horas após o desafio. Os animais foram pré-tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) 1 h antes do desafio com mBSA (30 µg/cavidade). Os animais falso-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. (A-B) Cortes histológicos longitudinais da articulação fêmuro-tibial de camundongos C57BL/6 foram obtidos 24 horas após o desafio com o antígeno, imagem representativa de pelo menos seis animais diferentes por grupo. A área destacada (retângulo) foi representada em maior amplificação na imagem ao lado (200 ×; barra = 50 µm). Coloração de Hematoxilina e Eosina (A) ou safranina (B). (C) Classificação subjetiva para os seguintes parâmetros: hiperplasia sinovial (formação de *pannus*), infiltrado inflamatório, presença de hemácias no tecido e degradação da cartilagem. As pontuações de todos os parâmetros foram somadas para dar o índice de artrite (IA). Os resultados foram expressos como média ± erro padrão da média (EPM) de 6 a 8 animais por grupo, representativo de um experimento independente. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+).


A
7 dias
Sham
Salina
mBSA
Dexa.
GM
100 x ; barra 50 µm
200 x; barra 50 µm

B


7 dias
Sham
Salina
mBSA
Dexa.
GM
100 x ; barra 50 µm
200 x; barra 50 µm

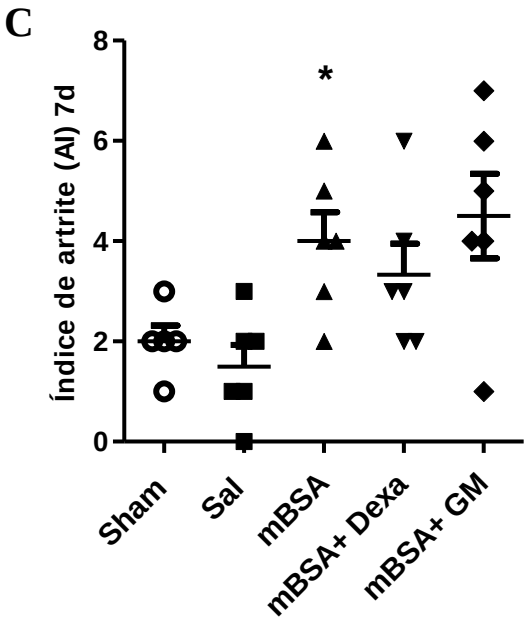


Figura 4.4: Histopatologia qualitativa e quantitativa do efeito do GM sobre a inflamação articular induzida por antígeno 7 dias após o desafio. Os animais foram tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) durante 7 dias consecutivos após o desafio i.a. com mBSA. Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. (A-B) Cortes histológicos longitudinais da articulação fêmuro-tibial de camundongos C57BL/6 foram obtidos 7 dias após o desafio com o antígeno, imagem representativa de pelo menos seis animais diferentes por grupo. A área destacada foi representada em maior amplificação na imagem ao lado (200 ×; barra = 50 µm). Coloração de Hematoxilina e Eosina (A) ou safranina (B). (C) Classificação subjetiva para os seguintes parâmetros: hiperplasia sinovial (formação de *pannus*), infiltrado inflamatório, presença de hemácias no tecido e degradação da cartilagem. As pontuações de todos os parâmetros foram somadas para dar o índice de artrite (IA). Os resultados foram expressos como média ± erro padrão da média (EPM) de 5 ou 6 animais por grupo, representativo de um experimento independente. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+).

4.2.3 EFEITO DO GM NA PRODUÇÃO DE MEDIADORES INFLAMATÓRIOS NA ARTRITE EXPERIMENTAL INDUZIDA POR ANTÍGENO

Uma vez que a inflamação é largamente dirigida por mediadores inflamatórios, o próximo passo foi investigar o efeito do GM na produção de algumas citocinas e quimiocinas envolvidas no modelo experimental de artrite induzida por antígeno. Logo, as concentrações das quimiocinas CCL-2/MCP-1 e CCL-1/MIP-1α e da citocina IL-17 foram determinadas no lavado articular, enquanto que, a concentração de TNF-α foi avaliada no extrato da articulação fêmuro-tibial. Todas as concentrações dos mediadores foram determinadas por ELISA.

Como pode ser observado na Figura 4.5, a imunização e desafio com mBSA induziu um aumento significativo na produção das quimiocinas CCL-2/MCP-1, CCL-3/MIP-1α e das

citocinas IL-17 e TNF-α 7 h após o desafio. O tratamento oral com o GM reduziu as concentrações de CCL-2, CCL-3, IL-17 e TNF-α, assim como a dexametasona, 7 h após o desafio i.a. com mBSA (Figura 4.2 A-D).

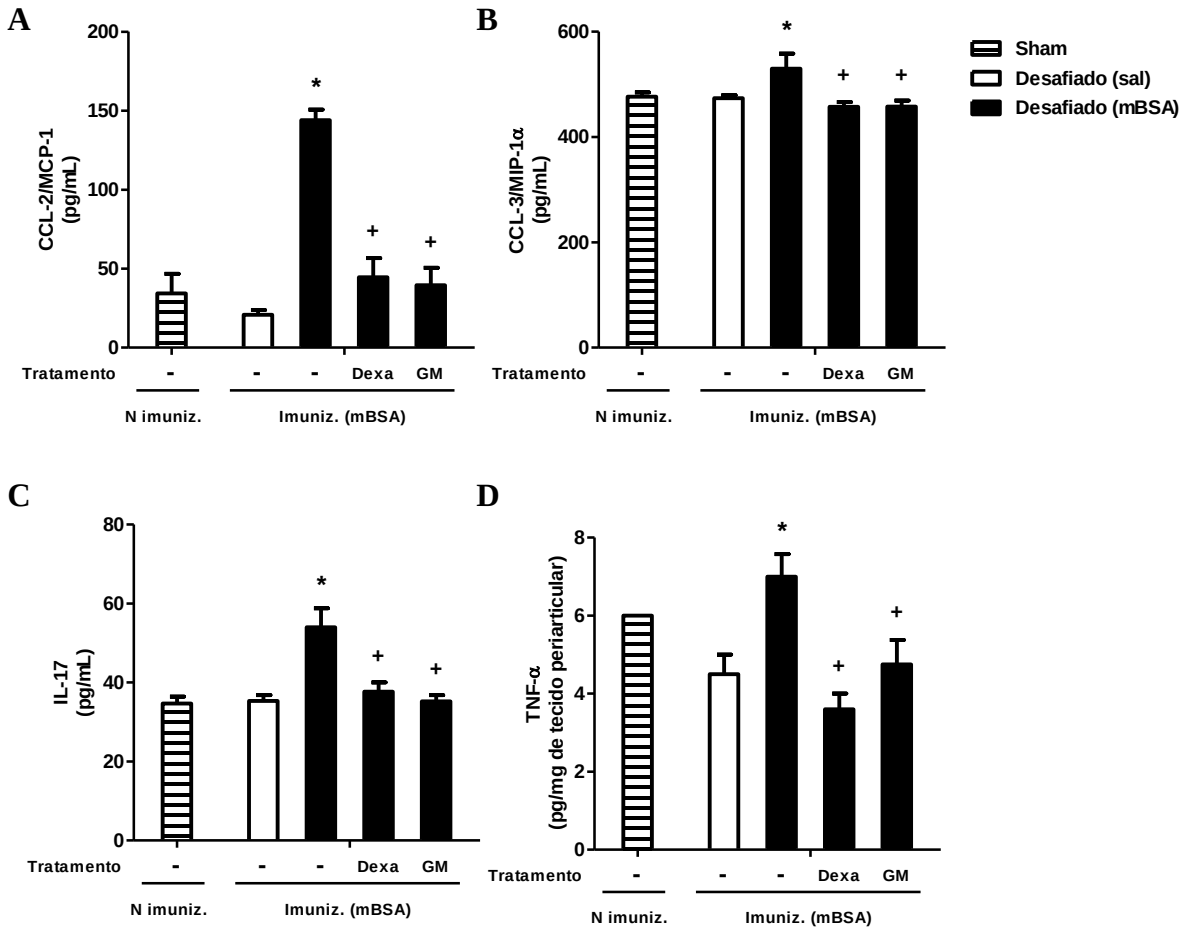


Figura 4.5: Efeito do tratamento oral com o GM sobre a produção de mediadores inflamatórios na artrite induzida por antígeno. Os animais foram pré-tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) 1 h antes do desafio com mBSA (30 µg/cavidade). Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. A concentração das quimiocinas CCL-2/MCP-1 (A), CCL-3/MIP-1α (B), das citocinas IL-17 (C) e TNF-α (D) foram determinadas no sobrenadante livre de células dos lavados recolhidos da articulação sinovial (A-C) ou dos extratos da articulação fêmuro-tibial (D) por ELISA. A coleta das amostras foi realizada 7 horas após o desafio com mBSA. Os resultados foram expressos como média ± erro padrão da média (EPM) de 6 animais por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+). N imuniz.= não-imunizado; Imuniz. (mBSA) = imunizado.

4.2.4 EFEITO DO GM NA LIBERAÇÃO DE DNA NO LAVADO SINOVIAL DE CAMUNDONGOS SUBMETIDOS A AIA

A formação de NETs na AR está entre os vários mecanismos pelos quais os neutrófilos causam danos nos tecidos e promovem a autoimunidade (Khandpur *et al.*, 2013). Vários estudos têm demonstrado um alto grau de correlação entre a formação espontânea de NETs em neutrófilos circulantes e presentes nas articulações da AR, e os níveis de DNA circulante livre (Khandpur *et al.*, 2013; Sur Chowdhury *et al.*, 2014; Pérez-Sánchez *et al.*, 2017). Foi avaliado, então, o efeito da administração oral do GM na liberação de DNA extracelular no lavado de camundongos submetidos a AIA nos tempos de 7 e 24 h após o desafio i.a. com mBSA.

Como pode ser observado na Figura 4.6, ocorreu um aumento da liberação de DNA no sobrenadante dos lavados da articulação fêmuro-tibial dos camundongos imunizados e desafiados com mBSA tanto em 7 h quanto em 24 h após o desafio, o que sugere a formação de NETs nesse modelo. O pré-tratamento oral com o GM (7 mg/kg) reduziu a liberação do DNA extracelular nos lavados dos camundongos submetidos a AIA em ambos os tempos avaliados, assim como o tratamento com a dexametasona (10 mg/kg) (Figura 4.6 A-B).

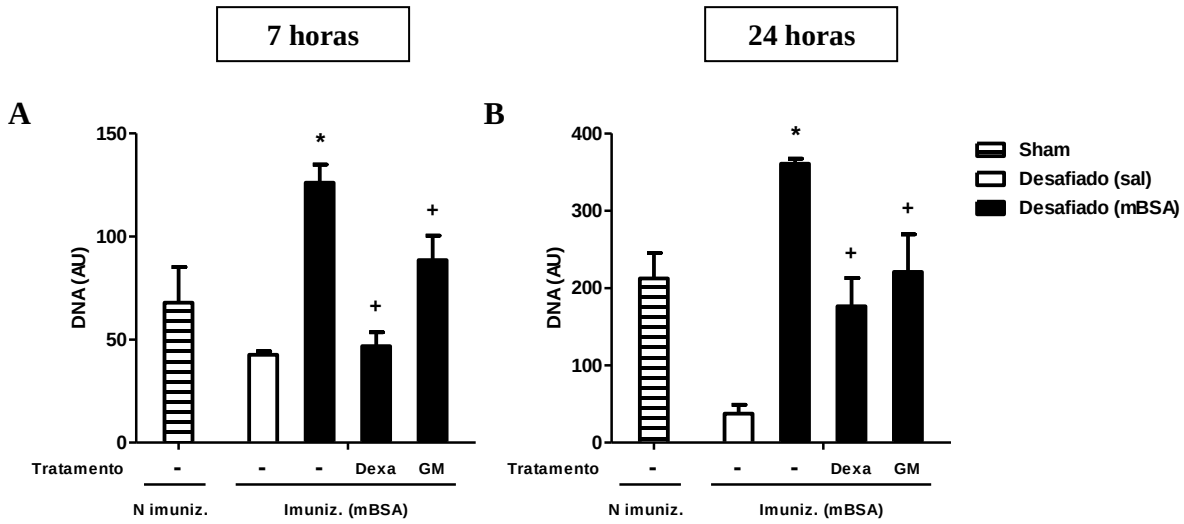


Figura 4.6: Efeito do tratamento oral com o GM sobre a liberação de DNA extracelular na artrite induzida por antígeno. Os animais foram pré-tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) 1 h antes do desafio com mBSA (30 µg/cavidade). Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. A concentração de DNA foi determinada no sobrenadante livre de células dos lavados recolhidos da articulação sinovial através do kit Quanti-it™PicoGreen® dsDNA. As análises foram realizadas 7 ou 24 horas após o desafio com mBSA. Os resultados foram expressos como média ± erro padrão da média (EPM) de 6 animais por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado

foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+). N imuniz.= não-imunizado; Imuniz. (mBSA) = imunizado.

4.2.5 EFEITO DO GM NA RESPOSTA IMUNE HUMORAL NA ARTRITE EXPERIMENTAL INDUZIDA POR ANTÍGENO

Os anticorpos desempenham um papel importante na patogênese da AR e em modelos experimentais da doença, portanto, foi investigado a presença de anticorpos séricos anti-mBSA em vista de seu possível papel no desenvolvimento da AIA. Os níveis de IgG anti-mBSA foi determinada no soro dos animais 7 h, 24 h ou 7 dias após o desafio i.a., por meio do teste de ELISA. A Figura 4.7 mostra os níveis de IgG no soro dos animais da linhagem C57BL/6. Observamos que nos camundongos imunizados a quantidade de anticorpos IgG anti-mBSA foi elevada em todas as diluições do soro e em todos os tempos avaliados (Figura 4.7 A-C). Em contrapartida, a titulação do soro para IgG anti-mBSA obtido de animais não-imunizados (*sham*) foi baixa (Figura 4.7). O pré-tratamento oral com o GM (7 mg/kg) não foi capaz de reduzir os níveis séricos de IgG anti-mBSA 7 h após o desafio, assim como a dexametasona (Figura 4.7 A), no entanto, 24 h após a injeção i.a. de mBSA, o GM e a dexametasona reduziram de forma significativa os níveis séricos de IgG anti-mBSA (Figura 4.7 B). O tratamento diário com o GM (7 mg/kg), durante 7 dias consecutivos, não alterou os níveis de anticorpos anti-mBSA em comparação ao grupo imunizado e desafiado sem tratamento. Já o tratamento diário (durante 7 dias) com a dexametasona (10 mg/kg) diminuiu os níveis séricos de IgG anti-mBSA (Figura 4.7 C).

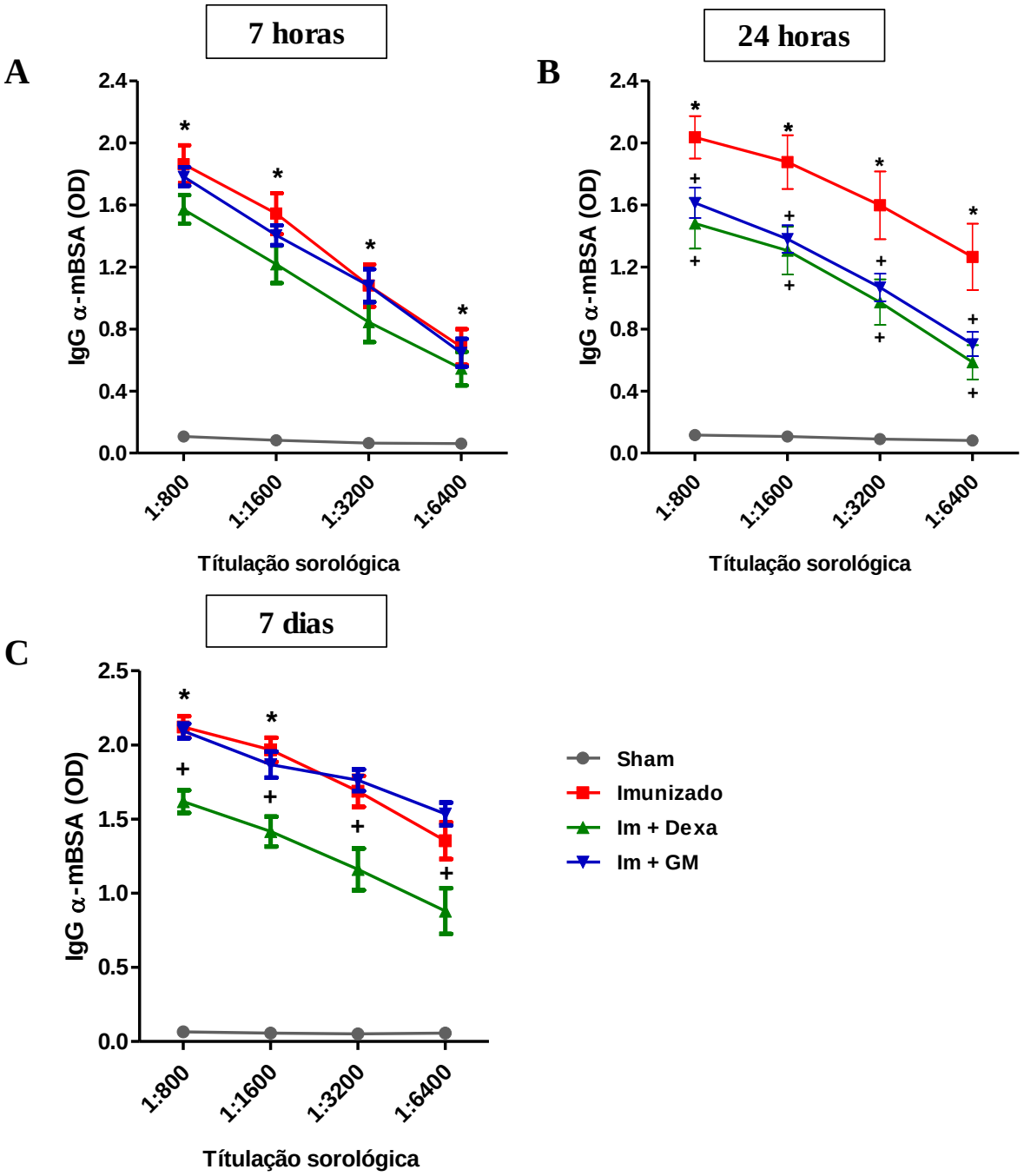


Figura 4.7: Efeito do tratamento oral com o GM sobre os níveis séricos de anticorpos anti-mBSA na induzida por antígeno. (A-B) Os animais foram pré-tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) 1 h antes do desafio com mBSA (30 µg/cavidade). (C) Os animais foram tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) durante 7 dias consecutivos após o desafio i.a. com mBSA. Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. Os níveis dos anticorpos IgG específicos anti-mBSA foram medidos por ELISA nos soros dos camundongos submetidos a AIA. As amostras foram coletadas em 7 h, 24 h ou 7 dias após o desafio com mBSA e avaliadas nas diluições de 1:800 até 1:6400. Os resultados foram expressos como média ± erro padrão da média (EPM) de 6 animais por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+).

4.2.6 EFEITO DO GM SOBRE PARÂMETROS BIOQUÍMICOS FISIOLÓGICOS EM CAMUNDONGOS SUBMETIDOS A AIA

Com o objetivo de estudar a ação tóxica do GM sobre parâmetros bioquímicos fisiológicos nos animais submetidos a artrite, foi avaliado o efeito do GM sobre a concentração de uréia, ácido úrico e as transaminases hepáticas AST e ALT em animais submetidos a AIA que foram tratados com GM (7 mg/kg) ou com dexametasona (10 mg/kg) durante sete dias consecutivos. Conforme observado na Figura 4.8 A ocorreu um leve aumento nas concentrações de uréia BUN nos animais imunizado com relação aos animais do grupo *sham,* ou com os valores de referência estabelecido para esse parâmetro bioquímico (18-29 mg/dL), segundo dados fornecidos pelo serviço de controle da qualidade animal ICTB/Fiocruz. Não foram observadas alterações na concentração de uréia nos grupos tratados com GM ou dexametasona (Figura 4.8 A). Em relação ao ácido úrico, observou-se um discreto aumento na sua concentração nos camundongos imunizados em relação ao grupo controle *sham* (Figura 4.8 B). Nos animais tratados com a dexametasona esse aumento foi um pouco mais evidente, já os animais tratados com o GM tiveram as concentrações similares aos do grupo imunizado (Figura 4.8 B). As transaminases hepáticas, aspartato aminotransferase (AST) e alanina aminotransferase (ALT), permaneceram dentro dos valores de referência considerados para cada parâmetro (AST= 59-247 U/L; ALT= 28-132 U/L). Entretanto, a dexametasona foi capaz de induzir um aumento significativo na concentração de AST em comparação aos grupos imunizado e desafiado com o mBSA e tratado com o GM (Figura 4.8 C-D).

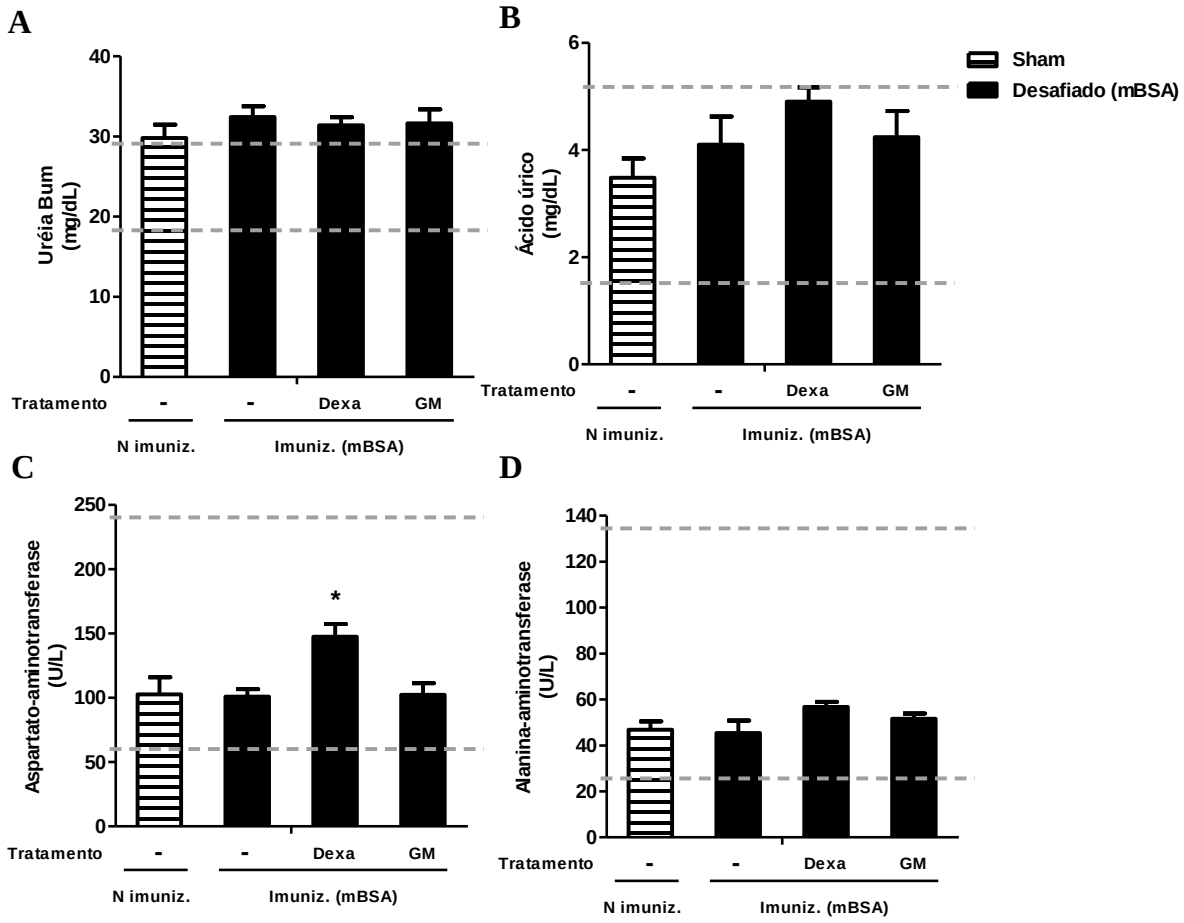


Figura 4.8: Efeito do tratamento oral com o GM sobre parâmetros bioquímicos fisiológicos. (A-D) Os animais foram tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) durante 7 dias consecutivos após o desafio i.a. com mBSA. Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. Os níveis séricos de úreia BUN, ácido úrico, AST e ALT nos soros dos camundongos submetidos a AIA foram quantificados através da metodologia de química seca pelo equipamento Vitros 250. As amostras foram coletadas 7 dias após o desafio com mBSA. Os resultados foram expressos como média ± erro padrão da média (EPM) de 6 animais por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos foram representados por (*). Tracejado cinza indica os valores de referência de cada parâmetro bioquímico (Uréia BUN = 18-29 mg/dL; ácido úrico = 1,7-5,4 mg/dL; AST= 59-247 U/L; ALT= 28-132 U/L). N imuniz.= não-imunizado; Imuniz. (mBSA) = imunizado.

4.2.7 EFEITO DO GM NA REABSORÇÃO ÓSSEA ARTICULAR NA ARTRITE INDUZIDA POR ANTÍGENO

A erosão óssea induzida pela formação aumentada de osteoclastos é um processo patológico debilitante na AR (Gravallese, 2002). A formação excessiva de osteoclastos desestabiliza o equilíbrio entre a formação e a erosão óssea, perturbando assim o metabolismo ósseo para a perda óssea patológica, culminando no aumento da incapacidade e morbidade em pacientes com AR (Zhang *et al.*, 2005). A expressão da enzima TRAP é um marcador da função dos osteoclastos e da intensidade da reabsorção óssea (Ballanti *et al.*, 1997). Desta forma, foi avaliado a ativação dos osteoclastos e consequentemente a reabsorção óssea na AIA, bem como os efeitos do GM nesse processo. Camundongos foram tratados durante 7 dias consecutivos com o GM (7 mg/kg; v.o.) ou com a dexametasona (10 mg/kg; i.p.) e as articulações sinoviais foram coletadas para avaliar a presença dos osteoclastos através da coloração de TRAP.

Na Figura 4.9 A, observamos um aumento significativo do número de células positivas para TRAP nos camundongos imunizados e desafiados com mBSA em comparação aos controles *sham* e as articulações dos camundongos imunizados, mas desafiados i.a. com salina. O tratamento com o GM reduziu o número de células positivas para TRAP nos camundongos imunizados, assim como a dexametasona. A análise quantitativa (Figura 4.9 B) das células positivas para TRAP confirmou que o GM inibiu eficientemente a presença dos osteoclastos no tecido da articulação fêmuro-tibial.

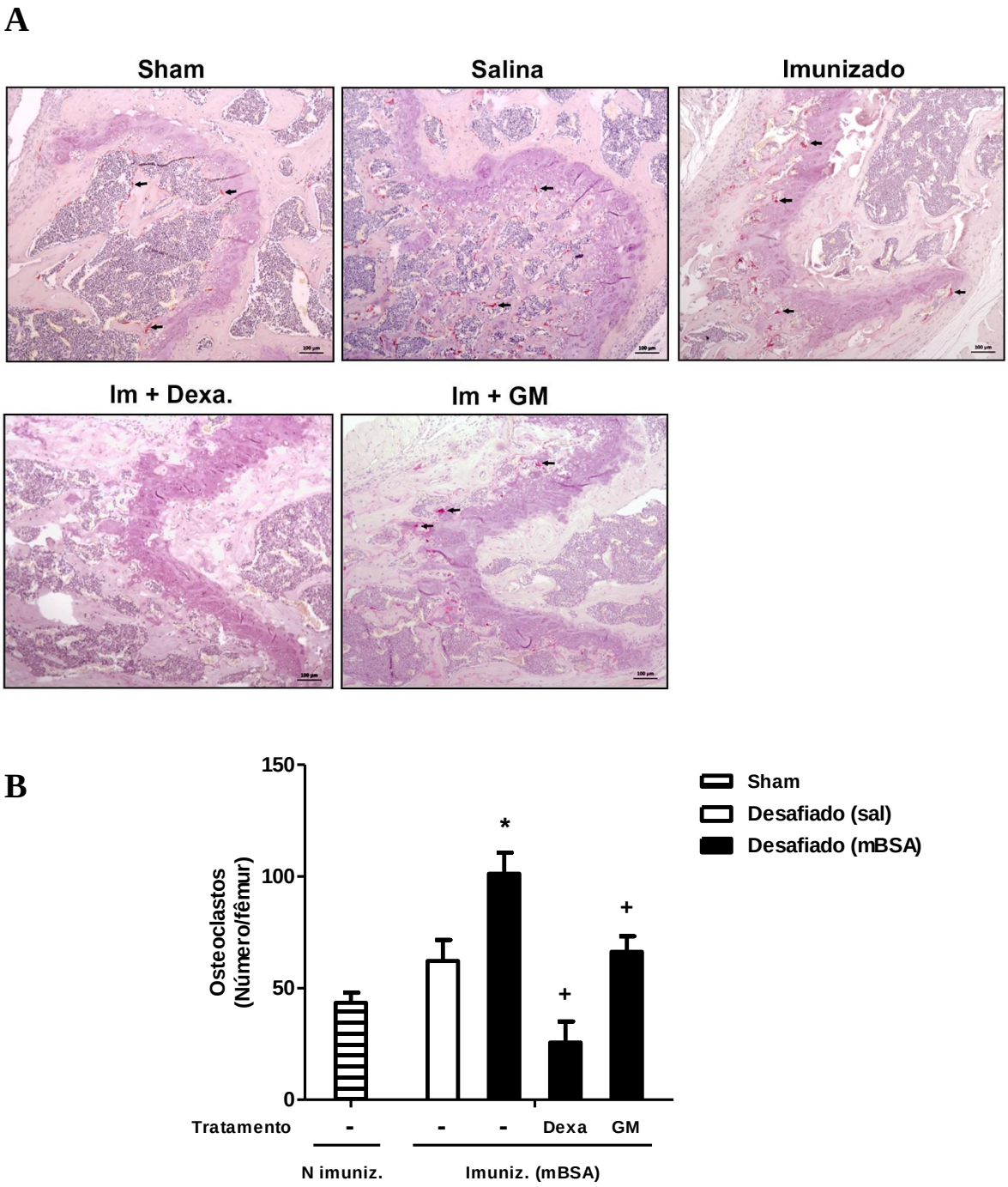


Figura 4.9: Histopatologia qualitativa e quantitativa do efeito do GM sobre a presença de osteoclastos na artrite induzida por antígeno 7 dias após o estímulo. (A) Cortes histológicos longitudinal da articulação fêmuro-tibial de camundongos C57BL/6, imagem representativa de pelo menos cinco animais diferentes por grupo (barra de aumento = 100 µm). Os animais foram tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) durante 7 dias consecutivos após o desafio i.a. com mBSA. Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. As articulações fêmuro-tibial foram coradas com kit TRAP 387A e as células positivas para TRAP aparecem como vermelho-púrpura e estão indicadas por uma seta. As análises foram realizadas 7 dias após o desafio com mBSA. Os resultados foram expressos como média ± erro padrão da média (EPM) de 5 animais por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os

grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+). N imuniz.= não-imunizado; Imuniz. (mBSA) = imunizado.

4.2.8 EFEITO DO GM NA EXPRESSÃO PROTEICA DE MARCADORES DE ATIVAÇÃO DE OSTEOCLASTOS NA ARTICULAÇÃO DE CAMUNDONGOS SUBMETIDOS A AIA

Em seguida, foi avaliado se o GM interfere nas vias de diferenciação e ativação dos osteoclastos nos tecidos das articulações fêmuro-tibiais dos camundongos submetidos a AIA. Diferente do que foi observado na marcação de TRAP nos tecidos articulares, anteriormente, na Figura 4.10 A, não se observa diferença significativa na expressão proteica do TRAP entre os grupos *sham*, imunizados e desafiados i.a. com salina ou com mBSA. Entretanto, o tratamento com o GM e com a dexametasona reduziram significativamente o aumento da expressão do TRAP em comparação ao grupo imunizado e estimulado com mBSA (Figura 4.10 A).

Está bem estabelecido que a expressão de genes marcadores de osteoclastos é regulada principalmente pelo fator de transcrição NFATc1. Assim, realizamos um *western blot* dos extratos das articulações fêmuro-tibial para avaliar se o GM estaria interferindo no principal regulador da osteoclastogênese, o NFATc1. Como mostrado na Figura 4.10 B, o desafio i.a. com mBSA foi capaz de induzir o aumento na expressão de NFATc1 em comparação ao grupo *sham*. Por outro lado, o GM não reduziu esse aumento de forma significativa (Figura 4.10 B).

Foi investigado o efeito do GM na capacidade de reabsorção óssea, avaliando a expressão da MMP-9 e da protease catepsina K (CTSK). Na Figura 4.10 C observa-se que a expressão da pro-MMP-9 (precursor da MMP-9) aumentou nos animais *sham* e imunizados desafiados com salina. Já nos animais imunizados e desafiados com mBSA a expressão da pro-MMP-9 teve uma significativa redução. O tratamento diário dos animais com o GM aumentou a expressão da pro-MMP-9 em comparação aos animais imunizados (mBSA) (Figura 4.10 C). De forma inesperada, a expressão da MMP-9 ativa está elevada nas articulações dos animais imunizados desafiados com salina (Figura 4.10 D). E como esperado, a expressão da MMP-9 ativa aumentou nos animais imunizados e desafiados com mBSA. Enquanto que, o tratamento com o GM reduziu significativamente a expressão da MMP-9 (Figura 4.10 D). Com relação a CTSK (catepsina K), não foi observado diferença significativa da sua expressão nos grupos controle (*sham*, imunizado sal ou imunizado mBSA). Entretanto, o GM reduziu a expressão da CTSK em comparação ao grupo controle imunizado mBSA (Figura 4.10 E).

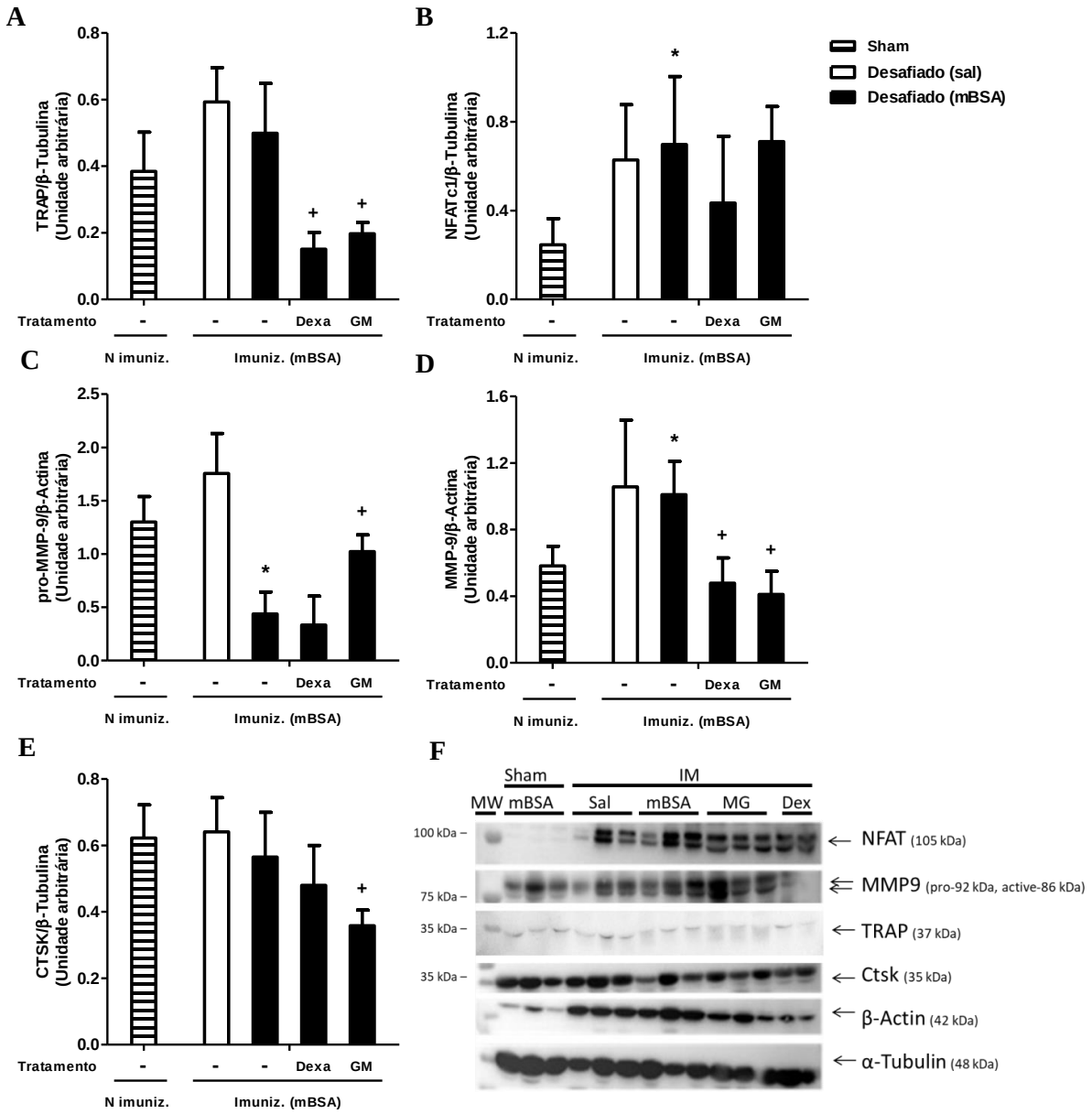


Figura 4.10: Efeito do tratamento oral com o GM na expressão de proteínas envolvidas nas vias de diferenciação e ativação dos osteoclastos. Os animais foram tratados com GM (7 mg/kg; v.o.) ou dexametasona (10 mg/kg; i.p.) durante 7 dias consecutivos após o desafio i.a. com mBSA. Os animais não-imunizados (*sham*) também foram desafiados i.a. com 30 µg de mBSA. As articulações fêmuro-tibiais foram coletadas e processadas para *western blot*. Os lisados totais das células foram resolvidos por SDS-PAGE e marcados para TRAP (A), NFATc1 (B), pro-MMP-9 (C), MMP-9 ativa (D) e catepsina K (CTSK) (E). As imagens representativas dos *westerns blots* está apresentada na figura (F), enquanto as análises densitométricas estão representadas nos gráficos. As amostras foram coletadas 7 dias após o desafio com mBSA. Os resultados foram expressos como média ± erro padrão da média (EPM) de 6 animais por grupo, representativo de um experimento independente. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos estimulado e não-estimulado foram representados por (*); diferenças entre os grupos tratados e não tratados foram representados por (+). N imuniz.= não-imunizado; Imuniz. (mBSA) = imunizado.

4.2.9 EFEITO DO GM NA OSTEOCLASTOGÊNESE MEDIADA POR RANKL *IN VITRO*

Foi investigado o efeito do GM na osteoclastogênese *in vitro*. Pré-osteoclastos derivados da medula óssea foram cultivados em meio osteoclastogênico (M-CSF 30 ng/mL e RANKL 10 ng/mL) com diferentes concentrações do GM (3, 10, 30 e 100 µM). Os osteoclastos, identificados como células multinucleadas positivas para TRAP, coradas de vermelho-púrpura, foram quantificados após 96 h de cultura. As células multinucleadas foram contadas com o auxílio de um microscópio de luz. Como pode ser observado na Figura 4.11 A e B, o GM suprimiu a diferenciação dos osteoclastos induzida por RANKL, uma vez que reduziu o número de células positivas para TRAP de forma dose-dependente, como pode ser observado nas figuras representativas de cada grupo (Figura 4.11 A) e na análise quantitativa expressa no gráfico (Figura 4.11 B). A incubação dos osteoclastos com diferentes concentrações do GM foi capaz de reduzir a área ocupada por essas células em todas as concentrações testadas de forma dose-dependente (Figura 4.11 C). Foi investigado se essa redução no número de osteoclastos poderia ser devido a uma diminuição na viabilidade dessas células induzida pelo GM. Na Figura 4.11 D, observa-se que apenas a concentração de 100 µM do GM está associada a uma diminuição na viabilidade celular. As outras concentrações testadas do GM (3, 10 e 30 µM) não interferiram na viabilidade dos osteoclastos (Figura 4.11 D**)**.

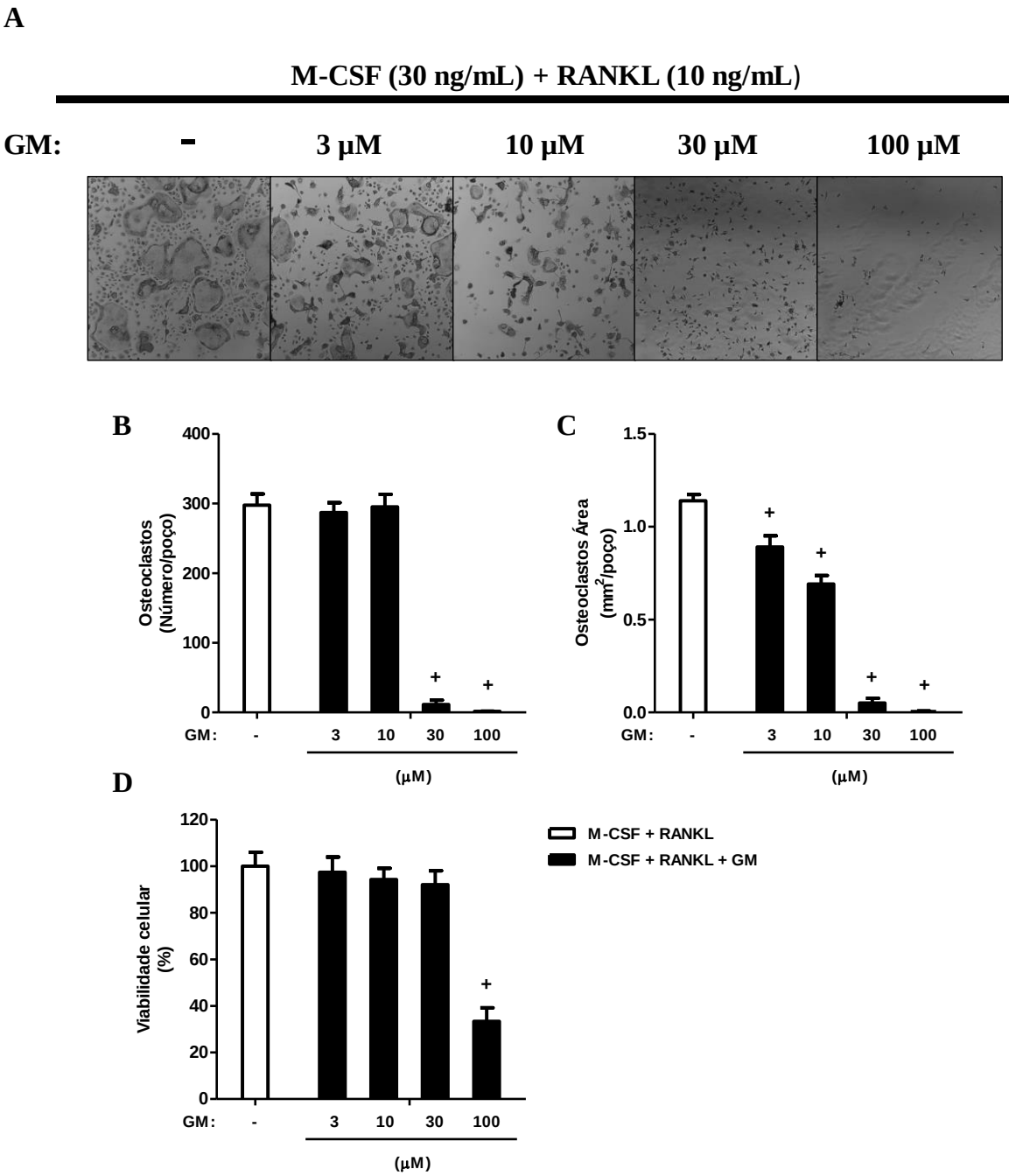


Figura 4.11: Efeito do GM na diferenciação e viabilidade celular dos osteoclastos *in vitro*. Pré-osteoclastos foram cultivados em meio osteoclástico (M-CSF 30 ng/mL e RANKL 10 ng/mL) e diferentes concentrações do GM (3, 10, 30 e 100 µM) por 96 horas. (A-B) As células foram fixadas e coradas com um kit comercial específico para TRAP e quantificadas com o auxílio de um microscópio de luz. (C) Para determinar a área dos osteoclastos, as imagens foram analisadas usando o software *ImageJ*. (D) A viabilidade dos osteoclastos foi determinada pelo método de MTT. Os resultados foram expressos como média ± erro padrão da média (EPM) de 5 poços por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos tratados e não tratados foram representados por (+).

4.2.10 EFEITO DO GM NA EXPRESSÃO DE GENES MARCADORES DE DIFERENCIAÇÃO DE OSTEOCLASTOS INDUZIDOS POR RANKL

Em seguida, foi examinado o efeito do GM na expressão de genes marcadores da osteoclastogêne por qPCR *in vitro*. Diferente do que foi observado na expressão de TRAP nas articulações sinoviais dos camundongos submetidos a AIA, a incubação do GM (30 μM) não reduziu de forma significativa a expressão do mRNA de Acp5 (TRAP), gene marcador específico da diferenciação dos osteoclastos (Figura 4.12 A). Como os resultados da expressão de NFATc1 na articulação fêmuro-tibial não foram muito conclusivos, foi avaliada a expressão desse importante fator de transcrição nos osteoclastos tratados com GM *in vitro*. Na Figura 4.12 B pode ser observado um aumento expressivo do mRNA de NFATc1 nos osteoclastos estimulados com M-CSF e RANKL e a incubação dessas células com o GM atenuou significativamente esse aumento (Figura 4.12 B). Foi avaliado a expressão de outros dois genes induzidos pela ligação de RANKL ao seu receptor com consequente diferenciação dos osteoclastos: a integrina-β3 o e receptor de calcitocina (CTR). O nível desses genes foi detectado em um nível mais baixo em osteoclastos tratados com o GM quando comparado com os osteoclastos não tratados (Figura 4.12 C e D).

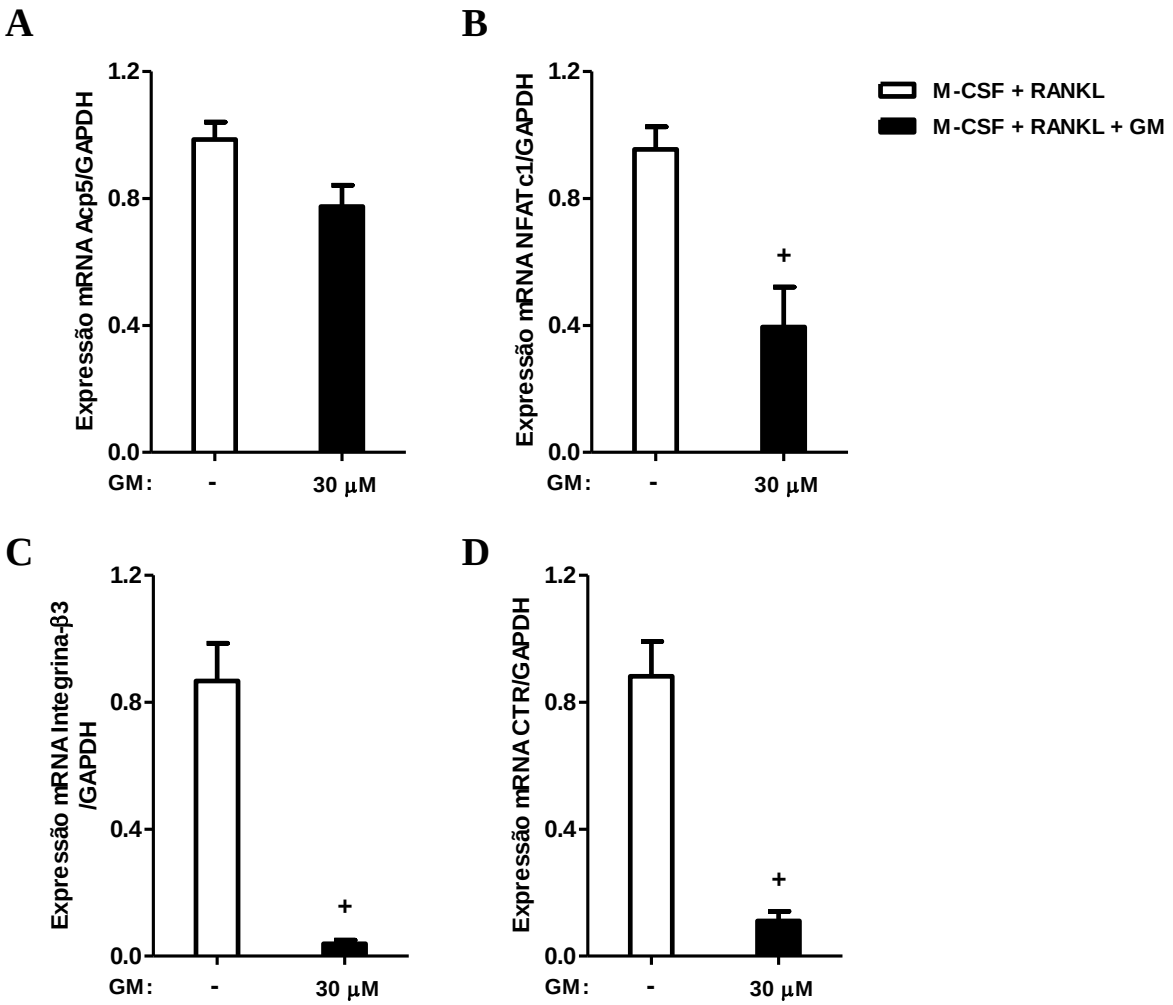


Figura 4.12: Efeito do GM na expressão de mRNA de marcadores de osteoclastos *in vitro*. Pré-osteoclastos foram cultivados em meio osteoclástico (M-CSF 30 ng/mL e RANKL 10 ng/mL) e incubados ou não com GM (30 μM) por 48 horas. (A-D) As células foram coletadas e os lisatos foram processados em tampão apropriado após 48 h de incubação para a quantificação da expressão gênica de Acp5 (A), NFATc1 (B), integrina-β3 (C) e CTR (D) pelo método de PCR quantitativo em tempo real. Os resultados foram expressos como média ± erro padrão da média (EPM) de 5 poços por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos tratados e não tratados foram representados por (+).

4.2.11 Efeito do GM sobre a expressão do marcador de autofagia LC3 em osteoclastos estimulados com RANKL

Estudos indicam que a autofagia desempenha um papel importante na reabsorção óssea por osteoclastos. Tem sido relatado que proteínas envolvidas na via autofágica são importantes na regulação da osteoclastogênese, indicando que esse processo participa tanto na formação quanto na reabsorção óssea. Dessa forma, foi avaliada a expressão dos marcadores de autofagia LC3 I e LC3 II em osteoclastos estimulados com RANKL e tratados com o GM (30 μM). Como

pode ser observado na Figura 4.13 A-B, o GM induziu um aumento na expressão dos marcadores autofágicos LC3 I e LC3 II em comparação as células estimuladas com RANKL sem tratamento.

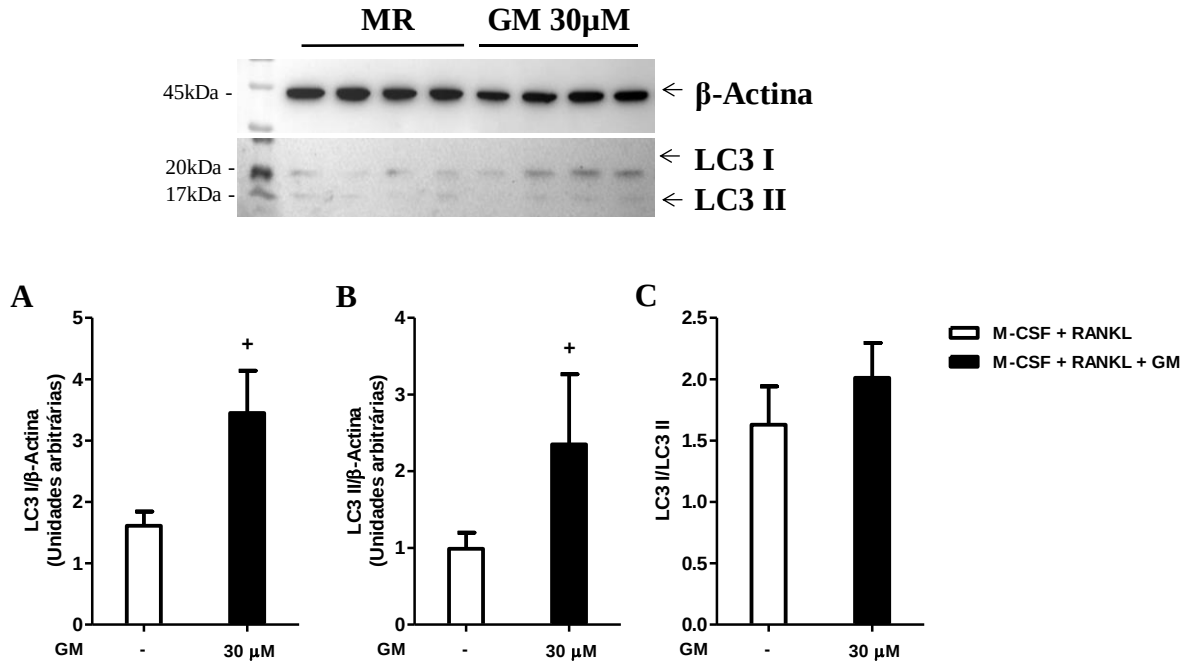


Figura 4.13: Efeito do GM na expressão do marcador de autofagia LC3 em osteoclastos *in vitro*. Pré-osteoclastos foram cultivados em meio osteoclástico (M-CSF 30 ng/mL e RANKL 10 ng/mL) e incubados ou não com GM (30 µM) por 72 horas. As proteínas celulares foram coletadas e processadas para *western blot*. Os lisados totais das células foram resolvidos por SDS-PAGE e marcados para LC3 I (A) e LC3 II (B). Os resultados foram expressos como média ± erro padrão da média (EPM) de 4 poços por grupo, representativo de dois experimentos independentes. As diferenças estatisticamente significativas (teste *one-way* ANOVA e *Student-Newman-Keuls*; $p \leq 0,05$) entre os grupos tratados e não tratados foram representados por (+).

5 DISCUSSÃO

Neste estudo, foi demonstrado que o galato de metila (GM) um derivado de ácidos fenólicos encontrado em várias espécies de plantas apresenta um efeito notável sobre a resposta inflamatória *in vivo* em modelos de artrite experimental, inibindo diversos parâmetros, tais como a formação de edema articular, acúmulo de leucócitos no sítio inflamatório e a produção de mediadores químicos. Além disso, foi avaliado o efeito desse composto *in vitro*, a fim de compreender o mecanismo pelo qual o GM inibe a migração dos neutrófilos e a ativação de macrófagos mediada principalmente via TLR-2.

Compostos fenólicos são uma das principais classes de produtos naturais estudados no contexto da AR. Eles são metabólitos secundários de plantas que normalmente desempenham um papel protetor contra a radiação ultravioleta ou patógenos (Sung *et al.*, 2019). Numerosos estudos mostram que dietas ricas em polifenóis exercem efeitos cardioprotetores, anticancerígenos, antidiabéticos e antienvelhecimento (Pandey e Rizvi, 2009). Os polifenóis tem potenciais atividades benéficas na AR devido as suas numerosas propriedades capazes de interferir com vias específicas da doença (Islam *et al.*, 2016). Nesse trabalho, foi demonstrado que o GM, um composto fenólico, apresenta propriedades anti-inflamatórias, em modelos experimentais de artrite e nas vias de sinalização envolvidas na AR, comprovando seu potencial terapêutico para estudos mais aprofundados dessa substância na patogênese da doença humana.

Modelos experimentais em animais são amplamente utilizados em estudos da patogênese da artrite reumatoide. Apesar das limitações inerentes de todos os modelos, a utilização deles para o estudo da AR tem agregado conhecimento as pesquisas sobre os mecanismos da inflamação, da destruição da cartilagem e da reabsorção óssea, contribuindo com os avanços na intervenção terapêutica dessa doença (Feldmann *et al.*, 1998; Asquith *et al.*, 2009; Bevaart *et al.*, 2010). A artrite experimental induzida por zimosan (AIZ) possui características semelhantes com as observadas na AR humana, embora tenha uma menor dependência da resposta imune mediada por linfócitos (Asquith *et al.*, 2009). Dentre as principais características

pode-se destacar: e formação de edema articular, a infiltração de células imunes para a cavidade sinovial, a produção dos principais mediadores inflamatórios envolvidos na doença e a incapacitação articular (Da Rocha *et al.*, 2004; Penido *et al.*, 2006; Guerrero *et al.*, 2008). No modelo de artrite induzida por antígeno (AIA), os animais são imunizados com uma substância catiônica, como a albumina de soro bovino metilada (mBSA). Ao ser injetado por via intra-articular o antígeno se liga à cartilagem, que possui carga negativa, os anticorpos ao reconhecerem o antígeno formam complexos imunes que ativam localmente a resposta imunológica resultando na destruição articular (Bendele, 2001; Svensson *et al.*, 2013).

O zimosan estimula a fagocitose, a produção de citocinas, quimiocinas, espécies reativas de oxigênio e nitrogênio, além de induzir a migração de células e o aumento da permeabilidade vascular (Gantner *et al.*, 2003; Yuhki *et al.*, 2008), nesse último ocorre o extravasamento de líquido e proteínas do plasma para o tecido induzindo a formação de edema (Sherwood e Toliver-Kinsky, 2004). A injeção intra-articular de zimosan induziu um aumento no diâmetro da articulação do joelho (edema) na primeira hora após o estímulo alcançando seu efeito máximo em 24 horas (Figura suplementar 1: artigo 1). O pré tratamento oral com o GM (7 mg/kg) foi capaz de reduzir o edema articular tanto em 6 quanto em 24 horas após o estímulo (Figura 2 A-B: artigo 1). O aumento do diâmetro articular induzido pelo zimosan é o resultado de uma cascata de eventos que incluem a ativação do sistema complemento, a degranulação de mastócitos, a geração de metabólitos do ácido araquidônico (como leucotrienos, prostanóides e PAF) e a produção de NO (Gegout *et al.*, 1995; Da S Rocha *et al.*, 2002; Conte *et al.*, 2008; Dimitrova *et al.*, 2010; Linke *et al.*, 2012). Além disso, foi observado que no modelo de edema de pata induzido por zimosan existe o envolvimento das aminas vasoativas como a histamina e a bradicinina, PAF e PGE_2 (Artigo submetido para o *Journal of Ethnopharmacology:* anexo 1).

Cavalher-Machado e colaboradores (Cavalher-Machado *et al.*, 2008) relataram que o pré-tratamento de mastócitos com o GM reduz a liberação de histamina e a degranulação dessas células após o estímulo com C48/80 ou a sensibilização passiva com IgE. Outros resultados obtidos no laboratório de Farmacologia Aplicada (Farmanguinhos) demonstraram que o GM também foi capaz de inibir o edema de pata induzido por histamina, bradicinina e PAF (Artigo submetido para o *Journal of Ethnopharmacology:* anexo 1). Além disso, a administração oral do GM reduziu a produção de PGE_2 na cavidade sinovial em 6 e 24 horas de reação (Figura 5 A-B: artigo 1). A inibição da produção de prostaglandina pelo GM também foi relatada por Kim e colaboradores (Kim *et al.*, 2006) que demonstraram uma falha na produção de PGD_2 por

mastócitos devido a uma redução da atividade da enzima COX-2 pelo GM, mas não pela sua expressão. Diferente do trabalho anteriormente citado, foi observado que o GM inibe a expressão de COX-2 em macrófagos estimulados *in vitro* (Figura 7C: artigo 1). A divergência entre os dois resultados pode ser explicada pela diferença dos tipos celulares utilizados nos dois estudos, e também pela diferença dos mediadores utilizados para induzir a expressão da enzima. Além disso, um estudo recente mostrou, através de experimentos químicos e estudos *in silico*, que o GM é um protótipo molecular para terapias anti-inflamatórias baseadas na inibição da COX e LOX (C S *et al.*, 2018). Neste estudo, sugere-se que a inibição da produção de PGE_2 *in vivo* é devido à diminuição da expressão da enzima COX-2. Contudo, mais estudos são necessários para afirmar que essa redução também é decorrente da ação do GM sobre a atividade da COX-2. Em conjunto, estes resultados sugerem que o efeito anti-edematogênico do GM pode estar direta ou indiretamente envolvido com a modulação da produção e ação de mediadores lipídicos, bradicinina, histamina e PAF na inflamação induzida por zimosan.

O estímulo i.a. de zimosan ou o desafio com mBSA levou a um aumento acentuado no número de neutrófilos no espaço articular (Figura 2: artigo 1; Figura 4.2). Os neutrófilos são as células mais abundantes presentes no fluido sinovial de pacientes com AR. Essas células amplificam as respostas inflamatórias e contribuem para a perpetuação da inflamação nas articulações (Cross *et al.*, 2005). A ativação dos neutrófilos induz à reorganização do citoesqueleto, a liberação do conteúdo dos seus grânulos, a geração de espécies reativas de oxigênio e nitrogênio, a produção de mediadores inflamatórios, incluindo citocinas e mediadores lipídicos, e o aumento da fagocitose (Cascão *et al.*, 2010; Wright *et al.*, 2010; Németh e Mócsai, 2012). Suo e colaboradores (Suo *et al.*, 2014), demonstraram que a depleção ou inibição das funções dos neutrófilos leva a uma redução significativa da formação de edema, 2-8 horas após a injeção de zimosan.

No presente trabalho, foi demonstrado que o pré-tratamento com o GM reduz o acúmulo de leucócitos na cavidade articular após o estímulo com zimosan ou o desafio com mBSA. O efeito observado na redução do número de células foi principalmente devido à redução do número de neutrófilos para na cavidade, o que foi confirmado através da análise histopatológica do tecido da articulação fêmuro-tibial (Figura 3: artigo 1). Para esclarecer se o GM inibe diretamente a migração dos neutrófilos, foi realizado um ensaio de quimiotaxia, onde observou-se que o GM foi capaz de reduzir de forma significativa a migração dos neutrófilos na presença da quimiocina CXCL-1/KC (Figura 6 A: artigo 1). De forma interessante, o pré-tratamento com o GM reduziu a migração dos neutrófilos apenas nas concentrações mais baixas (0,1 e 1 µM).

Eler e colaboradores (Eler *et al.*, 2013) mostraram que o GM tem uma certa lipofilicidade, o que facilita seu acesso a vários ambientes intracelulares. Neste trabalho, foi observado que na maior concentração o GM não conseguiu inibir a quimiotaxia dos neutrófilos, e esse efeito inesperado pode ser devido a ligação do GM a moléculas não específicas dentro da célula, desencadeando a ativação de vias de sinalização. Desse modo, é evidente que o GM tem um potente efeito inibitório na migração dos neutrófilos. No entanto, parece que *in vitro* em concentrações mais altas o GM interage com diferentes alvos moleculares modificando os efeitos observados em concentrações mais baixas.

O CXCL-1/KC induz a quimiotaxia dos neutrófilos através da sua ligação os receptores de sete domínios transmembranares (CXCR1 e CXCR2) localizados na superfície dos neutrófilos (Wu *et al.*, 2014). Esses receptores são acoplados à proteína G (GPCRs), que são compostos pelas subunidades α, β e γ. A subunidade Gβγ ativa fosfatidilinositol 3-quinase (PI3K) que conduz à fosforilação de fosfoinositídeo (PI) para formar PI-trifosfato (PI3P), que por sua vez ativa a proteína quinase B (Akt/PKB), bem como GTPases (Futosi *et al.*, 2013). Essa sinalização regula a polimerização de F-actina que controla a migração dos neutrófilos (Stillie *et al.*, 2009). Um estudo mostrou que o GM reduz significativamente a migração de células do glioma através da inibição da fosforilação de Akt e da formação de adesão focal (Lee *et al.*, 2013). Com base nesses dados, supõem-se que o GM pode interferir na via de sinalização dos GPCRs, tais como CXCR1/CXCR2.

Para comprovar essa hipótese foi realizado um ensaio de adesão celular uma vez que, as vias de sinalização intracelular dos GPCRs ativam integrinas de superfície que participam da cascata de adesão dos neutrófilos (Dimasi *et al.*, 2013). Foi observado que o tratamento dos neutrófilos com o GM inibiu sua adesão às células endoteliais ativadas com TNF-α (Figura 6 B-C: artigo 1). Esses resultados suportam a hipótese de que o GM pode estar interferindo na via de sinalização do receptor CXCL-1, impedindo assim a ativação, a migração e a expressão de moléculas de adesão nos neutrófilos, consequentemente, impedindo a entrada dessas células aos locais de inflamação como na cavidade sinovial.

Um outro papel importante dos neutrófilos na patogênese da AR é a NETose, que inicialmente foi descrita como um mecanismo antimicrobiano, mas dados recentes já sugerem que a NETose pode contribuir para a fisiopatologia das doenças autoimunes (Fattori *et al.*, 2016). A citrulinação de proteínas constitui a principal modificação pós-traducional que gera novos antígenos reconhecidos por anticorpos em pacientes com doenças autoimunes,

especialmente na AR (Pruijn, 2015). No líquido sinovial de pacientes com AR, a NETose contribui para a liberação de PAD-4, e consequentemente, geração de antígenos citrulinados (Spengler *et al.*, 2015). Com o objetivo de examinar o papel do GM sobre esse processo, foi avaliado a presença das NETs de forma indireta através da liberação de DNA extracelular no lavado dos animais que foram submetidos a AIA e tratados com o GM. De fato, o pré-tratamento dos camundongos com o GM foi capaz de reduzir a liberação de DNA extracelular no modelo de AIA (Figura 4.6), entretanto, para avaliar se isso pode ser devido a uma redução do número dos neutrófilos nas articulações (foi demonstrado anteriormente) ou se seria um efeito direto do GM sobre os neutrófilos é necessário que outros ensaios sejam realizados.

A produção de anticorpos contra antígenos próprios ou exógenos é uma característica marcante na AR e pode ser responsável pelo início e pela perpetuação dos processos inflamatórios dessa doença (Chaiamnuay e Bridges, 2005). Um dos fatores de ativação de neutrófilos mais importantes na AR parece ser imunocomplexos contendo IgG (Cross *et al.*, 2005). Sendo o modelo da AIA desencadeado pela deposição de imunocomplexos e consequente ativação do sistema complemento, que resulta na inflamação aguda (Bräuer *et al.*, 1988; Inada *et al.*, 1997), é provável que a produção de anticorpos nesse modelo experimental seja um fator importante para o influxo de células para a cavidade articular. Aqui, foi demonstrado que o GM só foi capaz de reduzir significativamente a produção de IgG anti-mBSA 24 h após o desafio i.a. com mBSA (Figura 4.7), entretanto, reduziu o número de neutrófilos na cavidade articular 7 h após o desafio (Figura 4.2), sugerindo que não só a presença dos anticorpos na articulação estimula a migração dos neutrófilos, mas outros fatores também podem estar envolvidos nesse processo. O acúmulo de leucócitos no foco inflamatório é um fenômeno complexo, que depende da interação leucócito-endotélio, moléculas de adesão e geração local de mediadores químicos, tais como as citocinas. Fatores esses que foram atenuados pelo tratamento dos camundongos ou das células com o GM.

As vias de sinalização ativadas em resposta ao estímulo com zimosan ou o antígeno levam a produção de muitas citocinas, quimiocinas e mediadores lipídicos pró-inflamatórios que são detectados em pontos iniciais nos modelos murinos de inflamação (Ajuebor *et al.*, 1999; Inada *et al.*, 2009). Nesse estudo, foi observado um aumento na produção das citocinas TNF-α, IL-6, IL-1β, IL-17 das quimiocinas CXCL-1, CCL-2, CCL-3 e dos mediadores lipídicos LTB_4 e PGE_2 na cavidade sinovial após a injeção i.a. de zimosan ou mBSA (Figuras 4 e 5: artigo 1; Figura 4.5). O pré-tratamento dos animais com o GM reduziu a produção de todos os mediadores inflamatórios mencionados.

Estratégias terapêuticas que bloqueiam ou antagonizam citocinas pró-inflamatórias tais como TNF-α, IL-6 ou IL-1β têm sido utilizadas na clínica para o tratamento da AR e se revelaram benéficas prevenindo a progressão da doença em pacientes (Cohen *et al.*, 2002; Nishimoto *et al.*, 2004; Alonso-Ruiz *et al.*, 2008). Experimentalmente, essas citocinas desempenham um papel crítico na hipernocicepção inflamatória, impulsionando a produção de fatores quimioatraentres de neutrófilos e aumentando a expressão de moléculas de adesão celular em modelos de artrite (Hickey *et al.*, 1997; Verri *et al.*, 2006; Kelly *et al.*, 2007; Cunha *et al.*, 2008). A inibição dos receptores de TNF-α e IL-1 (TNFR1 e IL-1R1, respectivamente) reduziu a migração dos neutrófilos no modelo de AIA (Sachs *et al.*, 2011). Além disso, Sachs e colaboradores (2011) mostraram que o bloqueio do influxo de neutrófilos com fucoidan diminuiu a produção de TNF-α e IL-1β. Assim, TNF-α e IL-1β são necessários para o acúmulo dos neutrófilos nas articulações, mas os neutrófilos também são essenciais para a produção completa dessas citocinas. Diversos trabalhos têm demonstrado também a participação da IL-17 nos múltiplos eventos inflamatórios durante a AR, através da liberação de vários mediadores inflamatórios, os quais estão envolvidos com a migração neutrofílica, erosão óssea e destruição tecidual (Witowski *et al.*, 2004). O papel da IL-17 na inflamação e destruição articular também já foi demonstrado em diferentes modelos experimentais de artrite (Lubberts *et al.*, 2002; Koenders *et al.*, 2005; Pinto *et al.*, 2010).

Múltiplos quimioatraentes podem induzir diretamente a migração dos neutrófilos. Chou e colaboradores (Chou *et al.*, 2010) demonstraram que o LTB_4 é um mediador crítico para a migração inicial dos neutrófilos no modelo de artrite murina K/BxN. No modelo de artrite induzida por zimosan ocorre uma liberação significativa de LTB_4 1 h após a injeção do estímulo (Da Rocha *et al.*, 2004). Como o acúmulo de neutrófilos na cavidade sinovial na artrite induzida por zimosan começa 2 h após o estímulo (Figura suplementar 1 C: artigo 1), é provável que a produção do LTB_4 preceda o influxo dos neutrófilos. Entretanto, o tratamento com fucoidan ou anticorpos anti-neutrófilos inibe a produção de LTB_4 induzida por zimosan 7 h após o estímulo i.a. (Guerrero *et al.*, 2008). Esses dados mostram que, embora as células residentes, como os sinoviócitos, podem ser os responsáveis pela liberação imediata de LTB_4, os neutrófilos são responsáveis pela liberação posterior desse mediador. Esses neutrófilos recrutados pelo LTB_4 produzem citocinas como IL-1β, a qual atua em células residentes induzindo a produção de quimiocinas ligantes dos receptores CXCR-1 e CXCR-2 (incluindo CXCL-1) (Guerrero *et al.*, 2012). Esses dados mostram a existência de uma cascata envolvendo lipídios-citocinas-quimiocinas que impulsionam o recrutamento dos neutrófilos e o desenvolvimento da artrite.

Nesse estudo, foi demonstrado que o pré-tratamento com o GM reduz a produção de LTB_4, CXCL-1/KC e IL-1β no lavado da articulação sinovial após o estímulo com zimosan (Figura 4 e 5: artigo 1), assim como também reduz a migração massiva de neutrófilos. É provável que o GM module a cascata lipídios-citocinas-quimiocinas e assim reduza a migração dos neutrófilos para o sítio da inflamação.

A interação do zimosan com macrófagos residentes (sinoviócitos do tipo A) nas articulações é o primeiro passo para o início da artrite induzida por zimosan (Pettipher e Salter, 1996; Young *et al.*, 2001). Macrófagos residentes nos tecidos apresentam um papel crucial no reconhecimento de patógenos e subsequente ativação endotelial e recrutamento de células (Medzhitov, 2008). Macrófagos ativados tem a habilidade de fagocitar e destruir agentes infecciosos e liberar mediadores inflamatórios, tais como TNF-α, IL-1β, IL-6, e IL-12, os quais contribuem para a ativação do sistema imune (Davies *et al.*, 2013). Apesar de contribuir com a resposta protetiva do hospedeiro, macrófagos também estão envolvidos no dano tecidual e perda da função em múltiplas patologias em casos onde a resposta inflamatória não é adequadamente controlada, como é o caso da AR. Assim, a liberação excessiva de mediadores inflamatórios e espécies reativas de oxigênio e nitrogênio pelos macrófagos desencadeia a destruição tecidual e muitas vezes contribui para a progressão da doença (Wynn *et al.*, 2013). Na tentativa de elucidar o mecanismo molecular pelo qual o GM diminui a produção de mediadores inflamatórios, foram utilizadas nesse estudo diferentes linhagens de macrófagos como as linhagens J774A.1 e RAW264.7 e culturas primárias de macrófagos derivados da medula óssea (BMDM) de camundongos.

A incubação das diferentes linhagens de macrófagos com o GM inibiu a produção/liberação de IL-6, TNF-α, CXCL-1/KC e NO induzidos pelo estímulo com zimosan e/ou INF-ɣ, $Pam3CSK_4$ e LPS. É importante ressaltar, que o efeito inibitório do GM na produção de mediadores inflamatórios nos macrófagos não está associado à redução da viabilidade celular (Figura 8 A-B: artigo 1; Figuras 1, 3 e 5: artigo 2).

O fator de transcrição NF-κB é muito estudado como um alvo de novos fármacos anti-inflamatórios como parte da resposta imune inata e adaptativa. Além disso, a atividade do NF-κB está aumentada em várias doenças inflamatórias incluindo a artrite reumatoide. A ativação do NF-κB por meio de receptores do tipo *toll* envolve uma reação em cascata pela ativação de MyD88/IRAK/TRAF6/TAK-1/IKK, seguida da degradação de IκB-α, ativação do NF-κB e liberação de mediadores inflamatórios (Li e Verma, 2002). Uma vez que a produção de

citocinas como IL-6, TNF-α, CXCL-1/KC, e a expressão das enzimas COX-2 e iNOS são principalmente dependentes da ativação do NF-κB (Hayden e Ghosh, 2008), foi hipotetizado que o efeito inibitório do GM na produção desses mediadores poderia ser devido à inibição da via de sinalização do NF-κB. Para testar essa hipótese foram utilizadas diferentes abordagens experimentais tais como: (i) quantificação da translocação da subunidade p65 do NF-κB para o núcleo e avaliação da degradação do IκB-α em BMDM estimuladas com $Pam3CSK_4$ por *western blot*; (ii) ou macrófagos RAW 264.7 carreando de forma estável o gene da luciferase controlado pelo promotor do NF-κB. Assim, quando o NF-κB se liga ao seu promotor ativando a transcrição gênica, a luciferase é transcrita e acumula no citoplasma das células podendo então ser quantificada através da conversão do seu substrato, luciferina, em luz, que é quantificada através da luminescência.

Na busca do mecanismo de ação do GM, diferentes estímulos foram utilizados para identificar vias de sinalização onde o GM atua. Cinco diferentes estímulos foram utilizados para induzir a ativação e sinalização do NF-κB em macrófagos. Notavelmente, a pré-incubação de macrófagos RAW 264.7 reduziu a emissão de luminescência induzida pelo zimosan, $Pam3CSK_4$ e LPS (Figuras 1, 4 e 5: artigo 2). Por outro lado, a incubação das células RAW 264.7 com o GM não reduziu a atividade de NF-κB induzida pelo PMA (ativador da proteína quinase C [PKC]) (Figura 5: artigo 2). Até aqui, esses resultados sugerem um possível mecanismo do GM nas vias de sinalização de receptores do tipo *toll*, mas não em outras vias. Entretanto, mais experimentos são necessários para comprovar essa hipótese.

Além do NF-κB também foi analisado a translocação de outro fator de transcrição, o AP-1, uma vez que, esse fator também e conhecido por estar envolvido nas respostas inflamatórias. O AP-1 é um ativador da transcrição sequência-específico composto por membros da família do c-Jun e c-Fos. É uma importante proteína reguladora envolvida no crescimento, diferenciação, transformação e apoptose celular, e também pode contribuir para a resposta inflamatória e imunológica (Silvers *et al.*, 2003). Na resposta inflamatória, o AP-1 regula a produção de citocinas como TNF-α, IL-1 e IL-12 (Giri *et al.*, 2009). Foi observado uma redução da translocação nuclear de c-Jun e c-Fos em macrófagos estimulados com $Pam3CSK_4$ e incubados com GM, sugerindo uma possível modulação do AP-1 pelo GM (Figura 6: artigo 2).

Para investigar o mecanismo molecular subjacente a inibição da translocação nuclear de c-Jun e c-Fos, foi avaliado o efeito do GM sobre a ativação das MAPKs. As MAPKs desempenham um papel importante na ativação de múltiplos genes inflamatórios, como o AP-

1. Os principais grupos de MAPKs incluem a quinase regulada por sinal extracelular (ERK), a quinase N-terminal c-Jun (JNK) e a quinase p38. Sabe-se que a MAPK p38 desempenha um papel importante na migração celular e na regulação positiva da expressão de mediadores como TNF-α, IL-1, IL-6 e IL-10 e enzimas como COX-2 e iNOS (Zarubin e Han, 2005; Yang *et al.*, 2014). A JNK medeia a regulação pós-traducional de citocinas através da fosforilação de c-Jun, uma das subunidades do AP-1. Por sua vez, a ERK1/2 regula a expressão de citocinas por mecanismos transcricionais e pós-transcricionais, além de participar dos processos de crescimento, diferenciação e sobrevivência celular (Gaestel *et al.*, 2007; Arthur e Ley, 2013). O tratamento dos macrófagos com o GM diminuiu a fosforilação de ERK1/2, JNK e p38 (Figura 6: artigo 2), sugerindo que a redução na liberação de citocinas após o tratamento com o GM também está associada com a ativação prejudicada de AP-1.

Esses resultados sugerem que as enzimas *upstream* que regulam a ativação transcricional do NF-κB e AP-1 podem ser diretamente alvos do GM. De fato, com base nos resultados obtidos foi demonstrado uma atividade supressora do GM prejudicando a degradação de IkB-α e ativação de ERK, JNK e p38 que estão envolvidos na modulação de NF-κB e AP-1. No entanto, a associação de TLRs e MyD88 estimula o recrutamento de membros da família das IRAKs que interagem com membros da famílias das TRAFs. Em sequência, TRAF ativa TAK1, que por sua vez ativa duas vias *downstream*, que são os complexos IκB-NF-κB e a cascata das MAPKs (Kawai e Akira, 2007). Não é possível descartar a possibilidade de que alguma dessas proteínas seja o alvo molecular do GM e, portanto, essa substância poderia interferir nas duas vias.

Apesar dos diversos mecanismos avaliados nesse estudo sobre o efeito do GM em diversos parâmetros da artrite experimental, o mecanismo de proteção do metabolismo ósseo ainda era incerto. Entretanto, recentemente Baek e colaboradores (Baek *et al.*, 2017) demonstraram pela primeira vez que o GM atenuou a diferenciação osteoclástica dependente de RANKL via sinalização de Akt e Btk-PLCγ2-Ca^{2+} e características de maturação dos osteoclastos incluindo estrutura de F-actina e atividade de reabsorção óssea *in vitro*. Com isso, pode-se supor que o GM interfere no metabolismo ósseo no modelo de artrite induzida por antígeno e assim proteger a articulação da erosão óssea que é característica da AR. Para avaliar essa hipótese, primeiramente foi avaliado se os osteoclastos estavam presentes na articulação 7 dias após o desafio i.a. de mBSA na AIA. Através da marcação de TRAP identificou-se a presença de osteoclastos no fêmur dos animais submetidos a AIA e foi observado ainda que o tratamento diário dos animais com o GM foi capaz de reduzir a presença dos osteoclastos

(Figura 4.9). Foram avaliados ainda alguns marcadores importantes da diferenciação dos osteoclastos com o objetivo de verificar se o GM estaria interferindo nesse processo *in vivo*. Entretanto, os resultados se mostraram inconclusivos, pois os controles experimentais não mostraram diferenças estatísticas entre si (Figura 4.10). Acredita-se que isso possa ser devido a uma possível degradação das amostras, uma vez que, o experimento e a retirada dos joelhos foram feitos no laboratório da Fiocruz (Rio de Janeiro), mas as amostras foram processadas para o WB na USP em Ribeirão Preto (São Paulo). Será necessário a repetição desses ensaios para confirmar os resultados obtidos até o momento.

Foi avaliado também o efeito do GM na osteoclastogênese *in vitro*. O GM suprimiu significativamente a osteoclastogênese induzida por RANKL, e induziu efeito citotóxico apenas na maior concentração (100 µM), que não foi utilizada nos experimentos seguintes (Figura 4.11). O GM inibiu efetivamente a formação de osteoclastos multinucleados, de forma dose-dependente, o que é demonstrado pela redução do número e área de células multinucleadas positivas para TRAP (Figura 4.11). Para iniciar o processo de comprometimento e diferenciação dos osteoclastos, a ligação de RANKL ao seu receptor RANK, localizado na membrana de células precursoras de osteoclastos, é requerida, levando a indução de várias cascatas de sinalização (Roodman, 2006; Boyce e Xing, 2007) que culminam com a expressão de vários genes marcadores relacionados a osteoclastos (Shinohara e Takayanagi, 2014). O tratamento dos pré-osteoclastos com GM reduziu a expressão de genes marcadores de osteoclastos como NFATc1 (Figura 4.12 B**).**

A interação RANKL-RANK resulta na ativação de fatores de transcrição osteoclastogênicos, como NF-κB em pré-osteoclastos, AP-1, e após o NFATc1, que induz a expressão de genes marcadores de osteoclastos (Boyce *et al.*, 2005; Yu *et al.*, 2011). Aqui, além de avaliar o efeito do GM na expressão de NFATc1 o efeito do GM no NF-κB e AP-1 também foi avaliado (Figuras 4 e 6: artigo 2). Os dados mostraram que a inibição da diferenciação dos osteoclastos induzida pelo GM pode ser devido a redução da expressão do NFATc1. O NFATc1 é o principal regulador da osteoclastogênese, uma vez que, regula vários genes específicos dos osteoclastos como o receptor de calcitocina (CTR) e a integrina-β3 que também tiveram sua expressão reduzida pelo tratamento das células com GM (Figura 4.12 C-D). A sinalização do NF-κB é induzida rapidamente em pré-osteoclastos em resposta a RANKL, o qual ocorre *upstream* a sinalização do NFATc1 e a formação dos osteoclastos. Como foi observado uma redução na via de sinalização do NF-κB é possível que o GM atue na diferenciação dos

osteoclastos pelo bloqueio do NF-κB em pré-osteoclastos e, consequentemente, na cascata de transcrição de NFATc1.

Um outro mecanismo que vem sendo descrito é que a inibição da autofagia em osteoclastos pode servir como um possível mecanismo terapêutico contra doenças ósseas em que há um aumento excessivo na reabsorção óssea, como é o caso da AR (Hocking *et al.*, 2012; Zhao *et al.*, 2012). De fato, foi recentemente observado em camundongos que a inibição farmacológica e genética da autofagia reduz a osteoclastogênese e a reabsorção óssea, inibindo a perda óssea causada pela ovariectomina ou pelo tratamento com glicocorticoide (Lin *et al.*, 2016). De acordo com os resultados obtidos de que o GM reduz a diferenciação dos osteoclastos e os marcadores de reabsorção óssea, foi investigado se os efeitos inibitórios do GM na osteoclastogênese poderia ser através de inibição do processo de autofagia. Conforme observado nos resultados obtidos, o GM não somente inibiu o processo autofágico, medido pela expressão de LC3 I e LC3 II (Figura 4.13), como ainda aumentou a expressão dessas proteínas autofágicas. Alguns estudos indicam que a ativação da autofagia pode estar associada ao processo de reparo de fraturas ósseas (Yang *et al.*, 2015; Zhou *et al.*, 2015). Nesse caso, foi proposto que o aumento da autofagia que ocorre após uma lesão óssea atuaria como um mecanismo de defesa das células ósseas contra o estresse celular, causado pela súbita redução ou interrupção do suprimento de nutrientes, devido a fratura óssea (Yang *et al.*, 2015). Assim, estudos indicam que a autofagia é um fator crucial para a manutenção da homeostase do tecido ósseo (Florencio-Silva *et al.*, 2017). No entanto, mais estudos são necessários para compreender melhor o papel do GM no processo autofágico.

Este conjunto de resultados demonstram que o GM apresenta um efeito direto em neutrófilos e na modulação de funções dos macrófagos, inclusive na sua capacidade de se diferenciar em osteoclastos. Isso está de acordo com os estudos que relatam que substâncias de origem natural apresentam múltiplos, porém definidos, alvos moleculares (Koeberle e Werz, 2014).

6 CONCLUSÃO

Tomados em conjunto, os resultados aqui apresentados demonstram um efeito anti-inflamatório importante do GM em modelos de inflamação, principalmente em modelos experimentais de artrite, devido à sua capacidade de modular o recrutamento dos neutrófilos, ativação de macrófagos e diferenciação de osteoclastos. Resultados esses que podem ser em parte explicados pelos efeitos do GM na modulação de fatores de transcrição como NF-κB e AP-1. Com base nesses achados, o presente trabalho fornece evidências e bases moleculares para o mecanismo anti-inflamatório do GM e demonstra o grande potencial dessa substância para o tratamento de doenças inflamatórias. Além disso, sugere-se que os efeitos anti-inflamatórios do GM podem ser mediados através de diferentes vias associadas ao processo inflamatório, corroborando com os estudos que demonstram que substâncias derivadas de produtos naturais possuem múltiplos alvos moleculares.

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8 ANEXOS

8.1 ANEXO I – PUBLICAÇÕES RELACIONADAS AO ASSUNTO DA TESE (2015-2019)

Chapter 5

Neutrophils in Rheumatoid Arthritis: A Target for Discovering New Therapies Based on Natural Products

Elaine Cruz Rosas, Luana Barbosa Correa and Maria das Graças Henriques

Additional information is available at the end of the chapter

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Abstract

Rheumatoid arthritis (RA) is a systemic autoimmune disorder with an important inflammatory component in joints. Neutrophils are the most abundant leukocytes in inflamed joints, and play an essential role in the initiation and progression of RA. Neutrophil effector mechanisms include the release of proinflammatory cytokines, reactive oxygen and nitrogen species (ROS and RNS), and granules containing degradative enzymes, which can cause further damage to the tissue and amplify the neutrophil response. Therefore, the modulation of neutrophil migration and functions is a potential target for pharmacological intervention in arthritis. The pharmacologic treatment options for RA are diverse. The current treatments are mostly symptomatic and have side effects, high costs, and an increased risk of malignancies. Because of these limitations, there is a growing interest in the use of natural products as therapies or adjunct therapies. Herbal products have attracted considerable interest over the past decade because of their multiple beneficial effects such as their antioxidant, anti-inflammatory, antiproliferative, and immunomodulatory properties. This chapter focuses on the role of neutrophils in the pathogenesis of arthritis and the action of substances from natural products as putative antirheumatic therapies.

Keywords: neutrophils, rheumatoid arthritis, herbal products, polyphenols, flavonoids, tetranortriterpenoids, inflammation

1. Introduction

Arthritis is an inflammatory joint disorder that can cause edema, pain, and loss of function. The most common types of arthritis are osteoarthritis, gout, and rheumatoid arthritis [1, 2]. Rheumatoid arthritis is a systemic, autoimmune disorder with an important inflammatory

component in which genetic and environmental risk factors contribute to disease development. Its prevalence in the world population is between 0.3 and 1%, and it affects three times more women than men [3, 4].

The pathophysiology of RA is complex and appears to be initiated when the adaptive immune system (cellular or humoral) recognizes self-joint antigens as non-self, which triggers a variety of distinct inflammatory effector mechanisms, including the recruitment of leukocytes [5–8].

RA is characterized by intense inflammatory processes and joint damage that are mediated by the influx of immune system cells to the synovial space such as neutrophils, macrophages, and lymphocytes [1, 2]. A critical factor that contributes to tissue damage is the excessive production of inflammatory mediators by resident and/or infiltrated cells. Among the primary mediators involved in joint damage are free radicals, enzymes that degrade the matrix, and pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, as well as chemokines such as CXCL-8, lipid mediators, such as leukotriene B_4 (LTB_4) [9, 10], and endothelin (ET) [11, 12]. Inflamed synovial tissue is invasive and called pannus, which can be formed by synovial cell proliferation, angiogenesis, and the accumulation of macrophages, lymphocytes, and neutrophils [13].

Neutrophils are crucial cells that have significant roles in diverse inflammatory diseases, including acute, chronic, autoimmune, infectious, and non-infectious conditions [14]. The most well-known effector function of neutrophils is their role in innate immunity. However, recent studies have identified neutrophils as active cells during adaptive immunity, facilitating the recruitment and activation of antigen-presenting cells or directly interacting with T cells. Neutrophils are the most abundant leukocytes in inflamed joints, and the importance of these cells in the initiation and progression of human RA as well as in murine models has been demonstrated [15–18]. Therefore, neutrophils play an essential role in joint inflammation, and the modulation of neutrophil functions is considered a potential target for pharmacological intervention in arthritis [19–21].

The pharmacologic treatment options for arthritis are diverse. The current treatments are mostly symptomatic and include non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs (DMARDs), and biologic therapies. High costs and an increased risk of malignancies limit the use of these agents, in addition to the potential side effects that all therapies possess. Plant-derived products, such as polyphenols, sesquiterpenes, flavonoids, and tetranortriterpenoids, which are herbal metabolites with anti-inflammatory activity, may provide new therapeutic agents and cost-effective treatments [22, 23]. This chapter focuses on the role of neutrophils in the pathogenesis of arthritis and the action of substances from natural products as putative antirheumatic therapies.

2. Role of neutrophils in rheumatoid arthritis

2.1. Neutrophil trafficking from blood to the synovial cavity

Neutrophil recruitment is an important stage in the inflammatory development process, including autoimmune diseases such as RA. Among the circulating cells, neutrophils are the first ones to reach the synovium and are the most abundant cells in the synovial fluid [24]. In this section,

we discuss the cascade of events that culminates in neutrophil entry into inflamed joints. The leukocyte recruitment cascade involves the following commonly recognized steps: capture, rolling, firm adhesion, and finally transendothelial migration.

Neutrophil release from the bone marrow to the circulating blood occurs immediately after the first signal of inflammation, serving to increase the number of neutrophils available for recruitment into the tissue in response to inflammation [25]. The mobilization of neutrophils from the bone marrow is orchestrated by the hematopoietic cytokine granulocyte colony-stimulating factor (G-CSF). G-CSF mobilizes neutrophils indirectly by shifting the balance between CXCR4 and CXCR2 ligands [26]. In response to the release of inflammatory mediators such as TNF-α and IL-17, the adjacent vascular endothelium becomes activated. Cell surface proteins of the selectin family termed E- and P-selectin and their ligands (L-selectin) mediate this initial neutrophil capture. Neutrophil rolling through the endothelium facilitates their contact with chemotactic factors that promotes neutrophil activation [27]. Chemokines (CXCR-1 or 2 ligands, such as IL-8), the C5a fragment of the complement system, and leukotriene B_4 (LTB_4) are responsible for neutrophil mobilization to the synovial fluid [28–30].

Firm adhesion is mediated by interactions between β_2 integrins (LFA-1, CD11a/CD18, and MAC-1, CD11b/CD18) and their ligand (ICAM-1). Integrins are usually in an inactive state on neutrophil and become activated after the triggering of G protein-coupled receptors such as chemokine receptors [31]. The binding of integrins to their ligands activates signaling pathways in neutrophils stabilizing adhesion and initiating cell motility [32, 33]. This signaling also regulates actin polymerization, which controls the direction of neutrophil movement [34, 35]. The final stage in the adhesion cascade is the ultimate migration of the neutrophil from the vasculature into the inflamed tissue. Passage through the endothelial cell layer occurs both paracellularly (between endothelial cells) and by a transcellular route (over the endothelial cell). Paracellular migration of neutrophils is mediated by binding to endothelial proteins that target neutrophils to intercellular junctions and facilitate their passage through them. To reach the inflamed joint, neutrophils must pass over the basal membrane, which occurs through the degradation of extracellular matrix molecules by proteases stored inside the cells, such as matrix metalloproteinases (MMPs) and serine proteases [14].

In inflammatory foci, neutrophils find immune complexes on the synovium that bind to Fcγ receptors on the neutrophil membrane, triggering their degranulation and reactive oxygen species (ROS) production [36]. In RA pathology, oxidative stress is a result of inadequate ROS release by neutrophils [37]. Oxygen radicals cause DNA damage and oxidation of lipids, proteins, and lipoproteins and may be involved in immunoglobulin mutations that lead to rheumatoid factor (RF) formation [38, 39]. Moreover, proteins from neutrophil degranulation are found at high concentrations in the RA synovial fluid and could be responsible for cartilage and tissue damage, activation of cytokines and soluble receptors, inhibition of chondrocyte proliferation and activation of synoviocytes proliferation and invasion [40–43]. In addition, activated neutrophils also generate chemoattractants (such as IL-8 and LTB_4) that promote further neutrophil recruitment and amplify the inflammatory response (see **Figure 1**).

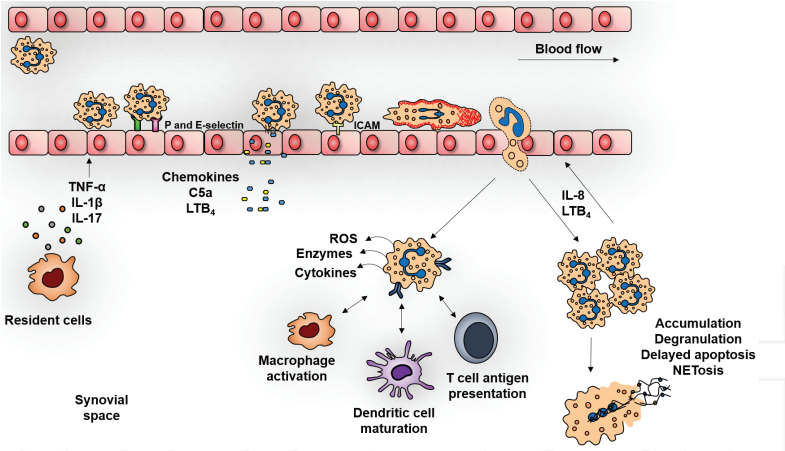

Figure 1. Overview of the role of neutrophils in arthritis. Neutrophils leave blood vessels after chemotactic signals from inflamed tissues that promote the firm adhesion of neutrophils to endothelial cells mediated by adhesion molecules, which induce neutrophil activation and actin filament formation followed by transendothelial migration toward the inflammatory foci. Immune complexes and proinflammatory molecules activate neutrophils, which then produce ROS and release enzymes responsible for cartilage destruction. Activated neutrophils communicate with other cells of the immune system through the secretion of cytokines and chemokines and by antigen presentation in conjunction with MHC class II. Neutrophils can undergo a special form of cell death called NETosis. This results in the release of a complex of nuclear and granule molecules called NETs contributing to tissue damage. Activated neutrophils also generate chemoattractants (such as IL-8 and LTB_4), forming a positive-feedback loop that promotes further neutrophil recruitment and amplifies the acute inflammatory response. Finally, effective neutrophil apoptosis is required for the resolution of inflammation. However, delayed neutrophil apoptosis occurs in the inflamed joint, which results in persistent inflammation and tissue damage due to the continued release of ROS, granule enzymes, and cytokines.

2.2. Neutrophil action in rheumatoid arthritis

Neutrophils are key cells in articular inflammation that are abundant in the synovial fluid and pannus of patients with active RA [44], a typical knee joint may have 2×10^9 cells, of which 90% are neutrophils [24]. These cells are mobilized to synovial tissue by chemoattractant mediators, such as CXCL1, CXCL2, endothelin (ET)-1, and leukotriene B_4, a process in which resident macrophages play a central role [11, 45, 46].

For many years, the major contribution of neutrophils to the pathology of RA was thought to be their cytotoxic potential, since neutrophils participate in the pathogenesis of arthritis by promoting the inflammatory process and cartilage degradation, as well as bone resorption. However, neutrophils are now recognized to have an active role in orchestrating the progression of inflammation through regulating the functions of other immune cells [47, 48], and current research has shown that these cells are involved in RA onset [49, 50].

In the synovial cavity, activated neutrophils exhibit an increased expression of plasma membrane receptors such as major histocompatibility complex (MHC) class II molecules and present antigens to T lymphocytes, an immune function that they share with macrophages and dendritic cells (DCs) [51]. In addition, the interaction of neutrophils with other cells induces the secretion of MMP-8 and MMP-9, and a repertoire of cytokines (IL-1β, IL-12, IL-18, IL-23,

and TNF-α) and chemokines (CCL-2, CCL-4, CCL-5, and CXCL-8), including TNF ligand superfamily member (RANKL) [52, 53] and TNFSF13B (also known as BLyS or BAFF) [54], which are implicated in the activation of osteoclasts and B lymphocytes, respectively, regulate the function of other immune cells [48, 55–57].

Neutrophils from patients with RA are functionally very different from those isolated from healthy individuals. RA blood neutrophils are already primed for ROS production [58] and striking differences in gene and protein expression exist between peripheral blood neutrophils from patients with RA and their healthy counterparts [18], including higher levels of membrane-expressed TNF and myeloblastin (also known as PR-3 or cANCA antigen) in RA [59].

In RA patients, neutrophils can be activated by immune complexes, such as RF or anti-citrullinated protein antibodies (ACPAs), both within the synovial fluid and deposited on the articular cartilage surface [60]. These complexes engage Fcγ receptors and thereby trigger neutrophil activation, which release ROS and RNS [61, 62], collagenases, gelatinases, neutrophil myeloperoxidase (MPO), elastase, and cathepsin G into the synovial fluid and joints [14, 55, 56, 63] due to frustrated phagocytosis [60].

2.2.1. Pain in rheumatoid arthritis and neutrophils

One of the most prevalent symptoms of RA is the increase in sensitivity to joint pain (hyperalgesia), which causes movement limitations. Despite its clinical relevance, strategies for the treatment of arthralgia remain limited. In animal models, hyperalgesia (inflammatory pain) is defined as hypernociception (a decreased nociceptive threshold) [64]. It is broadly accepted that articular hypernociception results mainly from the direct and indirect effects of inflammatory mediators on the sensitization (increased excitability) of primary nociceptive fibers that innervate the inflamed joints [65–67]. Prostaglandins and sympathetic amines are the key mediators of this process. Furthermore, other mediators, such as the cytokines TNF-α, IL-1β, IL-6, and IL-17 play a crucial role in the pathogenesis of arthritis, increasing the recruitment of neutrophils into the joint and driving the enhanced production of chemokines and degradative enzymes [68–70]. In addition, endothelin-1 (ET-1), acting directly or indirectly, also sensitizes primary nociceptive neurons [71–74].

During the inflammatory process, the migrating neutrophils participate in the cascade of events leading to mechanical hypernociception, by mediating the release of hyperalgesic molecules (such as MPO, MMPs, hypochlorite, superoxide anion, and PGE_2) capable of activating nociceptive neurons and causing pain [17, 75–78].

Indeed, decreased inflammation and joint destruction have been directly correlated with reduced neutrophil influx into the joints, as observed in mouse models by means of antibody blockade or the gene deletion of chemoattractant receptors such as CXCR1, CXCR2, and BLT1 (LTB_4 receptor) [15, 79]. Therefore, the blockade of neutrophil migration could be a target in the development of new analgesic drugs [77].

2.2.2. Citrullinated autoantigens and NETs in rheumatoid arthritis

Citrullination is the natural posttranslational conversion of arginine to citrulline mediated by peptidyl arginine deiminases (PADs), enzymes present in macrophages, dendritic cells, and

neutrophils. Experimental evidence indicates that citrullination is involved in the breakdown of immune tolerance and may generate neoantigens (neoAgs) that become additional targets during epitope spreading [80]. Citrullinated residues stimulate the production of anti-citrullinated protein antibodies (ACPAs) in predisposed individuals. It has been observed that ACPAs can be present for several years before any clinical signs of arthritis appear [81–83]. A substantial increase in the number and titer of many antibodies against posttranslationally modified proteins is also seen shortly before the onset of arthritis. Citrullinated Ags have increased immunogenicity and arthritogenicity, and their presence in arthritic joints correlates with disease severity [80, 84–86].

Osteoclasts are dependent on citrullinating enzymes for their normal maturation and display citrullinated antigens on their cell surface in a non-inflamed state. In humans, the binding of ACPAs to osteoclasts in the bone compartment induces IL-8 secretion. In turn, IL-8 sensitizes and/or activates sensory neurons by binding to CXC chemokine receptor (CXCR) 1 and CXCR2 on peripheral nociceptors [87–90], producing IL 8 dependent joint pain that is associated with ACPA-mediated bone loss.

IL-8 release contributes to the chemoattraction of neutrophils [49], which play critical roles in initiating and maintaining joint-inflammatory processes that have been described in experimental arthritis [36, 91]. However, the exact roles that neutrophils play in the posttranslational modification of proteins and disease initiation and progression in RA remain unclear. Recent evidence suggests that, among the various mechanisms by which neutrophils cause tissue damage and promote autoimmunity, aberrant formation of neutrophil extracellular traps (NETs) could play important roles in the pathogenesis of RA [50].

NETs are released during a process of cellular death named NETosis. NETosis occurs with neutrophils upon contact with bacteria, fungi [92], or under several inflammatory stimuli. This process is associated with changes in the morphology of the cells, which eventually lead to cell death with extrusion of NETs [93, 94]. This process requires calcium mobilization, reactive oxygen species (ROS) produced by NADPH oxidase, neutrophil chromatin decondensation mediated by neutrophil elastase (NE) and myeloperoxidase (MPO), and chromatin modification via the citrullination of histones by peptidyl arginine deiminase 4 (PAD4) [95–99]. NETs are a network of extracellular fibers, which contain nuclear compounds as DNA and histones and that are covered with antimicrobial enzymes and granular components, such as MPO, NE, cathepsin G, and other microbicidal peptides [93, 94]. In the extracellular environment, NET fibers entrap microorganisms, and their enzymes and granular substances reach locally high concentrations and are thus able to cleave virulence factors and kill microorganisms [95, 100, 101].

Although NETs play a key role in the defense against pathogens, they may cause undesirable effects to the host, which has increased the interest in the role of neutrophils and NETs in autoimmunity. Augmented NET formation was first described in preeclampsia and ANCA-associated vasculitis and followed by the description in a series of autoimmune conditions, including psoriasis, systemic lupus erythematosus (SLE), antiphospholipid antibody syndrome (APS), and RA [50, 100, 102–105]. Neutrophil extracellular traps are an obvious source of nuclear material. Among these are a range of cytoplasmic and extracellular citrullinated antigens, well-established

targets of the ACPAs found in RA [50, 100]. The protein contents of NETs not only serve as targets for autoantibody and immune complex formation but also induce further NETosis, resulting in a harmful positive-feedback loop. These factors form an inflammatory microenvironment that may trigger a strong autoimmune response in individuals with the corresponding susceptibility [106, 107]. Pro-inflammatory cytokines, such as TNF-α and IL-17, as well as autoantibodies stimulate the formation of NETs and affect their protein composition [50]. Additionally, NETs have been shown to stimulate autoimmunity via the production of interferons and activation of the complement cascade. Interferons activate both the innate and adaptive immune systems, inducing a Th1 immune response and stimulating B cells toward the generation of autoantibodies [108]. The deposition of NETs observed in various inflammatory pathologies is associated with the circulating cell-free DNA (cfDNA) levels in biological fluids, such as plasma and serum, from patients [100, 101, 109]. Therefore, circulatory cfDNA could eventually be utilized as a marker of NETs in these pathologies, while the determination of the DNA levels might facilitate the monitoring of disease activity and assessment of the effectiveness of a selected therapeutic strategy.

Neutrophils have been traditionally viewed as short-lived cells that die at sites of inflammation; however, some evidence suggests that they can prolong their life span upon specific stimuli and transmigrate away from inflammatory loci [48, 110, 111]. Conditions within the synovial joint, such as hypoxia [112] and the presence of antiapoptotic cytokines (including TNF, granulocyte-macrophage colony-stimulating factor (GM CSF), and IL 8) [113, 114], can increase neutrophil survival for up to several days [115, 116], which contributes to enhanced tissue damage.

As described above, neutrophils play an essential role on innate and adaptive immunity in RA physiopathology, contributing to tissue lesions in RA, and therefore represent a promising pharmacological target in RA. Pharmacological strategies that inhibit or reduce neutrophil mobilization or activation could be successful in RA treatment.

3. Neutrophils as therapeutic targets

Animal models have been extensively used in studies of RA pathogenesis. Despite the inherent limitations of all animal models, several rodent models have greatly contributed to the overall knowledge of important processes/mediators in the generation of inflammation, cartilage destruction, and bone resorption. In addition, the pharmaceutical industry has used these models for testing potential anti-arthritic agents, leading to important advances in therapeutic interventions for this destructive disease [117]. Such models include collagen-induced arthritis, collagen antibody-induced arthritis, zymosan-induced arthritis, the methylated BSA model, and genetically manipulated or spontaneous arthritis models such as the TNF-α-transgenic mouse, K/BxN mouse, and Skg mouse [118]. Many of these models show that neutrophils are the first immune cells to enter the arthritic joint, and that early measures of joint inflammation correlate with neutrophil infiltration [45, 119, 120]. In this section, we highlight pharmacological approaches targeting neutrophil recruitment and activity, which present a therapeutic benefit to patients with RA.

The current treatments available to RA patients include glucocorticoids, non-steroidal anti-inflammatory drugs, and disease-modifying antirheumatic drugs. Only disease-modifying agents—and to some extent glucocorticoids—can impede or halt the inflammatory and destructive disease processes [121]. With a more complete understanding of the immune-inflammatory events that occur in the pathogenesis of RA, scientists have developed therapeutic strategies that include monoclonal antibodies and receptor constructs, which target specific soluble or cell-surface molecules of interest. Biological agents such as monoclonal antibodies and recombinant proteins that target TNF-α, CD20, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), and the IL-1 receptor as well as therapies based on the blockade of T-cell and B-cell functions have shown efficacy in controlling the physical signs and pain associated with RA [122, 123].

Many interventions used to treat RA exert inhibitory effects on neutrophil responses in inflammation. However, non-steroid anti-inflammatory drugs (NSAIDS), DMARDs, and biologics do not specifically target neutrophil function [124].

Most NSAIDs inhibit the action of the cyclo-oxygenase-1 and -2 (COX-1 and -2) enzymes, which metabolize arachidonic acid into inflammatory mediators of the prostaglandin family. NSAIDs have been shown to inhibit neutrophil adherence, decrease degranulation and oxidant production, inhibit neutrophil elastase activity, and induce neutrophil apoptosis [125–127]. Corticosteroids induce anti-inflammatory signals by several mechanisms; a major one may be to reduce the expression of cytokine-induced genes. They enter all cells and bind to the cytoplasmic steroid receptor, and then this complex translocates to the nucleus where it is recognized by specific DNA sequences. The major effect of binding to DNA is the suppression of transcription by opposing the activation of the transcription factors AP-1 and NF-κB [128]. Corticosteroids have been shown to inhibit neutrophil degranulation and ROS production, decrease production of inflammatory mediators, and prevent neutrophil adhesion and migration into RA joints [44, 129–131]. The most widely used DMARD in clinic settings is methotrexate, a compound that blocks folic acid metabolism. Its benefits in RA include the stimulation of neutrophil apoptosis [116], inhibition of the NF-κB pathway [132], and reduced adhesion molecule expression and LTB_4 production [133], consequently decreasing neutrophil recruitment and ROS production [134].

Anti-TNF-α therapies are also widely used for the treatment of RA patients. TNF primes the neutrophil respiratory burst, upregulates the expression of adhesion molecules, cytokines and chemokines, and at high local concentrations can stimulate ROS production in adherent neutrophils [135–138]. Three different TNF inhibitors are available for RA patients who fail to respond adequately to standard DMARD therapy. Infliximab and adalimumab are monoclonal antibodies against TNF, whereas etanercept is a TNFRII fusion protein. All three drugs sequester soluble TNF [139]. Reports regarding the direct effect of anti-TNF agents on neutrophils have been published, and these drugs have been shown to decrease the mobilization of neutrophils from the peripheral blood to inflamed joints [140], decrease *ex vivo* neutrophil ROS production [20], and reduce neutrophil chemotactic and adhesive properties [141].

Tocilizumab, a monoclonal antibody that blocks the soluble and tissue-expressed IL-6 receptor, is also proving to be a highly effective biologic agent in RA treatment [142]. Neutrophils are a major source of soluble IL-6 receptors, which they shed in large quantities when activated, and their accumulation in high numbers within the synovial joint could contribute significantly to IL-6 signaling within the synovium through trans-signaling [143]. *In vivo* therapeutic blockade of IL-6 with tocilizumab induces transient neutropenia caused by apoptosis or phagocytosis of apoptotic neutrophils but does not impair antibacterial neutrophil functions [144].

Despite the clinical efficacy of these therapies, many patients do not exhibit significant responses or discontinue treatment because of adverse effects. In addition, the limited availability of biological agents in developing countries, the need for parenteral administration of these products, and the high cost restrict access to such therapies for many RA patients worldwide, and this promotes a continuous search for new therapeutic targets and the development of new drugs [145]. Due to these limitations, interest has grown in the use of alternative treatments and herbal therapies for arthritis patients [146, 147] (**Table 1**).

Therapy	Effect on neutrophil response	Reference
Non-steroidal anti-inflammatory drugs (NSAIDS)	Inhibit neutrophil adherence, decrease neutrophil degranulation and ROS production, inhibit neutrophil elastase activity, and induce neutrophil apoptosis	[125–127]
Corticosteroids	Inhibit neutrophil degranulation and ROS production, decrease the production of inflammatory mediators, and prevent neutrophil adhesion and migration into RA joints	[44, 129–131]
Disease-modifying antirheumatic drugs (DMARDs)	Stimulate neutrophil apoptosis, inhibit the NF-κB pathway, and reduce adhesion molecule expression, LTB_4 production, neutrophil recruitment, and ROS production	[116, 132–134]
TNF-α inhibitors	Decrease neutrophil mobilization from the peripheral blood to inflamed joints and reduce *ex vivo* neutrophil ROS production and neutrophil chemotactic and adhesive properties	[20, 140, 141]
IL-6 inhibitor	Induce transient neutropenia caused by apoptosis or phagocytosis of apoptotic neutrophils but not impair antibacterial neutrophil functions	[144]

Table 1. Current therapeutic targets for arthritis and their effect on neutrophils.

4. Plant-derived molecules as emerging therapies for arthritis

Current arthritis treatments result in unwanted side effects and tend to be expensive, and natural products devoid of such disadvantages offer a novel opportunity. The use of natural products represents a promising alternative to treat rheumatic diseases, in particular by acting as therapeutic adjuvants to reduce the daily doses of conventional drugs that RA patients administer [148–150]. In this section, we highlight future perspectives in the treatment of RA with natural compounds, mainly herbal compounds, to minimize the harmful effects of the over-activation of neutrophils.

Decreased inflammation and joint destruction have been directly correlated with reduced neutrophil influx into the joints, as observed in mouse models by means of antibody blockade or the gene deletion of chemoattractant receptors such as CXCR1, CXCR2, and BLT1 (LTB_4 receptor) [15, 79]. The prospect of new drugs obtained from herbal products (or from structures of herbal products) plays a compelling role in drug discovery and development [151].

As previously mentioned, pharmacologic treatment options for arthritis are diverse and present several side effects. Furthermore, the high costs and increased risk of malignancies limit the use of such agents. Because of these limitations, there is a growing interest in the use of natural products as therapies or adjunct therapies [22]. Plant-derived products such as polyphenols, sesquiterpenes, flavonoids, and tetranortriterpenoids, which are herbal metabolites, are considered to have potential activity to block inflammation, and they may provide new therapeutic agents and cost-effective treatments [22, 23]. These natural products have attracted considerable interest over the past decade because of their multiple beneficial effects, such as their antioxidant, anti-inflammatory, antiproliferative, and immunomodulatory properties. In this section, we discuss the plant-derived products that have been most studied in RA experimental models and/or clinical trials (**Table 2**).

4.1. Quercetin

Quercetin (**Figure 2a**) is the major dietary flavonol found in fruits, vegetables, and beverages, such as tea and red wine [152]. Several epidemiological and experimental studies support the antioxidant, anti-inflammatory, antiangiogenic, antiproliferative, and proapoptotic effects of this molecule [153–155]. Preclinical studies on primary cells and animal models, as

Compound	Chemical class	Arthritis experimental model	Reference
Quercetin	Flavonoid	Adjuvant-induced arthritis	[156]
Methyl gallate	Polyphenol	Zymosan-induced arthritis	[171]
Gedunin	Tetranortriterpenoid	Zymosan-induced arthritis	[176]
Epigallocatechin gallate	Polyphenol	Collagen-induced arthritis	[179]
Curcumin	Polyphenol	Collagen-induced arthritis	[191]

Table 2. Herbal products that exhibit anti-arthritic potential in animal models.

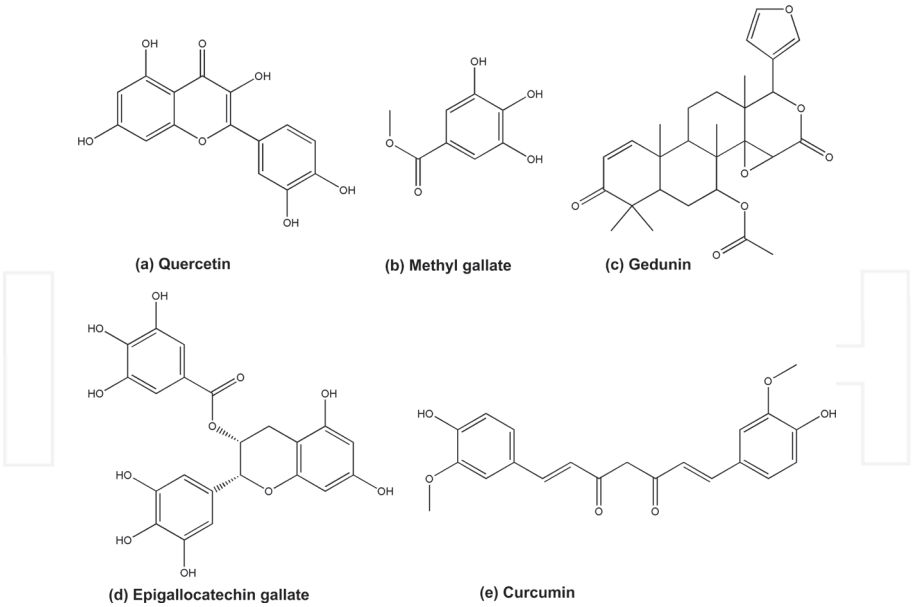

Figure 2. Chemical structure of (a) quercetin, (b) methyl gallate, (c) gedunin, (d) epigallocatechin gallate, and (e) curcumin.

well as clinical studies, suggest an inhibitory action of quercetin in RA. Quercetin has been reported to lower the levels of IL-1β, C-reactive protein, and monocyte chemotactic protein-1 (MCP-1), and restore plasma antioxidant capacity. In addition, quercetin increased the expression of hemeoxygenase-1 in the joints of arthritic rats. Finally, quercetin inhibited the twofold increase in NF-κB activity observed in joints after arthritis induction [156].

There are divergent data on the effect of quercetin in neutrophils. For instance, *in vitro*, quercetin inhibited myeloperoxidase activity [157] but had no effect on lipopolysaccharide-induced neutrophil surface expression of the adhesion molecules L-selectin (CD62L) and β2 integrin (CD11b/Mac1), [158] which are related to rolling and firm adhesion, respectively [159]. In paw edema induced by carrageen, quercetin did not inhibit the increase in myeloperoxidase, which is used as a marker of neutrophil recruitment [160]. Therefore, it seems unlikely that quercetin would inhibit neutrophil recruitment [158]. On the other hand, quercetin inhibits the fMLP-induced increase in intracellular calcium, [158] which is necessary for actin polymerization and consequently neutrophil migration [159]. In addition, *in vitro*, quercetin blocked human neutrophil mobilization through the inhibition of the cellular signaling responsible for actin polymerization in association with the down-regulation of adhesion molecules [161], indicating that treatment with this flavonoid is a conceivable approach to control excessive neutrophil recruitment during inflammation and to prevent neutrophil-mediated tissue lesions [162] (**Table 3**).

4.2. *Schinus terebinthifolius* and methyl gallate

S. terebinthifolius Raddi (Anacardiaceae) is a native plant from South America. It has been used in folk medicine as teas, infusions, or tinctures, as an anti-inflammatory, febrifuge, analgesic,

Compound	Molecular targets/mechanisms	Reference
Quercetin	Inhibits IL-1β, C-reactive protein, and MCP-1 levels. Restores plasma antioxidant capacity, increases HO-1 expression, and inhibits NF-κB activity in joints Inhibits myeloperoxidase activity in neutrophils and blocks neutrophil mobilization	[156, 157, 161]
Methyl gallate	Reduces edema formation, total leukocyte accumulation, neutrophil migration and IL-6, TNF-α, CXCL-1, IL-1β, LTB_4, and PGE_2 production in zymosan-induced arthritis. Impairs neutrophil chemotaxis and adhesion	[171]
Gedunin	Attenuates zymosan-induced articular edema, neutrophil migration, hypernociception, and the production of IL-6, TNF-α, LTB_4, and PGE_2 and prevents increases in lipid bodies. Decreases neutrophil shape changes, chemotaxis, and lipid body formation	[176]
Epigallocatechin gallate	Ameliorates the severity of arthritis and regulates the expression of cytokines, chemokines, MMPs, ROS, NO, COX-2, and PGE_2. Affects neutrophil functionality and inhibits IL-8 and MIP-3α expression	[179–184, 186–189]
Curcumin	Suppresses collagen-induced arthritis by reducing cellular infiltration, synovial hyperplasia, cartilage destruction, and bone erosion. Blocks neutrophil recruitment	[191, 193]

Table 3. Major molecular targets and anti-arthritic mechanisms of herbal products.

and depurative agent and to treat urogenital system illnesses [163]. Scientific reports demonstrated that *S. terebinthifolius* extracts and fractions are rich in polyphenols and display antioxidant, antibacterial, and antiallergic properties in different experimental models [164–166]. The HPLH chromatograms of hydroalcoholic extracts from *S. terebinthifolius* leaves (ST-70) reveal that methyl gallate (MG, **Figure 2b**) is one of the major polyphenol components of the ST-70 extract [167]. Methyl gallate has been extensively studied because of its antioxidant, antitumor, and antimicrobial activities [168–170]. Pharmacological studies have shown that ST-70 and MG also have an anti-inflammatory effect and may have potential activity against arthritis. Pretreatment with ST-70 or MG markedly reduced knee-joint thickness, total leukocyte (mainly neutrophil) infiltration, and reduced the production of inflammatory mediators associated with arthritis such as CXCL-1/KC, IL-6, TNF-α, IL-1β, LTB_4, and PGE_2. ST-70 and MG also inhibited murine neutrophil chemotaxis induced by CXCL-1/KC *in vitro*, and

MG impaired the adhesion of these cells to TNF-α-primed endothelial cells [167, 171]. These results provide some evidence that MG inhibits neutrophil activation and adhesion molecules expression and consequently prevents the neutrophil entry into inflammatory sites (**Table 3**).

Moreover, unlike potassium diclofenac, the long-term oral administration of ST-70 does not induce lethality or gastric damage in mice, which suggests that ST-70 could be used to treat inflammatory conditions such as arthritis with less toxicity [167].

4.3. *Carapa guianensis* and gedunin

C. guianensis Aublet is a member of the Meliaceae family that is widely used in folk medicine in Brazil and other countries surrounding the Amazon rainforest [172]. Anti-inflammatory and analgesic activities are among the most remarkable properties attributed by ethnopharmacological research to the oil extracted from *C. guianensis* seeds, mainly for rheumatic pain and arthritis [172, 173]. *C. guianensis* oil and six different tetranortriterpenoids (TNTP) isolated from the oil were able to significantly inhibit zymosan-induced knee joint edema formation and protein extravasation. TNTP pretreatment inhibited the increase in total leukocyte and neutrophil numbers in the synovial fluid. TNTP also impaired the production of TNF-α, IL-1β, and CXCL-8/IL-8, and significantly inhibited the expression of the NF-κB p65 subunit [174].

Gedunin (**Figure 2c**) is a natural tetranortriterpenoid isolated from vegetal species of the Meliaceae family and is known to inhibit the stress-induced chaperone heat shock protein (Hsp) 90 [175]. Mouse pretreatment and posttreatment with gedunin impaired zymosan-induced edema formation and total leukocyte influx mainly due to the inhibition of neutrophil migration and reduced articular hypernociception. Gedunin also reduced the *in situ* expression of preproET-1 mRNA and IL-6, TNF-α, LTB_4 and PGE_2 production and prevented increases in the number of lipid bodies in synovial leukocytes [176]. Lipid bodies are important sites for the synthesis and storage of lipid mediators and they increase in number during inflammatory responses [177]. In neutrophils, gedunin impaired ET-1-induced shape changes, blocked ET-1- and LTB_4-induced chemotaxis, decreased ET-1-induced lipid body formation and impaired neutrophil adhesion to TNF-α-primed endothelial cells [176]. The combined *in vitro* and *in vivo* effects of gedunin reveal its potential as an anti-arthritic candidate, especially its direct effect on key cells involved in articular inflammation such as neutrophils (**Table 3**).

4.4. Epigallocatechin gallate

Epigallocatechin gallate (EGCG, **Figure 2d**) is one of the main components of green tea [178]. It has antioxidative, anti-inflammatory, antitumor, and chemopreventive properties. The potential disease-modifying effects of green tea on arthritis have been reported; for example, in a mouse model of RA, the induction and severity of arthritis was ameliorated by the prophylactic administration of green tea polyphenols [179]. Subsequent studies suggested that EGCG possesses remarkable potential to prevent chronic diseases like OA and RA [180–184]. The anti-inflammatory and anti-arthritic effects of EGCG are supported by *in vitro* and *in vivo* data indicating that EGCG can regulate the expression of cytokines, chemokines, MMPs,

ROS, nitric oxide (NO), COX-2, and PGE_2 in cell types relevant to the pathogenesis of RA [179–184]. In *in vivo* studies, EGCG was found to inhibit inflammation in mouse models by affecting the functioning of T cells and neutrophils [185, 186]. IL-8 is the most powerful chemo-attractant for neutrophils in the target tissue. EGCG is a very effective inhibitor of IL-1β and of TNF-α-induced IL-8 and macrophage-inflammatory protein-3α (MIP-3α) expression in different cell types [187–189]. These *in vitro* and *in vivo* observations indicated the efficacy of EGCG and demonstrate that it can modulate multiple signal transduction pathways in a fashion that suppresses the expression of inflammatory mediators that play a role in the pathogenesis of arthritis (**Table 3**).

4.5. Curcumin

Curcumin (**Figure 2e**) is a yellow-colored polyphenol found in the rhizome of turmeric. It has antioxidant, anti-inflammatory, antiapoptotic, and anticarcinogenic properties [190]. Oral administration of curcumin suppressed type II collagen-induced arthritis (CIA) in mice by reducing cellular infiltration, synovial hyperplasia, cartilage destruction, and bone erosion. Moreover, the production of MMP-1 and MMP-3 was inhibited by curcumin in CIA and in TNF-α-stimulated RA fibroblast-like synoviocytes (RA-FLS) and chondrocytes [191].

In vitro, it has been reported that curcumin decreases IL-1β-induced expression of the pro-inflammatory cytokine IL-6 and vascular endothelial growth factor (VEGF) in RA-FLS [192]. In addition, curcumin blocks neutrophil recruitment through the inhibition of cellular signaling responsible for actin polymerization in association with the down-regulation of adhesion molecules [193]. It has also been shown to induce apoptosis of RA-FLS (which are resistant to apoptosis) by increasing the expression of the proapoptotic protein Bax and down-regulating the expression of the antiapoptotic protein Bcl-2 [190]. Some molecular mechanisms related to curcumin have been identified. In a human synovial fibroblast cell line (MH7A) stimulated with IL-1β, curcumin blocked the activation of the NF-κB pathway and induced deactivation of the ERK-1/2 pathway [192]. In addition, this polyphenol inhibited activating phosphorylation of protein kinase Cδ (PKCδ) in CIA, RA-FLS, and chondrocytes. Curcumin also suppressed JNK and c-Jun activation in those cells [191].

In a clinical trial with RA patients, curcumin reduced reported pain, tenderness, and swelling of joints [194]. A curcumin-based medicine, Meriva®, demonstrated efficacy in clinical trials with patients with osteoarthritis by reducing reported pain [195]. In another clinical trial, treatment with Meriva® reduced stiffness and physical signs of RA (treadmill test) along with IL-1, IL-6, and VCAM-1 production [196] (**Table 3**).

5. Conclusion

In RA, neutrophils are key cells that are recognized to play an active role in orchestrating the progress of inflammation, through the release of pro-inflammatory cytokines, ROS, RNS, and NETs, which potentially affect the activities of both neutrophils and other cell types, such as resident mononuclear cells and chondrocytes. In addition, neutrophils participate in the

cascade of events leading to mechanical hypernociception. Therefore, neutrophils participate in the pathogenesis of arthritis by promoting the inflammatory process, degradation of cartilage, and bone resorption. The modulation of neutrophil migration and functions in RA can be considered a potential target for pharmacological intervention in arthritis. The pharmacologic treatment options for arthritis are diverse. High costs and an increased risk of malignancies limit the use of these agents, in addition to the potential for side effects that all therapies possess. Nevertheless, herbal metabolites with anti-inflammatory activity and inhibitory action in neutrophils may provide new therapeutic agents and cost-effective treatments.

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CHAPTER 28

Antiinflammatory Properties of *Schinus terebinthifolius* and Its Use in Arthritic Conditions

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1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease of an autoimmune nature characterized by autoantibodies to immunoglobulin G (rheumatoid factor) and citrullinated proteins. If insufficiently treated, RA can lead to accumulating joint damage and irreversible disability.[1] Arthritis represents a major health problem, and its global burden is rising at an alarming rate. Most epidemiological studies in RA have been done in Western countries, showing a prevalence of RA in the range of 0.5%–1.0% in white individuals.[2] In the United States, arthritis affects nearly 46 million people and by 2030 the number of patients with arthritis is expected to rise to 67 million.[3,4] In the Western world and the United States, arthritis and related conditions have been identified as the third-largest contributor to direct health expenditures (behind cardiovascular disease and neurological disorders).[1] Conventional treatment options for arthritis are mostly symptomatic and include nonsteroidal antiinflammatory drugs (NSAIDs),[5] cyclooxygenase-2 (COX-2) inhibitors, and intraarticular therapies with glucocorticoids[6] for pain relief, but these fail to block the progression of the disease. The intention of this treatment in RA patients is to control pain and swelling, delay disease progression, minimize disability, and improve quality of life.[7] Unfortunately, these agents are also associated with gastrointestinal[8] and cardiovascular adverse events.[6] Disease-modifying antirheumatic drugs (DMARDs) are used to treat the clinical and radiological course of RA but have serious side effects.[9] The use of biologics (antibodies or soluble receptors for interleukin (IL)-1), IL-6, and tumor necrosis factor (TNF-α) has recently been introduced for treating RA, but these are also not universally effective.[10–12] Additionally, higher costs and increased risk of malignancies limit the use of such agents in many populations.[13]

New approaches to safer and more available drugs are urgently needed. Ethnopharmacological and ethnobotanical research has been approached by scientists to select

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medicinal plants, thus increasing the interest in using herb derivatives as a therapy or adjuvant therapy to prevent the development of arthritis and other chronic diseases. Studies from folk medicine have been a consistent source of antiinflammatory drugs for decades.[14] Considering arthritis as an inflammatory disease, agents derived from plants that can modulate the expression of proinflammatory signals clearly have potential against this disease.[15]

Schinus terebinthifolius Raddi is a shrub or small tree that belongs to the Anacardiaceae family, and is native to South America (Peru, Argentina, Paraguay, and Brazil). In Brazil, this tree is known as "aroeira-vermelha," "aroeira-pimenteira," the Brazilian pepper tree,[16,17] and by several other names, and it spreads from Pernambuco to Rio Grande do Sul.[18] This species adapts easily to climate change. It establishes itself through an extensive geographical distribution through plasticity of the species. Currently, *Schinus terebinthifolius* Raddi is a forest species that has been prominent in the culinary field, but is used primarily in the medicinal fields. Its therapeutic potential is due to its secondary metabolites, which have been responsible for the growing scientific research with this plant. This species has been frequently studied from a chemical viewpoint, and the presence of several constituents has been established, including phenols[19] such as pentagalloyglucose (a precursor of many complex structures of tannins)[20] and flavonoids.[21,22] *Schinus terebinthifolius* has been used in folk medicine as teas, infusions, or tinctures as well as an antiinflammatory, febrifuge, analgesic, and depurative agent.[23]

This chapter will review the effects of *Schinus terebinthifolius* and its main compounds on inflammation and arthritis.

2. *SCHINUS TEREBINTHIFOLIUS* RADDI—NOMENCLATURE, FOLK MEDICINE, AND CHEMICAL PROPERTIES

Schinus terebinthifolius Raddi (Figure 28.1) is popularly known as aroeira, aroeira-pimenteira, aroeira-da-praia, aroeira precoce, aroeira negra, aroeira branca, aroeira vermelha, aroeira mansa, aroeira-do-brejo, aroeira-do-sertão, fruto de raposa, fruto de sabia, coração de bugre, cambuí, bálsamo, aroeira-do-campo, and aroeira-de-sabiá (in Brazil).[24] There are several plant species known by the name of aroeira or arrueira, such as *Myracrodruon urundeuva* (aroeira-da-serra), *Lithraea molleoides* (aroeira-brava), and *Pistacia lentiscus* L. (arrueira). *Schinus terebinthifolius* Raddi has officially been in the Brazilian Pharmacopeia since its first publication.

Aroeira is known in several countries around the world, and it has names such as the Brazilian pepper tree; "brasilianischer pfeffer" and "peruanischer pfeffer" (Germany); "Pimentero del Brasil" and "turbinto" (Spain); "Faux poivrier" and "poivre rose" (France); "Christmas berry," "Brazilian pepper," "Florida holly," and "peppertree" (United States); cobal (Cuba); cambuí (Mexico); and chichita (Argentina).[25] As for the taxonomic synonyms, the *Schinus terebinthifolius* Raddi is also known as *Schinus*

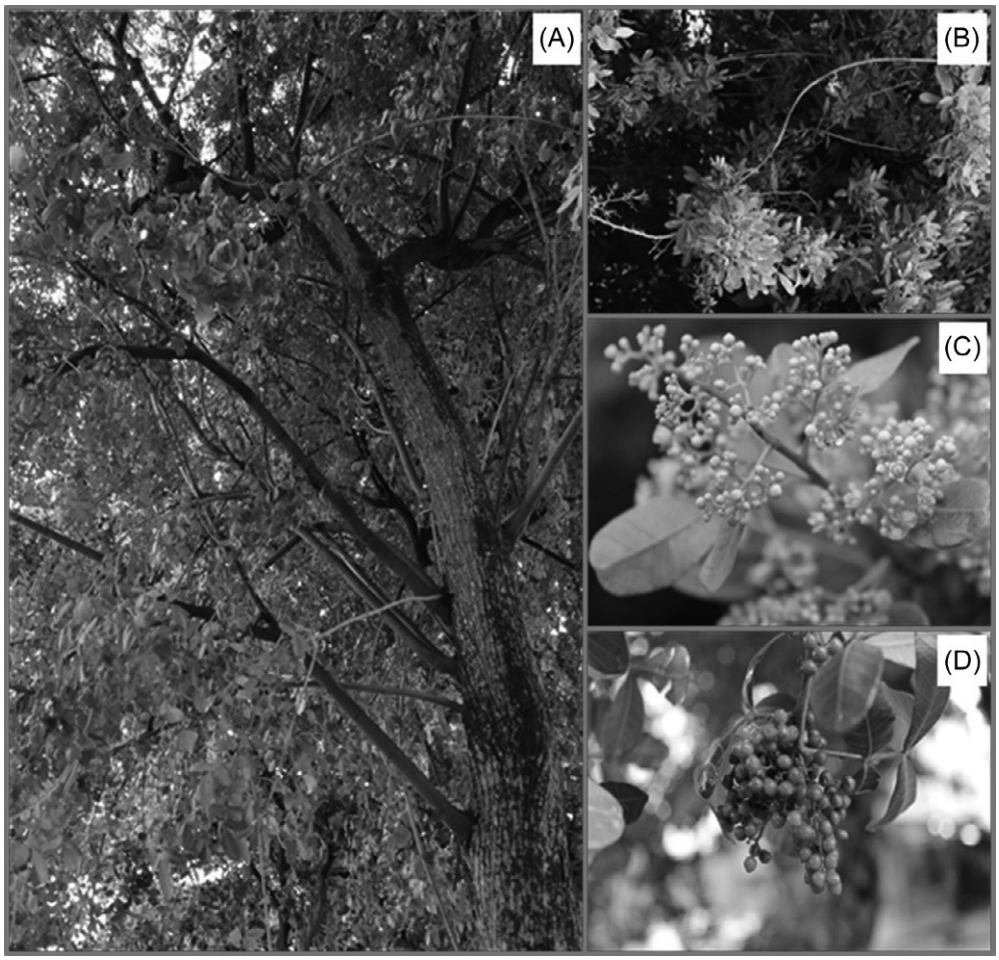


Figure 28.1 Brazilian pepper trees, *Schinus terebinthifolius* Raddi (aroeira-da-praia; aroeira vermelha) and its important parts: bark (A), leaves (B), flours (C) and fruits (D).

mucronulata, *Schinus weinmanniifolius*, *Schinus riedeliana*, *Schinus selloana*, *Schinus damaziana*, *Schinus raddiana*, *Astronium juglandifolium* Griseb, and *Astronium urundeuva*.[26]

Although *Schinus terebinthifolius* has no significant commercial value, it has been used in the restoration of degraded areas and especially to gallery forests or to stabilize sand dunes. The wood is used for posts, fuelwood, and charcoal[27,28] and the plant is also used as a feed supplement for animals. It is also used to obtain essential oils and resins, which are used in leather tanning, pharmaceuticals, cosmetics, the perfumery industry, or the strengthening of fishing nets.[26] Besides, the fruits are highly appreciated as a condiment in foreign cooking, mainly in Europe, where it is used as a spice, either alone or in mixtures with pepper.[29] In France, it is used as a type of sweet pepper.[30]

While the fruits of *Schinus terebinthifolius* are commonly used as spices, the bark, leaves, and roots are traditionally applied in folk medicine. The therapeutic action of *Schinus terebinthifolius* was first mentioned by the Dutch Piso during his visit to Brazil Northeast (1637–44). This plant is among the 39 medicinal species cited by naturalists such as von Martius and St. Hilaire in the 19th century, and they are listed in the first Brazilian Pharmacopeia. In 1912, Alfredo da Matta cited the use of 51 decoctions and peeled resins as antifebrile and antirheumatic, respectively. In 1920, Chernoviz describes the use of bath peel decoction against edema in the legs.[31]

In folk medicine, this plant has been used as a treatment for ulcers, respiratory problems, wounds, tumors, diarrhea, skin ailments, and arthritis[16,32] as well as an antiseptic, antiinflammatory, balsamic, and hemostatic agent.[33,34] The decoction of bark, flowers, stalks, fruits, and leaves is used for the treatment of tumors, leprosy, and infections in the genitourinary system such as cystitis and urethritis.[35] The biological properties of *Schinus terebinthifolius* are due to its secondary metabolites, which are a source of bioactive compounds.[36]

Plant-derived phenolic compounds have received considerable attention due to their beneficial effects on human health, such as protective actions against chronic degenerative diseases (cataracts, macular degeneration, neurodegenerative diseases, and diabetes mellitus), cancer, cardiovascular diseases, and others,[37] which have been ascribed to their antioxidant activity.[38] Phytochemical studies of *Schinus terebinthifolius* showed that it is rich in phenolic compounds that are responsible for the antiinflammatory action, among other activities. Besides, extracts from different parts of aroeira have a therapeutic effect due to terpenes. According to de Lima et al.,[39] the presence of high levels of monoterpenes in this species is responsible for its activity in treating respiratory disorders, mycosis, and invasive candida infections.

Masticadienoico acids and 3α-masticadienoico (schinol) are triterpenoids isolated from *Schinus terebinthifolius* berries, and they were characterized as active site-directed specific competitive inhibitors of phospholipase A_2, which suggests an antiinflammatory activity.[40] The extraction of essential oils is done by solvents or steam distillation from berries or leaves. The main compounds are α-phellandrene (34.38%), β-phellandrene (10.61%), α-terpineol (5.60%), α-pinene (6.49%), β-pinene (3.09%), *p*-cymene (7.34%), and γ-cadinene (18.04%).[23] The percentage distribution of the substances in the essential oil may differ from the geographical placement of the plants[41–43] as well as seasonality.[44]

Chemical studies of the extracts from the bark of the *Schinus terebinthifolius* indicated the presence of anthraquinones, xanthones, and steroids[39] in addition to catechin, tannins, terpenes, flavonoids, and saponins such as bauerenone, α-amyrin, α-amyrenone, gallic acid, agathisflavone, and luteolin.[25] Regarding the stem bark extracts, extensive use is reported in Brazil for the treatment of cutaneous inflammation and wound healing.[45, 46] Chemical studies showed that polyphenolics and flavonoids are the major

constituents of the extracts from *Schinus terebinthifolius* leaves.[41, 47] Compounds such as phenolic derivatives (gallic acid and methyl and ethyl gallates) and flavonoids (trans-catechin, kaempferol, quercitrin, afzelin, myricetin, myricetrin, and quercetin) were isolated from the leaves and displayed antioxidant, cytotoxic, and antiinflammatory activities.[19,30,48]

3. ETHNOPHARMACOLOGICAL AND PHARMACOLOGICAL PROPERTIES

Almost all parts of *Schinus terebinthifolius*, including leaves, bark, fruits, seeds, resins, and oleoresin (or balsam), have been used medicinally by indigenous peoples from different regions.[49] The combination of popular knowledge and studies with extracts obtained from different parts of the aroeira resulted in works about the antioxidant, antiallergic, antimicrobial, antiinflammatory, and antiulcer properties as well as the wound-healing action.[20,25,50–54]

Extracts from the bark and leaves of *Schinus terebinthifolius* are widely used in treating human diseases caused by microorganisms. Among the reports that show the antimicrobial action of aroeira, we highlight the work of Uliana et al.,[54] which reported that the ethanolic extract of the leaf exhibited antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Currently, it is available as a commercial drug, Kronel, which is a gel produced from the aqueous extract of the bark from *Schinus terebinthifolius*. It is used in the treatment of cervicitis, vaginitis, and cervicovaginitis, fighting inflammatory and infectious urogenital processes.

Carlini et al.[34] reported that bark extracts showed a marked protective effect against gastric ulcerations in rats. *Schinus terebinthifolius* extracts can raise both the pH and the volume of the gastric content. Also, these extracts reduced gastric hemorrhage and intestinal transit. These results corroborate the widespread belief that decoctions of *Schinus terebinthifolius* are useful for the treatment of gastric disorders and contribute to the appearance of another medicine, Kios. Kios was also developed from popular knowledge and pharmacological studies, and was produced from the dried extract of the bark of *Schinus terebinthifolius*. It acts in the treatment of gastritis and poor digestion symptoms.

The antiinflammatory potential of *Schinus terebinthifolius* has been identified since Jain and collaborators[40] showed the specific inhibition of phospholipase A_2 by 3α-masticadienoico (schinol) and acid masticadienoico. However, most studies focus on the antimicrobial properties of aroeira. In studies conducted by Cavalher-Machado and collaborators,[20] methyl gallate and 1,2,3,4,6-pentagalloylglucose, which are phenolic acid derivatives isolated from *Schinus terebinthifolius* leaves, showed important antiinflammatory and antiallergic properties by inhibiting edema formation, mast cell degranulation, and eosinophil influx as well as decreasing eosinophilotactic mediator production. In addition, treatment with these phenolic compounds inhibited histamine paw edema to the same extent as the antiallergic drug promethazine. Some reports

suggest mechanisms of action in combating the inflammatory response of extracts from *Schinus terebinthifolius*. Recently, it was showed that extracts and essential oil from the leaves and fruits inhibited NO production by macrophages and the ability to scavenge free radicals.[48,54,55]

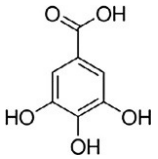
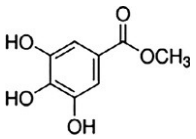
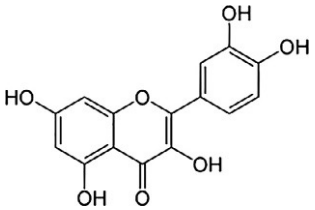
There are reports in which patients with rheumatologic diseases obtained great relief after warm baths with a bark decoction. This action was later explained by Carvalho et al.,[25] who described a study in which the plant inhibited the activity of xanthine oxidase, which is a mechanism of action like that of allopurinol, a drug formally used in the treatment of the disease. Recently, it was shown that the hydroalcoholic extract from *Schinus terebinthifolius* leaves presents an important antiinflammatory property in an arthritis experimental model. This was due to a marked inhibition of neutrophil mobility and reduced inflammatory mediator production induced by zymosan in knee synovial joints, such as chemokine CXCL-1/KC, interleukin (IL)-1α, IL-6, and tumor necrosis factor (TNF)-α.[48]

Despite its many uses in folk medicine, there are few scientific studies to support the antiinflammatory and antirheumatic potential of *Schinus terebinthifolius* that use in vitro experimentations and animal models of arthritis. However, *Schinus terebinthifolius* is a source of many bioactive compounds that are responsible for its biological properties. In the following section, we highlight some of these compounds and emphasize their important actions (Table 28.1) through experimental evidence. We also present arguments for the future development of *Schinus terebinthifolius* as a potential therapeutic entity for the treatment of inflammatory diseases.

4. GALLIC ACID

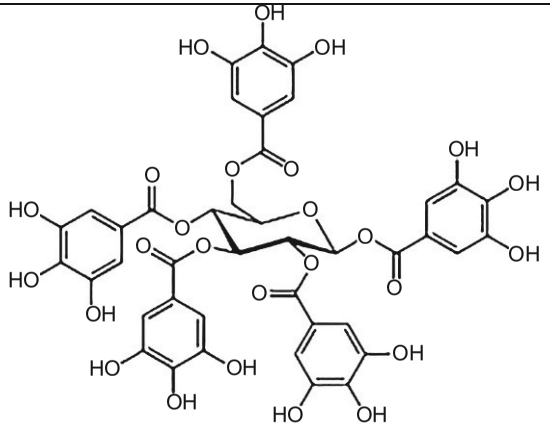
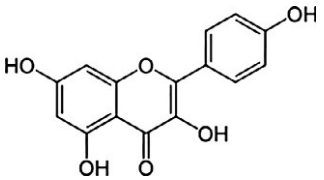
Gallic acid (GA) is a member of the hydroxybenzoic acids found in methyl acetate fraction and hydroalcoholic extract from *Schinus terebinthifolius* leaves.[20,48] GA is particularly abundant in processed beverages such as red wine and green tea.[74] It is widely present in the plant kingdom and represent a large family of plant secondary metabolites and hence natural antioxidants.[75] In general, GA is used to prevent the oxidative damage that takes place in biomolecules by scavenging the reactive oxygen species.[76,77] Other biological studies show that GA has various properties, including antifungal, antimicrobial, and anticancer activities.[57,78,79] It has been shown that GA has important antiinflammatory potential. GA suppressed the levels of NO, PGE_2, and IL-6 in LPS-induced activation of RAW264.7 cells.[56] Another study showed that GA could inhibit the production of IL-6 and IL-8 induced by *Fusobacterium nucleatum* in human mouth epithelial cells as well as the growth of *F. nucleatum*, indicating that GA is a good candidate for controlling periodontal disease.[57] In addition, it was reported that GA significantly decreased the cell viability of fibroblast-like synovial cells (FLS) from patients with rheumatoid arthritis. These cells are key participants of inflammatory signals in the inflamed rheumatoid synovium, mediating

Table 28.1 *Schinus terebinthifolius* compounds and its action on inflammation and arthritis

Compound	Chemical structure	Molecular targets/mechanisms	References
Gallic acid		↓ levels of NO, PGE_2 and IL-6 in LPS-induced activation of RAW264.7 cells	56
		↓ production of IL-6 and IL-8 induced by *F. nucleatum*	57
		↓ cell viability of synovial fibroblast	58
		↓ levels of IL-1, IL-6 CCL-2, CCL-7, COX-2, and MMP-9 on RA FLS	58
Methyl gallate		↓ articular knee-joint thickness and leukocyte infiltration in ZIA	59
		↓ production of CXCL-1, IL-6, TNF-α, IL-1β, LTB_4, and PGE_2	59
		↓ neutrophil chemotaxis and adhesion	60
		↓ RANKL dependente osteoclastic differentiation and maturation	60
Quercetin		↓ NF-κB signaling	61
		↓ hyperalgesia	62
		↓ gout arthritis reducing joint edema, leukocyte infiltration, IL-1β, PGE_2, and NO production	63
		↓ ZIA pain, edema, leukocyte recruitment and production/expression of inflammatory molecules/enzymes	64
		↓ severity of clinical signs of CIA	65

Continued

Table 28.1 *Schinus terebinthifolius* compounds and its action on inflammation and arthritis—cont'd

Compound	Chemical structure	Molecular targets/mechanisms	References
Pentagalloyl glucose		↓ IL-8 mRNA expression and secretion stimulated by PMA or TNF-α	66
		↓ COX-2 and iNOS activity in RAW 264.7 cells stimulated by LPS	67
		↓ ICAM-1 and VCAM-1 expression induced by TNF-α	68
		↓ TNF-α and IL-6 secretion by IL-1β stimulated synoviocytes	69
		↓ activation of NF-κB via inhibition of IKK activity	70
Kaempferol		↓ COX-1 and COX-2 enzymes in vitro cell free assay systems	71
		↓ LPS-induced MAPK pathway in cell line THP-1	72
		↓ proliferation of synovial fibroblast unstimulated and IL-1β-stimulated	73
		↓ mRNA and protein expression of MMP-1, MMP-3, COX-2 and PGE2 induced by IL-1β on synovial fibroblast	73

direct tissue damage and persistent cellular infiltration in the disease.[80] GA treatment also suppresses the levels of proinflammatory cytokines IL-1 and IL-6, chemokines CCL-2 and CCL-7, cyclooxygenase-2 (COX-2), and matrix metalloproteinase-9 (MMP-9) on RA FLS.[58] This study indicates that GA may reduce the inflammatory response by limiting the abnormal proliferation of RA FLS. Also, the suppressed levels of several critical proinflammatory mediators from RA FLS suggest that GA may offer a novel therapeutic or joint protection approach for the treatment of RA.

5. METHYL GALLATE

Methyl gallate (MG) is a strong antioxidant phenolic compound that is found in various plant species such as *Meliaceae* species, *Galla Rhois*, and seed coats of *Givotia rottleriformis* Griff.[57,81] The HPLH chromatograms of hydroalcoholic extracts from *Schinus terebinthifolius* leaves reveal that methyl gallate is one of the major polyphenol components of this extract.[48] Methyl gallate has been extensively studied because it possesses a lot of biological activity such as antiplatelet action, protection of DNA damage against oxidative stress,[82] reduction of lung injury induced by phosgene,[83] attenuation of diabetic oxidative stress, and antiapoptotic activity.[83] Pharmacological studies have shown that MG also has an antiinflammatory effect with potential action against arthritis.[59] Pretreatment with MG markedly reduced knee-joint thickness, total leukocyte (mainly neutrophil) infiltration, and the production of inflammatory mediators associated with arthritis such as CXCL-1/KC, IL-6, TNF-α, IL-1β, LTB_4, and PGE_2. MG also inhibited murine neutrophil chemotaxis induced by CXCL-1/KC in vitro, and MG impaired the adhesion of these cells to TNF-α-primed endothelial cells.[59] Moreover, MG attenuates RANKL-dependent osteoclastic differentiation and osteoclast maturation, including the F-actin structure and bone resorbing activity in vitro. This suggests that MG is a candidate to treat osteoporosis and decrease bone destruction that occurs in patients with rheumatoid arthritis.[60] These results provide some evidence that MG could be used to treat inflammatory diseases such as arthritis.

6. QUERCETIN

Quercetin, a bioflavonoid presenting low toxicity,[84] is the most common flavonoid in nature and presents prominent antioxidant properties, including oxygen radical scavenging, reduction of lipid peroxidation, and metal ion chelation.[85,86] Quercetin is found in large quantities in many plant foods, including apples, tea, and onions, and forms a significant part of the daily dietary intake of polyphenols.[87] Quercetin is present in the alcoholic extract from the leaves and bark of *Schinus terebinthifolius*.[48,54] It has been the subject of numerous studies regarding its biological actions, mainly because of its antioxidant and antiinflammatory properties.[62,88,89] Evidence shows that quercetin inhibits

proinflammatory cytokine (e.g., TNF-α) expression by suppressing NF-κB signaling,[61,90] reduces paw edema[91] and hyperalgesia,[62,92] diminishes neutrophil recruitment by inhibiting the cellular signaling responsible for actin polymerization,[93] and increases the production of antiinflammatory cytokines such IL-10.[94] In the context of arthritis, quercetin inhibits gout arthritis by reducing MSU-induced knee joint edema, hyperalgesia, leukocyte infiltration, IL-1β, prostaglandin E_2, nitric oxide production, and COX-2 expression.[63,95] Quercetin also inhibits zymosan induced-arthritis pain, edema, leukocyte recruitment, and production/expression of inflammatory molecules/enzymes.[64] Furthermore, this bioflavonoid effectively inhibits synoviocyte proliferation and angiogenesis in an inflammatory process associated with arthritis.[96] Lastly, it was recently shown that quercetin diminished the severity of clinical signs of collagen-induced arthritis (CIA) and protected cartilage and bone from destruction. Protection against CIA was associated with decreased circulating levels of TNF-α, IL-1β, IL-17, and MCP-1. Interestingly, quercetin was shown to be a better antiinflammatory, immunosuppressive, and protective therapy than methotrexate, a widely used treatment for RA, using the CIA model.[65] All these results support the therapeutic potential of quercetin in joint inflammation and that this flavonoid merits further investigation toward clinical development.

7. PENTAGALLOYL GLUCOSE

Pentagalloyl glucose (PGG) is a derivative of phenolic acid found in green tea, red wine, and nuts. Pentagalloyl glucose is found in hydroalcoholic extract and in the ethyl acetate fraction from *Schinus terebinthifolius* leaves.[20,48] Reports of the biological activity of PGG are numerous. In vivo and in vitro studies have reported beneficial effects of PGG in the prevention of several major human diseases, including cancer (prostate, lung, sarcoma, breast, leukemia, melanoma, and liver), diabetes, and bacterial diseases.[97] PGG also has aroused particular scientific interest because it is one of the most potent antioxidants in tannins.[98] Other studies show that PGG has high antiinflammatory activity. PGG inhibited IL-8 mRNA expression and secretion in human cell lineage monocytic (U937) stimulated with PMA or TNF-α.[66] In macrophages (RAW 264.7 cells) stimulated by LPS, PGG significantly inhibited the COX-2 and inducible nitric oxide synthase (iNOS) activity as well as NO production.[67] Moreover, PGG suppressed the expression levels of adhesion molecules, including the intracellular cell adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1) induced by TNF-α.[68] PGG also inhibited the secretion of TNF-α and IL-6 by IL-1β-treated rat synoviocytes.[69] Mechanistic studies showed that PGG may exert its antiinflammatory actions by suppressing the activation of NF-κB via inhibition of IKK activity.[66,70] Although there are no reports showing that PGG has an antiinflammatory effect in experimental models of arthritis, there is sufficient evidence to prove the potential antiinflammatory properties of PGG for use in therapy and prevention of several inflammatory diseases, including arthritis.

8. KAEMPFEROL

A member of the flavonoids, kaempferol is abundantly found in beverages such as teas, broccoli, apples, citrus fruits, strawberries, beans, and onions.[99] Kaempferol is widely distributed in different genera such as *Delphinium*, *Camellia*, *Berberis*, *Citrus*, *Brassica*, *Allium*, *Malus*, etc.[100] Kaempeferol was identified in the hydroalcoholic leaf extract of *Schinus terebinthifolius*.[48] Kaempferol has been reported to have antioxidant, antiinflammatory, and immunomodulatory properties in vitro and in vivo.[101] Moreover, it has been demonstrated to invoke several different mechanisms on the regulation of cancer cells. Kaempferol is not only a potent promoter of apoptosis, but it also modifies a host of cellular signaling pathways.[102] Regarding its antiinflammatory effects, it has already been reported that kaempferol inhibits COX-1 and COX-2 enzymes in in vitro cell free assay systems[71] and prevents nitric oxide production induced by LPS in J774 and RAW 264.7 cells, thereby lowering the inflammatory response.[103,104] Kaempferol was shown to suppress the expression of the LPS-induced MAPK pathway in cell line THP-1, which in turn reduced the inflammatory burden by inhibiting the production of monocyte-derived chemokine and IL-8.[72] There is also accumulating evidence indicating the role of kaempferol in attenuating NF-κB-mediated inflammation in various model systems.[105–107] The effectiveness of kaempferol is also demonstrated by its ability to inhibit the proliferation of both unstimulated and IL-1β-stimulated fibroblast-like synovial cells (FLS) in vitro as well as the mRNA and protein expression of MMP-1, MMP-3, COX-2, and PGE_2 induced in these cells by IL-1β.[73] The same study showed that kaempferol also inhibits the phosphorylation of ERK1/2, p38, and JNK as well as the activation of NF-κB induced by IL-1β.[73] These results indicate that kaempferol should inhibit synovial fibroblast proliferation in rheumatoid arthritis as well as the production of MMPs (matrix metalloproteases), COX-2, and PGE_2, which are involved in joint inflammation and destruction in this disease.

9. CONCLUSION

Many studies in the literature demonstrate that natural substances derived from plants provide new approaches to the development of therapeutics for inflammatory diseases. Although plant-derived products offer much promise, they require extensive investigation in various preclinical and clinical settings to prove their usefulness. The chemical investigation of *Schinus terebinthifolius* has revealed many secondary metabolites from this species with significant activities. Bioactive components include monoterpenes, sesquiterpens, triterpenes, fatty acids, and phenols. Because of the presence of these compounds, significant analgesic, antioxidant, antitumor, and antiinflammatory activities of extracts obtained from *Schinus terebinthifolius* have been reported in the literature. In this chapter, we reviewed experimental evidence documenting the antiinflammatory

effect of *Schinus terebinthifolius* and some substances found in its extracts. Numerous in vitro and in vivo studies have demonstrated that compounds present in *Schinus terebinthifolius* extract possess antiinflammatory activity. Some of them are directed to experimental models of arthritis or in cells involved in the pathophysiology of this disease. Therefore, *Schinus* and the compounds present in its extracts display an important antiinflammatory effect, suggesting a putative use of this herb for the development of phytomedicines to treat inflammatory diseases, including arthritis.

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