JULIA BAGATIM DE SOUZA

A MARESINA 2 REDUZ A DOR NOS MODELOS DE CARRAGENINA, ADJUVANTE COMPLETO DE FREUND, VÍRUS CHIKUNGUNYA E SUA PROTEÍNA E2 EM CAMUNDONGOS

Londrina
2022

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Dissertação de mestrado apresentada ao programa de pós-graduação em Ciências da Saúde da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestra em Ciências da Saúde.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Júnior

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Londrina, 26 de setembro de 2022.

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RESUMO

A resposta inflamatória é um mecanismo de defesa do organismo. A inflamação descontrolada pode causar lesões teciduais bem como efeitos crônicos, como a dor. A Maresina 2 (MaR2) é um mediador lipídico especializado em pró-resolução (SPM), derivado do ácido graxo ômega-3 que exibe efeitos anti-inflamatórios e pró-resolução. O objetivo deste estudo foi investigar o potencial efeito anti-inflamatório e analgésico da MaR2 em modelo de dor e inflamação induzidos pela carragenina e adjuvante completo de Freund (CFA). Nesse trabalho também foi avaliado o efeito da MaR2 em um modelo de dor articular induzido pelo vírus chikungunya inativado (CHIKVi) e sua proteína envelope recombinante, rE2. No primeiro conjunto de experimentos, foi avaliado o efeito do tratamento intratecal (i.t.) com a MaR2 sobre a hiperalgesia mecânica e térmica induzidas por estímulo intraplantar (i.pl.) com a carragenina e CFA . Num segundo conjunto de experimentos, foi avaliado o efeito da MaR2 via intraperitoneal (i.p.) sobre o recrutamento de leucócitos em um modelo de peritonite induzida pela carragenina. As células do peritônio também foram coletadas para determinar o efeito da MaR2 sobre o estresse oxidativo por fluorescência de 4,5-diaminofluoresceína diacetato (DAF-2DA) e 2,7-dihidrodiclorofluoresceína (DCF), e avaliar atividade antioxidante mediada pelo fator nuclear derivado de eritróide 2 (Nrf2) por imunofluorescência. Por fim, avaliamos o efeito da MaR2 (via i.p.) na hiperalgesia mecânica induzida pelo estímulo intraarticular com o CHIKVi e E2r. Os neurônios do gânglio da raiz dorsal (DRG) de camundongos naive foram coletados e processados para avaliação da ativação neuronal por influxo de cálcio. O tratamento com a MaR2 via intratecal foi capaz de reduzir a hiperalgesia mecânica e térmica induzida pela carragenina e CFA. A MaR2 i.t. também reduziu o recrutamento celular na pata dos animais estimulados com carragenina. O tratamento intraperitoneal com a MaR2 reduziu a contagem total e diferencial (polimorfonucleares e mononucleares) de leucócitos induzido pela carragenina (i.p.). O tratamento via i.p. com a MaR2 também reduziu a quantidade de espécies reativas de oxigênio e nitrogênio induzidas pela carragenina, e esse mecanismo parece ser depende da ativação do fator nuclar antioxidadante Nrf2. O tratamento intraperitoneal com a MaR2 reduziu de maneira dose e tempo dependente a hiperalgesia mecânica induzida pelo vírus CHIKVi e sua proteína rE2. Por fim, a MaR2 (i.p.) diminuiu a ativação neuronal induzida pelo CHIKVi e rE2. De maneira geral, observamos que a MaR2, utilizada em diferentes vias de tratamento foi capaz de desempenhar um efeito anti-inflamatório, analgésico e antioxidante em baixas doses, demonstrando um potencial terapêutico para esse mediador lipídico.

Palavras-chave: dor; maresina-2; Nrf2; CHIKV.

BAGATIM-SOUZA, Julia. **Maresin 2 reduces pain induced by carrageenan, Complete Freund's adjuvant, Chikungunya virus and its protein E2 mice.** 2022. 73 p. Dissertation (Master's degree in Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2022.

ABSTRACT

Inflammation is the body's defense mechanism against pathogens. Uncontrolled inflammation can cause tissue damage as well as harmful effects such as pain. Maresin 2 (MaR2) is a specialized pro-resolution lipid mediator (SPM), derived from omega-3 fatty acid that exhibits anti-inflammatory and pro-resolution effects. The aim of this study was to investigate the effect of MaR2 in models of pain and inflammation induced by carrageenan and complete Freund's adjuvant (CFA). This work also evaluated the effect of MaR2 in a model of joint pain induced by inactivated chikungunya virus (iCHIKV) and its protein envelope, E2. In the first set of experiments, the effect of intrathecal (i.t.) treatment with MaR2 was evaluated in mechanical and thermal hyperalgesia induced by carrageenan and CFA (intraplantar route, i.pl.). In a second set of experiments, the effect of intraperitoneal (i.p.) MaR2 on leukocyte recruitment in a carrageenan-induced peritonitis model was evaluated. Peritoneal cells were also collected to determine the effect of MaR2 on oxidative stress by fluorescence of 4,5-diaminofluorescein diacetate (DAF-2DA) and 2,7-dihydrodichlorofluorescein (DCF), and to evaluate the antioxidant activity by determination of the factor erythroid-derived nuclear 2 (Nrf2). Finally, we evaluated the effect of MaR2 (via i.p.) in a joint pain model induced by iCHIKV and recombinant E2 (rE2). Dorsal root ganglion (DRG) neurons were processed for neuronal assessment by calcium influx. Treatment with MaR2 intrathecal route reduced both mechanical and thermal hyperalgesia induced by Cg and CFA. A MaR2 i.t. also demontrated a reduction in cell recruitment in the paw stimulated with carrageenan. In addition, treatment with MaR2 intraperitoneal route reduced total and differential (polymorphonuclear and mononuclear cells) leucocyte couting induced by carrageenan peritonitis. In the same experiment, MaR2 also demontrated a reduction in reactive oxygen species and reactive nitrogen species induced by carrageenan, and this mechanism seems to be dependent on the activation of Nrf2 antioxidized signiling. Treatment of MaR2 i.p. reduced in a dose- and time- dependent manner, the mechanical hyperalgesia induced by iCHIKV and rE2. Finally, we found that MaR2 reduced DRG neuron activation induced by iCHKV and rE2, possibly acting in TRPV1 positive neurons. In general, we observed that treatment with MaR2 by different routes displayed an anti-inflammatory, analgesic and antioxidant effect in low doses, demonstrating the therapeutic potential of this lipid mediator.

Key words: pain; maresin-2; Nrf2; CHIKV.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AINES	Anti-inflamatório não esteroidal
AXL	Receptor de tirosina kinase
CD147	Cluster de diferenciação 147
CD209	Cluster de diferenciação 209
CFA	Adjuvante completo de Freund
CGRP	Peptídeo relacionado ao gene da calcitonina
CHIKV	Virus Chikungunya
COX	Cicloxigenase
CXCL	Ligante CXC
DHA	Ácido docosahexaenoico
EPA	Ácido eicosapentaenoico
ERO	Espécie reativa de oxigênio
ERN	Espécie reativa de nitrogênio
G-CSF	Fator estimulante de colônias de granulócitos
GM-CSF	Fator estimulador de colônias de granulócitos e macrófagos
GPCR	Receptores acoplados a proteínas G
IL	Interleucina
IFN-α	Interferon-alfa
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
LXA	Lipoxina A
LXB	Lipoxina B
Ly6G	Locus G6D do complexo do antígeno linfocitário 6
MaR	Maresina
MCP-1	Proteína quimiotática de monócitos 1
mRNA	RNA mensageiro
MRXA8	Proteína 8 associada à remodelação de matriz
NET	Armadilhas extracelulares dos neutrófilos
NF-κB	Fator nuclear kappa B
NLRP3	Proteína 3 que contém domínio de pirina da família NLR
Nrf2	fator nuclear eritróide 2
PD	Protectina

PBQ	fenil-p-benzoquinona
ORF	fase de leitura aberta
RvD	Resolvina da série D
RvE	Resolvina da série E
SPM	Mediadores lipídicos especilizados em pró-resolução
TNF-α	Fator de necrose tumoral-alpha
TRPA1	Receptor de Potencial Transitório Subfamília A, membro 1
TRPV1	Receptor de Potencial Transitório Subfamília V, membro 1
TRPV3	Receptor de Potencial Transitório Subfamília V, membro 3
TRPV4	Receptor de Potencial Transitório Subfamília V, membro 4

SUMÁRIO

1 INTRODUÇÃO

1.1 INFLAMAÇÃO

A resposta inflamatória é um mecanismo natural de defesa dos tecidos frente a uma agressão, seja ela um patógeno, uma toxina ou dano tecidual. A resposta inflamatória aguda é rápida e de curta duração, sendo caracterizada pelos sinais cardinais da inflamação. São eles: calor, rubor, edema, dor e, caso esses sinais persistam, eles podem levar ao quinto sinal cardinal: a perda de função do tecido ou órgão inflamado (Zigterman & Dubois, 2022).

O edema é proveniente do extravasamento de líquido e proteínas plasmáticas, provocando um aumento de fluido no tecido intersticial (Sherwood & Toliver-Kinsky, 2004). A presença de exsudato implica no aumento de permeabilidade dos pequenos vasos sanguíneos, provocando calor e rubor no local inflamado (Freire & Van Dyke, 2013). Essa vasodilatação é induzida pela ação de vários mediadores, sobretudo a histamina (Jutel, Blaser, & Akdis, 2006).

Os mediadores mais importantes da inflamação aguda são as aminas vasoativas (como a histamina, mencionada anteriomente), os produtos lipídicos (prostaglandinas e leucotrienos), as citocinas (inteleucinas, quimiocinas, fatores de crescimento) e os produtos da ativação do complemento (L. Chen et al., 2018). Em resposta a secreção de várias citocinas, incluindo o fator de necrose tumoral (TNF), a interleucina-1 (IL-1) e quimiocinas, ocorre um influxo de células imunes no local inflamado (que também contribui para a edemaciação do tecido lesado) **}**(Abdulkhaleq et al., 2018). As prostaglandinas e leucotrienos, também denominados de eicosanoides, são produzidos a partir do ácido araquidônico, presente nos fosfolipídios da membrana (Freire & Van Dyke, 2013). Esses mediadores lipídicos clássicos também atuam iniciando o tráfego de leucócitos requerido na defesa do hospedeiro, além de estimular as alterações do fluxo sanguíneo, que proporciona influxo dessas células nos tecidos **}**(Serhan, Chiang, & Dalli, 2015).

O influxo de células imunes no local inflamado tem como função eliminar os agentes causadores do processo inflamatório **}**(Kourtzelis, Mitroulis, von Renesse, Hajishengallis, & Chavakis, 2017). Os leucócitos mais importantes nas reações inflamatórias são aqueles capazes de realizar fagocitose, em especial, os neutrófilos e os monócitos/macrófagos (Butterfield, Best, & Merrick, 2006). Uma vez

recrutadas, sob ação de citocinas e quimiocinas os leucócitos, se aderem e migram através dos espaços endoteliais (movimento denominado de diapedese) em direção ao local da lesão ou da infecção (Ley, Laudanna, Cybulsky, & Nourshargh, 2007). Os neutrófilos predominam no infiltrado inflamatório durante as primeiras horas , devido a sua superioridade em quantidade no sangue (Rosales, 2018). Além disso, os neutrófilos responderem mais rapidamente às quimiocinas e se ligam mais firmemente às moléculas de adesão que são expressas nas células endoteliais **}**(Mocsai, Walzog, & Lowell, 2015). Na sequência do processo inflamatório, os neutrófilos são substituídos pelos monócitos a partir de 24 a 48 horas (Butterfield et al., 2006). Os monócitos são leucócitos derivados da medula óssea que circulam no sangue e no baço, que quando recrutados para os tecidos, são capazes de se diferenciar em macrófagos e células dendríticas (S. Chiu & Bharat, 2016). Essas células possuem uma alta atividade fagocitica contra patógenos ou toxinas **}**(Aderem & Underhill, 1999).

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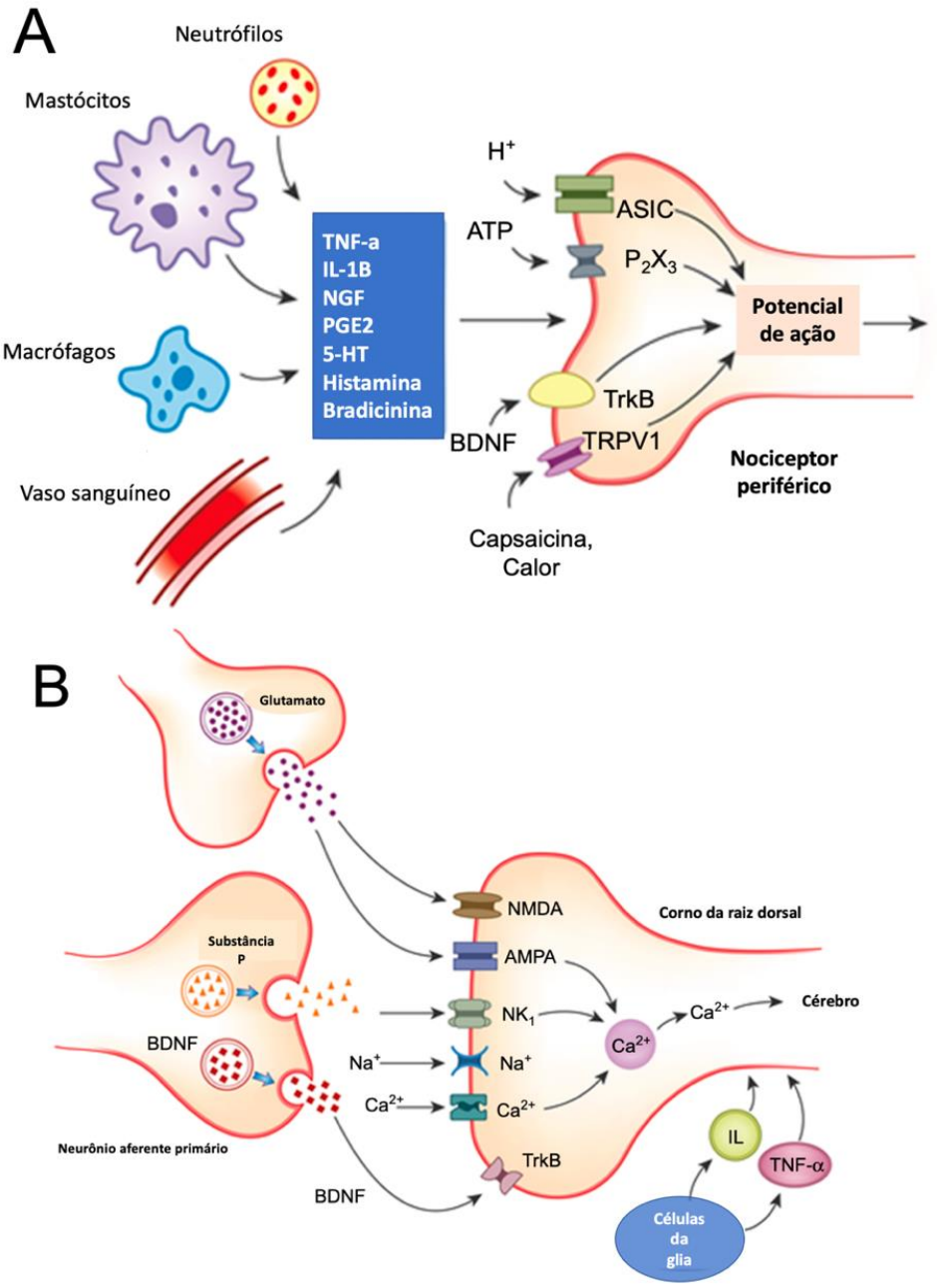
1.2 DOR

A nocicepção (do latim *nocere*, que significa “ferir”) é um tipo de percepção modulada pelo sistema somatossensorial, que coloca o indivíduo em relação com o ambiente, conferindo a ele capacidade de detectar estímulos potencialmente nocivos (Basbaum, Bautista, Scherrer, & Julius, 2009). O processamento das sensações somáticas é dependente de impulsos que surgem da estimulação dos receptores específicos, que detectam uma grande variedade de estímulos nocivos (St John Smith, 2018). Os nociceptores encontram-se distribuidos amplamente na pele, tecidos profundos (incluindo músculos e articulações) e na maioria dos órgãos viscerais **}**(Yam et al., 2018). Sequencialmente ao fenômeno sensitivo-doloroso, ocorre a transformação dos estímulos ambientais, físicos ou químicos, em potenciais de ação que migra através das fibras nervosas periféricas, passando pela medula até o sistema nervoso central (SNC), gerando a consciência de dor (Dubin & Patapoutian, 2010) (**Figura 1**).

Figura 1 – Mecanismo de sensibilização periférica e central.



(A) Ativação de nociceptores periféricos em resposta a estímulos, como calor, lesão ou distúrbios mecânicos leva ao recrutamento de células da imunidade inata, como neutrófilos, macrófagos e mastócitos bem como o aumento do influxo sanguínio no local. O recrutamento celular bem como vasodilatação inicia a liberação de mediadores químicos (citocinas pró-inflamatórias, eicosanoides, histamina, dentre outros) no local da lesão (sensibilização periférica). **(B)** A dor persistente ou inflamação causam ativação e disparo repetitivo em nociceptores aferentes de fibra C, que desencadeiam a liberação de glutamato no corno da raiz dorsal da medula espinal (sensibilização central). Isso é acompanhado pela liberação da substância P, BDNF e neurocininas, que causam despolarização persistente da membrana celular. Adicionalmente, levam a ativação de receptores AMPA ou NMDA por glutamato que estimula as células da glia e subsequentemente induz a liberação de COX 1 e 2, óxido nítrico e outros mediadores pró-inflamatórios (por exemplo: TNF-α, IL-1β, IL-6). Fonte: Adaptado de Dureja *et al.,* 2017.

Em suma, na dor ocorre a sensibilização dos nociceptores e essa

sensibilização causa hiperalgesia (uma resposta aumentada a um estímulo normalmente doloroso) ou alodinia (dor devido a um estímulo que normalmente não é doloroso), fenômenos que envolvem a percepção da dor (componente emocional e sensação nociceptiva) (Verri et al., 2006). Logo, o conceito de dor envolve 2 componentes, nocicepção e percepção. A percepção de dor é uma função integradora modulada por emoções e condições psicológicas relacionadas à historia do indivíduo (Verri et al., 2006).

A dor pode ser classificada em três tipos – dor nociceptiva, dor neuropática e dor inflamatória, com base em três características: sintomas, mecanismos e síndromes **}**(Yam et al., 2018).

A dor nociceptiva, também denominada de dor fisiológica, consiste em evento agudo, tem como função primordial proteger a integridade do organismo (Basbaum et al., 2009). Por isso, a dor nociceptiva possui um alto limiar. Quando ocorre mudança de uma dor alto limiar para uma dor de baixo limiar, a dor deixa de ser uma resposta fisiológica, passando a denominar-se dor patológica. A dor patológica, por sua vez, envolve desconforto e sensibilidade anormal na sintomatologia do paciente acometido, podendo ser classificada como dor inflamatória ou dor neuropática, depedendo da sua origem (Wieseler-Frank, Maier, & Watkins, 2004).

A dor inflamatória é resultante da sensibilização periférica dos nociceptores nos tecidos inflamados, sendo induzida por mediadores inflamatórios como TNF-α, IL-1β e prostaglandinas (Q. Xu & Yaksh, 2011). A dor inflamatória também envolve a sensibilização dos neurônios do corno da raiz dorsal e sensibilização central (Z. Z. Xu & Ji, 2011). Além disso, a dor inflamatória também é capaz de induzir hipersensibilidade mecânica e térmica (Fattori et al., 2019). A dor neuropática, em contrapartida, é causada por uma lesão ou doença do sistema nervoso somatossensorial **}**(Yam et al., 2018).

A dor inflamatória pode ser classificada em dois tipos: dor crônica e aguda **}**(Yam et al., 2018). A dor inflamatória aguda é normalmente intensa e ocorre por um curto período de tempo, que se inicia como resposta a estímulos nocivos normalmente mediados pelas fibras Aδ. No entanto, a inflamação prolongada, induz a dor inflamatória crônica, que dura além do período esperado de cicatrização, que é tipicamente mediado por fibras C **}**(Yam et al., 2018). A dor crônica é um problema de saúde pública mundial, apresentando uma prevalência elevada, além de estar

associada à baixa qualidade de vida e à condições como a depressão }(Smith et al., 2001).

Apesar do enorme progresso no campo da terapêutica da dor nos últimos anos, a dor continua sendo altamente prevalente (Yong, Mullins, & Bhattacharyya, 2022) . Embora diversos medicamentos sejam usadas para tratar a dor, 3 em particular – acetaminofeno (Paracetamol), anti-inflamatórios não esteroides (AINEs) e opióides – são mais frequentemente usados, juntamente com adjuvantes, como relaxantes musculares e anticonvulsivantes (Nalamachu, 2013). O uso prolongado de acetaminofeno, AINEs ou inibidores seletivos da COX-2, está associado a efeitos gastrointestinais graves, distúrbios hepáticos e insuficiência renal, bem como supressão imunológica }(Chiang & Serhan, 2017; Ripamonti, 2012).

Apesar de serem a base da terapia analgésica, capazes controlar a dor de intensidade moderada a intensa, os opioides são conhecidos pelos seus graves efeitos colaterais, como depressão respiratória, comprometimento motor e dependência (Sehgal, Manchikanti, & Smith, 2012).

O tratamento adequado da dor requer uma compreensão das características da dor, incluindo a gravidade e a natureza da dor e/ ou doença. Para isso é necessário um conhecimento das eficácias dos agentes disponíveis em diferentes modelos de dor e inflamação, bem como a identificação de seus efeitos adversos (Nalamachu, 2013).

1.2.1 Modelos de Dor e Inflamação

Atualmente, os modelos animais para o estudo da dor avaliam dois parâmetros: 1) hiperalgesia e 2) nocicepção evidente/dor manifesta }(Martinez et al., 2016; Pavao-de-Souza et al., 2012).

A hiperalgesia é o aumento da sensibilidade aos estímulos nociceptivos, causada pela sensibilização, direta ou indireta por mediadores inflamatórios, dos nociceptores e, para ser detectada, precisa da estimulação dos nociceptores por estímulos mecânicos ou térmicos, por exemplo }(Pavao-de-Souza et al., 2012; Verri et al., 2006).

Na dor manifesta, um estímulo nociceptivo induz comportamentos

evidentes, tais como sacudidas e lambidas da pata, sem que haja outros estímulos externos (mecânicos ou térmicos, por exemplo) }(Martinez et al., 2016; Pavao-de-Souza et al., 2012). Esse comportamento evidente ocorre porque os estímulos nociceptivos induzem a rápida produção de mediadores endógenos que ativam os neurônios nociceptivos primários. Para os modelos de estudo da dor manifesta são utilizados estímulos químicos, como ácido acético, fenil-p-benzoquinona (PBQ) e formalina; ou biológicos, como o zymosan; ou até uma uma mistura de agentes químicos e biológicos, como o adjuvante completo de Freund (CFA) (Martinez et al., 2016).

Os modelos de hiperalgesia e nocicepção evidente/comportamento manifesto de dor são amplamente utilizados para avaliar a atividade de novos candidatos de drogas analgésicas e seus mecanismos de ação (Martinez et al., 2016).

A carragenina é um mucopolissacarídeo extraído das paredes celulares das algas vermelhas, capaz de promover supressão severa da resposta imune tanto *in vivo* quanto *in vitro*, e de induzir inflamação e dor inflamatória em modelos animais }(Thomson & Fowler, 1981; Z. Z. Xu et al., 2010).

O edema de pata e a peritonite induzido pela carragenina são testes bem estabelecidos e amplamente usado para avaliar a inflamação em modelo animal, testar atividade anti-inflamatória de diversas drogas, bem como determinar os mecanismos envolvidos na inflamação }(Fehrenbacher, Vasko, & Duarte, 2012; Prajapati, Maheriya, Jani, & Solanki, 2014). Ambos constituem modelos animais agudos e simples para avaliação da dor no local da inflamação (Prajapati et al., 2014). A inflamação induzida pela carragenina é mediada por diversos mediadores como a histamina, prostaglandinas, citocinas pró-inflamatórias, como TNF-α, IL-1β e IL-33, e recrutamento e infiltração de neutrófilos locais, produzindo, entre outros mediadores, radicais livres derivados do oxigênio }(Prajapati et al., 2014; Zarpelon et al., 2013). A carragenina também pode induzir a liberação de NET (Neutrophil extracellular traps) e espécies reativas de oxigênio (EROs) no modelo de peritonite }(Barth et al., 2016).

O adjuvante completo de Freund (CFA) é constituído por bactérias inativadas, geralmente de *Mycobacterium tuberculosis* ou espécies *Mycobacterium butyricum*, e para a composição do adjuvante há adição de óleo mineral, garantindo que após sua administração ocorra a formação de granulomas ricos em macrófagos e células imunocompertentes no local }(Armentero, Levandis, Nappi, Bazzini, & Blandini,

2006).

O modelo animal inflamatório induzido pelo CFA tem sido muito utilizado em estudos de dor inflamatória devido sua capacidade de induzir uma resposta inflamatória prolongada (Fattori et al., 2019). O CFA é um adjuvante que, quando administrada via intraplantar em murinos, causa dor inflamatória local grave, além de hiperemia e edema }(Fehrenbacher et al., 2012).

1.3 VIRUS CHIKUNGUNYA

O virus chikungunya (CHIKV) é uma arborvirose que pertence a família Togaviridae e gênero Alphavirus, assim como o vírus Mayaro, vírus O'Nyong-nyong e vírus Ross River }(LaBeaud et al., 2015). O CHIKV foi primeiro identificado na Tanzânia, por volta de 1952-1953 (Robinson, 1955). O nome “chikungunya” deriva de uma palavra do dialeto Makonde, que significa “aquilo que se dobra”, referindo-se à posição curvada que os pacientes infectados desenvolvem, devido as dores articulares severas (Kondekar &, Gogtay., 2006). Sua transmissão aos humanos ocorre através da picada de mosquitos fêmeas das espécies Aedes aegypti e Aedes albopictus }*(Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007).*

Por muitos anos, o vírus Chikungunya causou surtos esporádicos na África e Ásia, até 2004 quando se espalhou para aproximadamente 60 países em todo o mundo (Schwartz & Albert, 2010). Em 2005-2006, foi reportado um surto de febre chikungunya em algumas ilhas do Oceano Índico }(Kariuki Njenga et al., 2008). O vírus foi reportando na Europa pela primeira vez, durante um surto na Itália em 2007 (Tilston, Skelly, & Weinstein, 2009). Em dezembro de 2013, foi anunciado a reemergencia do CHIKV nas Américas, quando o Centro Nacional de Referência para Arboviroses da França diagnosticou os primeiros casos locais de chikungunya em na ilha de Saint Martin }(Yactayo, Staples, Millot, Cibrelus, & Ramon-Pardo, 2016). A partir de então, o vírus se disseminou para aproximadamente 45 países na América do norte, central e do sul }(Yactayo et al., 2016). De acordo com a atualização epidemiológica da Organização Pan-Americana da Saúde (OPAS) de dezembro de 2021, foram notificados um total de 1.324.108 casos de arboviroses, incluindo dengue, chikungunya e Zika vírus (OPAS/OMS, 2021). Desses, 131.630 eram casos de chikungunya (OPAS/OMS, 2021).

1. 3. 1 Genoma e estrutura viral

O genoma do CHIKV é composto por um RNA de fita simples e cadeia positiva (Cho et al., 2008). Seu genoma é composto por duas regiões codificantes (ORFs – do inglês, *Open Reading Frames*), ORF 5' e ORF 3' separados por uma junção não codificante (Silva & Dermody, 2017). A ORF 5' codifica quatro proteínas não estruturais (nsP1, nsP2, nsP3, and nsP4) que constituem a RNA replicase; e a OFR 3' codifica seis estruturas proteicas: o capsídeo, as proteínas envelope 1 (E1), 2 (E2) e 3 (E3) , a 6K e a proteína Transfame (Silva & Dermody, 2017).

A estrutura viral é esférica, com aproximadamente 70 nm de diâmetro, formada por 240 cópias da proteína do capsídeo e circundada por um envelope composto por uma bicamada lipídica. (R. V. D. Cunha & Trinta, 2017). Dentro do envelope encontram-se as glicoproteínas E1 e E2, em forma de trímero (R. V. D. Cunha & Trinta, 2017). As proteinas E1 e E2 carregam os principais epítopos virais e participam da ligação e entrada do vírus nas células-alvo, onde E2 é responsável pela ligação ao receptor e E1 – pela fusão da membrana **}**(Tanabe et al., 2018; Weger-Lucarelli et al., 2015). Tanto E1 quanto E2, interagem com proteínas humanas desencadeando uma resposta imune e inflamatória robusta, contribuindo para a artralgia crônica (Dudha et al., 2015). A proteína E3 é reponsável pela translocação para o retículo endoplasmático, e parece ser importante para a montagem e liberação de partículas virais para o exterior da célula **}**(Tanabe et al., 2018). A proteína transframe é produzida como resultado da extensão C-terminal da proteína 6K no quadro -1 **}**(Snyder et al., 2013).

1.3.2 Patogênese da infecção pelo vírus Chikungunya.

O vírus chikungunya pode infectar células epiteliais, células endoteliais, fibroblastos e macrófagos derivados de monócitos (R. V. D. Cunha & Trinta, 2017). Ao encontrar a célula-alvo, a glicoproteína E2 do vírus CHIKV se liga ao receptor de membrana Mxra8 **}**(Basore et al., 2019; Song et al., 2019). Essa ligação ativa a via sinalização interna mediada pela clatrina, que promove a endocitose do vírus **}**(R. C. Lee et al., 2013). Após a formação do endossoma, ocorre a acidificação no pH do meio, levando as moléculas de clatrina a se dissociam das vesículas endossomais, promovendo mudanças conformacionais nas proteínas do envelope viral, que levam à fusão da membrana viral com a membrana endossomal (Fields & Kielian, 2013). Nesse momento, o nucleocapsídeo do vírus é liberado no citoplasma, e o RNA viral inicia a tradução das quatro proteínas do complexo de replicação viral (nsP1–nsP4)

(Constant et al., 2021). O RNA subgenômico é traduzido nas proteínas estruturais: capsídeo, pE2 (precursor de E2 e E3), E1 e 6K (Constant et al., 2021). No citoplasma, as proteínas do capsídeo se associam para formar o nucleocapsídeo, que incorpora o RNA genômico durante o processo de maturação. As proteínas precursoras do envelope são transportadas para o retículo endoplasmático e complexo de Golgi, onde sofrem modificações pós-transducionais para formar o heterodímero E1-E2 }(Yap et al., 2017). Essas proteínas são transportadas para a membrana da célula hospedeira, onde o vírus é então liberado por brotamento (Constant et al., 2021).

Outras moléculas como os glicosaminoglicanos, imunoglobulina de célula T e mucina, cluster de diferenciação 209 (CD209 ou DC-SIGN) e 147 (CD147), e o receptor de tirosina kinase AXL também são descritos como co-receptores de CHIKV, embora seus mecanismos não estejam elucidados (Constant et al., 2021).

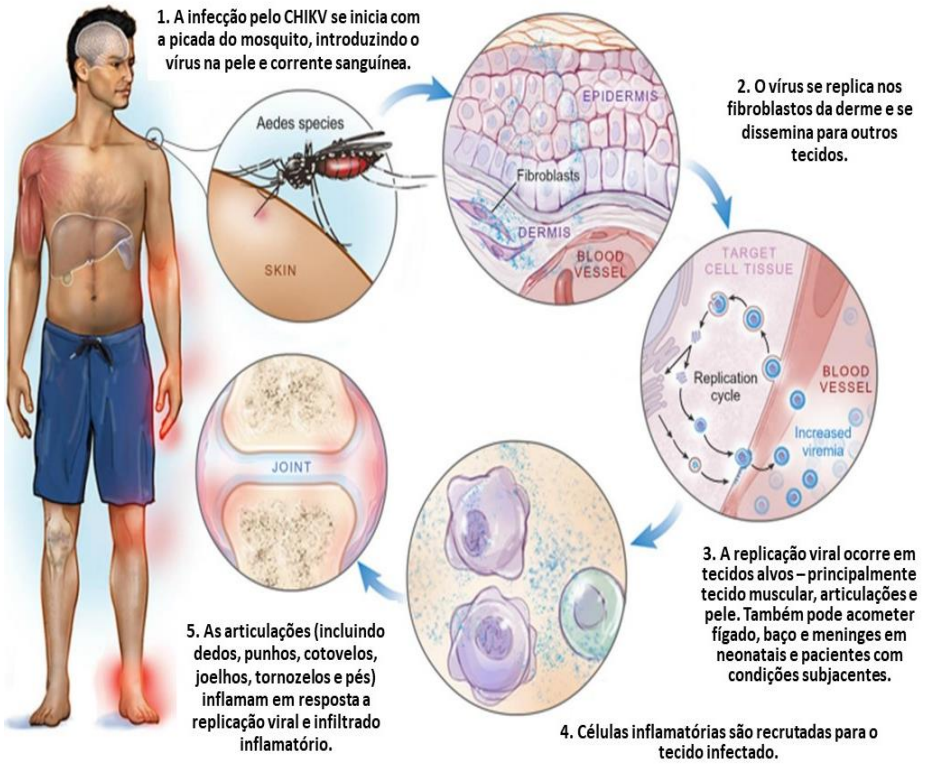
A infecção pelo vírus Chikungunya inicia uma resposta imune mediada por macrófagos (MØ), células natural killer (NK), neutrófilos, células dendríticas, basófilos e eosinófilos }(Tanabe et al., 2018). No início da fase aguda, há uma forte indução de interferons (IFN), como IFN-α, IFN-β, IFN-γ, ligante 10 de quimiocina com motivo C-X-C (CXCL10 ou IP10) e interleucina 1 β (IL-1β) (Chirathaworn, Chansaenroj, & Poovorawan, 2020). Outras citocinas, como a interleucina-6 (IL-6), fator estimulador de colônias de granulócitos (G-CSF), fator estimulador de colônias de macrófagos, (GM-CSF), proteína quimioatraente de monócitos 1 (MCP-1), fator de necrose tumoral alfa (TNF-α) também já foram descrita na infecção por CHIKV (Chirathaworn et al., 2020). Níveis altos de IL-1β, IL-17A, IL-27 e GM-CSF foram associados à gravidade da dor nas articulações (Chirathaworn et al., 2020). Além disso, baixos níveis de TNF-α, IL-2, IL-4 e IL-13 durante a fase inicial da infecção parecem ser marcadores preditivos de dor articular crônica }(Chang et al., 2018).

1.3. 3 Manifestações clínicas

A resposta imune mediada pela infecção do vírus CHIKV causa uma doença febril (por isso também é denominada de febre chikungunya) geralmente com dor de cabeça, náusea, vômitos, mialgia (dor nos músculos), exantema (erupções avermelhadas na pele), severa artralgia (dores articulares) (**Figura 2**) (R. V. D. Cunha & Trinta, 2017). A maior parte dos sintomas pode ser confundido com a dengue ou Zika. Após a fase inicial a doença pode evoluir em duas etapas subsequentes: fase subaguda e crônica. Alguns pacientes relatam a persistencia das dores articulares

após a fase aguda, caracterizando o início da fase subaguda, com duração de até três meses (Schwartz & Albert, 2010). Quando a duração desses sintomas persiste além dos três meses, atinge-se a fase crônica da doença. É importante ressaltar que os fatores genéticos e imunológicos determinam a cronicidade dos sintomas artríticos, embora estes ainda não tenham sido totalmente compreendidos.

Figura 2 – Etapas da infecção por CHIKV



Fonte: Adaptado de (Couderc & Lecuit, 2015).

Estudos mostram que até 82% dos pacientes infectados com CHIKV são sintomáticos }(Imad et al., 2021). Considerando a ausência de um tratamento eficaz contra a doença e que a maioria desses pacientes necessitarão de atendimento, a infecção por CHIKV pode gerar uma sobrecarga aos serviços de saúde. Além disso, o período debilitante causado pelas dores agudas, reafirma a importância de se intensificar a busca por novos candidatos a fármacos ou vacinas contra o vírus Chikungunya.

Um estudo conduzido por Segato-Vendrameto e colaboradores, utilizou o vírus CHIKV inativado e sua proteína recombinate E2 (rE2) em diversas doses, via intrarticular, para demonstrar seus mecanismos relacionados à dor. De fato, observou-se que o vírus inativado e a rE2 foram capazes de induzir hiperalgesia sob

estimulação mecânica e térmica. Em termos de mecanismo, o vírus inativado e a rE2 induziram hiperalgesia atráves da ativação de neurônios do gânglio da raiz dorsal e ativação de canais TRPV1 (SEGATTO-VENDRAMETO et al., 2019).

1. 4 MEDIADORES LIPÍDICOS PRÓ RESOLUÇÃO

Em um ambiente ideal, a resposta inflamatória aguda deve ser autolimitada, visando o retorno da homeostasia do tecido, processo denominado de resolução **}**(Chiang & Serhan, 2017; Serhan et al., 2015). O processo inflamatório agudo, quando não resolvido, pode progredir para o desenvolvimento da inflamação crônica, fibrose (reparo pela substituição do tecido conjuntivo) ou formação de abscessos (Ueha, Shand, & Matsushima, 2012).

No passado, sabia-se que a resolução da inflamação estava relacionada a fagocitose de neutrófilos por macrófagos teciduais, além da participação de outras moléculas, como por exemplo, componentes do complemento, citocinas, quimiocinas e certos eicosanóides, cuja função era promover o recrutamento de leucócitos do sangue para o tecido inflamado (Serhan & Petasis, 2011). Acreditava-se que a remoção do estímulo inflamatório impedia a produção de quimioatractivos, e isso impedia o recrutamento de novos leucócitos, e esses mecanismos passivos culminavam no fim dos eventos da inflamação **}**(Kourtzelis et al., 2017).

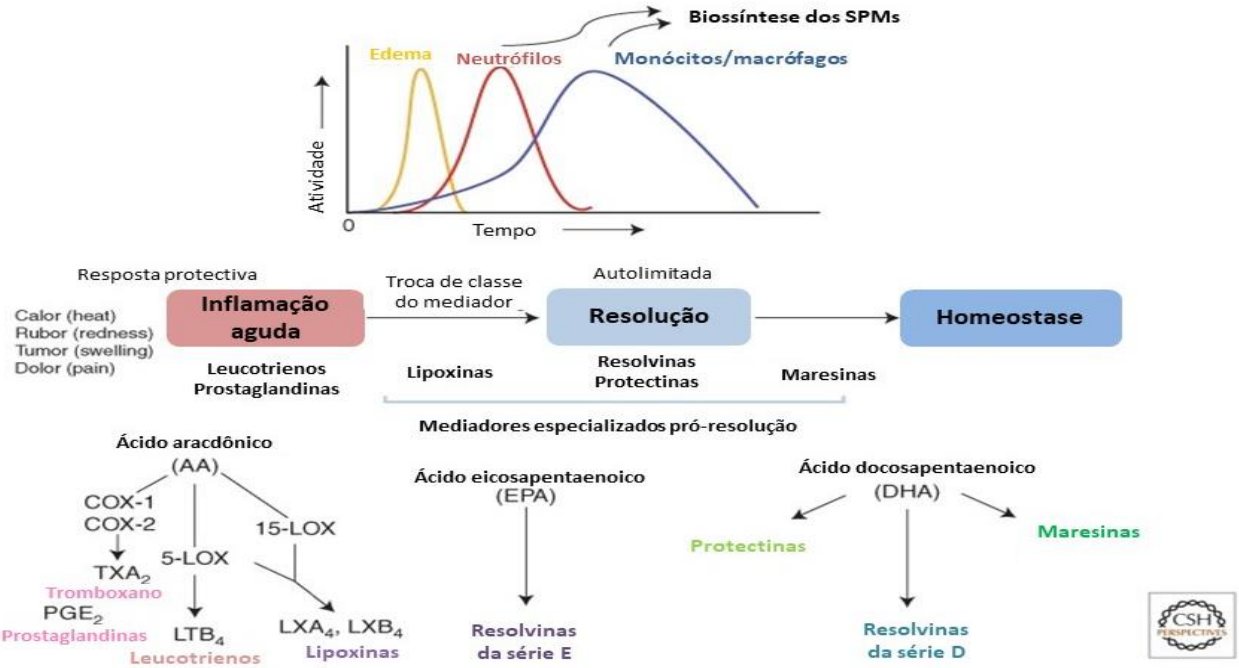
Hoje, sabe-se que as populações de macrófagos podem ter fenótipos diferentes dependendo dos estímulos. Os macrófagos presentes em diferentes tecidos são polarizados de acordo com a mudanças em seu ambiente, formando diferentes subtipos de macrófagos, como macrófagos M1 e macrófagos M2. Os macrófagos M1, também chamados de macrófagos classicamente ativados ou inflamatórios, são polarizados por moléculas como o LPS, IFN-γ e fator estimulador de colônias de granulócitos e macrófagos (GM-CSF) (Yunna et al., 2020). Os macrófagos M1 são caracterizados pela produção de altos níveis de citocinas pró-inflamatórias (IL-1β, TNF-α, IL-12 , IL-18 e IL-23), alta produção de espécies reativas de nitrogênio e oxigênio intermediários e promoção de respostas Th1 (Davis et al., 2013). Já os macrófagos M2, alternativamente ativado ou cicatrizante, atuam promovendo a remodelação de tecidos, regulação imune e atividade fagocítica eficaz. São polarizados por complexos imunológicos, componentes de complemento, células apoptóticas, fator estimulador de colônias de macrófagos (MCSF), IL-4, IL-13, IL-10 e TGF-β e em respostas a helmintos e fungos (Nair, Cochrane and Allen et al., 2003). Os macrófagos, portanto, são importantes células efetoras que orquestram a gravidade,

longevidade e eventual resultado de doenças causadas por inflamação (Motwani and Gilroy., 2015). Um desequilibrio entre as proporções de macrófagos M1/M2 pode desempenhar um papel patogênico (Funes et al., 2018).

Posteriomente surgiram evidências de que, em exsudatos inflamatórios, ocorriam interações célula-célula, que levavam à biossíntese de sinais ativos que limitavam o recrutamento de neutrófilos para o tecido, promoviam a fagocitose de neutrófilos apoptóticos por macrófagos e por consequência promoviam um retorno do tecido à homeostase **}**(Serhan et al., 2015). Essas descobertas demonstraram, portanto, que a resolução da inflamação é um processo regulado por mecanismos ativos **}**(Serhan et al., 2015).

Na resolução ativa da inflamação, foi descoberto um processo chave: a troca de classe de mediador lipídico. Enquanto no início da inflamação aguda ocorre a síntese dos iniciadores clássicos, como as prostaglandinas e os leucotrienos, na resolução a síntese desses mediadores é alterada, passando a produzir mediadores especializados em resolução pró ativa (SPMs) **}**(Serhan et al., 2015) (**Figura 3**).

Figura 3 – Biossíntese de mediadores lipídicos no recrutamento de células na resolução de inflamação aguda.



Fonte: Adaptado de Serhan et al., 2015.

Foram identificados, nos últimos anos, três novas familias de potentes mediadores endógenos pró-resolução, denominados de resolvinas,

protectinas, e maresinas }(Serhan et al., 2002; Serhan et al., 2009). Cada família é estruturalmente distinta e biossintetizada separadamente por substratos do precursor ácido graxo ômega-3. A resolução da inflamação também conta com as lipoxinas, derivadas do ácido araquidônico (derivado do ácido graxo ômega-6) }(Chiang & Serhan, 2017) (**Figura 3**).

Anteriormente, sabia-se que o ácido graxo ômega-3 era substrato para a biossíntese de anti-inflamatórios potentes e mediadores pro-resolução }(Hong, Gronert, Devchand, Moussignac, & Serhan, 2003; Serhan et al., 2002). Agora, sabe-se que os ácidos eicosapentaenóico (EPA) e docosahexaenóico (DHA), ambos derivados do ômega-3, são precursores dos mediadores que ativam mecanismos pró-resolução, os SPMs. O EPA dá origem as resolvinas da série E, enquanto o DHA da origem as resolvinas da série D, protectinas e maresinas (**Figura 3**).

Os SPMs já identificados são as resolvinas da série E (RvE): RvE1, RvE2 e RvD3; resolvinas da série D (RvD): RvD1, RvD2, RvD3, RvD4, RvD5 e RvD6 (Serhan & Petasis, 2011); protectinas (PD): PD1 e PDX; maresinas: MaR1 e MaR2 }(Serhan et al., 2012).

1.4. 1 Ações dos SPMs

Os SPMs são capazes de ativar receptores acoplados à proteína G (GPCR), que amplificam e transduzem sua resposta tecidual }(Serhan et al., 2015). Essas moléculas possuem ações anti-inflamatórias e pró-resolução e podem agir por mecanismos distintos }(Chiang & Serhan, 2017). No processo de resolução, os SPMs atuam em diversos aspectos como: a limitação da infiltração leucocitária, fazendo a contra-regulação de mediadores pró-inflamatórios, reduzindo dor, atuando na captação de neutrófilos apoptóticos e restos celulares, e também promovendo a regeneração dos tecidos }(Serhan et al., 2015) }(Chiang & Serhan, 2017).

Embora os SPMs sejam produzidos localmente e atuam como autacóides para respostas inflamatórias agudas, estudos recentes indicam que essas moléculas também podem alcançar níveis circulantes e promover efeitos prolongados }(Mas, Croft, Zahra, Barden, & Mori, 2012; Oh, Pillai, Recchiuti, Yang, & Serhan, 2011). Diante disso, os SPMs tem sido alvos para o estudo de muitas condições relacionadas a processos inflamatórios. Diversos estudos já demonstraram que os SPMs possuem potentes ações pró-resolução e anti-inflamatórias em diversos modelos de doenças animais }(Serhan et al., 2015) e relacionas à dor }(Fattori et al., 2019; Serhan et al.,

2012; Z. Z. Xu & Ji, 2011).

1.4.2 SPMs em Contexto de Doença

Os mediarores lipídicos especializados pró resolução são biossintetizados dentro de exudatos resolutivos a fim de regular as repostas inflamatórias. Os SPMs podem ser biosintetisados por leucócitos humanos encontrados nesses exsudatos inflamatórios em escalas de picogramas a nanogramas }(Serhan et al., 2015). Já foram identificados altos níveis de SPMs associados a condições patológicas no leite materno (Weiss et al., 2013), fluido sinovial (Giera et al., 2012), plasma humano }(Colas, Shinohara, Dalli, Chiang, & Serhan, 2014; Mas et al., 2012; Psychogios et al., 2011) e tecido adiposo (Claria, Dalli, Yacoubian, Gao, & Serhan, 2012).

Estudos têm encontrado SPMs, em baixas doses, relacionados condições patológicas associadas à inflamação, como por exemplo, na doença de Alzheimer }(X. Wang et al., 2015), na colite }(Vong et al., 2012); na Diabetes tipo 2 }(Gutierrez et al., 2012) e obesidade (Claria et al., 2012); na asma }(Miyata et al., 2013); em doenças cardiovasculares }(Ho et al., 2010); na esclerose múltipla (Pruss et al., 2013), bem como nas doenças inflamatórias clássicas, como na artrite reumatoide (Giera et al., 2012) e na periodontite }(Fredman et al., 2011).

1.4.3 Potencial Terapêutico dos SPMs na Dor

Recentemente, têm-se evidenciado o papel dos SPMs na antinocicepção. Xu e colaboradores (2010) demonstraram que a RvE1 e RvD1 atuam diminuindo a dor inflamatória induzida por formalina, carragenina e CFA sem afetar a percepção da dor basal, tanto via mecanismos centrais (administração intratecal) quanto periféricos (administração intraplantar).

Outro estudo demonstrou que a administração intraplantar de RvD1 reduziu a dor inflamatória em camundongos e inibiu a atividade de vários canais iônicos receptores de potencial transitório (TRP), como TRPA1, TRPV3 e TRPV4, mas não TRPV1 }(Bang et al., 2010). Huang, Wang, Serhan, and Strichartz (2011) evidenciaram que injeções intratecais de RvD1 em ratos reduzem a dor cirúrgica no pós-operatório.

Lima-Garcia et al. (2011)} demonstram que o tratamento repetido por

6 dias com a resolvina D1 desencadeada pela aspirina (ATRvD1), e seu precursor, o 17 (R)-ácido hidroxi-docosahexaenóico, desencadeou potentes efeitos anti-hiperalgésicos, reduzindo a dor inflamatória na artrite induzida por adjuvante em ratos. Com isso, os autores ainda compararam a eficácia antinociceptiva do 17(R) HDoHE com a de analgésicos utilizados comercialmente como a indometacina (inibidor de COX), morfina (agonista do receptor opióide), gabapentina (antiepiléptica/anticonvulsivante) e dexametasona (anti-inflamatório esteroidal).

}Hu et al., 2012, demonstraram que o tratamento intratecal com LXA4 e LXB4 conseguiu reduziu a alodinia mecânica na dor óssea induzida por câncer através da dimunição da expressão das citocinas pró-inflamatórias IL-1β e TNF-α. Nesse estudo, eles também observaram que receptor da lipoxina estava colocalizado principalmente com os astrócitos na medula, demonstrando uma modulação neuroimunedo desse mediador lipídico. Curiosamente, um estudo descobriu que o tratamento intratecal com RvD5 reduziu a dor da segunda fase induzida pela formalina (lamber e vacilar) em camundongos machos, mas não em camundongos fêmeas (Luo, et al. 2019).

1.4.4 Maresina-2

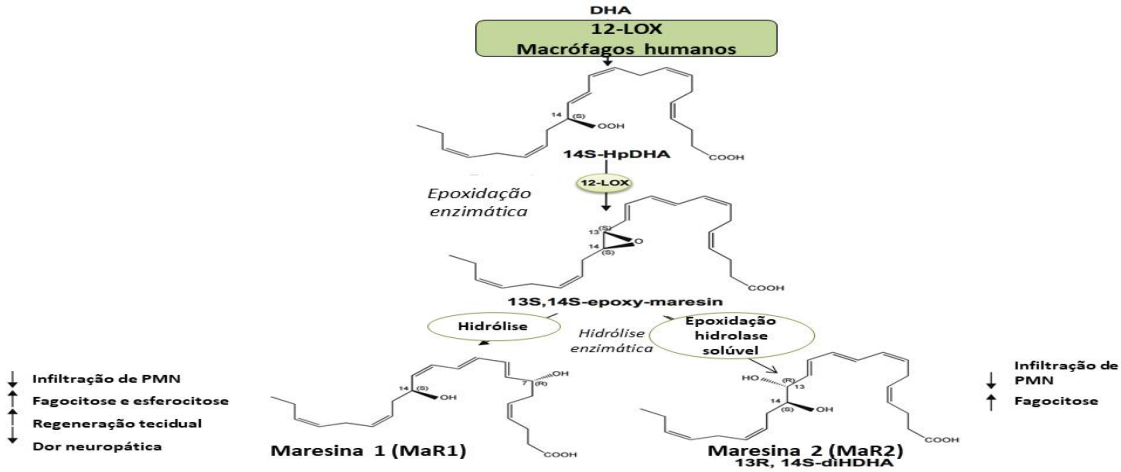
As maresinas são uma família de mediadores lipídicos, biossintetizados por macrófagos humanos, a partir do ácido docosahexaenóico (DHA) via 12-lipoxigenase humana (12-LOX), que atuam na homeostase tecidual, na resolução da inflamação, na cicatrização e na defesa do hospedeiro }(Deng et al., 2014; Serhan et al., 2015; Serhan et al., 2009).

A MaR1 têm se mostrado promissora na terapêutica da dor. Estudos têm apontato que além do seu potencial anti-inflamatório e pró-resolução, a MaR1 também exibe potentes ações regenerativas e antinociceptivas }(Fattori et al., 2019; Serhan et al., 2012). A MaR1 mostrou-se capaz de reduzir parâmetros de dor inflamatória induzida por carragenina e CFA, além de reduzir a ativação do NF-κB, a produção de IL-1β e TNF-α e ativação das células gliais da medula espinhal (Fattori et al., 2019). A MaR1 demonstrou potentes ações na regulação da resolução da inflamação, bem como na regeneração tecidual e reduzindo dor neuropática induzida por quimioterapia, atuando de forma dose-dependente em canais ionicos receptores de potencial transitório do tipo vaniloide 1 (TRPV1), em camundongos }(Serhan et al., 2012). A MaR1 também demonstrou uma ação protetora sobre a pele, frente a inflamação e estresse oxidativo causada pela irradiação UVB }(Cezar et al., 2019). A

maresina-1 demonstrou-se capaz de melhor dor neuropática induzida por ligadura do nervo subespinal (SNL), através da regulação de atividades gliais **}**(Gao et al., 2018).

A maresina-2 ou ácido 13R,14S-di-hidroxi-docosahexaenóico, foi primeiramente descrita por Deng e colaboradores em 2014. A biossíntese da maresina 2 envolve o processo intermediário da biossíntese da maresina-1, sendo produzida a partir do DHA por 12-LOX, seguido por conversão via epóxido hidrolase solúvel (sEH) **}**(Deng et al., 2014) (**Figura 4**).

Figura 4 - Biossíntese da maresina 1 e maresina 2.



Fonte: Adaptado de DENG et al., 2014.

Nesse mesmo estudo, a maresina-2 já demonstrou potentes ações anti-inflamatórias e pró-resolução. A dose de 1 ng de MaR2 foi capaz de reduzir em 40% a infiltração de neutrofílos no modelo de peritonite induzida por zymosan em camundongos **}**(Deng et al., 2014). Estudos mais recentes, demonstraram que é capaz de reduzir MaR2 o recrutamento de células imunes próximas às fibras CGRP+ na pele da pata, além de inibir a dor inflamatória induzida por LPS, capsaicina ou AITC, e inibir a ativação de TRPV1 e TRPA1 em neurônios DRG **}**(Fattori et al., 2022). A MaR2 também reduziu a expressão das proteínas ASC, MPO, Ly-6G, ICAM-1, NLRP3 e Caspase-1 no tecido pulmonar em um modelo animal de asma (Yu et al., 2022). Em conjunto, esses achados demonstraram que o MaR2 apresenta potentes propriedades anti-inflamatórias e analgésicas.

2 OBJETIVOS

Investigar o efeito analgésico do mediador lipídico pró-resolução, maresina-2 (MaR2), em modelos de dor e inflamação induzidos pela carragenina e adjuvante completo de Freund (CFA), e na dor articular induzida pelo vírus Chikungunya inativado e sua proteína recombinante E2, em camundongos.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito da MaR2 na hiperalgesia mecânica no modelo agudo com carragenina; e hiperalgesia mecânica e térmica no modelo crônico com CFA;
- Avaliar o efeito da MaR2 na diminuição do infiltrado celular na pata dos animais em resposta aos estímulos com carragenina;
- Avaliar o efeito da MaR2 sobre o recrutamento celular em modelo de peritonite com a carragenina;
- Avaliar o efeito da MaR2 no estresse oxidativo induzido por espécies reativas de oxigênio e nitrogênio por fluorescência de 4,5-diaminofluoresceína diacetato (DAF-2DA) e 2,7-dihidrodiclorofluoresceína (DCF), em células peritoneais estimuladas com carragenina;
- Avaliar o efeito da MaR2 na dor articular induzida pelo vírus Chikungunya inativado e sua proteína recombinante E2.
- Avaliar o efeito da MaR2 na ativação de neurônios do gânglio da raiz dorsal estímulado com vírus Chikungunya inativado e sua proteína recombinante E2.

3 ARTIGO PARA PUBLICAÇÃO I (INFLAMMOPHARMACOLOGY)

O presente trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista *Inflammopharmacology.* Os resultados parciais estão descritos no artigo intitulado “Maresin 2 reduces pain-like behavior, leukocyte recruitment and oxidative stress by enhancing Nrf2”.

RESEARCH PAPER

Maresin 2 reduces pain-like behavior, leukocyte recruitment and oxidative stress by enhancing Nrf2

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Abstract

The inflammatory response is a host defense mechanism. However, uncontrolled inflammation can lead to tissue damage and chronic pain. Maresin 2 (MaR2) is a recently found specialized pro-resolving lipid mediator (SPM) with anti-inflammatory and analgesic effect recent discovered. In this study, we demonstrate the effect of MaR2 treatment against inflammatory hyperalgesia and leukocyte recruitment in mice triggered by carrageenan. The animals were treated with MaR2 by intrathecal route 1 hour before the plantar stimulus with carrageenan and complete Freund's adjuvant (CFA). Animals were submitted to behavioral tests to determine mechanical hyperalgesia by electronic von Frey's method and thermal hyperalgesia by a hot plate apparatus. Paw skin were collected to determinate leukocyte recruitment by hematoxylin and eosin (H&E) stain. In a carrageenan-peritonitis model, animals received MaR2 intraperitoneally 1 hour before stimulus, and leukocyte recruitment was determinate by total and differential counts in Rosenfeld's stained slices. Peritoneum cells were collected to determinate oxidative stress by nitroblue tetrazolium, 4,5-diaminofluorescein diacetate (DAF-2DA) and 2,7-dihydrodichlorofluorescein (DCF) fluorescence, and immunofluorescence staining of nuclear factor erythroid 2-related factor 2 (Nrf2). MaR2 was able to reduce carrageenan- and CFA-induced hyperalgesia in a dose- and time-dependent manner. MaR2 also reduced leukocytes recruitment in the paw skin and in the peritoneum (total leukocytes, neutrophils and mononuclear cells). MaR2 prevented the production of reactive oxygen and nitrogen species induced by carrageenan peritonitis by activation of Nfr2 antioxidant transcriptional factor. Thus, this work highlights the efficacy of MaR2 as an anti-inflammatory and analgesic treatment in inflammatory pain and peritonitis by inducing the transcription factor Nrf2.

Key words: Maresin-2. SPMs. CFA. carrageenan. Nrf2. peritonitis.

1 Introduction

Inflammation is the immune response to pathogens, tissue damage and toxins. The inflammatory signaling pathways depend on the nature of the stimulus and cellular targets (receptors, enzymes). In general, acute inflammation will involve the activation of tissue resident immune cells such as macrophages that will produce pro-inflammatory cytokines and chemokines and oxidative stress. These inflammatory mediators will activate endothelial cells to express adhesion molecules and orchestrate the recruitment of neutrophils followed by mononuclear cells, thus, amplifying the inflammatory response (L. Chen et al., 2018). The recruitment of leukocytes is an important event since these cells will phagocyte the inflammatory agent and kill it, which in the case of infections is a highly crucial activity. However, excessive acute inflammation may cause extensive tissue lesion. Uncontrolled acute inflammation may also become chronic **}**(Furman et al., 2019). Indeed, chronic inflammatory response is a common characteristic of many diseases, including arthritis, obesity, colitis, atherosclerosis and Alzheimer's **}**(R. X. Wang, Zhou, Ma, Qiao, & Li, 2021).

Pain is a classical cardinal sign of the inflammatory process and can become pathological when not properly treated (Verri et al., 2006). Pain is one of the most frequent complaints that brings patients to the clinic. Patients that surfer with chronic pain have low quality of life **}**(Lame, Peters, Vlaeyen, Kleef, & Patijn, 2005). Inflammatory pain results from peripheral sensitization of nociceptors in inflamed tissues, which is induced by inflammatory mediators such as TNF-α, IL-1β and prostaglandins (Q. Xu & Yaksh, 2011). Chronic peripheral inflammatory pain involves the sensitization of the primary afferent neurons who's cellular bodies are in the dorsal root horn and also central sensitization that involved a neuroinflammatory produces with the activation of glial cells (Z. Z. Xu & Ji, 2011) (Wieseler-Frank et al., 2004).

The resolution of the acute inflammatory process was demonstrated to be an active process regulated by omega-3 fatty acid-derived molecules, the specialized pro-resolving lipid mediators (SPMs) **}**(Fattori, Zaninelli, Rasquel-Oliveira, Casagrande, & Verri, 2020). Maresins are a family of SPMs biosynthesized from human docosahexaenoic acid (DHA) via 12-lipoxygenase (12-LOX) by macrophages. Thus, maresins are endogenous molecules produced during the resolution phase of inflammation **}**(Serhan et al., 2009). The levels of maresins and other SPMs is negatively correlates with disease activity in chronic conditions (Zaninelli, Fattori, & Verri, 2021). Maresin-2 (MaR2) or 14S-dihydroxy-docosahexaenoic (13R, 14S-diHDHA) was identified in inflammatory exudate and produced by resolution macrophages **}**(Deng et al., 2014). At 1 ng, MaR2 reduced neutrophil infiltration in zymosan mouse peritonitis by approximately 40% and at 10 pM enhanced human macrophage phagocytosis of zymosan particles by approximately 90% (Deng et al., 2014). MaR2 demonstrated analgesic mechanisms by targeting nociceptor TRPV1 and TRPA1 activation, and CGRP release in mice (Fattori et al., 2022). Thereby, despite the identification of MaR2 and initial triage of its activity, much remains to be determined about its biological activities and whether it has a potential therapeutic application.

Herein, we aimed to evaluate the potential anti-inflammatory and analgesic effect of the SPM MaR2 in models of inflammatory pain and peritonits with carrageenan and a chronic inflammatory model with complete Freund's adjuvant (CFA).

2 Materials and methods

2.1 Animals

All experiments were performed in accordance with the International Association for Study of Pain guidelines and with the approval of the Londrina State University Ethics Committee on Animal Research and Welfare (process number 11145.2016.54). In this study, we used healthy male Swiss mice from Londrina State University, Paraná, Brazil. Mice were randomly assigned and housed in standard clear plastic cages, kept in light/dark cycle of 12:12 h with ad libitum food and water. Behavioral testing was performed in a room maintained at a temperature of 21°C ± 1°C. The investigators were blinded to the treatments. All efforts were made to minimize the number of animals used and their suffering. Animals were euthanized with isoflurane anesthesia (5% in oxygen using a precision vaporizer) followed by decapitation as a confirmation method.

2.2 Experimental procedures

For inflammatory hyperalgesia, mice were treated once with 1, 3, or 10 ng of MaR2 (Cayman Chemical, Ann Arbor, MI, EUA) or vehicle (10% ethanol) via intrathecal route (10 µl, between L4 and L6 spinal segments) and under isoflurane anesthesia (3% in oxygen using a precision vaporizer), 1h before intraplantar injection of carrageenan (Santa Cruz Biotechnology, Dallas, TX, USA) (300 µg/ 20 µl/ paw). For inflammatory hyperalgesia induced by CFA (10 µl/ paw), animals were only pre-treated with 10ng of MaR2 also by intrathecal route. Mechanical and thermal hyperalgesia were evaluated at this time-points: 1, 3, 5 after carrageenan injection and 1, 3, 5, 7h and daily until day 6 after CFA stimulus. Paw skin from animals stimulated with carrageenan were collect and processed for hematoxylin and eosin staining to determinate leukocyte recruitment. Separately, mice were treated with 0,01; 0,1 and 1 ng of MaR2 via intraperitoneal (i.p.) 1 h before i.p. injection of carrageenan (1mg/ 200 µL/ cavity). We did not observe additional effect with higher doses (data not shown). Peritoneal washes were collected 5h after carrageenan administration to determinate leukocyte recruitment (total and differential leukocyte counting), oxidative stress (determination of total ROS and RNS) by DCFDA and DAF2-DA assay, superoxide anion production (NBT count of NBT-positive cells) and transcription factor immunostaining (Nrf2 immunofluorescence). Time points and drug doses chosen were previously standardized by other studies of our laboratory **}**(Fattori et al., 2019; Fattori et al., 2022; Mizokami et al., 2016; Zucoloto et al., 2017).

2.3 Mechanical hyperalgesia

Mechanical hyperalgesia was evaluated by the electronic version of von Frey's test. In a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15-30 min before the beginning of the test. This test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic anesthesiometer, Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5-mm2 polypropylene tip. The pressure required to evoke the paw withdrawal movement by the animal is automatically registered in grams (g). The animals were tested before (baseline) and in time points described in 2.2 topic after the carrageenan and CFA stimulation. The results are expressed as delta (Δ) withdrawal threshold (in g), which was calculated by subtracting the mean measurements at each time points previously, from the baseline values mean measurements. The investigators were blinded to the treatment.

2.4 Thermal hyperalgesia

Heat thermal hyperalgesia was performed using a hot plate at 52°C ± 1°C (Insight, Ribeirão Preto, SP, Brazil), as previously described }(Pinho-Ribeiro et al., 2016). The reaction time was registered when one of the following responses (endpoints) were observed: clear paw flinching, paw licking, or jumping. Measures were taken before and after stimulus at the same intervals described for the mechanical hyperalgesia experiment. The results were expressed as mean of the absolute values obtained in the analysis. The maximum time to remain on the hot plate is 20 seconds, to avoid tissue damage. The investigators were blinded to the treatment.

2.5 Hematoxylin and Eosin (H&E) staining

After mechanical and thermal hyperalgesia experiment, hind paw was dissected (5h after carrageenan stimulus) and fixed with 10% paraformaldehyde in PBS. Tissue were embedded in paraffin and posteriorly processing with hematoxylin and eosin (H&E) staining. Images were acquired in a conventional light microscope (40x objective). Analyses were performed on ImageJ 1.44 software for Windows using the threshold tool and performed on RGB images as previously described }(Lourenco-Gonzalez et al., 2019). Leukocyte recruitment was determinate by % of area selected. Images were analyzed and scored by an experimenter blinded to the treatment.

2.6 Cell Counts

Leukocyte recruitment into the peritoneal cavity was assessed 5 h after administration of carrageenan (1 mg/ 200µL/ cavity, ip.). Peritoneal cavities were washed with 1 mL of a solution containing phosphate-buffered saline (PBS), 1 mM ethylenediamine tetraacetic acid (EDTA) and BSA 0.5%. Total leukocyte counts were performed after dilution of peritoneal exudate in Turk solution (2% acetic acid) using a Neubauer chamber. Differential cell counts were performed using the Fast Panoptic Kit for histological analysis (Laborclin, Pinhais, PR, Brazil) to distinguish polymorphonuclear (PMNs) from mononuclear cells under a light microscope (Olympicus Optical Co., Hamburg, Germany). The results are expressed as number of cells × 10^6 per cavity.

2.7 NBT Assay

For assessment of NBT-positive cells, the peritoneal exudate was harvested 5 h after carrageenan. A 15 µL amount of peritoneal wash was incubated with 15 µL of nitroblue tetrazolium (NBT) (1 mg/ mL) for 30 min. Cells were then stained for counts using the Fast Panotic Kit for histological analysis (Laborclin, Pinhais, PR, Brazil), and the values are expressed as percent of the number of NBT-positive cells (×10^6) per cavity.

2.8 Total ROS and RNS detection

The H2DCF-DA and DAF-2 DA fluorescent assay was used to detect and quantify intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively. Peritoneal exudates were harvested 5 h after carrageenan (1mg/cavity). Briefly, 500 µL of cell suspension were seeded on to Nunc™ Glass Bottom Dishes and incubated with 500 Dulbecco's Modified Eagle Medium (DMEM) overnight, under controlled temperature (37 °C). Peritoneum cells were loaded with 10 µM of H2DCF-DA or DAF2-DA, incubated for 40 min and washed with Hank’s Balanced Salt Solution

(HBSS). Image processing was performed in a Confocal Microscope (TCS SP8, Leica Microsystems). Total intracellular ROS and RNS detection was analyzed from the mean fluorescence (LAS X Software, Leica Microsystems).

2.9 Immunofluorescence staining

Cells from the peritoneum were collected 5 hours after carrageenan stimulus and fixed in a solution of paraformaldehyde 4%. Cells were then centrifuged (10 min, 4 °C, 1.2 rpm) and blocked with (PBS, 5% BSA, 0.5% Triton) for 1 h on ice. After centrifugation, cells were incubated with rabbit anti-mouse Nrf2 (1:200, Santa Cruz Biotechnology, sc-722) overnight. After two washes in PBS, cells were incubated with the secondary antibody for Nrf2 Alexa Fluor 488 goat anti-rabbit (1:500, Invitrogen, A11008) for 2 hours on room temperature. The images and analyzes were performed using a Confocal Microscope (TCS SP8, Leica Microsystems, Mannheim, Germany).

2.10 Data and statistical analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad Prism software version 6.0). Results are presented as means ± SEM of measurements made with 6 mice per group. Each experiment was conducted twice. Two-way ANOVA, followed by the Tukey post-test, were used to analyze data from multiple-moment experiments (mechanical and thermal hyperalgesia). One-way ANOVA were used followed by the Tukey post-test for experiments with specific times. Significant statistical differences were considered when $P < 0.05$.

Results

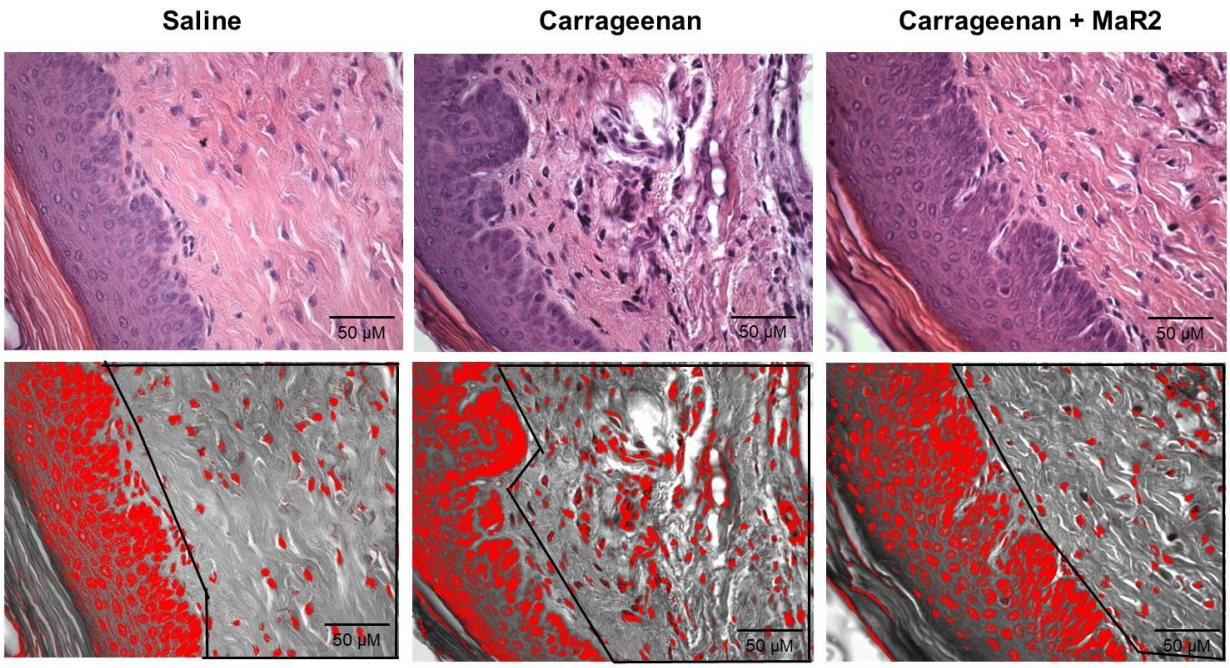
MaR2 reduces carrageenan-induced mechanical and thermal hyperalgesia.

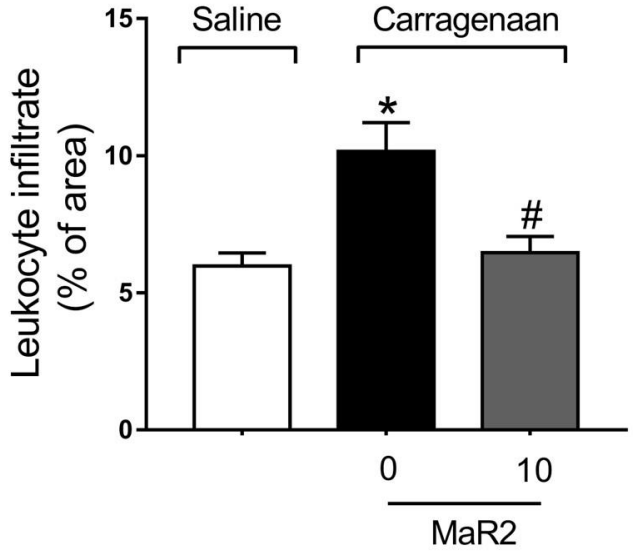
In this study, we performed a dose-response curve (1, 3, or 10 ng, intrathecal) with MaR2 1 hour after stimulus with carrageenan injection (300µg/20µL/paw). Mechanical and thermal hyperalgesia were observed 1, 3, and 5 h after stimulus. Carrageenan induced mechanical and thermal hyperalgesia, and treatment with MaR2 at 3 and 10 ng reduced both parameters (Fig. 1A and B) in all time points. The dose of 10ng was the most effective dose in mechanical hyperalgesia, being statistically different from dose of 1 ng in the first time points. For that, the dose of 10 ng was chosen for the following experiments.

Figure 1. MaR2 reduces carrageenan-induced inflammatory pain. Intensity of mechanical (A) and thermal (B) hyperalgesia was evaluated 1, 3, and 5 h after stimulus using an electronic von Frey and hot plate apparatus, respectively. Results are presented as mean ± SEM; n = 6 mice per group per experiment (*P < 0.05 vs saline group; #P< 0.05 vs vehicle group, &P< 0.05 vs Carrageenan + MaR2 1ng group. One-way ANOVA followed by Tukey's posttest).

MaR2 decreases carrageenan-induced leukocyte recruitment in the paw skin.

Next, to access the effect of MaR2 in leukocyte recruitment in the paw, mice were treated with 10 ng of MaR2 (i.t.) 1 hour before carrageenan plantar stimulus and hematoxylin and eosin staining were performed. Paw was collected 5 hours after carrageenan stimulus, and we could observe that treatment with MaR2 reduced leukocyte infiltrate in stimulated paw.





792

793 **Figure 2.** MaR2 decreases leukocyte recruitment to the paw skin. Five hours after
794 intraplantar injection of carrageenan (300 µg/paw), the hind paw skin was dissected for
795 histopathological analysis by H&E staining using a light microscope (original
796 magnification 40x). The representative total score of leukocyte recruitment is
797 determinate by % of area selected according to the representation using ImageJ
798 software. Results are presented as mean ± SEM; n = 6 mice per group per experiment
799 (*P < 0.05 vs saline group; #P < 0.05 vs vehicle group, one-way ANOVA followed by
800 Tukey's posttest).

801

MaR2 reduces complete Freund's adjuvant (CFA)-induced mechanical and thermal hyperalgesia with a long-lasting effect.

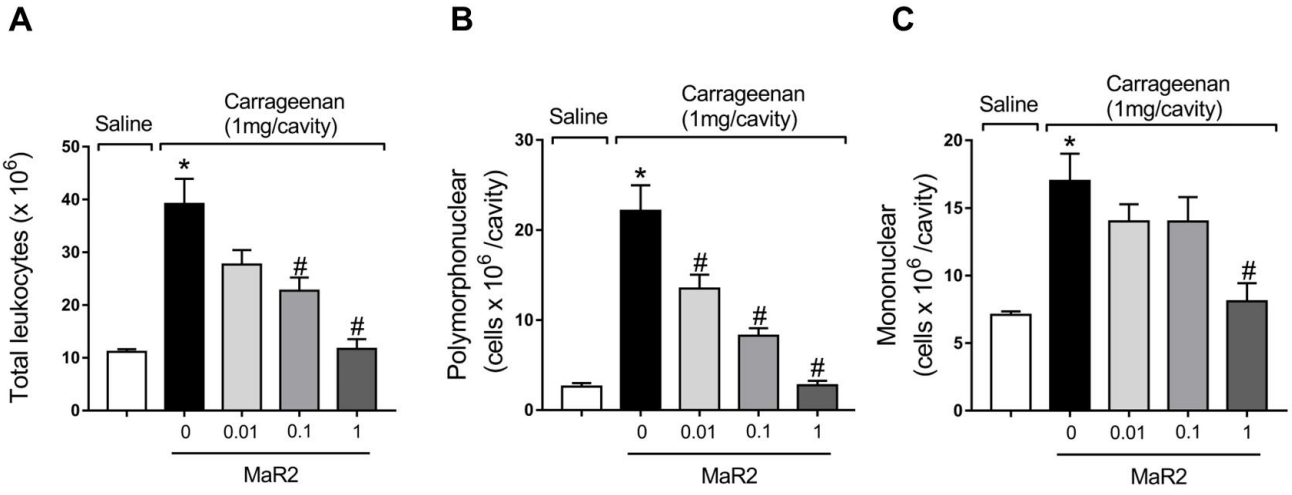
Previous work showed MaR1 has a long lasting effect (Fattori et al., 2019). We next sought to determine whether MaR2 has a prolonged effect either. For that, we used a chronic inflammatory hyperalgesia model with CFA. Mice were treated with 10 ng of MaR2 (i.t.) and mechanical and thermal hyperalgesia were evaluated. Similar to what was observed with carrageenan stimulus, here MaR2 at 10 ng effect lasted until day 2 for thermal hyperalgesia and until day 4 for mechanical hyperalgesia (Fig. 3A and B). However, as the intensity and duration of inflammation caused by CFA exceeds the carrageenan paw inflammation, MaR2 presented lessened analgesic activity.

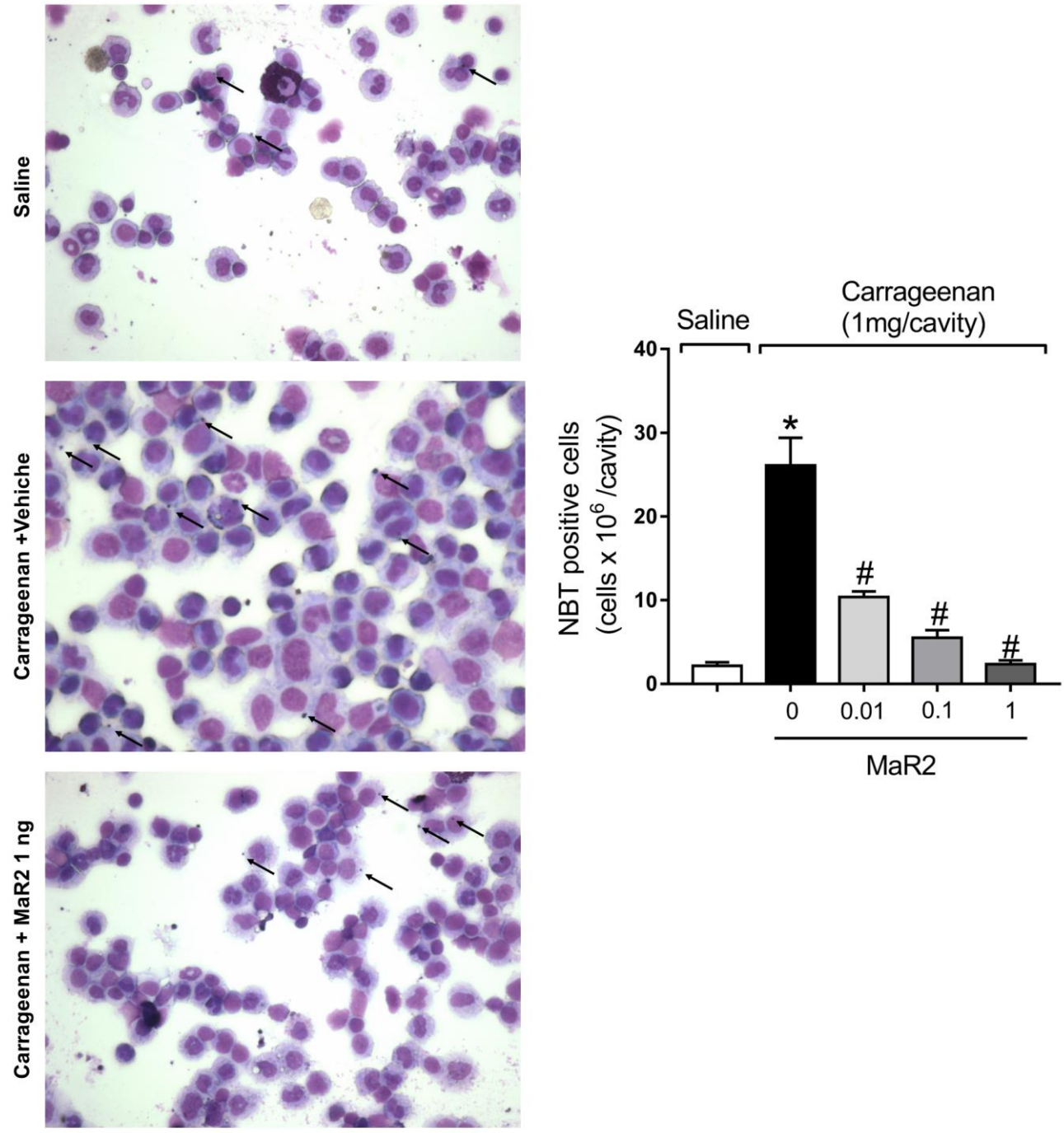
Figure 3. MaR2 reduces CFA-induced inflammatory pain. Intensity of mechanical (A) and thermal (B) hyperalgesia was evaluated 1, 3, 5, 7 h and daily 1-6 days after stimulus using an electronic von Frey and hot plate apparatus, respectively. Results are presented as mean ± SEM; n = 6 mice per group per experiment (*P < 0.05 vs saline group; #P < 0.05 vs vehicle group, two-way ANOVA followed by Tukey's posttest).

MaR2 reduces carrageenan-induced leukocyte recruitment and superoxide anion production in the peritoneal cavity.

We decided then analyze whether MaR2 would reduce leukocyte recruitment at peritoneal cavity. For that, we performed a peritonitis model with intraperitoneal injection of carrageenan (1 mg/cavity). A dose–response curve was performed with 0.01, 0.1 and 1 ng of MaR2, intraperitoneal route. We choose to work with low doses of MaR2 based on previously findings that showed MaR2 at 1 ng reduced leukocyte

recruitment in zymosan-induced peritonitis }(Deng et al., 2014). MaR2 at 0.1 and 1 ng reduced total and polymorphonuclear (Fig. 4A and B) leukocyte recruitment in the peritoneal cavity, but only 1ng reduced mononuclear cells recruited by carrageenan (Fig. 4C). All doses of MaR2 reduced the number of NBT positive cells (Fig. 4D), demonstrating a possible antioxidant activity of MaR2.





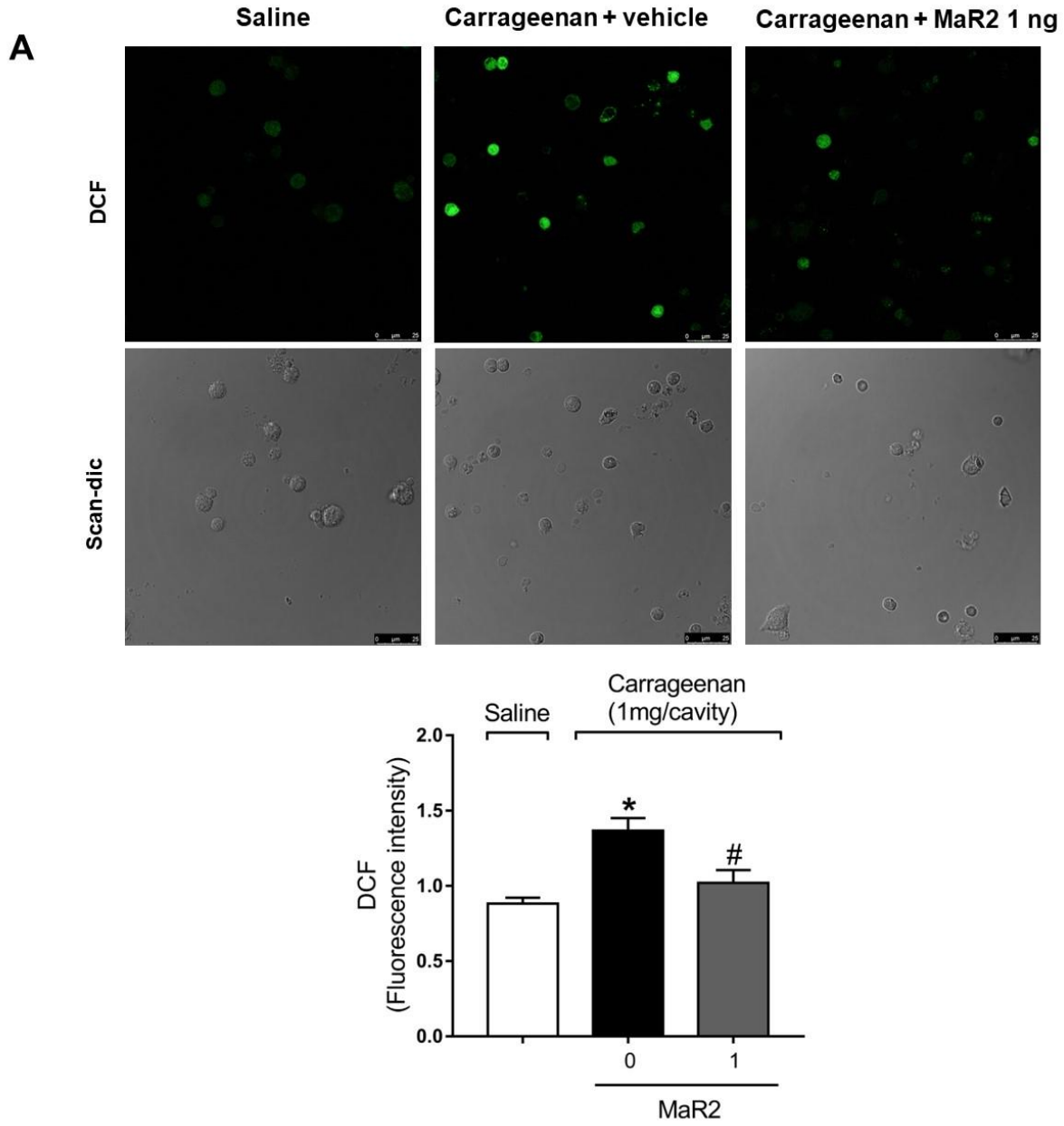
833

834 **Figure 4.** MaR2 reduces carrageenan-induced leukocyte recruitment and superoxide
835 anion production in the peritoneal cavity. Peritoneal wash was collected 5 h after
836 carrageenan for analysis of recruitment of total leukocytes and polymorphonuclear and
837 mononuclear cells (A). Total leukocyte recruitment was counted under a light
838 microscope (400× magnification). NBT-positive cells in the peritoneal exudate were
839 determined 5 h after carrageenan treatment (D). Representative pictures of NBT-

positive cells are indicated by arrows in panel D. Results are presented as mean ± SEM; n = 6 mice per group per experiment (*P < 0.05 vs saline group; #P < 0.05 vs vehicle group, one-way ANOVA followed by Tukey's posttest).

MaR2 reduces carrageenan-induced ROS and RNS production.

Oxidative stress is directely linked to many inflammatory disease. Other SPMs demonstrated potential antioxidant properties }(Cezar et al., 2019; X. Chen et al., 2016; Cox et al., 2015; Rodriguez et al., 2021). Here, to evaluate ROS and RNS production we used DCFDA and DAF2-DA, which are molecules that upon oxidation generate fluorescence product (DCF and DAF, respectively) proportional to overall intracellular ROS and RNS levels. We observed a significant decrease in carrageenan-induced ROS and RNS production in peritoneal cells of the group treated with MaR2 (Fig 5A and B, respectively). These results indicated that MaR2 reduces oxidative stress in the carrageenan peritonitis.


A
Saline
Carrageenan + vehicle
Carrageenan + MaR2 1 ng
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MaR2

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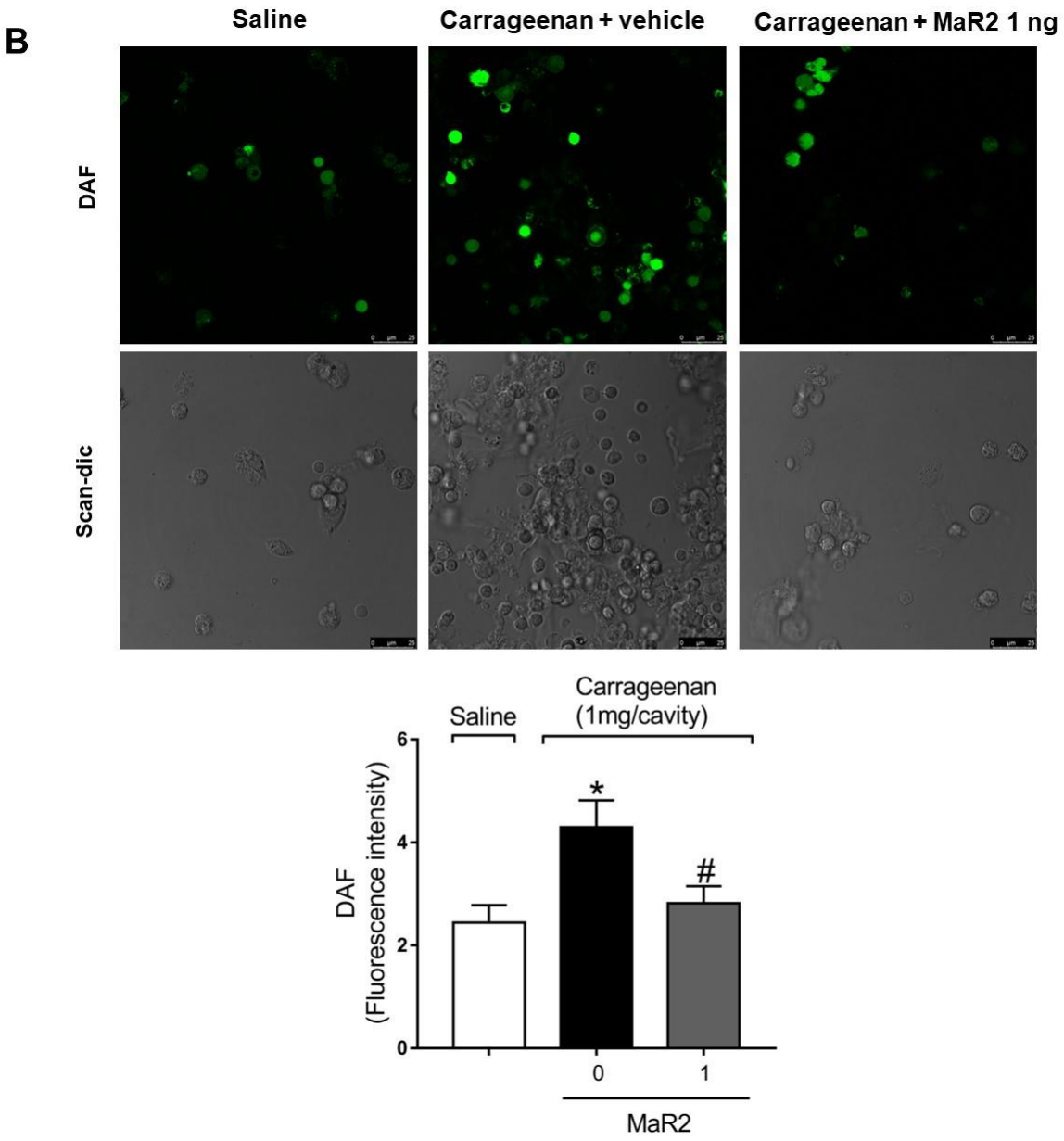
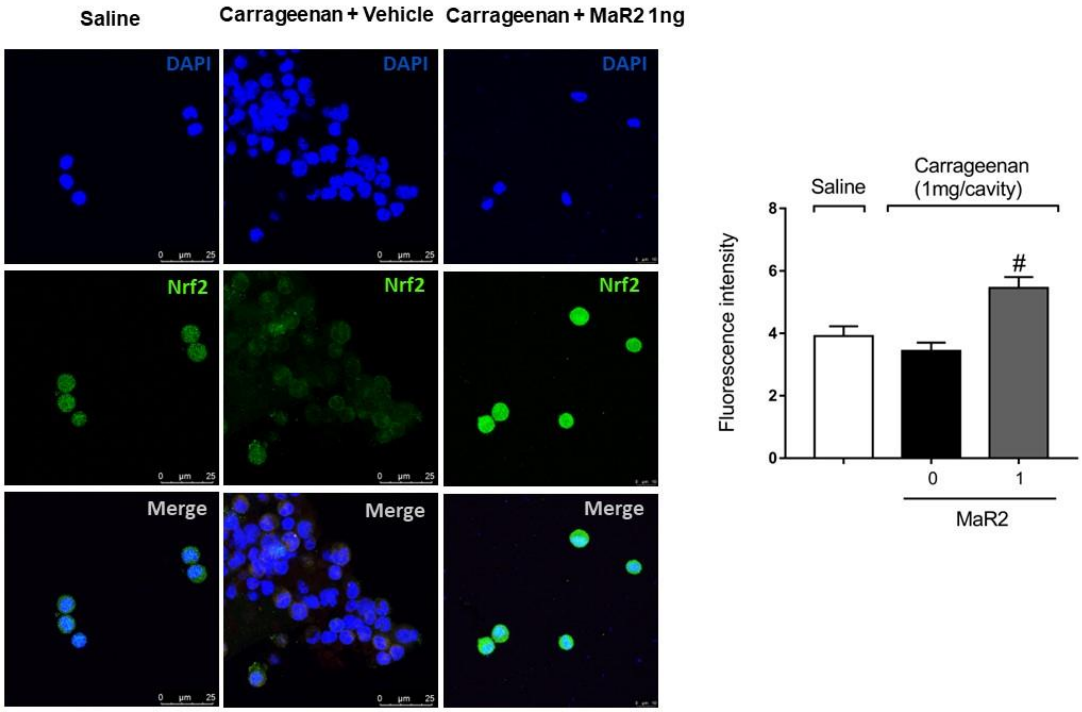


Figure 5. Total ROS (A) and RNS (B) levels were analyzed by the DCF-DA and DAF2-DA assay, respectively using the bright field and green channel in a confocal microscope at 20× magnification. DCF and DAF fluorescence intensity indicates ROS production and was quantified by LAS X software (Leica). Results are presented as mean ± SEM; n = 6 mice per group per experiment (*P < 0.05 vs saline group; #P < 0.05 vs vehicle group, one-way ANOVA followed by Tukey's posttest).

MaR2 induces Nrf2 antioxidant signaling.

To address a possible pathway by which MaR2 could perform an antioxidant effect, we analyzed Nrf2 staining in peritoneal cells by immunofluorescence. MaR2 induced an increase in Nrf2 expression compared to the saline and carrageenan group (Fig. 6). These data demonstrate that MaR2 can be preventing ROS and RNS oxidative stress by activating Nrf2 intracellular signaling.



876

877 **Figure 6.** MaR2 induces Nrf2-dependent antioxidant signaling. Mice were treated with
878 MaR2 (1ng, i.p.) or vehicle (saline i.p.) 1 h before carrageenan injection. Confocal
879 immunofluorescence analysis in peritoneal cells was performed 5 h after carrageenan
880 stimulus to determine Nrf2 staining. Results are presented as mean ± SEM of six mice
881 per group per experiment and are representative of two separate experiments. (*P <
882 0.05 vs saline group; #P < 0.05 vs vehicle group, one-way ANOVA followed by Tukey's
883 posttest).

884

885 **Discussion**

886 Maresin 2 (MaR2) is a lipid mediator that due to its precursor, synthesis and
887 structure was classified as belonging to the specialized pro-resolution lipid mediators
888 (SPM) family. However, its biological activities are indeed not poorly known. MaR2
889 reduces neutrophil recruitment triggered by zymosan, but if this activity is dose-
890 dependent was not determined. Furthermore, MaR2 reduces macrophage
891 phagocytosis of opsonized zymosan with higher potency than MaR1. On the other
892 hand, MaR1 presented higher potency than MaR2 when it comes to phagocytosis of
893 apoptotic neutrophils (efferocytosis) by macrophages in vitro }(Deng et al., 2014).
894 These results suggest that the activity of MaR2 might be more prone to an anti-
895 inflammatory activity than a pro-resolution compared to MaR1 since efferocytosis is a
896 characteristic pro-resolution phenomenum }(Serhan, 2014). However, again, this is
897 something that was not pursued in the literature. In the present study, we combined
898 different models of pain and inflammation and demonstrated that treatment with the
899 MaR2 displays a significant anti-inflammatory and analgesic effect in mice by a
900 mechanism involving the inhibition of oxidative stress in mice.
901 We first demonstrated that MaR2 reduces carrageenan- and CFA-induced
902 inflammatory hyperalgesia behaviors in mice. Carrageenan is a polysaccharide
903 obtained from red seaweeds used as an animal model of inflammation and pain }(T. M.
904 Cunha et al., 2005). In carrageenan-induced hyperalgesia, the intrathecal treatment

with MaR2 showed a dose-dependent response and decreased leukocyte recruitment at the paw skin of the animals. These results suggest that MaR2 might be exerting its activity in part by down regulating the communication between neurons and immune cells. In fact, we have demonstrated that MaR2 reduces the neuronal activation by capsaicin (a TRPV1 agonist) and AITC (Allyl isothiocyanate, a TRPA1 agonist) observed as calcium levels and the release of the neuropeptide CGRP (Fattori et al., 2022). We have also demonstrated that intrathecal MaR1 presents a similar activity in terms of inhibiting CGRP release by primary afferent neurons, which results in reduced recruitment of leukocytes to the inflamed paw skin (Fattori et al., 2019). Thus, it seems that modulating the neuro-immune communication to reduce pain and inflammation is a common biological activity of maresins. However, MaR1 and MaR2 are, obviously, different molecules. Indeed, they have different functions. MaR1 has been shown to reduce the neuronal activation of TRPV1, but not TRPA1 **}**(Serhan et al., 2012) an effect that occurs through a G-protein coupled receptor that was later identified as being LGR6 **}**(Chiang, Libreros, Norris, de la Rosa, & Serhan, 2019). MaR2 instead, inhibits the neuronal activation of TRPV1 and TRPA1 suggesting that it may have a different receptor(s) and/or interact differently with the receptor in a manner its activity is not the same as MaR1.

The complete Freund's adjuvant (CFA) is a suspension of heat killed mycobacterium in paraffin oil and mannide monooleate that is used for animal models of chronic inflammation and pain, including models of arthritis pain **}**(Manaheji, Jafari, Zaringhalam, Rezazadeh, & Taghizadfarid, 2011; Martinez et al., 2016; Pinho-Ribeiro et al., 2016). A single MaR2 intrathecal treatment reduced CFA-induced mechanical hyperalgesia for four days and thermal hyperalgesia for 2 days. This approach allowed to observe that MaR2 has a prolonged analgesic effect. As prior data indicate that MaR2 inhibits both TRPV1 and TRPA1 **}**(Fattori et al., 2022). Thus, we can speculate that MaR2 has a more pronounced effect over TRPA1 than TRPV1 since the analgesia in terms of mechanical stimulation was more prolonged and higher than in thermal hyperalgesia.

It is worth mentioning that intrathecal treatment with other SPMs derived from docosahexaenoic acid has promising results as well. Intrathecal treatment with MaR1 reduced CFA-induced astrocyte and microglia activation and decreased the production of TNF-α and IL-1β and NF-κB activation (Fattori et al., 2019). Another study reported that intraplantar or intrathecal administration of RvD1 in mice potently reduces inflammatory pain behaviors induced by intraplantar injection of formalin, carrageenan or CFA **}**(Z. Z. Xu et al., 2010). Intrathecal RvD1 also reduced gout arthritis pain and inflammation by disrupting the communication between primary afferent neurons and immune cells. The inhibition of CGRP release by RvD1 treatment diminished the monosodium urate crystals phagocytosis by macrophages, which resulted in the dampening of IL-1β release, an essential pro-inflammatory cytokine in gout arthritis pathology **}**(Zaninelli et al., 2022). Intrathecal RvD2 administration reduced mechanical and thermal hyperalgesia in a fibromyalgia-like model **}**(Klein et al., 2014). A study found that intrathecal RvD5 treatment reduced formalin-induced second phase pain (licking and flinching) (Luo, Gu, Tao, Serhan, & Ji, 2019). Indeed, the neuronal inhibition and even the targeting of neuroinflammation and neuro-immune interactions by the administration of SPMs by intrathecal route represents a potential analgesic approach to treat chronic pain (Fattori et al., 2019).

During the inflammatory response, peripheral innate immune cells such as neutrophils, mast cell and macrophages, release mediators that act on peripheral nerve terminals producing peripheral nociceptor sensory neuron sensitization, which is observed as hyperalgesia **}**(Pinho-Ribeiro, Verri, & Chiu, 2017). Given the fundamental role of leukocytes recruitment in the emerge and maintenance of inflammation and pain, we further investigated whether MaR2 could alleviate carrageenan peritonitis

since the access to cells in the peritoneal cavity avoids long procedures to isolate cells that could interfere with activity and is a reliable model of inflammation. Deng et al. previously found that MaR2 a low dose (1ng) reduced zymosan-induced neutrophil recruitment to the peritoneal cavity **}**(Deng et al., 2014). According to that, we choose to perform a dose-response (0,01 - 1 ng) curve using 1 ng as the highest dose, intraperitoneal route this time. Tissue or cavity resident immune cells will be the first to be activated. The peritoneal cavity presents macrophages, mast cells and natural killer cells. Cavity resident cells produce inflammatory mediators in response to inflammatory stimulation. These inflammatory mediators will orchestrate the recruitment of neutrophils and mononuclear cells. MaR2 reduced in a dose-dependent manner the recruitment of neutrophils and mononuclear cells triggered by carrageenan. It remains to be determined if MaR2 can reduce the production of inflammatory mediators such as cytokines upon carrageenan stimulus, which would add to explain the reduction of leukocyte recruitment. In LPS inflammation, MaR2 reduces the production of cytokines and chemokines **}**(Fattori et al., 2022), which indicates that inhibiting cytokine production is a consistent mechanism of local action of MaR2 among inflammatory models. Considering Resolvin D1-treated animals showed a significant increase in plasma LXA4 (Bathina and Das, 2021), we could speculate that MaR2 could activate other SPMs receptor and biosynthesis, thus promoting the local anti-inflammatory effects as well.

Reactive oxygen (ROS) and nitrogen (RNS) are involved in inflammation development, which includes the recruitment of leukocytes and pain **}**(Hackel et al., 2013). An example is that the injection of a superoxide anion donor induces leukocyte recruitment **}**(Maioli et al., 2015), ROS inhibition dysregulates the neutrophil recruitment **}**(Marriott et al., 2008), and ROS can activate nociceptor sensory neurons causing pain **}**(I. Lee, Kim, Kim, Chung, & Chung, 2007; Taylor-Clark, 2015). Here, we observed that MaR2 clearly reduced ROS and RNS production in peritoneal cells stimulated with carrageenan using fluorescent probes. Since MaR2 does not present chemical groups that would confer an antioxidant effect per se, we speculated that it would, instead, trigger antioxidant responses of the leukocytes. In this line, we observed that MaR2 enhanced the transcription factor nuclear factor-2 erythroid related factor-2 (Nrf2). Nrf2 regulates cellular redox status through endogenous antioxidant systems with simultaneous anti-inflammatory activity (Staurengo-Ferrari et al., 2018). Nrf2 activity is normally repressed by the cytosolic protein Keap-1 (Kelch-like ECH-associated protein 1) that regulates its ubiquitination and proteasome degradation. Thus, enhanced Nrf2 can be a result of increased expression or reduced degradation. A minor proportion of Nrf2 constantly escapes the repression by Keap-1 and translocate to the cell nucleus to bind to antioxidant responsive elements (ARE) up-regulating the transcription of antioxidant molecules/enzymes such as reduced glutathione, superoxide dismutase and catalase. Therefore, enhancing Nrf2 is a mechanism to enhance endogenous antioxidant responses (Staurengo-Ferrari et al., 2019; Hohmann et al., 2020)

Nrf2 function goes beyond oxidative stress, it regulates inflammation. Nrf2 reduces the production of ROS and inflammatory cytokines in LPS-stimulated neutrophils. In macrophages, Nrf2 down-regulates the expression of toll-like receptor 4, the activation of the transcription factor NFκB and the production of inflammatory cytokines. As a consequence, Nrf2 deficiency enhances the inflammatory response caused by LPS in the context of sepsis and pleurisy (Hohmann et al., 2020). The properties against oxidative stress through activation of Nrf-2 reported herein corroborate with previous studies with other SPMs. MaR1 showed an antioxidant activity by activation of the Nrf2-mediated HO-1 signaling pathway in a model of lung I/R injury, **}**(Sun, Wu, Zhao, & Wang, 2017). Other studies demonstrated that MaR1 prevented liver fibrosis **}**(Rodriguez et al., 2021) and DSS-induced colitis by activating Nrf2 signaling **}**(Qiu, Li, Zhao, & Li, 2020). SPMs, such as Resolvin D1 and Lipoxin A4

also activate Nrf-2 signaling in several models of inflammatory diseases }(X. Q. Chen, Wu, Zhou, & Tang, 2013; Han et al., 2016; Jin et al., 2014; Li et al., 2020; Mostafa & Satti, 2020; Ye et al., 2019). Thus, inducing/activating Nrf2 seems a preserved function of SPMs, and we are showing it for the first time for MaR2.

Conclusions

The findings in this work demonstrate that MaR2 reduces acute and prolonged inflammatory pain, and inflammation. The activity of MaR2 is related to the induction of Nrf2 resulting on reduction of ROS and RNS. The role of MaR2 in regulating oxidative balance through Nrf2, to our knowledge, is a novel function of this SPM.

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4 ARTIGO PARA PUBLICAÇÃO II (NEUROSCIENCE LETTERS)

O presente trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista *Neuroscience Letters*. Os resultados parciais estão descritos no artigo intitulado de "Maresin 2 reduces articular pain induced by inactivated Chikungunya virus and its envelope protein".

RESEARCH PAPER

Maresin 2 reduces articular pain induced by inactivated Chikungunya virus and its envelope protein.

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Abstract

Chikungunya virus (CHIKV) is an emerging alphavirus responsible for large outbreaks of acute and chronic arthritis throughout Africa, Southeast Asia and America. However, treatment for chronic pain caused by CHIKV remains a challenge. The specialized pro resolving mediators (SPMs) are endogenous molecules termed resolvins, protectins, maresins, and lipoxins that acts in the inflammatory cascades promoting resolution of inflammation. The use of SPMs for treatment of chronic inflammation and pain has drawn attention in the past decade due its efficiency and safety. Here, we evaluated the lipid mediator Maresin 2 (MaR2) as treatment for pain induced by CHIKV and its E2 envelope protein. For that, we injected inactivated Chikungunya virus (iCHIKV) and recombinant E2 (rE2) in the knee joint of Swiss mice and treated with MaR2, via intraperitoneal. In a separated experiment, we collected DRGs neurons from naive C57/BL6 mice, then stimulated with iCHIKV, rE2 or Mock and treated with MaR2 to determinate calcium influx. MaR2 reduced mechanical hyperalgesia induced by both iCHIKV and rE2. MaR2 also reduced the influx of calcium induced by iCHIKV and rE2 in DRG neurons. Curiously, a subset of these neurons was responsive to capsaicin (Transient Receptor Potential Vanilloid 1 (TRPV1) agonist). Taken together, these results demonstrated for the first time that MaR2 can reduce pain induced by CHIKV and E2, preventing neuronal activation.

Key words: Maresin 2. Chikungunya virus. E2 protein. Pain. TRPV1.

1 Introduction

Chikungunya virus (CHIKV) is a arboviruses that was first identified in Tanzania in 1952 (Robinson, 1955). The virus belongs to the genus of Alphaviruses, which is characterized by a single-stranded, positive-sense RNA }(Khongwichit, Chansaenroj,

Chirathaworn, & Poovorawan, 2021). CHIKV infection is characterized by high fever, skin rash, severe joint pain (arthralgia) and polyarthritis }(Khongwichit et al., 2021; Soulaphy et al., 2013). The transmission to humans occurs mainly by infected *Aedes aegypti* mosquitoes, but in 2006 a mutation in the E1 protein facilitated enhanced replication and transmission in *Aedes albopictus* mosquitoes }(Tsetsarkin et al., 2007).

Chikungunya viruses are mainly prevalent in tropical/subtropical regions }(Caglioti et al., 2013). According to Pan American Health Organization (PAHO) epidemiological update of December of 2021, a total of 1,324,108 cases of arboviral diseases were reported, including dengue, chikungunya and Zika virureses (PAHO / WHO, 2021). Of those, 131,630 were chikungunya cases (PAHO / WHO, 2021).

After transmission, the virus likely replicates in fibroblasts, mesenchymal cells, and osteoblasts }(Fox & Diamond, 2016). CHIKV induces an inflammatory response mediated by cytokines, such as IFN-α/β, IFN-γ,TNF-α, IL-6 and chemokines, which recruits neutrophils, monocytes/macrophages, natural killer (NK) cells and CD4+ and CD8+ T cells }(Gardner et al., 2010; Morrison et al., 2011). Moreover, CHIKV infection results in monocyte chemoattractant proteins (MCP) -induced cellular infiltration in the inflamed joints, which can cause bone loss }(W. Chen et al., 2015).

In mice, CHIKV infection causes a biphasic pattern of swelling in the ipsilateral inoculated foot with a small peak between 2–3 days post-infection (dpi) and a second, larger peak at 6–7 dpi }(Gardner et al., 2010). In the first peak, viral replication results in cell death, cytokine production, and tissue edema }(Caglioti et al., 2013). While in the second peak, the infection is taken from the blood to tissues, being associated with the increase of inflammatory cells into joints causing more edema, myositis, and synovitis }(Fox & Diamond, 2016).

CHIKV is composite by an enveloped, which contains a positive-sense single stranded RNA genome (Cho et al., 2008). The genome comprises two open reading frames (ORFs), ORF1 and ORF2, flanked by a 5′ cap and a polyadenylated tail at the 3′UTR (Silva & Dermody, 2017). These ORFs encode polyprotein precursors of four nonstructural (nsP1, nsP2, nsP3, and nsP4) that constitute the RNA replicase; and six structural proteins: capsid, envelope proteins E1, E2, and E3, 6K and transframe proteins (Silva & Dermody, 2017).

The infection is usually self-limiting, however, after the acute phase, some patients reports persistence of articular, musculoskeletal and neuropathic pain, with possible post-illness destructive arthropathy }(Brighton & Simson, 1984; Schilte et al., 2013). The articular commitment caused by chikungunya infection, in its different phases, causes significant physical disability, and impacts directly in the quality of life of affected patients }(Soumahoro et al., 2009). The disability caused by the disease in economically active groups, increases the magnitude of the problem for the affected population.

Currently, there are no antiviral therapy or vaccines available to prevent CHIKV infection }(Fox & Diamond, 2016). Despite the inflammatory component in the pain caused by the disease, NSAIDs aren't recommended due to the risk of complications associated with severe forms of Chikungunya (hemorrhage and renal insufficiency) }(Brito et al., 2016). Considering that therapies for pain presents many side effects when used for long periods, and CHIKV infection led to chronic painful symptoms, the search for an effective and safe analgesic becomes necessary.

The mechanism behind the resolution of inflammation once thought to be a passive process, now is known to be an active process mediated by endogenous bioactive molecules derived from omega-3 and omega- 6 fatty acid }(Serhan et al., 2015). These molecules so-called Specialized lipid mediators include resolvins, maresins, and protectins (derived from ω-3 polyunsaturated fatty acids) and lipoxins (derived from ω-6 PUFAs) }(Chiang & Serhan, 2017). SPMs have shown success when used as therapeutic drugs in different pathological models, including chronic pain models. }(Chavez-Castillo et al., 2021). Indeed, the production of SPMs was observed at the site of inflammation, alleviating the symptoms of arthritis }(Barden et al., 2016).

Considering there is no study addressing the use of SPMs as therapy for pain in Chikungunya virus infection, our study aimed to evaluate the effect of MaR2 on inactivated CHIKV- and rE2- induced hyperalgesia in mice.

2 Methods

2.1 Animals

All experiments were performed in accordance with the International Association for Study of Pain guidelines and with the approval of the Londrina State University Ethics Committee on Animal Research and Welfare (process numbers 056.2020). In this study, we used healthy male Swiss mice and C57BL/6 mice from Londrina State University, Paraná, Brazil. Mice were randomly assigned and housed in standard clear plastic cages, kept in light/dark cycle of 12:12 hr with ad libitum food and water. Behavioral testing was performed between in a room maintained at a temperature of 21°C ± 1°C. The investigators were blinded to the treatments. All efforts were made to minimize the number of animals used and their suffering. Animals were euthanized with isoflurane anesthesia (5% in oxygen using a precision vaporizer) followed by decapitation as a confirmation method.

2.2 Experimental procedures

Mice were stimulated with inactivated Chikungunya virus (iCHIKV) (100UFF/ 10µL/animal), recombinant E2 protein (100ng/ 10µL/ animal), Mock (10 µL) or saline (10 µL), intraarticular route in the knee joint. After 1 hour, animals were treated with 0.03, 0.3 or 3 ng of MaR2 (Cayman Chemical, Ann Arbor, MI, EUA) or vehicle via intraperitoneal (100 µL/ animal). Mechanical hyperalgesia was evaluated at time-points: 1, 3, 5, 7 hour after MaR2 treatment and daily until day 6 post iCHIKV stimulus and until day 3 for rE2 stimulus. DRGs (from L4 to L6 spinal cord segments) from naive C57BL/6 mice were collected, stimulated with iCHIKV or rE2 and treated with MaR2 or vehicle, to determinate neuron activation and TRPV1 activation through calcium imaging. The doses of iCHIKV and rE2 were previously standard by other study in our laboratory (SEGATTO-VENDRAMETO et al., 2019). The doses of MaR2 were chosen based on other studies in our laboratory using the SPMs **}**(Fattori et al., 2019; Fattori et al., 2022)

2.3 Chikungunya virus

The virus used in this experiment (CHIKV BR_2015/15010) was isolated from the serum of a patient with chikungunya disease from Northeast Brazil in 2015. The virus was amplified, titrated by foci-forming assay in C6/36 cells and inactivated using β-propiolactone (0.025%, 72h, 4ºC). The inactivated virus was concentrated by PEG 7%/NaCl 2.3% precipitation and purified using sucrose cushion. A noninfected control (Mock) was prepared in the same manner from β-propiolactone inactivated C6/36 cell-culture supernatant.

2.4 Recombinant E2 protein

The CHIKV recombinant E2 protein (rE2) was expressed by *Drosophila* S2 cells. S2 cells were co-transfected with the plasmids pMt/Bip/V5-HisA (Invitrogen) containing the gene of the E2 protein from CHIKV and pCoBlast. The transfected cells were selected

with 25 µg/mL of blasticidin and cultured in SF900II (Invitrogen) medium with 25 mg/mL gentamicin (Gibco). The rE2 protein expression was induced by 700 mM of $CuSO_4$ for 24 h, and the protein was purified from the cell-culture supernatant by immobilized metal *affinity chromatography* (using Ni-NTA agarose resin). A Mock control was prepared from the supernatant of S2 cells induced by 700 mM of $CuSO_4$ and purified following the same protocol.

2.5 Mechanical hyperalgesia

Mechanical hyperalgesia was evaluated by the electronic version of von Frey's test. In a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15-30 min before the start of testing. This test consists of evoking a tibio-tarsal reflex with a hand-held force transducer (electronic anesthesiometer, Insight, Ribeirão Preto, SP, Brazil) adapted with a with a non-nociceptive tip probe with area size of 4.15 mm2. The results were expressed as the flexion-elicited withdrawal threshold which is automatically registered in grams (g). The animals were tested before (baseline) and in time points described in 2.2 topic after the MaR2 treatment. The results calculated through the difference between the average of 2 measures after the treatment (baseline) and the average of 2 measures before the treatment. The investigators were blinded to the treatment.

2.6 Calcium imaging

Calcium imaging were performed as previously described (Fattori et al., 2019). After dissection, DRGs were processed and loaded with 1.2 µM of Fluo-4AM in Neurobasal-A medium, incubated for 30 min 37°C, washed with HBSS, and imaged in a Confocal. To assess neuron activation, DRG were stimulated with mock, iCHIKV or rE2 and treated with 3ng of MaR2 or vehicle. DRGs plates were recorded for 7 min, which was divided in 1 min of initial recording (0-s mark, baseline values), following by stimulation with mock, iCHIKV or rE2 and treatment with MaR2 or vehicle for 4 min at the 60-s mark, and capsaicin (1 µM, TRPV1 agonist) for 2 min at the 300-s mark. Calcium influx was analysed from the mean fluorescence measured with the LAS X Software (Leica Microsystems).

2.7 Data and statistical analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad Prism software version 6.0). Results are presented as means ± SEM of measurements made with 6 mice per group. Each experiment was conducted twice. Two-way ANOVA, followed by the Tukey post-test, were used to analyze data from multiple-moment experiments (mechanical and thermal hyperalgesia and edema). One-way ANOVA were used followed by the Tukey post-test for experiments with specific times. Significant statistical differences were considered when $P < 0.05$.

3 Results

3.1 Maresin 2 inhibits inactivated Chikungunya virus (iCHIKV) induced mechanical hyperalgesia.

To evaluate the efficacy of Maresin 2 treatment in iCHIKV articular pain, we injected 100FFU of iCHIKV, mock (10 µL) or saline (10 µL) in the knee joint of mice. One hour after stimulus, animals received treatment with 3, 0.3 or 0.03 ng of MaR2 or vehicle (10% ethanol), intraperitoneally (i.p.). We observed that all doses reduced mechanical hyperalgesia induced by iCHIKV, starting in the first hour lasting until the 7th hour after treatment. Only the dose of 3 ng demonstrated analgesia until the 4th day after treatment, which demonstrates that the effect of MaR2 was dose dependent. The dose of 3 ng of MaR2 was choose for the following experiments. Experimental groups were compared to Mock group as control and this one was compared to saline group.

Figure 1. MaR2 reduces mechanical hyperalgesia induced by inactivated Chikungunya virus. Animals received intra-articular (i.a.) injection of iCHIKV (100 FFU, 10 µL), Mock (control, 10 µL) or saline (10 µL). One hour before, animals were treated with MaR2 at 0.03, 0.3 and 3 ng (i.p.) or vehicle (10% ethanol).. The mechanical hyperalgesia was evaluated 1 – 7 hours after treatment and daily during six days. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; #P<0.05 compared to iCHIKV group. ANOVA followed by Tukey's test.

3.2 Maresin 2 inhibits mechanical hyperalgesia induced by recombinant E2 protein from Chikungunya virus.

In a second moment, we evaluate whether MaR2 could reduce mechanical hyperalgesia induced by recombinant E2 protein (rE2). Animals were injected intra-articular with 100ng of rE2 one hour before treatment with 3 ng of MaR2 (i.p. route) or vehicle (10% ethanol). We observed that MaR2 reversed the mechanical hyperalgesia induced by rE2. This time, the analgesic effect of MaR2 started only after the 3rd hour of treatment and lasted until the 7th hour. Experimental groups were compared to Mock as control and this one was compared to saline group.

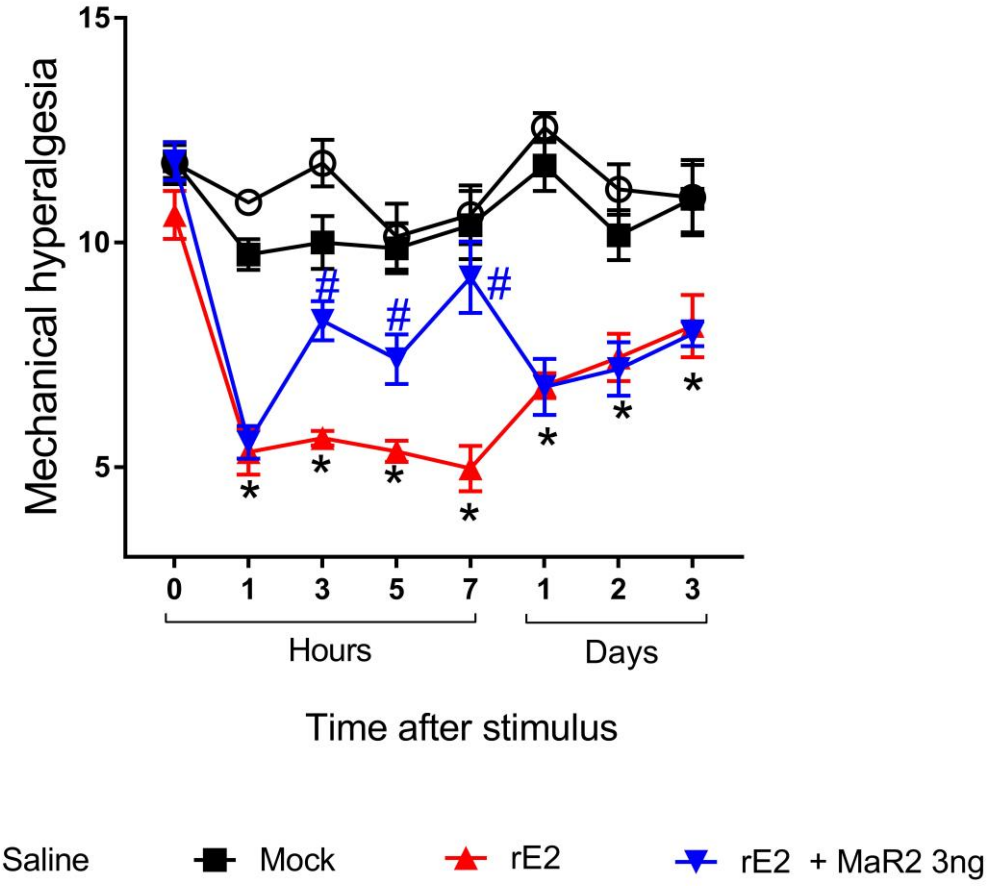

Figure 2. MaR2 reduces mechanical hyperalgesia induced by rE2 protein from Chikungunya virus. Animals received intra-articular (i.a.) injection of rE2 (100 ng, 10µL), Mock (control, 10 µL) or saline (control, 10 µL). The mechanical hyperalgesia was evaluated 1- 7 hours after treatment with MaR2, and daily during three days after treatment. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; #P<0.05 compared to rE2 group. ANOVA followed by Tukey's test.

3.3 MaR2 reduces iCHIKV- and rE2- induced activation of DRG neurons.

Activation of DRG neurons can be observed through an increase in calcium influx (Blake et al., 2018; Chiu et al., 2013). Considering DRG neurons from iCHIKV and rE2-stimulated mice presented a higher baseline level of calcium (Segato-Vendrameto et al., 2019), we decided to evaluate whether MaR2 could prevent iCHIKV and rE2 neuron activation. For that, DRG neurons from naïve animals were collected and

plated. DRGs were stimulated with mock, iCHIKV or rE2 and treated with MaR2 (3ng/mL) or vehicle. We observed that iCHIKV and rE2 induced higher influx of calcium. The treatment with MaR2 reduced both iCHIKV and rE2 calcium influx in the DRG neurons. In addition, a subset of DRG neurons that responded to the virus and its protein also responded to capsaicin (transient receptor potential V1 (TRPV1) agonist).

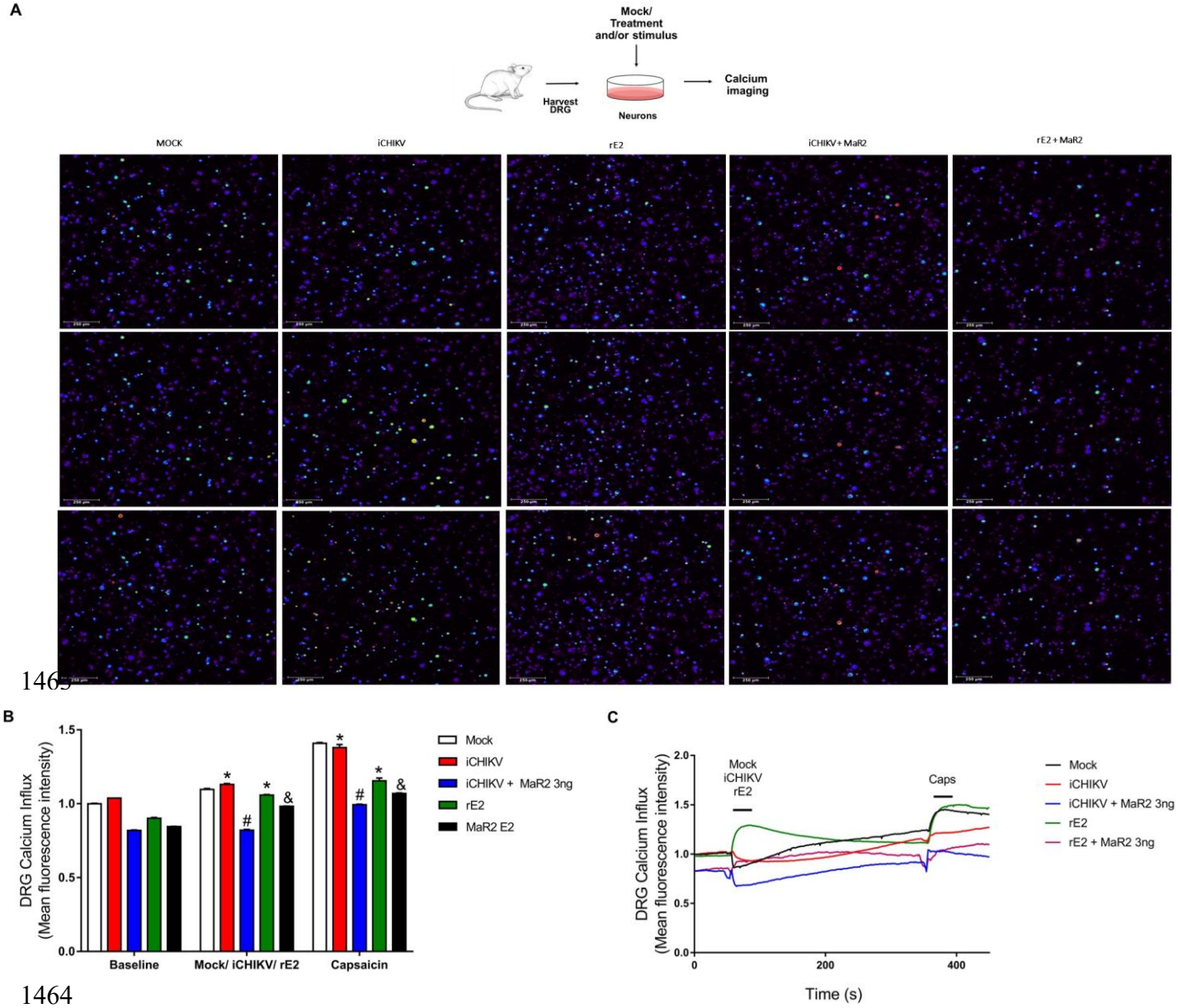

Figure 3. MaR2 reduces DRG neurons activation induced by iCHIKV and rE2 protein. DRG neurons from naïve animals were collected and plated for calcium imaging using Fluo-4AM. DRGs were stimulated with mock, iCHIKV or rE2 and treated with MaR2 (3ng) or vehicle (10% ethanol). Panel A displays representative fields of baseline fluorescence of DRG neurons without stimulus, after stimulus with mock, iCHIKV or rE2 and treatment with MaR2 3 ng/mL, and after stimulus with capsaicin (TRPV1 agonist). Panel B shows the mean fluorescence intensity of calcium influx on the baseline (0-60 seconds mark), after stimulus + treatment (60-300 seconds mark), and after capsaicin (300-420 seconds mark). Results are expressed as mean ± SEM, n

= 4 DRG plates (each plate is a neuronal culture pooled from 6 mice) per group per experiment, two independent experiments (*p < 0.05 vs. mock; #p < 0.05 vs. iCHIKV; &p <0.05 vs. rE2, one-way ANOVA followed by Tukey's post-test). 288x220mm (96 x 96 DPI).

Discussion

Alphaviruses such as Chikungunya virus cause acute and chronic arthralgia associated with inflammatory immune responses **}**(Lin et al., 2020). A robust cytokine response during acute infection is correlated with a decreased incidence of chronic joint pain and lower levels of TNFα, IL-2, IL-4, and IL-13 during acute infection seems to be a predictive of chronic joint pain **}**(Chang et al., 2018). CHIKV-infected patients suffer from rheumatic manifestations that last months to years, causing a significant impact in their quality of life **}**(Soumahoro et al., 2009).

Considering pain in CHIKV infection has inflammatory components (cytokines, chemokines, immune cells recruitment, joint swelling and pain) **}**(Fox & Diamond, 2016), in this study we presented Maresin 2 (MaR2), a specialized pro resolving lipid mediator as a possible therapy for pain in Chikungunya infection. Indeed, preclinical and clinical studies demonstrated that SPMs therapy have been successively in chronic pain related disorders, including migraine, rheumatic diseases, neuropathies and others **}**(Chavez-Castillo et al., 2021).

MaR2 is a pro-resolving lipid mediators biosynthesized from docosahexaenoic acid (DHA) by macrophages **}**(Serhan et al., 2009). Recent studies demonstrated that MaR2 reduced neutrophil recruitment in a model of zymosan peritonitis and immune cell recruitment close the $CGRP^{+}$ fibers in the paw skin **}**(Deng et al., 2014; Fattori et al., 2022). MaR2 also inhibited inflammatory pain induced by LPS, capsaicin or AITC, and inhibited TRPV1 and TRPA1 activation in cultured DRG neurons **}**(Fattori et al., 2022). Another study demonstrated that MaR2 reduced the expression of ASC, MPO, Ly-6G, ICAM-1, NLRP3 and Caspase-1 proteins in lung tissue in an animal asthma model **}**(Yu et al., 2022). Taken together, these findings have demonstrated that MaR2 displays potent anti-inflammatory and analgesic properties.

Using a model of articular joint pain with inactivated CHIKV, we observed that the virus induced mechanical hyperalgesia, and treatment with MaR2 successfully reversed the mechanical hyperalgesia, longing for 4 days with a single treatment (figure 1).

The E2 envelope protein plays a critical role in viral infection, replication and potentially cell tropism **}**(Weger-Lucarelli et al., 2015). CHIKV envelope proteins, both E1 and E2, interacts with human proteins triggering a robust immune and inflammatory response contributing to chronic arthralgia (Dudha et al., 2015). Here, we observed that E2 protein induced mechanical hyperalgesia, corroborating with the previous findings that showed that E2 protein is involved in Chikungunya virus-induced joint pain (SEGATO-VENDRAMETO et al.,2019). MaR2 reduced E2-induced hyperalgesia as well, although the treatment lasted until the 7^{th} hour (Figure 2).

Dorsal root ganglion is an aggregate of cell bodies from neurons that carry sensory information from the periphery to the spinal cord **}**(Kim et al., 2016). Over stimulation of DRG neurons cause heightened pain sensitivity, often leading to chronic pain **}**(Kim et al., 2016). The activation of DRG neurons can be measured by calcium influx **}**(I. M. Chiu et al., 2013). Here, we cultured DRG neurons from naïve animals, stimulated with iCHIKV or rE2 and treated with MaR2. Indeed, iCHIKV and rE2 induced calcium influx, demonstrating both activates sensorial neurons (figure 3). In addition, a subset of these DRG neurons that responded to the virus and its protein also responded to capsaicin, a agonist of transient receptor potential vanilloid 1 (TRPV1), which modulate pain responses (Yang & Zheng, 2017). Indeed, therapies that antagonizes TRPV1 channels have been studied for the treatment of conditions associated with chronic pain **}**(Bamps, Vriens, de Hoon, & Voets, 2021). In summary,

we found that MaR2 reversed DRG activation induced by both iCHIKV and rE2 by reducing calcium influx in DRG neurons (figure 3).

A study using primary human fibroblast-like synoviocyte (HFLS) cultures infected with CHIKV revealed that supernatants cultures not only induced migration of primary human monocytes, but also drove monocytes/ macrophages into osteoclast-like cells **}**(Phuklia et al., 2013). These differentiated osteoclast-like cells produced high levels of TNF-α and IL-6, that are principal mediators of arthritis **}**(Phuklia et al., 2013). Of interest, Resolvin E1 (RvE1), another SPM, significantly suppressed Receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation and bone resorption (Funaki et al., 2018). Considering this, the use of other SPMs may represent interesting therapies for CHIKV infection.

Conclusion

Taken together, our findings demonstrated that MaR2, a specialized lipid mediator, reduced pain responses induced by inactivated Chikungunya virus and its envelope protein, the E2, acting in the reduction of DRG neuron activation. Here, we brought for the first time the use of a lipid mediator, MaR2, as a treatment for pain induced by Chikungunya virus.

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5 CONCLUSÃO

No presente trabalho, foi demonstrado que o mediador lipídico MaR2, reduz dor e inflamação de natureza aguda e crônica, por diferentes vias de tratamento. O tratamento intratecal com a MaR2 reduziu dor inflamatória induzida por estímulo intraplantar com a carragenina e CFA. A hipótese de mecanismo para esse efeito, seria que este mediador possivelmente atua nos canais TRPV1 e TRPA1 nos neurônios do ganglio da raiz dorsal, como descrito na literatura, bloqueando a transmissão do estímulo doloroso para a via ascendente e desse modo promovendo uma analgesia observada. O tratamento intraperitoneal com a MaR2, por sua vez, também demonstrou-se efetivo na redução dos parâmetros inflamatórios induzidos pela carragenina. Dessa vez, a MaR2 possivelmente estaria atuando de maneira direta na redução das células imunes, e também na redução da produção de espécies reativas de oxigênio e nitrogênio. Nesse trabalho, foi observado a atividade da MaR2 na ativação do fator antioxidante Nrf2. O papel do MaR2 na regulação do equilíbrio oxidativo através do Nrf2, até onde se sabe, é uma ação inédita deste mediador lipídico. Por fim, utilizando um modelo de dor articular induzida pelo vírus Chikungunya e sua proteína envelope, E2, observou-se que o tratamento intraperitoneal com a MaR2 reduziu os parâmetros de dor nesse modelo. Além disso, a MaR2 reduziu a ativação dos neurônios do gânglio da raiz dorsal que foram induzidos pelo vírus e sua proteína, reafirmando, novamente, o papel analgésico da MaR2 através dos neurônios do gânglio da raiz dorsal.

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ANEXOS

ANEXO A – Cópia do Parecer da Comissão de Ética no Uso de Animais (Nº 152/ 2016)

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 152/2016 **Londrina, 15 de Julho de 2016.**

Prezado Pesquisador,

Certificamos que o projeto intitulado **"Avaliação dos mecanismos analgésicos e anti-inflamatórios dos mediadores lipídicos pró-resolução resolvina D5 (RvD5), maresina 2 (Mar2), protectina (PD1), 15-epi-lipoxina A4 (ATLA4) em modelos de dor e inflamação"**, protocolo CEUA n° **11145.2016.54**, sob a responsabilidade de **Waldiceu Aparecido Verri Junior**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **05/07/2016**.

O objetivo do projeto investigar os mecanismos dos resolvina D5 (RvD5), maresina 2 (Mar2), protectina (PD1), 15-epi-lipoxina A4 (ATLA4) em modelos de dor induzidos por formalina, carragenina, adjuvante completo de Freund (CFA) e LPS. Os animais serão divididos em gaiolas de polipropileno padrão medindo 41 X 34 X 16 CM (Insight®) no biotério de acordo com os grupos experimentais (máximo de 12 animais por gaiola), com livre acesso à água e ração e serão adaptados aos ambientes e condições experimentais com pelo menos 1 hora de antecedência em relação aos experimentos. Os procedimentos de cuidado e manuseio de animais estarão de acordo com as diretrizes da Associação Internacional de Estudo da Dor (IASP). Para o tratamento intratecal, os animais serão anestesiados com isoflurano, via inalatória. Os animais são eutanasiados por inalação de isoflurano (3% em O_2) seguido de decaptação para coleta das amostras. GI 2.

Vigência do Projeto	01/01/2017 a 01/12/2022
Espécie/linhagem	Camundongo heterogênico / Swiss
Nº de animais	1296
Peso/Idade	20-25 g / 2 meses
Sexo	Machos
Origem	Biotério Central / UEL
Amostras a serem coletadas	Tecido plantar cutâneo, lavado peritoneal

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente,

Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador do Projeto
Departamento de Ciências Patológicas / Centro de Ciências Biológicas
Com cópia para Coord. do Biotério Central/UEL; Chefe do Departamento de Ciências Patológicas e Diretor(a) do Centro de Ciências Biológicas

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 3371-4000 PABX - Fax 3328-4440 - Caixa Postal 10.011 - CEP 86057-970 – Internet http://www.uel.br
LONDRINA - PARANÁ - BRASIL

ANEXO B – Cópia do Parecer da Comissão de Ética no Uso de Animais (Nº 144/ 2020)

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 144/2020

Londrina, 19 de novembro de 2020.

Prezado (a) professor (a),

Certificamos que o projeto intitulado: **"Avaliação do efeito analgésico dos lipídeos pró-resolução em modelo murino de dor e infecção pelos vírus Dengue e Chikungunya e pela proteína E2 do vírus Chikungunya"** protocolo CEUA n° **056.2020** sob a responsabilidade de **Waldiceu Aparecido Verri Junior**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) em reunião realizada em **17/11/2020.**

Este projeto tem por objetivos avaliação dos potenciais analgésico e mecanismos de ação do tratamento intratecal com 15-Epi-lipoxina A4 (15-epi-LXA4), Resolvina D1, D2 e D5 (RvD1, RvD2 e RvD5), Maresina 2(MaR2), e Protectina DX (PDX) em modelo murinho de dor induzida pelos vírus Dengue e Chikungunya e pela proteína E2 do vírus Chikungunya. **Grau de invasividade: 3.**

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	01/01/2021 a 01/01/2025
Espécie/ linhagem/ raça	Camundongo heterogênico/ Swiss
N° de animais	**4.608**
Peso/ Idade	20-25 g/ 2 meses
Sexo	Machos
Origem	Biotério Central da Universidade Estadual de Londrina
Amostras a serem coletadas	Medula espinal, gânglio da raiz dorsal.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Em cumprimento às exigências do Conselho Nacional de Controle de Experimentação Animal (CONCEA), em até 30 dias da finalização do projeto de pesquisa ou extensão envolvendo o uso de animais (verificar período de vigência expresso neste ofício), é necessário encaminhar relatório da descrição de uso de animais para ceua@uel.br, conforme modelo disponível no site da CEUA: http://www.uel.br/comites/ceua/pages/relatorio-de-projetos.php.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente.

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Ilmo.(a) Sr.(a)
Prof. (a) Dr (a). Waldiceu Aparecido Verri Junior
Responsável pelo projeto
C/C para a Chefia do Departamento de Ciências Patológicas/ CCB
C/C para a Direção do Centro de Ciências Biológicas/ CCB
C/C para o Biotério Central da Universidade Estadual de Londrina/ CCB

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