Universidade Estadual de Londrina

VICTOR FATTORI

O MEDIADOR LIPÍDICO PRÓ-RESOLUÇÃO MARESINA-1 INIBE NEURÔNIOS CGRP-POSITIVOS:

CONTROLE DA DOR INFLAMATÓRIA E ALTERNATIVA NÃO-HORMONAL PARA O TRATAMENTO DA ENDOMETRIOSE

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Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior

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"Perhaps few persons who are not physicians can realize the influence of which long-continued and unendurable pain can have upon both body and mind".

Silas Weir Mitchell, *Injuries of Nerves and Their Consequences*, 1872.

FATTORI, Victor. **O mediador lipídico pró-resolução Maresina-1 Inibe neurônios CGRP-positivos:** controle da dor inflamatória e alternativa não-hormonal para o tratamento da endometriose. 2020. 222 f. Tese de Doutorado (Programa em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2020.

RESUMO

Maresina-1 (MaR1) é um mediador lipídico pró-resolução (SPM) com efeito anti-inflamatório potente. Assim, o objetivo geral dessa tese foi avaliar o efeito da Maresina-1 em silenciar nociceptores, podendendo assim, atuar no controle da dor inflamatória e como alternativa não hormonal para o tratamento da endometriose. Espcificamente, os objetivos dessa tese foram 1) avaliar o efeito analgésico e anti-inflamatório Maresina-1 em modelo de dor e inflamação induzidos pelo adjuvante completo de Freund (CFA) e carragenina; 2) padronizar um modelo não cirúrgico de dor associada à endometriose; 3) avaliar o efeito da MaR1 no modelo de endometriose. Ademais, essa tese oferece revisões sobre o efeito analgésico dos SPMs e sobre importância da capsaicina e TRPV1 na modulação da dor e inflamação. No primeiro trabalho, demonstramos que a MaR1 reduziu a hiperalgesia mecânica e térmica induzida por carragenina e CFA e o recrutamento de neutrófilos e macrófagos próximo a fibras $CGRP^+$ na pele da pata. Além disso, a MaR1 reduziu ativação das células da glia e produção de IL-1β e TNF-α e na medula espinal. No gânglio da raiz dorsal (DRG), MaR1 reduziu a expressão do RNAm de *Nav1.8* e *Trpv1*, e a liberação de CGRP. Os efeitos analgésicos e anti-inflamatórios duradouros da MaR1 (5 dias com um único tratamento) sugerem que os SPMs se tornar uma nova classe de drogas importantes para o tratamento da dor inflamatória. Para o segundo trabalho, nos propusemos a estabelecer um modelo não cirúrgico de dor associada à endometriose em camundongos. A endometriose é uma doença inflamatória que afeta aproximadamente 10% das mulheres e que cursa com dor pélvica ou abdominal debilitante. Além de requererem cirurgia, os modelos existentes não induzem a dor espontânea, que é o principal sintoma de pacientes com endometriose. A endometriose foi induzida não cirurgicamente pela injeção intraperitoneal de fragmentos dissociados de útero de camundongo doador em camundongo receptor. Nosso modelo produz mudanças neuronais e comportamentais (como presença de dor evocada e dor espontânea) que mimetizam a doença em mulheres. Esse modelo também responde a fármacos clinicamente ativos (letrozol e danazol) e pode ser usado, portanto, para avaliar o efeito de novas terapias. Dessa forma, o próximo objetivo foi investigar o efeito da MaR1 no modelo de endometriose. O tratamento com a MaR1 não só reduz a dor abdominal, mas também diminuiu a porcentagem de camundongos com lesões e o tamanho das lesões. Parte desse efeito está relacionado com a inibição da ativação de neurônios $CGRP^+$, como observado pela diminuição da colocalização de CGRP com NF-κB por imunofluorescência no DRG. Em conclusão, demonstramos que a MaR1 apresenta um efeito analgésico duradouro (5 dias após um único tratamento) no modelo de dor induzida por CFA que está relacionado a sua capacidade em bloquear a ativação de nociceptores $TRPV1^+$ e a liberação do neuropeptídeo CGRP. Ademais, padronizamos um modelo não-cirúrgico de dor associada à endometriose que é sensível a fármacos clinicamente ativos, como letrozol e danazol. Demonstramos também que a MaR1 reduz não apenas a dor abdominal, mas também a porcentagem de camundongos com lesões e o tamanho

das lesões endometrióticas. Parte desse efeito está relacionado com a habilidade da MaR1 em reduzir a ativação de nociceptores $CGRP^{+}$. Esses dados indicam que a MaR1 é uma molécula promissora para o tratamento de doenças inflamatórias, como a endometriose.

Palavras chaves: dor abdominal, dor crônica, resolução da inflamação, ômega-3, analgesia, dor espontânea

FATTORI, Victor. **The pro-resolving lipid mediator Maresin-1 Inhibits CGRP-positive neurons:** reduction of inflammatory pain and non-hormonal alternative for the treatment of endometriosis. 2020. 222 p. PhD Thesis (Experimental Pathology Program) – Londrina State University, Londrina, 2020.

ABSTRACT

Maresin-1 (MaR1) is a specialized pro-resolving lipid mediator with potent anti-inflammatory activity. We aimed at determining whether MaR1 can silence nociceptors to reduce inflammatory pain and emerge as a non-hormonal alternative for endometriosis treatment. Specifically, we aimed at 1) address the analgesic and anti-inflammatory effect of MaR1 in the carrageenan or complete Freund's Adjuvant (CFA) models; 2) set out a non-surgical model of endometriosis-associated pain, and 3) investigate the effect of MaR1 in the endometriosis model. This thesis also brings two reviews about 1) the analgesic (without immunosuppressive) effects of SPMs and 2) the role of capsaicin and TRPV1 in modulating inflammatory response. In the first original research work, MaR1 reduced carrageenan- and CFA-induced mechanical and thermal hyperalgesia, and neutrophil and macrophage recruitment proximal to $CGRP^+$ fibers in the paw skin. Moreover, MaR1 reduced spinal cord glial cells activation and TNF-α and IL-1β production. In the DRG, MaR1 reduced CFA-induced *Nav1.8* and *Trpv1* mRNA expression, calcium influx in $TRPV1^+$ nociceptors, and capsaicin-induced release of CGRP by DRG neurons. The enduring analgesic and anti-inflammatory of MaR1 suggest that the SPMs may become a new class of drugs important for the treatment of inflammatory pain. For the second original research work, we set out to establish a murine model of endometriosis-associated pain. Endometriosis is an estrogen-dependent inflammatory disease that affects approximately 10% of women. Debilitating pelvic or abdominal pain is one of its major clinical features. Current animal models of endometriosis-associated pain require surgery either to implant tissue or to remove the ovaries and do not induce spontaneous pain, which is the primary symptom of patients with chronic pain, including endometriosis. Endometriosis was induced non-surgically by injecting intraperitoneally a dissociated uterine horn into a recipient mouse. In conclusion, we have established a model of endometriosis-associated pain that recapitulates neuronal and behavioral changes in women with endometriosis. In addition, this model responds to clinically active drugs and can be used, therefore, to screen for novel therapies. Therefore, we next addressed whether MaR1 reduces endometriosis-associated pain. We showed that MaR1 reduces the activation of a subpopulation of nociceptors that are responsible for producing neuropeptides such as CGRP. We also demonstrated that MaR1 not only reduced pain but also the number of mice with endometriotic lesions, indicating it is a promising drug for the treatment of endometriosis. In conclusion, we demonstrated that MaR1 presents a long-lasting analgesic effect (5 days after a single treatment) in the CFA-induced pain model which is related to the its ability to block the activation of $TRPV1^+$ nociceptors and the release of the neuropeptide CGRP. Furthermore, we set out a non-surgical pain model associated with endometriosis that is sensitive to clinically active drugs such as letrozole and danazol. In terms of new molecules, treatment with MaR1 reduced not only abdominal pain but also the percentage of mice with lesions and lesion size. Part of that effect is related to MaR1's ability to reduce the activation of $CGRP^+$ nociceptors. These data

indicate that MaR1 is a promising molecule for the treatment of inflammatory diseases such as endometriosis.

Keywords: abdominal pain, chronic pain, resolution of inflammation, omega-3 fatty acids, pain treatment, spontaneous pain

SUMÁRIO

1 INTRODUÇÃO

O livro *Treatise of Man* (1664) de René Descartes foi um marco no entendimento da fisiologia da dor no mundo moderno. Utilizando seu icônico diagrama de um garoto próximo a uma fogueira, Descartes proprunha que o fogo ativa fibras em locais periféricos para posterior transmissão ao cérebro. O reconhecimento dessa informação promoveria uma ação de retirada do pé de próximo do estímulo nocivo (no caso de Descartes, as partículas de fogo). Para Descartes, a dor era apenas uma consequência de uma ativação linear periférica para o cérebro (MOAYEDI; DAVIS, 2013), definição essa que não é errônea, apenas incompleta. Muito se evoluiu desde então, e parte dessa evolução na compreensão dos mecanismos da dor passa pela descoberta do receptor TRPV1 (receptor de potencial transitório subfamília V, membro 1) (CATERINA *et al.*, 1997). Esse trabalho demonstrou que o reconhecimento de estímulos nocivos pelos nociceptores se dava através da expressão de canais em seus terminais. O artigo número 5 dessa tese (item 3.5) publicado no periódico *Molecules* revisa de forma minuciosa a importância da capsaicina (principal componente da pimenta e agonista de TRPV1) e TRPV1 na modulação da dor e inflamação (FATTORI *et al.*, 2016). O conhecimento gerado decorrente dessa revisão foi essencial para auxiliar no desenho experimental do artigo publicado no periódico *British Journal of Pharmacology* (FATTORI *et al.*, 2019) e do artigo aceito no periódico *Pain* (item 3.2, ambos fazem parte dessa tese).

Durante a Guerra Civil dos Estados Unidos (1861-65), Silas Weir Mitchell, um médico contratado pelo país, fez relatos poderosos sobre como a dor impactava em soldados com ferimentos de balas (MITCHELL; MOREHOUSE; KEEN, 1864). Em seu livro *Gunshots Wounds, and Other Injuries* escrito em 1864, ele descreveu que mesmo após a remoção das balas, a dor frequentemente persistia por

longos períodos (MITCHELL; MOREHOUSE; KEEN, 1864) Em um dos relatos, o soldado descreve a dor: “como se um ferro quente de passar roupas estivesse sob a palma da mão, com um peso em cima, e como se a pele estive sendo arrancada das pontas dos dedos” (MITCHELL; MOREHOUSE; KEEN, 1864). Assim, é possível concluir que a dor crônica afeta negativamente a vida das pessoas, pois limita a realização de tarefas cotidianas.

A endometriose é uma doença que afeta até 10% das mulheres e sua principal característica clínica é dor abdominal crônica e debilitante (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015). Essa dor pélvica crônica e debilitante contribui de forma significativa para o desenvolvimento de sintomas depressivos (GAMBADAURO; CARLI; HADLACZKY, 2018) e em mulheres adolescentes está associada a maiores taxas de ansiedade, depressão e problemas com o trabalho ou outras atividades diárias (GALLAGHER *et al.*, 2018). No que tange à dor, os modelos atuais de endometriose em roedores não são capazes de reproduzir o fenótipo observado em mulheres. Dessa forma, o desenvolvimento de modelos que mimetizem de forma mais fidedigna a doença é essencial para a descoberta de novos fármacos para o tratamento da endometriose. O artigo número 2 dessa tese (item 3.2) descreve a padronização de um modelo não cirúrgico de dor associada à endometriose.

A descoberta dos mediadores lipídicos pró-resolução (*Specialized Pro-Resolving Lipid Mediators*, SPMs) mudou o paradigma de como a inflamação termina. Hoje sabemos que a resolução da inflamação é um processo ativo e dependente da produção de SPMs. O artigo número 3 dessa tese (item 3.3) traz uma revisão extensiva sobre os efeitos dos SPMs no controle da dor e infecção, tendo em vista que essa classe de moléculas inibe dor sem efeito imunossupressor (FATTORI

et al., 2020). A Maresina-1 é um SPM derivado do ácido docosahexaenóico (DHA) que age diretamente em neurônios TRPV1^{+} e CGRP^{+} (FATTORI *et al.*, 2019; SERHAN *et al.*, 2012) para produzir um efeito analgésico duradouro (FATTORI *et al.*, 2019).

Assim, o objetivo geral dessa tese foi avaliar o efeito da Maresina-1 em silenciar nociceptores, podendendo assim, controlar a dor inflamatória e atuar como alternativa não hormonal para o tratamento da endometriose. Espcificamente, os objetivos foram 1) avaliar o efeito analgésico e anti-inflamatório da Maresina-1 em modelo de dor e inflamação induzidos pelo adjuvante Complete de Freund (CFA) e carragenina; 2) padronizar um modelo não cirúrgico para estudar dor associada à endometriose em camundongos; 3) avaliar o efeito do SPM Maresina-1 no modelo de endometriose.

2 REVISÃO DA LITERATURA

2.1 Neurônios Nociceptivos e Suas Heterogeinedades

Ao longo da evolução os organismos foram selecionados para sentir e detectar estímulos externos com o objetivo de se protegerem dos perigos potenciais do ambiente. Essa habilidade depende da capacidade dos circuitos neurais em receber e processar essas informações. Para isso, o sistema nervoso somatossensorial está densamente distribuído no corpo. A percepção desses estímulos externos como temperatura, pressão, dor e etc, se dá pela disposição de células especializadas para tal. Essas células são chamadas de nociceptores, cujos corpos celulares encontram-se no gânglio da raiz dorsal (DRG) ou trigêmio. Em organismos evoluídos, esses neurônios estão posicionados na pele, mucosas e tecidos linfóides primários e secundários para modular, além da percepção de estímulos, a imunidade. Para o reconhecimento de estímulos potencialmente nocivos, os nociceptores expressam receptores de alto limiar em suas porções terminais (BASBAUM *et al.*, 2009; CHIU; VON HEHN; WOOLF, 2012; PINHO-RIBEIRO; VERRI; CHIU, 2017). Após ativados, há o aumento do influxo de íons, como o cálcio, permitindo a despolarização dos nociceptores. Os nociceptores apresentam diferentes modalidades sensoriais que estão baseadas na expressão desses canais. Enquanto que, por exemplo, o receptor TRPV1 é ativado por calor nocivo (temperaturas maiores que 43 °C), o TRPM8 (receptor de potencial transitório subfamília M, membro 8) responde ao frio inócuo e nocivo (entre 10-15 °C com ativação parcial a partir de 26 °C) (CROSSON *et al.*, 2019; DUBIN; PATAPOUTIAN, 2010). Os ASICs (canais iônicos sensíveis à acidez) são ativados por diminuição no pH extracelular, enquanto o Piezo2 é necessário para a nocicepção mecânica, mas não para a sensação de toque suave (CROSSON *et al.*, 2019). De modo geral, os canais TRP são bastante

promíscuos, podendo ser ativados por estímulos diferentes. O TRPV1, por exemplo, além do calor, também é ativado por produtos químicos irritantes exógenos e endógenos, incluindo capsaicina (o componente pungente na pimenta) e baixo pH (BENARROCH, 2015; DUBIN; PATAPOUTIAN, 2010; FATTORI *et al.*, 2016). O TRPA1 (receptor de potencial transitório subfamília A, membro 1) responde a uma grande variedade de irritantes químicos, como óleo de mostarda (o ingrediente pungente da mostarda e do wasabi), alina (responsável pelo cheiro característico de alho), e cinemaldeído (responsável pelo cheiro característico da canela). Ademais, o TRPA1 é um sensor de EROs (espécies reativas de oxigênio), e responde também ao frio e pressão mecânica (BENARROCH, 2015; CROSSON *et al.*, 2019; DUBIN; PATAPOUTIAN, 2010). Esse vasto repertório permite que os nociceptores reconheçam de forma eficaz diversas ameaças ambientais.

De modo geral, há dois tipos de fibras nociceptivas: A e C, sendo que a primeira se subdivide em Aβ e Aδ (BRAZ *et al.*, 2014). Em condições fisiológicas, enquanto que as fibras A são mielinizadas e responsáveis pela percepção de toque leve na pele, as fibras C são não mielinizadas e responsáveis pela detecção de estímulos de alto limiar (capazes de gerar dor) (BRAZ *et al.*, 2014). Inicialmente, a classificação de neurônios sensoriais se dava através da determinação do diâmetro do axônio e se produziam ou não neuropetídeos, como CGRP (peptídeo relacionado ao gene da calcitonina) e substância P. Isso era feito através da utilização de marcadores como NF200 (*neurofilament-200*, marcador de neurônios mielinizados – diâmetro largo), $IB4^{+}$ (*isolectin B4*, marcador de neurônios não mielinizados e não peptidérgicos) e $CGRP^{+}$ ou $IB4^{-}$ (*calcitonin gene-related peptide*, marcador de neurônios não mielinizados e peptidérgicos) (WOOLF; MA, 2007). Atualmente, é possível identificar, no DRG por exemplo, milhares de neurônios aferentes primários

que, apesar de compartilharem algumas funções, apresentam grande heterogeneidade com modalidades sensoriais próprias e identidades moleculares distintas. Utilizando neurônios DRG que foram separados por citometria de fluxo (*FACS sorted*) de camundongos Nav1.8-Cre/TdTomato+ (expressa proteína vermelha fluorescente em neurônios Nav1.8^{+} – fibras C) e Parv-Cre/TdTomato (expressa proteína vermelha fluorescente em neurônios relevantes para propriocepção – fibras A), Chiu e colaboradores identificaram 6 clusteres de neurônios sensoriais (CHIU *et al.*, 2014). Dentre esses clusteres, é possível destacar os Grupos II, V e VI. O Grupo II consiste de neurônios *Trka^{hi}Nav1.8^{+}Trpv1^{+}Aquaporin^{+}*, que são relacionados com fibras C termosenssíveis, o Grupo V consiste de *Th^{+}Nav1.8^{+}Trka^{-}Trpv1^{-}*, que é característico de fibras C mecanossensíveis de baixo limiar. O Grupo VI são neurônios *Nbpp^{+}IL31^{+}* relacionados com coceira (CHIU *et al.*, 2014). É relevante destacar que nenhum neurônio de camundongos Parv-Cre/TdTomato foram positivos para os canais TRP (como o *Trpv1* e *Trpa1*, por examplo, relacionados a detecção de estímulos térmicos e químicos). Isso reflete a característica das fibras C em detectar estímulos químicos e térmicos de alto limiar (CHIU *et al.*, 2014). Utilizando a técnica de sequenciamento de RNA em células individuais (*single cell RNA-seq*), um estudo demonstrou que os nociceptores podem ser divididos inicialmente em 4 clusteres: NF (positivos para NF200, marcador de neurônios mielinizados), PEP (positivos para CGRP e substância P, neurônios peptidérgicos), NP (positivos para Mrgprd, neurônios não peptidérgicos e polimoidais) e TH (positivos para *tyrosine hydroxylase*, uma classe distinta de neurônios não mielinizados) (USOSKIN *et al.*, 2015). A análise mais detalhada desses nociceptores foi capaz de revelar a presença de múltiplos clusteres neuronais com 11 subtipos, sendo eles: três grupos distintos de neurônios mecanorreceptores de baixo limiar, dois proprioceptivos e seis tipos principais de

neurônios termossensíveis, sensíveis à coceira, neurônios mecanossensíveis e nociceptores de baixo limiar do tipo C. Todos esses com propriedades moleculares e operacionais marcadamente diferentes (USOSKIN *et al.*, 2015). Relevante para o contexto de dor, é possível destacar o cluster positivo para substância P PEP1, caracterizado por *Trka*$^{+}$*Tac1*$^{+}$*Cgrp*$^{+}$ (além de expressar *Trpv1*, *Nav1.8* e *9*) o cluster PEP2, caracterizador por *Fam19a1*$^{+}$*Calca*$^{+}$*Nefh*$^{+}$ (além de expressar *Nav1.8* e *9*) (USOSKIN *et al.*, 2015). Esses estudos ilustram a diversidade dos nociceptores e a complexidade envolvendo as sensações somáticas.

2.2 Fisiologia da Dor: Aspectos Gerais

A dor nociceptiva funciona como um dispositivo de alerta para chamar a nossa atenção para o perigo potencial no ambiente. Esse tipo de dor dispõe de um mecanismo de proteção que está intimamente ligado a geração de emoções negativas. Portanto, para ser eficaz, a dor nociceptiva deve desencadear uma resposta fisiológica que o hospedeiro não possa ignorar. De modo geral, o mecanismo ascendende da dor envolve o impulso nervoso percorrer o nociceptor primário até o corno dorsal da medula espinal, onde ocorre sinapses com as terminações dos nociceptores de segunda ordem. As fibras C e Aδ conduzem as informações para áreas nociceptivas específicas nas lâminas I e II da superfície do corno dorsal da medula espinal e também para lâmina V. Por outro lado, fibras mielinizadas Aβ transmitem informações, como toque leve ou estímulos mecânicos inócuos, para estruturas no corno dorsal da medula espinal nas lâminas III e IV (TODD, 2010). Essa comunicação entre o nociceptor primário e secundário é mediada por neurotransmissores excitatórios (glutamato, por exemplo), neuropeptídeos e citocinas que são liberados pelo neurônio nociceptivo primário e células da glia na fenda

sináptica (BRAZ *et al.*, 2014; SCHOLZ; WOOLF, 2002). Como recurso compensatório, dispomos de mecanismos inibitórios da dor (mecanismos descendentes). Esses envolvem a liberação de mediadores inbitórios (GABA, canabinóides e β-endorfina, por exemplo) de modo a limitar a despolarização dos nociceptores ativados (MILLAN, 2002). Por fim, a percepção é o fornecimento da informação referente ao estímulo inicial (através de sinapses no córtex somatossensorial) sobre a localização e a intensidade do estímulo nocivo. Para o componente emocional do processo doloroso, ocorrem sinapses na região da amígdala (BASBAUM *et al.*, 2009; BRAZ *et al.*, 2014).

Embora desagradável, a dor é o comportamento mais importante preservado durante o processo de evolução. Indivíduos diagnosticados com insensibilidade congênita à dor, apresentam expectativa de vida reduzida uma vez que não conseguem diferenciar os estímulos nocivos de não nocivos. Tal importância do aspecto emocional da dor, que o comitê de taxonomia da IASP (Associação Internacional para o Estudo da Dor) define a dor como "uma experiência sensorial e emocional desagradável associada a danos reais ou potenciais nos tecidos, ou descrita em termos de tais danos". Reconhece-se agora, também, que o humor, a função cognitiva, as memórias, o estado de humor, a atenção (BUSHNELL; CEKO; LOW, 2013), e as expectativas quanto à intensidade do estímulo (WIECH *et al.*, 2014) também moldam a percepção da dor. Desse modo, cada indivíduo interpreta a dor de acordo com suas experiências, o que torna inconcebível a dissociação do caráter emocional para seu entendimento.

Há também evidências de como a empatia modula a percepção da dor do próprio indivíduo e dos outros. O que se pensava ser exclusivo dos primatas superiores (possivelmente apenas humanos), novas evidências demonstram que os roedores também podem mostrar empatia (LANGFORD *et al.*, 2006; SMITH *et al.*,

2016). Através de “sugestões olfativas” (*olfactory cues*) dentro do ambiente social, camundongos *naive* apresentam aumento de comportamentos relacionados à dor após contato com “camundongos colegas de gaiolas” (*cage mate mice*) injetados com estímulos que causam dor. Esses dados indicam que os camundongos podem mostrar uma resposta empática quando em contato com *cage mate mouse* que está com dor (LANGFORD *et al.*, 2006; SMITH *et al.*, 2016). Corroborando estes dados, um estudo recente em ratos demonstra que uma pequena população de neurônios no córtex cingulado anterior (ACC) são ativados enquanto testemunha dor em outros, porém não são ativados em situações de medo (*foot shooks* e enquanto ouve *fear-conditioned sound*) (CARRILLO *et al.*, 2019). Esses dados indicam que a empatia é um comportamento conservado durante a evolução e relevante para a compreensão atual da dor.

Ademais, evidências recentes demonstram que as células periféricas de Schwann também podem iniciar comportamentos dolorosos induzidos por um estímulo mecânico em camundongos (ABDO *et al.*, 2019). O cruzamento de camundongos da linhagem Sox10-Tomato com camundongos da linhagem *channelrhodopsin-2(ChR2)-enhanced YFP* dá origem à camundongos Sox10-ChR2 que são sensíveis a estímulo luminoso. A ativação optogenética das células $Sox10^{+}$ (marcador celular de Schwann) demonstram que sua ativação é suficiente para induzir o disparo neuronal periférico e produzir dor mecânica (ABDO *et al.*, 2019).

A mudança na compreensão de como a dor ocore passa pela descoberta do receptor TRPV1 (CATERINA *et al.*, 1997). Antes dessa descoberta, a injeção intradérmica de capsaicina (principal componente da pimenta e agonista seletivo de TRPV1) era usada para produzir hipersensibilidade primária e secundária a estímulos nocivos e inócuos, tanto em macacos quanto em ratos (BAUMANN *et al.*,

1991; SLUKA; WILLIS, 1997). Trabalhos seminais demonstraram que a capsaicina é capaz de ativar os nociceptores, aumentando o influxo de íons, como o cálcio, nos neurônios DRG (OH; HWANG; KIM, 1996; WOOD *et al.*, 1988). Anos mais tarde, a clonagem do TRPV1 esclarece o mecanismo pelo qual a capsaicina induz a dor (CATERINA *et al.*, 1997). Este trabalho é um marco no que se refere aos mecanismos da dor, pois demonstrou que a capsaicina ativa diretamente receptores TRPV1 expressos pelos nociceptores para produzir dor. Mais importante ainda, essa descoberta mudou nossa compreensão dos mecanismos da dor, pois demonstra que receptores expressos por nociceptores detectam estímulos ambientais resultando na despolarização dessas células especializadas e consequentemente produzindo dor.

2.2.1 Dor Patológica

A sensibilização periférica e central e alterações nos fenótipos das células imunológicas fazem parte da compreensão contemporânea da dor patológica persistente. Enquanto que a dor nociceptiva é desencadeada pela ativação de nociceptores de alto limiar, na dor inflamatória há uma mudança nesse padrão de ativação. A diminuição do limiar de ativação dos neurônios promove hipersensibilidade mecânica e térmica, em que estímulos não nocivos podem, agora, gerar dor (FATTORI, VICTOR *et al.*, 2017; VERRI *et al.*, 2006; WOOLF; SALTER, 2000). Esse fenômeno é conhecido como hiperalgesia (aumento da dor devido a um estímulo que normalmente provoca dor) ou alodinia (sensibilidade a um estímulo que não provoca dor) (FATTORI, VICTOR *et al.*, 2017; VERRI *et al.*, 2006; WOOLF; SALTER, 2000).

Durante a inflamação, um evento chave para dor é o recrutamento de células imunológicas inatas periféricas para o foco inflamatório (PINHO-RIBEIRO; VERRI; CHIU, 2017). A presença de neutrófilos é um denominador comum no que se

refere a dor inflamatória e desempenham um papel fundamental na manutenção da mesma (CUNHA *et al.*, 2008). Dentre outros mediadores, neutrófilos recrutados produzem EROs, PGE_2 e citocinas pró-inflamatórias como IL-1β, TNF-α e IL-33 (VERRI *et al.*, 2006). No modelo de inflamação plantar por carragenina, foi demonstrado que uma cascada de citocinas pró-inflamatórias ocorre para produção da dor. A cascata se inicia com a liberação da alarmina IL-33 (ZARPELON *et al.*, 2013) a qual estimula a produção sequencial de 1) TNF-α, IL6, IL-1β e PGE_2; e 2) TNF-α, CXCL1, IL-1β e aminas simpáticas (CUNHA *et al.*, 2005; VERRI *et al.*, 2006). Essa cascata de produção de citocinas ocorre em outros modelos de dor, como por exemplo na artrite. No modelo de artrite induzida por antígeno, a IL-15 induz a produção sequencial de IFN-γ, ET-1 e PGE_2 para induzir dor inflamatória articular (VERRI *et al.*, 2007). Atuando em receptores específicos, esses mediadores inflamatórios como a PGE_2, histamina e citocinas são os principais responsáveis pela redução do limiar neuronal e pela produção de sensibilização periférica (PINHO-RIBEIRO; VERRI; CHIU, 2017). Ao se ligarem em seus receptores, essas moléculas ativam kinases (PKA e PKC, por exemplo), que por sua vez, fosforilam canais de iônicos expressos pelos nociceptores (TRPV1, TRPA1, Nav1.8; por exemplo) (VERRI *et al.*, 2006). Ademais, além de estimular a produção de outras citocinas, IL-33 (LIU *et al.*, 2016), IL-1β (BINSHTOK; BEAN; WOOLF, 2007) e TNF-α (JIN; GEREAU, 2006) podem também ativar diretamente os neurônios. Utilizando cultura de neurônios DRG, foi possível demonstrar que a IL-33, por exemplo, ativa aproximadamente 85% de neurônios $TRPV1^+$ e 68% $TRPA1^+$ (LIU *et al.*, 2016). A ativação dessas subpopulações neuronais foi demonstrada através do aumento no influxo de cálcio, que é um indicador de ativação dessas células. Aumentando a lista de mediadores, um estudo recente demonstrou que os neurônios reconhecem o imunocomplexo de

colágeno tipo 2 para produzir dor em modelo de artrite (BERSELLINI FARINOTTI *et al.*, 2019). Agindo sobre os receptores FcγRI e FcγRIIb expressos pelos neurônios TRPV1^{+} do DRG, o imunocomplexo de colágeno tipo 2 aumenta a excitabilidade neuronal e liberação do neuropetídeo CGRP para produzir dor (BERSELLINI FARINOTTI *et al.*, 2019).

A inflamação crônica induz neuroplasticidade que modifica o fenótipo e a função do nociceptor no sistema nervoso central. Esse fenômeno é descrito como sensibilização central e é reconhecido como a principal causa de dor patológica persistente. Sua descoberta pelo Prof. Clifford Woolf representou uma mudança no paradigma do entendimento sobre a dor crônica (WOOLF, 1983). A sensibilização central foi inicialmente descrita como um aumento da transmissão sináptica excitatória e diminuição da transmissão sináptica inibitória no corno dorsal da medula espinal (WOOLF, 1983). O entendimento contemporâneo, no entanto, sugere que citocinas, quimiocinas e fatores de crescimento liberados pelas células residentes microglia, astrocitos e oligodendrócitos, contribuem para plasticidade neuronal na sensibilização central (FATTORI, VICTOR *et al.*, 2017; JI; CHAMESSIAN; ZHANG, 2016; PINHO-RIBEIRO; VERRI; CHIU, 2017). Enquanto neurotransmissores como glutamato, GABA e glicina produzem efeitos sinápticos na faixa de μM; citocinas, quimiocinas e fatores de crescimento liberados por astrocitos, microglia e oligodendrócitos fazem o mesmo em concentrações de nM (COULL *et al.*, 2005; GAO *et al.*, 2009; KAWASAKI *et al.*, 2008). Em um modelo de dor neuropática, por exemplo, os oligodendrócitos do corno dorsal da medula espinal são a principal fonte de IL-33 que estimula a produção de IL-1β e TNF-α por microglia e astrocitos (ZARPELON *et al.*, 2016). A produção dessas moléculas pró-inflamatórias pela microglia ocorre em um mecanismo dependente da MAPK p38 e em astrocitos em um mecanismo dependente de JNK

(FATTORI, VICTOR *et al.*, 2017; JI; CHAMESSIAN; ZHANG, 2016; PINHO-RIBEIRO; VERRI; CHIU, 2017). As MAPKs também podem ser ativadas por IL-1β, TNF-α e IL-33, resultando em produção adicional de mediadores importantes na sensibilização central, como IL-1β, TNF-α, IL-6, PGE_2 e BDNF (FATTORI, VICTOR *et al.*, 2017; JI; CHAMESSIAN; ZHANG, 2016; PINHO-RIBEIRO; VERRI; CHIU, 2017).

2.2.2 Reprogramação Neuronal Após Lesão

A lesão dos terminações periféricas de neurônios induz o reprogramação transcricional de genes que são essenciais para iniciar o crescimento dos axônios, mas ao mesmo tempo, induz hiperexcitabilidade neuronal patológica que é subjacente à dor. Através da técnica de *single cell RNA-seq*, é possível verificar a formação de novos clusteres de neurônios expressando novos marcadores após lesão de nervo (HU *et al.*, 2016; NGUYEN; LE PICHON; RYBA, 2019; SUN *et al.*, 2019). Em um modelo de transecção do nervo infraorbital em camundongos, é possível verificar a rápida formação (2 dias após lesão) de um novo *cluster* de neurônios (NGUYEN; LE PICHON; RYBA, 2019). Esse novo *cluster* é caracterizado por neurônios positivos para *Atf3* (marcador de lesão neuronal), *Sox11* (fator de transcrição que regula crescimento neuronal), e *Gal* (galanina, um neuropetídeo), dentre outros (NGUYEN; LE PICHON; RYBA, 2019). Um outro estudo, utilizando um modelo de ligação do nervo ciátivo (*spinal nerve ligation*) em ratos, demonstra a formação de uma nova população de neurônios positivas para diversos marcadores como *Gal*, *Atf3*, *Serpina3n* (inibidor de serina protease) e *Hspb1* (proteína de choque térmico) (SUN *et al.*, 2019). Portanto, em resposta a estímulos nocivos, novos clusteres de neurônios são formados na tentativa de controlar os danos. No entanto, esse fenômeno frequentemente leva a modificações maladapativas que são inerentes à dor. Devido à

grande heterogeneidade dos neurônios (abordada na seção anterior), estudos avaliando individualmente essas mudanças transcripcionais possibilita o entendimento da programação de resposta de cada neurônio frente à lesão. Dessa forma, os estudos revisados nessa seção contribuem não só para o desenvolvimento de novas terapias para a dor, mas também para elucidação da biologia de neurônios sensoriais após a lesão.

2.3 Regulação da Resposta Imunológica por Nociceptores

Além dos mediadores mencionados anteriormentes, os nociceptores também podem reconhecer produtos derivados de patógenos, como estreptolisina S (PINHO-RIBEIRO *et al.*, 2018), α-hemolisina (CHIU *et al.*, 2013), e β-glucana (MARUYAMA *et al.*, 2017). Dessa forma, apesar de antes vistos como espectadores no contexto de infecções, estudos recentes demonstram que após a ativação por esses produtos derivados de patógenos, os nociceptores orquestram a inflamação neurogênica e a atividade das células imunes inatas e adaptativas de forma dependente do contexto. Por exemplo, enquanto em um contexto de infecção intestinal induzida por *Salmonella* enterica serovar Typhimurium, o bloqueio dos nociceptores piora a infecção (LAI *et al.*, 2019), na infecção por *Streptococcus pyogenes*, melhora o resultado da doença (PINHO-RIBEIRO *et al.*, 2018). Especificamente, a estreptolisina S derivada da bactéria *S. pyogenes* ativa diretamente os nociceptores estimulando a liberação do neuropeptídeo CGRP. Ao se ligar em seu receptor expresso por neutrófilos, o CGRP diminui a atividade bactericida dos neutrófilos (PINHO-RIBEIRO *et al.*, 2018). O bloqueio da liberação de CGRP, utilizando a toxina botulínica tipo A ou utilizando a linhagem de camundongos TRPV1-Cre/DTA (elimina neurônios $TRPV1^{+}$) reduz os danos causados pela bactéria (PINHO-RIBEIRO *et al.*, 2018). Nociceptores e células

imunes, portanto, evoluíram para comunicarem entre si (comunicação neuroimune) de modo a controlar as respostas inflamatória e do hospedeiro contra patógenos de uma forma complementar, e ao mesmo tempo distinta. Essa comunicação neuroimune é iniciada após a liberação de neuropeptídeos CGRP, VIP e substância P (PINHO-RIBEIRO; VERRI; CHIU, 2017). Um estudo recente demonstrou que a ativação de nociceptores TRPV1$^+$ é suficiente para iniciar uma resposta imune antecipatória na ausência de outros estímulos inflamatórios (COHEN *et al.*, 2019). A liberação de CGRP por nociceptores após ativação optogenética aumenta a produção de IL-17 TCRγδ que, em última instância, fornece imunidade do tipo 17 em áreas adjacentes ao local do estímulo e aumentam a defesa do hospedeiro contra *C. albicans* no tecido adjacente (COHEN *et al.*, 2019). Esse trabalho revela um novo papel para os nociceptores no controle da imunidade inata antecipatória em áreas adjacentes ao local da infecção.

Em modelos de doenças inflamatórias estéreis, a ablação dos nociceptores usando a linhagem de camundongo Nav1.8-Cre/DTA (elimina neurônios Nav1.8$^+$) ou QX-314 (uma molécula derivada da lidocaína que silencia os nociceptores) reduz o número de leucócitos recrutados no BALF (líquido de lavagem bronco-alveolar) nos modelos de rinite alérgica e asma (TALBOT *et al.*, 2015), indicando um papel para os neurônios no controle da inflamação. Os nociceptores também podem interagir com outras células residentes, como as células dendríticas, para regular a resposta inflamatória na pele (RIOL-BLANCO *et al.*, 2014). Após ativados, os nociceptores estimulam a liberação de IL-23 por células dendríticas que ativam linfócitos TCRγδ para produzirem IL-17. Essa citocina ainda contribui para o influxo de neutrófilos e macrófagos para o local da lesão. Corroborando, ablação de nociceptores Nav1.8$^+$ e TRPV1$^+$ reduz a inflamação na pele (RIOL-BLANCO *et al.*, 2014). Em conjunto, esses

estudos demonstram que os nociceptores são capazes de regular de forma ativa a inflamação e atividade de células do sistema imune.

2.4 ENDOMETRIOSE

A endometriose é uma doença inflamatória dependente de estrógeno que afeta de 5 a 10% das mulheres em idade reprodutiva nos Estados Unidos e de até 50% em mulheres inférteis (BULUN, 2009). A endometriose é caracterizada histologicamente pela presença ectópica de tecido (lesões) mimetizando o aparecimento de endométrio uterino. Essas lesões são definidas por glândulas tipo endometrial, estroma e hemossiderina; e também contêm quantidades variáveis de vasos sanguíneos, fibras nervosas, músculo e células imunes (BULUN, 2009). A menstruação retrógrada é a hipótese melhor aceita como o mecanismo primário pelo qual as lesões são formadas (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015). A menstruação retrógrada é definida pelo caminho reverso percorrido pelo fluxo sanguíneo menstrual. O transporte retrógrado (através das trompas de Falópio) faz com que haja depósito do sangue menstrual na cavidade peritoneal. Estudos recentes demonstram que mutações em genes comumente associados ao câncer também são encontradas lesões de mulheres com endometriose sem câncer (ANGLESIO *et al.*, 2017; SUDA *et al.*, 2018). Um estudo com 17 (ANGLESIO *et al.*, 2017) e outro com 54 pacientes (SUDA *et al.*, 2018) identificaram que mutações em genes como *KRAS* e *PIK3CA* em células endometrióticas epiteliais contribuem para a expansão clonal e formação de lesões. Dessa forma, a menstruação retrógrada contendo células epiteliais normais e com mutações em genes como *KRAS* e *PIK3CA* contribui para a formação de lesões em mulheres. Porém, em ambos estudos, apenas 20% das mulheres com lesões

apresentavam mutações para pelo menos 1 dos genes estudados. Dessa forma, outros mecanismos devem contribuir de forma relevante para a formação de lesões. Durante a menstruação retrógrada, DAMPs (padrões moleculares associados a danos) podem ser liberados e ativar células imunes residentes, incluindo macrófagos (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015). As células imunes ativadas produzem mediadores pró-inflamatórios e hiperalgésicos de forma dependente da NF-κB. Entre esses mediadores, as citocinas TNF-α, IL-1β e IL-33 contribuem para a dor e inflamação. Outros mediadores, como os fatores de crescimento NGF e BDNF produzidos pelas células imunológicas também desempenham um papel fundamental (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015). Em resposta a esses estímulos, os neurônios nociceptores aumentam e mantêm a inflamação através da liberação de neuropeptídeos, como a substância P e o CGRP (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015) além de quimicionas como CCL2 e CCL3 (GREAVES *et al.*, 2015). Esses mediadores contribuem para a formação de lesão, recrutamento de mais células imunes e dor (Figura 1) (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015). De fato, o TNF-α estimula a expressão do receptor para substância P (NK1R) e o bloqueio da interação da substância P com seu receptor NK1R reduz o número de células endometrióticas viáveis do estroma. Isso sugere que a substância P contribui para o crescimento e suporte da lesão endometriótica (MCKINNON *et al.*, 2013).

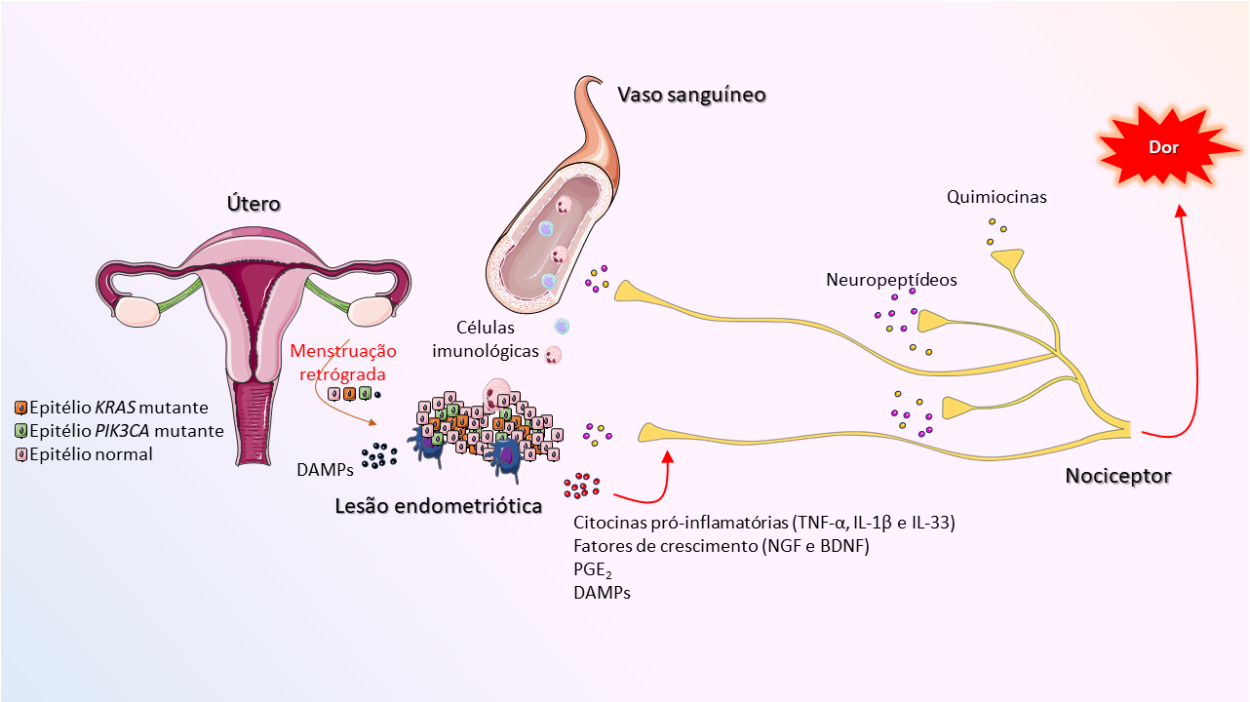

Figura 1. Fisiopatologia da endometriose. Durante a menstruação retrógrada, DAMPs e células do endométrio (mutadas ou normais) ativam células do sistema imunológico e neurônios. Após ativados, macrófagos e neutrófilos liberam mediadores pró-inflamatórios para ativar os neurônios, que por sua vez, liberam neuropeptídeos (CGRP e substância P) que promovem o suporte para a formação da lesão endometriótica. Fonte: próprio autor.

Outros fatores, como a dieta, podem influenciar no risco de desenvolver endometriose (HARRIS *et al.*, 2018; MISSMER *et al.*, 2010; YAMAMOTO *et al.*, 2018). Um estudo prospectivo com uma coorte com 2.609 mulheres diagnosticadas com endometriose demonstrou que o consumo de frutas e vegetais está inversamente correlacionado com o risco de desenvolver a doença. O consumo de pelo menos 3 frutas por dia diminuiu em 14% o rsico de desenvolver endometriose (HARRIS *et al.*, 2018). O consumo em associação de 5 porções de frutas e vegetais diminui o risco em 18% do desenvolvimento da doença (HARRIS *et al.*, 2018). Um outro estudo de coorte envolvendo 3.800 mulheres diagnosticadas com endometriose demonstrou que o consumo de carne vermelha também aumenta o risco de desenvolver a doença (YAMAMOTO *et al.*, 2018). Mulheres que consomem carne duas ou mais vezes por dia apresentam um aumento em 56% de chance de desenvolver endometriose quando comparadas com mulheres que consomem carne

uma vez na semana (YAMAMOTO *et al.*, 2018). Por outro lado, o consumo de peixe, frutos do mar ou ovos diminui o risco de desenvolvimento da endometriose (YAMAMOTO *et al.*, 2018). A utilização de dietas com alto teor de ômega-3 (precursor dos SPMs) diminui em 22% o risco de desenvolver endometriose em um estudo de coorte com 1199 mulheres diagonosticas com a doença (MISSMER *et al.*, 2010). Em conjunto, esses dados demonstram que mutações em genes específicos além da dieta podem contribuir de forma significativa para desenvolver endometriose.

Uma característica clínica chave da endometriose são as diferentes formas de dor, incluindo dor pélvica crônica, dismenorreia, dispareunia e disqueia (LAUX-BIEHLMANN; D'HOOGHE; ZOLLNER, 2015; MCKINNON *et al.*, 2015). A sensibilização cruzada de órgãos pélvicos (*pelvic organ cross-sensitization*) é um dos principais fatores que dificultam o diagnóstico de doenças que cursam com dor abdominal, como doenças relacionadas com órgãos pélvico-urinários ou ginecológicos (BRUMOVSKY; GEBHART, 2010). A dor na endometriose faz com que as mulheres afetadas percam, em média, 10,8 horas de trabalho semanais, principalmente como resultado da redução da eficácia durante o tempo de trabalho (NNOAHAM *et al.*, 2011). Em mulheres diagnosticadas com endometriose, a dor pélvica crônica debilitante também contribui para sintomas depressivos (GAMBADAURO; CARLI; HADLACZKY, 2018) e em mulheres adolescentes está associada a maiores taxas de ansiedade, depressão e problemas com o trabalho ou outras atividades diárias devido à saúde emocional (GALLAGHER *et al.*, 2018). Essa dor prolongada leva as pacientes com endometriose a diminuir tempo de trabalho, o envolvimento social, o exercício e o relacionamento com colegas e familiares (GROGAN; TURLEY; COLE, 2018). O dispêndio financeiro da endometriose no sistema de saúde dos EUA é substancial, com os custos anuais diretos e indiretos

estimados em US$ 12.419 por mulher afetada (ROGERS *et al.*, 2017).

No Brasil, há escassez de dados nesse sentido. De fato, uma revisão sistemática enfatizou a necessidade de mais estudos com enfoque nessa doença (SPIGOLON; AMARAL; BARRA, 2012). É importante ressaltar que o prejuízo não está relacionado apenas aos custos de exames e internações hospitalares. Há também o prejuízo indireto decorrente do afastamento do trabalho dessas mulheres jovens em seu período mais produtivo (SPIGOLON; AMARAL; BARRA, 2012). Em um quadro mais geral, um estudo prospectivo multicêntrico com 909 mulheres diagnosticadas com endometriose atendidas em 12 centros terciários de saúde distribuídos em 10 países avaliou os custos da doença em euros. Foram investigados custos com cuidado à saúde (diretos) e custos com perda de produtividade no trabalho (indiretos). A média de custos totais anuais por mulher foi de € 9579 euros (SIMOENS *et al.*, 2012). Ainda mais relevante, dois estudos conduzidos no Brasil demonstraram o impacto psicológico (além do impacto físico) da endometriose na vida das mulheres. A frequência de depressão pode ser de 86,5% a 92% (LORENCATTO *et al.*, 2002; SEPULCRI RDE; DO AMARAL, 2009), e a ansiedade em até 87,5% das pacientes avaliadas (SEPULCRI RDE; DO AMARAL, 2009). Esses estudos indiretamente apontam para a necessidade de caracterização e padronização de um modelo que mimetize da melhor maneira possível essa doença. As terapias atuais para dor associada à endometriose consistem em anti-inflamatórios não-esteróides (AINEs) e outros analgésicos, agentes hormonais e remoção cirúrgica das lesões (STRATTON; BERKLEY, 2011). Entretanto, o uso de AINEs a longo prazo tem efeitos colaterais significativos, incluindo a nefrotoxicidade (UNGPRASERT *et al.*, 2015). De fato, nosso grupo demonstrou que o AINE diclofenaco induz ativação do NF-κB para induzir danos renais em camundongos (FATTORI, V. *et al.*, 2017). As terapias hormonais

também proporcionam benefício limitado ou nenhum benefício a uma fração significativa de pacientes (BECKER *et al.*, 2017). Além disso, mesmo após a cirurgia, as recidivas de doença e dor são muito comuns (RIZK *et al.*, 2014). Portanto, ainda são necessárias novas terapias que proporcionem benefícios a longo prazo.

A utilização de cirurgia para implantar tecido ou remover ovários (ovariectomia) é comumente realizada para induzir endometriose em roedores (SIMITSIDELLIS; GIBSON; SAUNDERS, 2018). Os ovários são a principal fonte de estrógeno e uma vez que a endometriose é uma doença inflamatória dependente do estrógeno, a suplementação hormonal é necessária para manter o crescimento da lesão (SIMITSIDELLIS; GIBSON; SAUNDERS, 2018; TIRADO-GONZALEZ *et al.*, 2010). No entanto, as incisões cirúrgicas na pele e nos tecidos mais profundos aumentam o comportamento de guarda nos animais, indicando dor (LI *et al.*, 2014). A cirurgia também pode aumentar a atividade espontânea periférica e neuronal central, sugerindo que esses procedimentos influenciam o comportamento animal (XU; BRENNAN, 2009; 2010).

Infelizmente, nenhum modelo recapitula completamente todos os aspectos da endometriose, além de dor não ser frequentemente analisada (SIMITSIDELLIS; GIBSON; SAUNDERS, 2018). Quando feitas, essas medidas raramente refletem a experiência humana. Normalmente, o foco principal é a avaliação das respostas evocadas, sendo a hiperalgesia térmica o teste comportamental mais frequentemente utilizado nos modelos de endometriose de roedores (SIMITSIDELLIS; GIBSON; SAUNDERS, 2018; TIRADO-GONZALEZ *et al.*, 2010). Entretanto, a sensibilidade a estímulos térmicos é raramente relatada por mulheres com endometriose. Comumente as pacientes sentem dor na ausência de um estímulo, ou seja, dor espontânea contínua ou intermitente (FATTORI, VICTOR *et*

al., 2017). Assim, a falta de modelos que reproduzam o fenótipo de doenças contribue para a alta taxa de insucesso dos analgésicos em ensaios clínicos (ANDREWS *et al.*, 2016; WOOLF, 2010), incluindo os de endometriose (BECKER *et al.*, 2017). Baseado nisso, nós estabelecemos um novo modelo em camundongos para a dor associada à endometriose que produz mudanças neuronais e comportamentais consistentes com o fenótipo de doença nas mulheres. Validamos esse modelo demonstrando que fármacos utilizados na clínica como o letrozol (inibidor da aromatase) e danazol (androgênio) inibem tanto a dor espontânea como evocada. Esse artigo foi aceito no periódico *Pain* (artigo 2, item 4 dessa tese).

2.5 Resolução da Inflamação

As descobertas seminais do Prof. Sergio H. Ferreira e Sir John R. Vane esclarecem o mecanismo de ação da aspirina e de fármacos semelhantes à aspirina e como que esses mecanismos bloqueiam a dor inflamatória (FERREIRA, 1972; FERREIRA; MONCADA; VANE, 1971; VANE, 1971). Devido a estas grandes descobertas, para controlar a inflamação e dor, as abordagens terapêuticas concentraram-se principalmente na supressão, bloqueio ou inibição de mediadores pró-inflamatórios como a PGE_2 (FERREIRA; LORENZETTI; CORREA, 1978; MONCADA; FERREIRA; VANE, 1973). Apesar de eficazes, os anti-inflamatórios convencionais apresentam vários efeitos adversos, são imunossupressores e altamente associados a complicações infecciosas (ORLICKA; BARNES; CULVER, 2013). Da mesma forma, pacientes com doenças reumáticas, além do alto custo, frequentemente desenvolvem resistência a medicamentos imunobiológicos (como etanercept e Anakinra), limitando seu uso crônico (FATTORI; AMARAL; VERRI, 2016).

Os opióides constituem outra classe de medicamentos com amplo reconhecimento pelo seu efeito analgésico potente. No entanto, tem sido dada crescente atenção ao abuso e dependência de opiáceos, que é um grave problema de saúde pública nos EUA. De fato, os custos diretos dos cuidados de saúde podem ser 8,7 vezes mais elevados em pacientes dependentes de opiódes quando comparados com os não dependentes (KAYE *et al.*, 2017). Em geral, mesmo sob opióide-terapia, a dor neuropática, por exemplo, permanece um desafio para o tratamento, com apenas metade da população tratada apresentando uma redução significativa da dor e a resolução completa dos sintomas raramente é alcançada (SCHUTZ; ROBINSON-PAPP, 2013).

Durante muitos anos, se pensava que a inflamação era resolvida após uma simples diluição dos mediadores inflamatórios, levando ao restabelecimento da função dos tecidos. Mais de 20 anos após as descobertas de Ferreira e Vane, é reconhecido que lipídeos pró-resolução são produzidos para bloquear o recrutamento de neutrófilos e resolver a inflamação (SERHAN, 1994). Fica claro, portanto, que a inflamação excessiva ou descontrolada está associada a um desequilíbrio entre mediadores lipídicos pró-inflamatórios e SPMs (BUCKLEY; GILROY; SERHAN, 2014; SERHAN; CHIANG; VAN DYKE, 2008). Usando a LC-MS/MS (cromatografia líquida-espectrometria de massa) em células imunes de exsudados de inflamação autolimitante, Prof. Charles Serhan confirmou e identificou moléculas com capacidade de resolver ativamente a inflamação, as chamadas SPMs (BANNENBERG *et al.*, 2005; SERHAN *et al.*, 2000; SERHAN *et al.*, 2002).

Os SPMs são biosintetizados a partir do araquidônico (AA) e ácidos graxos ômega-3 ácido eicosapentaenóico (EPA), ácido docosapentaenóico (DPA), ou ácido docosahexaenóico (DHA) (Figura 2). O artigo 3 desta tese oferece uma revisão

os SPMs (item 3.3) (FATTORI *et al.*, 2020). Dadas as suas diferenças estruturais, os SPMs são divididos em diferentes famílias: as lipoxinas (LXs, derivadas de AA), as resolvinas (Rvs, derivadas de EPA [série E] ou derivadas de DHA [série D]), as protectinas (PDs, derivadas de DHA), ou as maresinas (MaRs, derivadas de DHA) (FATTORI *et al.*, 2020).

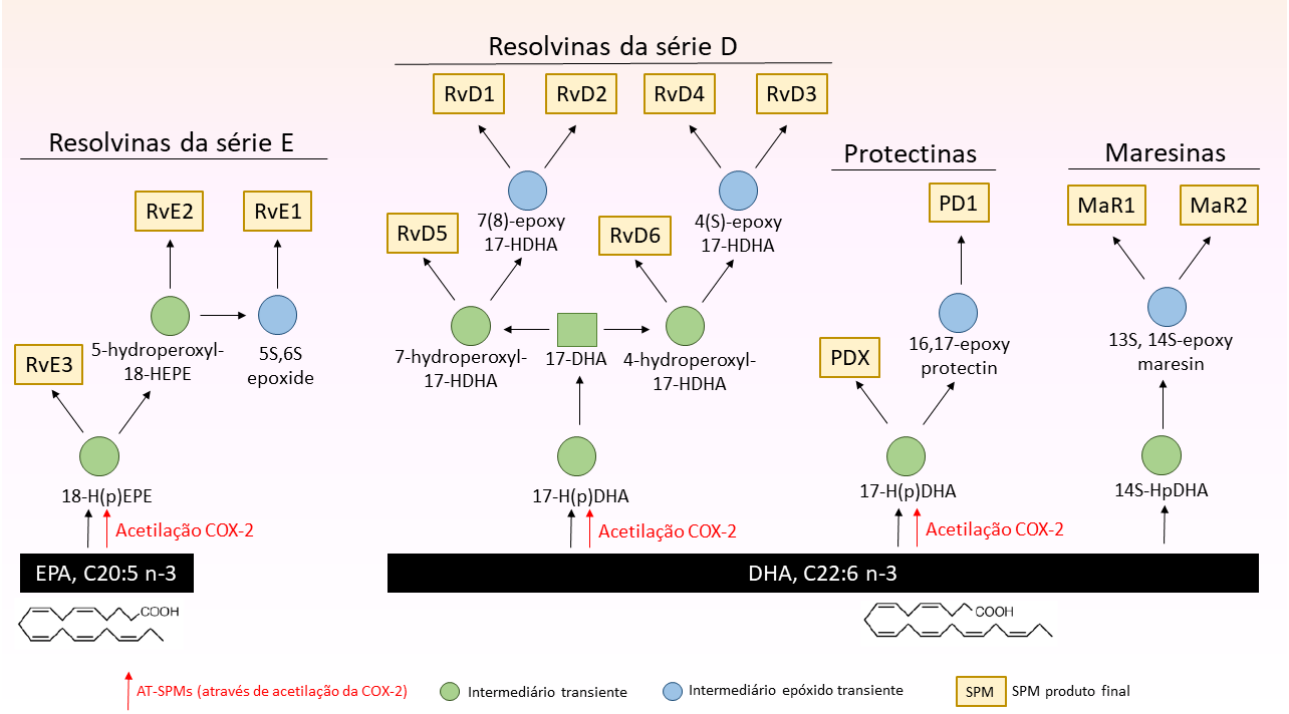

Figura 2. Rota biossintética para produção de SPMs. Figura esquemática com os intermediários e precursores para a produção endógena de SPMs a partir dos ácidos graxos ômega-3 EPA e DHA. As setas vermelhas indicam as rotas que são induzidas através da acetilação da COX-2, dando origem aosde Aspirin-triggered (AT) SPMs. Fonte: próprio autor.

Outra possível rota para produção de SPMs é através da acetilação da COX-2 (Figura 2) (CLARIA; SERHAN, 1995; FATTORI *et al.*, 2020; ROMANO *et al.*, 2015). A aspirina, por exemplo, acetila um resíduo de serina que é conservado no sitio ativo da COX-2, alterando a atividade enzimática do tipo COX para o tipo lipoxigenase. Esta acetilação permite a biossíntese de epímeros de SPM, os chamados SPMs acionados por aspirina (AT-SPMs, Figura 2) (CLARIA; SERHAN, 1995; FATTORI *et al.*, 2020; ROMANO *et al.*, 2015).

Para promover seus efeitos biológicos, as SPMs atuam sobre os

receptores de proteína G acoplada (GPCR). Os SPMs podem ser agonistas, antagonistas, inibidores, ou agonistas alostéricos de receptores específicos expressos por células imunes ou neurônios para modular a resposta do hospedeiro (FATTORI *et al.*, 2020). A resolução da inflamação é um mecanismo ativo e tempo-dependente que conta com uma mudança biossintética de mediadores pró-inflamatórios para pró-resolução, os SPMs (BANNENBERG *et al.*, 2005; LEVY *et al.*, 2001). Por exemplo, para promover a resolução no modelo de inflamação induzida pelo zymosan, a RvD1 aumenta a produção de IL-10 e induz a expressão de miR-21 12h e 24h após o zymosan (RECCHIUTI; SERHAN, 2012). Outros miRNAs controlados temporalmente (chamados miRNAs de resolução) pelo RvD1 são miR-146b, miR-208a e miR-219 (RECCHIUTI; SERHAN, 2012). Consequentemente, uma subpopulação de macrófagos $F4/80^{med}CD11b^{med}$ é recrutada 48 a 72h após estímulo com zymosan, que se correlaciona com a resolução da inflamação (BANNENBERG *et al.*, 2005). Portanto, estes trabalhos mostram que os SPMs resolvem a inflamação de forma dependente do tempo, promovendo a expressão de miRNAs hierarquicamente agrupados (BANNENBERG *et al.*, 2005). De acordo com esses trabalhos, SPMs isolados têm demonstrado eficácia dependente do tempo. Em um modelo de inflamação cutânea induzida por UVB em camundongos, o tratamento com LXA4 72h antes do estímulo melhora a eficácia dessa SPM (MARTINEZ *et al.*, 2018). Em um contexto de dor, enquanto o tratamento com RvD1 antes do desenvolvimento da alodinia tátil produz um efeito analgésico duradouro (até 30 dias), o tratamento com ele em pontos posteriores fornece analgesia limitada (HUANG *et al.*, 2011). Estes conjuntos de dados mostram que os SPM isolados demonstram eficácia dependente do tempo, que pode ser útil para o tratamento de doenças inflamatórias.

Conforme mencionado anteriormente, nociceptores regulam a

resposta inflamatória através da liberação de neuropeptídeos. Estudos recentes demonstram que nociceptores presentes no nervo vago de humanos e camundongos produzem SPMs, de maneira contexto-dependente. Enquanto que a estimulação elétrica do nervo vago humano aumenta os níveis de RvE1, RvD3, RvD4, RvD5, PD1, e MaR1, em camundongos, o vago produz RvD4, RvE1, RvE3 e 15-epi-LXA4 (SERHAN; DE LA ROSA; JOUVENE, 2018). No entanto, utilizando *E. coli* como estímulo, o nervo vago humano produz RvD6, MaR1 e 15-epi-LXA4, o nervo vago do camundongo produz apenas PD1 (SERHAN; DE LA ROSA; JOUVENE, 2018). Dessa forma, é posssível verificar que o nociceptor produz SPM de maneira contexto-dependente. Corroborando esses dados, a vagotomia reduz os níveis de SPMs e retarda a resolução da infecção automilitante por *E. coli* (DALLI *et al.*, 2017) e peritonite induzida por zimosan (MIRAKAJ *et al.*, 2014). Ademais, em humanos, a estimulação elétrica do nervo vago tem mostrado benefícios, como redução dos níveis de TNF-α e a gravidade da doença em pacientes com artrite (KOOPMAN; CHAVAN; MILJKO; GRAZIO et al., 2016). Portanto, esses dados destacam um possível papel na regulação da respostas imune através da produção de SPMs pelos nociceptores.

2.5.1 Maresina-1

As MaRs (MaR1 e MaR2) fazem parte de outra família de moléculas derivadas do DHA. Essas moléculas foram inicialmente descritas como produzidas por macrófagos, assim o nome *macrophage mediators in resolving inflammation – Maresin-1* (DALLI *et al.*, 2013; SERHAN *et al.*, 2012). A biossíntese endógena de MaR1 se inicia com a 14-lipoxigenação do DHA pela 12-LOX de macrófagos. Essa reação dá origem à molécula intermediária ácido 14*S*-HpDHA (14*S*-hydro(peroxy)-docosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoico) (DALLI *et al.*, 2013; SERHAN *et al.*,

2012). Uma hidrólise enzimática via ataque nucleofílico no carbono 7 da 14*S*-HpDHA produz, por fim, a MaR1 (Figura 3) (DALLI *et al.*, 2013; SERHAN *et al.*, 2012). Um outro estudo demonstrou que a interação entre neutrófilos e plaquetas é uma rota alternativa para biossíntese endógena de MaR1 (ABDULNOUR *et al.*, 2014). Nesse estudo, a co-incubação de neutrófilos com plaquetas estímuladas com PAF ou após incubação com 13*S*,14*S*-epoxy-maresin ou 14*S*-HpDHA derivadas de plaquetas, leva a produção de MaR1, sugerindo uma rota transcelular e alternativa para a biossíntese desse SPM (ABDULNOUR *et al.*, 2014).

Parte do efeito da MaR1 está relacionado com a inibição do recrutamento de neutrófilos para o foco inflamatório e mudança de perfil de macrófagos de M1 para macrófagos resolutivos (FATTORI *et al.*, 2020). Essa mudança de perfil está associada com o aumento da eferocitose de neutrófilos apoptóticos, por exemplo. Em um modelo de lesão da medula espinal (*spinal cord injury*), apesar da MaR1 não reduzir o recrutamento inicial de neutrófilos para a medula, ela aumenta o *clearance* dessas céulas e diminui em 50% a presença dessas céulas 7 dias após a lesão além de causar a redução de macrófagos Ly6C^{hi} (pró-inflamatório) e aumento em macrófagos Ly6C^{low} (anti-inflamatório) (FRANCOS-QUIJORNA *et al.*, 2017).

Para produzir seus efeitos imunoresolventes (capacidade de resolver inflamação sem efeito imunossupressor), a MaR1 ativa o receptor LGR6 (receptor 6 acoplado à proteína G contendo repetições ricas em Leucina) em neutrófilos e macrófagos (CHIANG *et al.*, 2019). Após ativados, há aumento da eferocitose e fagocitose de *Escherichia coli* e zymosan, por exemplo (CHIANG *et al.*, 2019). Vale ressaltar que o LGR6 é expresso por células da glia (microglia, astrocitos e oligodendrócitos), células imunes periféricas (macrófagos, neutrófilos, células NK,

etc.) e neurônios, o que explica seu efeito benéfico em diferentes modelos. Em modelo de inflamação de pele induzida por UVB, por exemplo, o tratamento com MaR1 inibe a produção de IL-1β e TNF-α, além de aumentar a atividade antioxidante na pele. Foi possível observar também, uma redução do recrutamento de neutrófilos e ativação de mastócitos (CEZAR *et al.*, 2019).

Em relação a modelos de dor, o tratamento com MaR1 reduz os comportamentos nociceptivos induzidos pela capsaicina e inibe o influxo de cálcio em neurônios induzido por capsaicina mas não AITC (isotiocianato de alila, agonista TRPA1) indicando um efeito seletivo para TRPV1 (SERHAN *et al.*, 2012). Essa seletividade é compartilhada por outros mediadores lipídicos. Enquanto que a RvD2 bloqueia TRPV1 e TRPA1, a RvE1 e RvD1 inibem seletivamente TRPV1 e TRPA1, respectivamente (PARK *et al.*, 2011). Nós demonstramos que um único tratamento intratecal com MaR1 apresenta efeito analgésico de longa duração (até 5 dias) no modelo de dor inflamatória induzida por CFA (FATTORI *et al.*, 2019). Enquanto na medula espinal MaR1 diminui a ativação de astrócitos e microglia, a ativação de NF-κB e a liberação de citocinas pró-inflamatórias (TNF-α e IL-1β); na periferia, a MaR1 reduz o número de leucócitos (células $CD11b^{+}$) próximos às fibras neuronais $CGRP^{+}$. Estendendo esses resultados, nosso grupo forneceu a primeira demonstração que um SPM reduz a liberação de neuropeptídeos por neurônios. Utilizando cultura de DRG, nós demonstramos que a MaR1 reduz liberação de CGRP após estímulo com a capsaicina (FATTORI *et al.*, 2019) Dessa forma, além de inibir a ativação de células imunes, a MaR1 controla a inflamação silenciando os nociceptores (FATTORI *et al.*, 2020).

3 METODOLOGIA, RESULTADOS E DISCUSSÃO

Os resultados obtidos que fazem parte dessa tese estão descritos em 5 artigos científicos, sendo que 4 deles já estão publicados e 1 está em andamento. Dentre os artigos publicados, 3 foram publicados em periódicos Qualis A1 e 1 em Qualis A2. Vale ressaltar que o artigo sobre endometriose (item 3.2) foi escolhido pelo Editor-Chefe di periódico *Pain* como destaque e capa da edição (anexo III):

3.1 "The Specialised Pro-Resolving Lipid Mediator Maresin 1 Reduces Inflammatory Pain with a Long-Lasting Analgesic Effect" (doi: 10.1111/bph.14647, PMID: 30830967) Foi Publicado No Periódico Internacional *British Journal of Pharmacology* (FI: 6,583, Qualis A1, Anexo I).

3.2 "Non-Surgical Mouse Model of Endometriosis-Associated Pain that Responds to Clinically Active Drugs" Foi Aceito No Periódico Internacional *Pain* (FI: 6,029, Qualis A1) (Doi: 10.1097/j.pain.0000000000001832, Anexo II e III).

3.3 "Specialized Pro-Resolving Lipid Mediators: a New Class of Non-Immunosuppressive and Non-Opioid Analgesic Drugs" (doi: 10.1016/j.phrs.2019.104549, PMID: 31743775) Publicado No Periódico Internacional *Pharmacological Research* (FI: 5,574, Qualis A1, Anexo IV)

3.4 "Maresin-1, a Specialized Pro-Resolving Lipid Mediator, as a Non-Hormonal Alternative for the Treatment of Endometriosis-Associated Pain". Artigo em Andamento.

3.5 "Capsaicin: Current Understanding of its Mechanisms and Therapy of Pain and Other Pre-Clinical and Clinical Uses" (doi: 10.3390/molecules21070844, PMID: 27367653) Publicado No Periódico Internacional *Molecules* (FI: 3,06, Qualis A2, Anexo V)

3.1 The specialized pro-resolving lipid mediator Maresin-1 reduces inflammatory pain with a long-lasting analgesic effect

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Running title: Maresin-1 reduces inflammatory pain

Abstract

Background: MaR1 is a specialized pro-resolving lipid mediator with anti-inflammatory and analgesic activities. In this study, we addressed the modulation of peripheral and spinal cord cells by MaR1 in inflammatory pain context.

Experimental approach: Mice were treated with MaR1 before intraplantar injection of carrageenan or complete Freund's Adjuvant (CFA). Mechanical hyperalgesia was assessed using the electronic von Frey and thermal hyperalgesia using a hot plate. Spinal cytokine production and NF-κB activation were determined by ELISA; and astrocytes and microglia activation by RT-qPCR and immunofluorescence. CGRP release by dorsal root ganglia (DRG) neurons was determined by EIA. Neutrophil and macrophage recruitment were determined by immunofluorescence, flow cytometry, and colorimetric methods. *Trpv1* and *Nav1.8* expression and calcium imaging of DRG neurons were determined by RT-qPCR and Fluo-4AM, respectively.

Key results: MaR1 reduced carrageenan- and CFA-induced mechanical and thermal hyperalgesia, and neutrophil and macrophage recruitment proximal to $CGRP^+$ fibers in the paw skin. Moreover, MaR1 reduced NF-κB activation, IL-1β and TNF-α production and spinal cord glial cells activation. In the DRG, MaR1 reduced CFA-induced *Nav1.8* and *Trpv1* mRNA expression and calcium influx, and capsaicin-induced release of CGRP by DRG neurons.

Conclusions: MaR1 reduced DRG neurons activation and CGRP release explaining, at least in part, its analgesic and anti-inflammatory effects. The enduring analgesic and anti-inflammatory effects and also post-treatment activity of MaR1 suggest that the specialized pro-resolving lipid mediators may become a new class of drugs important for the treatment of inflammatory pain.

Keywords: glial cells, omega-3 fatty acids, pain treatment, spontaneous pain, chronic pain

Bullet point summary

What is already known:

-MaR1 reduces neuropathic pain and capsaicin-induced pain-like behaviour.

-MaR1 reduces microglia and astrocyte activation.

What this study adds:

-MaR1 reduces inflammatory pain with long lasting analgesic effect.

-MaR1 reduces activation and CGRP release by DRG neurons and leukocyte counts nearby CGRP+ fibres.

Clinical significance:

-MaR1 presents efficacy at low doses and is active as pre- and post-treatment.

-The long-lasting analgesic effect of MaR1 might be useful for the treatment of chronic pain.

List of abbreviations

ALX/FRP2: N-formyl peptide receptor 2
CFA: Complete Freund's Adjuvant
CGRP: Calcitonin gene-related peptide
Chem23: Chemerin Receptor 23
DHA: Docosahexaenoic acid
DRG: Dorsal root ganglion
GABA: gamma-Aminobutyric acid
GFAP: Glial fibrillary acidic protein
GPCR: G protein-coupled receptors
IASP: International Association for Study of Pain
IBA-1: Ionized calcium-binding adapter molecule 1
MaR1: Maresin-1 (IUPAC: 7*R*,14*S*-dihydroxy-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-docosahexaenoic acid)
MPO: Myeloperoxidase
NAG: N-Acetyl-β-D-glucosaminidase
NMDA: N-Methyl-D-aspartic acid
NSAID: Nonsteroidal anti-inflammatory drug
PGE_2: Prostaglandin E_2
Rv: Resolvin
SCI: Spinal cord injury
sEPSC: Spontaneous excitatory postsynaptic current
SNL: Spinal nerve ligation
SPM: Specialized pro-resolving lipid mediators
TRPA1: Transient receptor potential cation channel subfamily A member 1
TRPV1: Transient receptor potential cation channel subfamily V member 1

1. Introduction

Nociceptive pain is essential for the maintenance of bodily integrity. Inflammatory pain, on the other hand, induces mechanical and thermal hypersensitivity, which occur in the absence of noxious stimuli. Therefore, it represents an important type of pain given it can be pathological if not properly managed. In fact, when inadequately managed the perception of acute pain can be worsened due to impairment in sleep (Sinatra, 2010; Alexandre *et al.*, 2017). That exacerbation of pain occurs due to sensitisation of specialized sensory neurons, namely nociceptors, leading to a state denominated as hyperalgesia (Verri *et al.*, 2006). The peripheral sensitisation is mediated by several inflammatory mediators, such as prostaglandin (PG) E_2, histamine, and cytokines released by immune cells such as mast cells, neutrophils, and macrophages (Fattori *et al.*, 2017b; Pinho-Ribeiro *et al.*, 2017). In the spinal cord, this sensitisation is mediated by cytokines, chemokines, and growth factors released by glial cells, namely as microglia, astrocytes, and oligodendrocytes (Scholz *et al.*, 2007; Zarpelon *et al.*, 2016; Fattori *et al.*, 2017a).

Over the past decade, the concept involving the resolution of the acute inflammatory process has changed. Compelling evidence has demonstrated that the resolution of inflammation is an active process (Serhan *et al.*, 2015; Chiang *et al.*, 2017; Serhan, 2017). Once thought to be a passive process, now is known to be tightly regulated by omega-3 fatty acid-derived molecules, so-called specialized pro-resolving lipid mediators (SPMs) (Serhan, 2017). Regarding pain, SPMs namely Resolvin (Rv) RvE1, RvE2, RvE3, RvD1, RvD2, and Maresin-1 (MaR1) were identified in human synovial fluids of patients with arthritis (Giera *et al.*, 2012). The presence of these mediators was negatively associated with pain score, indicating they possibly control pain (Giera *et al.*, 2012). Isolated SPMs have been successively used as therapeutic drugs in different models, including pain models (Chiang *et al.*, 2017). They possess efficacy at very low doses, usually in nanogram to microgram ranges (Serhan *et al.*, 2015). For instance, intrathecal treatment with RvE1 at 10 ng reduces inflammatory pain *via* spinal and peripheral mechanisms by acting on Chem23 receptor, which is expressed by DRG neurons and in the spinal cord (Xu *et al.*, 2010). Of interest, RvE1 reduced the second phase of formalin-induced overt pain-like behaviour in a dose 1000 times lower than morphine, indicating potent analgesic effect (Xu *et al.*, 2010). Their clinical relevance was demonstrated in a meta-analysis study showing that supplementation with omega-3 fatty acids for 3 to 4 months reduces patient-reported joint pain intensity, minutes of morning stiffness, and NSAIDs consumption (Goldberg *et al.*, 2007). SPMs act on G protein-coupled receptors (GPCR), which can be either selective

for a SPM or shared with other SPMs (Xu *et al.*, 2010; Park, 2015; Serhan *et al.*, 2015; Chiurchiu *et al.*, 2016; Chiang *et al.*, 2017; Zhang *et al.*, 2017; Gu *et al.*, 2018). Evidence suggests that MaR1 share the ALX/FPR2 receptor with D-series Rv and lipoxins (Zhang *et al.*, 2017; Gu *et al.*, 2018). However, ligand-binding assays are required to further confirm this interaction and whether the effect of MaR1 depends solely on agonism of ALX/FPR2. In trigeminal ganglion and DRG neurons, the inhibitory effects of MaR1 on capsaicin-induced TRPV1 currents were blocked after treatment with pertussis toxin, suggesting it can act on a Gαi-coupled GPCR pathway (Serhan *et al.*, 2012; Park, 2015). MaR1 is a docosahexaenoic acid (DHA)-derived SPM produced by macrophages and possesses potent anti-inflammatory activity, which is related mainly to the inhibition of neutrophil recruitment and stimulation of efferocytosis by macrophages (Serhan *et al.*, 2012; Serhan *et al.*, 2015; Francos-Quijorna *et al.*, 2017). Furthermore, in a model of spinal cord injury (SCI), endogenous production of MaR1 initiates only 14 days after injury and exogenous administration of it at early time points induces spinal cord recovery, indicating neuroprotective and pro-resolving effects (Francos-Quijorna *et al.*, 2017). MaR1 also presents analgesic activity in different models of pain (Serhan *et al.*, 2012; Park, 2015; Gao *et al.*, 2018; Zhang *et al.*, 2018). Treatment with MaR1 reduces capsaicin-induced overt pain like behaviour, vincristine-induced neuropathic pain (Serhan *et al.*, 2012), complete Freund's Adjuvant (CFA)-induced temporomandibular pain (Park, 2015), spinal nerve ligation (SNL)-induced neuropathic pain (Gao *et al.*, 2018), and tibial bone fracture-induced pain (Zhang *et al.*, 2018). However, to the date, there is no study addressing the modulation of DRG neurons and immune cells (in the periphery and spinal cord) by MaR1 in the context of peripheral inflammatory pain. For that purpose, we administrated MaR1 intrathecally in the carrageenan- and CFA-induced pain, which are two well-established experimental models for the screening of new drugs for pain relief.

2. Material and methods

2.1. Animals

All experiments were performed in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Londrina State University Ethics

Committee on Animal Research and Welfare (process numbers 4014.2015.70 and 12766.2015.54). In this study, it was used healthy male Swiss and LysM-eGFP mice (8 weeks of age, 25 ± 1g, [RRID:MGI:2654932]) from Londrina State University, Paraná, Brazil. Mice were randomly assigned and housed in standard clear plastic cages in light/dark cycle of 12:12h with *ad libitum* water and food. Behavioural testing was performed between 9 a.m. and 5 p.m. in a room maintained at a temperature of 21±1°C. Block randomisation method was used to randomise subjects into groups resulting in an equal sample sizes at all time-points. The investigators were blinded to the treatments. All efforts were made to minimise the number of animals used and their suffering. Animal studies are in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2015). Euthanasia was performed by isoflurane anaesthesia (5% in oxygen using a precision vaporiser) followed by decapitation as a confirmation method. A total of 588 Swiss mice and 72 LysM-eGFP were used in this study. No animals were excluded from statistical analysis.

2.2. Experimental procedures

Mice were treated once with 1, 3, or 10 ng of MaR1 (Cayman Chemical, Ann Arbor, MI, USA) or vehicle (10% ethanol) via intrathecal route (between L4-L6 spinal segments, 10 μL) and under isoflurane anaesthesia (3% in oxygen using a precision vaporiser), 20 minutes before intraplantar injection of carrageenan (100 μg/20μL/paw) or CFA (10μL/paw). The stock solution contained 1 ng/μL of MaR1 in 100% ethanol and was kept in a -80 °C freezer. Caution was taken to avoid exposure of MaR1 to air during the preparation for treatment. Previous report indicates that intrathecal delivery of 10% ethanol produces mild and transient analgesic effect, which is observed 30 min after delivery but not after one hour (Xu *et al.*, 2010). Based on that study, all behavioural experiments involving pre-treatment with MaR1 were performed one hour after the stimulus (1:20 h after MaR1 treatment). The exceptions were those experiments involving pre-treatment with MaR1 on the CFA-induced pain model, which were conducted one day after treatment. Mechanical and thermal hyperalgesias were evaluated 1-5 h after carrageenan injection or during seven days after CFA injection. The best dose of MaR1 (10 ng) was chosen for the following experiments based on mechanical and thermal hyperalgesia. Spinal cord was dissected three hours after carrageenan injection to determine cytokine production (TNF-α and IL-1β) and NF-κB activation by ELISA. Hind paw skin was dissected five hours after carrageenan injection to determine neutrophil and macrophage recruitment by immunofluorescence, flow cytometry, and enzymatic assays. In the other set of experiments, spinal cord was dissected three days after CFA injection to determine cytokine production

(TNF-α and IL-1β) and NF-κB activation by ELISA. To determine activation of glial cells, spinal cord was dissected three days after CFA injection. Hind paw skin was dissected three days after CFA injection to determine neutrophil and macrophage recruitment by immunofluorescence, flow cytometry, and enzymatic assays. Ipsilateral dorsal root ganglia (DRG from L4-L6 spinal cord segments) were also dissected three days after CFA injection to determine *Trpv1* and Nav1.8 mRNA expression by RT-qPCR and calcium imaging using confocal microscopy. For CFA-induced overt pain-like behaviour (flinches and time spent licking the paw), mice were treated once with 10 ng of MaR1 or vehicle (10% ethanol) *via* intrathecal one day before intraplantar injection of CFA (10 μL/paw). To assess the efficacy of the post-treatment with MaR1, mice received intrathecal treatment with MaR1 at 10 ng or vehicle (10% ethanol) one or three days after stimulus with CFA. In this set of experiment, all behavioural assays were performed 1:20 h post-treatment. CGRP release was determined using DRG neurons culture. Cells were treated with MaR1 (0.3, 1, or 3 ng/mL) before stimulus with capsaicin (500 nM, 1h).

2.3. Mechanical hyperalgesia

Mechanical hyperalgesia was evaluated by the electronic version of von Frey's filaments, as reported previously (Cunha *et al.*, 2004). In a quiet, temperature-controlled room, mice were gently placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic aesthesiometer, Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the plantar hind paw with a gradual increase in pressure. The upper limit pressure was 15 g. The end-point was characterized by the removal of the member followed by clear paw flinching or paw licking movements. After paw withdrawal, the intensity of the pressure was automatically recorded, with the final response value being obtained by the average of three measurements. The animals were tested before and after treatments. The results are expressed by delta (Δ) withdrawal threshold (in grams), which was calculated by subtracting the mean measurements at 1-5 h after carrageenan injection or 1-7 days after CFA injection, from the zero-time (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 55°C±1ºC (Insight, Ribeirão Preto, SP, Brazil), as previously described (Calixto-Campos *et al.*, 2015). The reaction time was registered when one of the following responses (end-points) were observed: clear paw flinching, paw licking, or jumping. The results were calculated by subtracting the values obtained 1-5 h after carrageenan injection or 1-7 days after CFA injection, from the zero-time (baseline values) measurement. The results are expressed by delta (Δ) withdrawal latency (in seconds). A cut-off of 20 seconds was set to avoid tissue damage (Fattori *et al.*, 2015). The investigators were blinded to the treatment.

2.5. Cytokine measurement

For cytokine production, spinal cord was homogenised into 500 µL of PBS buffer containing protease inhibitors. TNF-α and IL-1β levels were determined by ELISA using eBioscience kits (eBioscience, San Diego, CA, USA) accordingly with manufacturer instructions. Reading was performed at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results were expressed as picograms (pg) of each cytokine per mg of protein.

2. 6. NF-κB activation

Spinal cord was dissected into ice-cold lysis buffer (Cell Signaling Technology, Beverly, MA, USA). The homogenates were centrifuged (16,100 *g*, 10 min, 4°C) and the supernatants used to assess the levels of total and phosphorylated NF-κB p65 subunit by ELISA using PathScan kits #7836 and #7834, respectively (Cell Signaling Technology, Beverly, MA, USA) accordingly with manufacturer's instructions. The results are presented as the sample OD ratio (sample OD from total p65 subunit divided by sample OD phosphorylated p65 subunit) measured at 450nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland).

2.7. Myeloperoxidase (MPO) and N-Acetyl-β-D-glucosaminidase (NAG) activities

Neutrophil (MPO activity) and macrophage (NAG activity) recruitment were evaluated using a colorimetric assay as described previously (Ruiz-Miyazawa *et al.*, 2015). Samples of the hind paw skin were dissected into ice-cold 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and kept at -80°C until use. Samples were homogenised and centrifuged at 16,100 *g*, 2 min, 4°C. For MPO activity, 10 µL of the

supernatant was mixed with 200 µL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. Reading was performed after 15 minutes at 450nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results are presented as MPO activity (number of neutrophils × 10^4 per mg of protein). For NAG activity, 20 µL of supernatant was obtained as described for the MPO activity assay and added to a 96-well plate. The reaction was initiated by adding 2.24 mM of 4-nitrophenyl N-acetyl-β-D-glucosaminide. Then, the plate was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 100 µL of 0.2 M glycine buffer, pH 10.6. Reading was performed at 405 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results are presented as NAG activity (macrophages × 10^4 per mg of protein).

2.8. Overt pain-like behaviour

The total number of paw flinches and time spent licking the paw were counted during 30 min after injection CFA as previously described (Calixto-Campos *et al.*, 2015). Results were expressed as the total number of flinches or time spent licking the paw (in seconds) performed in 30 min. The investigators were blinded to the treatment.

2.9. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR).

DRG or spinal cord were dissected into TRIzol™ reagent and total RNA was isolated according to manufacturer's directions. The purity of total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA and qPCR were performed using GoTaq® 2-Step RT-qPCR System (Promega, Madison, WI, USA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Thermo Fisher Scientific, Waltham, MA, USA). The relative gene expression was determined using the comparative $2^{-(\Delta\Delta Ct)}$ method. The primers sequence used in this study were: *Trpv1*: sense: 5'-TTCCTGCAGAAGAGCAAGAAGC-3', *Trpv1* antisense: 5'-CCCATTGTGCAGATTGAGCAT-3'; *Nav1.8*: sense: 5'-GTGTGCATGACCCGAACTGAT-3', *Nav1.8*: antisense: 5'-CAAAACCCTCTTGCCAGTATCT-3'; *Gfap* sense 5'-GGCGCTCAATGCTGGCTTCA-3', antisense: 5'-TCTGCCTCCAGCCTCAGGTT-3'; *Iba-1* sense: 5'-ATGGAGTTTGATCTGAATGGAAAT-3', antisense: 5'-TCAGGGCAGCTCGGAGATAGCTTT-3'; *β-actin* sense: 5'-

AGCTGCGTTTTACACCCTTT-3', *β-actin* antisense: 5'-AAGCCATGCCAATGTTGTCT-3'. The expression of *β-actin* mRNA was used as a reference gene to normalize data.

2.10. Immunofluorescence staining

Spinal cord segments from region L4–L6 were dissected and post-fixed, and then they were replaced overnight with 30% sucrose. Spinal cord segments were embedded in optimum cutting temperature, and 15 or 40 µm (only for $CGRP^+$ fibres staining) sections were cut in a cryostat and processed for immunofluorescence. For microglia staining, sections were blocked and then incubated with primary antibodies anti-IBA-1 (1:400, cat #PA5-27436, Thermo Fisher Scientific, Waltham, MA, USA, [RRID:AB_2544912]). After that, sections for IBA-1 were stained with secondary antibody Alexa Fluor 488 (1:1000, cat #A-11008, Thermo Fisher Scientific, Waltham, MA, USA, [RRID:AB_143165]). For astrocyte staining, it was used an anti-GFAP antibody conjugated with Alexa Fluor 488 (1:1000, cat #MAB3402X, MilliporeSigma, Burlington, MA, USA, [RRID:AB_11210273]). In the hind paw skin, neutrophils were stained using anti-Ly-6G/Ly-6C (Gr-1) antibody conjugated with Alexa Fluor 488 (1:200, clone RB6-8C5, BioLegend, San Diego, CA, USA, [RRID: AB_313366]). Macrophages were stained using anti-CD68 (1:500, cat #ab125212, Abcam, Cambridge, MA, USA, [RRID: AB_10975465]) followed by the secondary antibody DyLight 594 (1:200, cat #DI-1594, Peterborough, United Kingdom, [RRID:AB_2336413]). $CGRP^+$ fibres were stained with anti-CGRP (1:500, cat #C8198, MilliporeSigma, Burlington, MA, USA, [RRID:AB_259091]) followed by the secondary antibody Alexa Fluor 647 (1:500, cat# ab150075, Abcam, Cambridge, MA, USA, [RRID:AB_2752244]) and leukocytes were stained with anti-CD11b (1:250, clone 5C6, cat #MCA711, Bio-Rad, Hercules, CA, USA, [RRID:AB_321292]) followed by the secondary antibody Alexa Fluor 594 (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, [RRID:AB_2340689]). The images and analysis were performed using a Confocal Microscope (TCS SP8, Leica Microsystems, Mannheim, Germany).

2.11. Flow cytometry

Hind paw skin was dissected, minced and then incubated for 2 h (37 °C, shaking) in 2mL of HEPES-buffered saline (MilliporeSigma, Burlington, MA, USA) containing collagenase P, as described previously (Pinho-Ribeiro *et al.*, 2018). After incubation, cells were gently dissociated and filtered through a 40 µm mesh and mixed washing buffer constituted by HBSS (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5% BSA. Cells were centrifuged

for 5 min at 300g, supernatant was discarded, and the pellet was resuspended in 500 µL of washing buffer. The cell suspension was incubated on ice with mouse FcR Blocking Reagent (Miltenyi Biotec, Cambridge, MA, USA) for 10 min, and then incubated for 30 min on ice with the following antibodies (BioLegend, San Diego, CA, USA): Zombie Aqua™ Fixable Viability Kit (1:1000), anti-CD45-PE (1:200, clone 30-F11, [RRID:AB_2563597]), anti-CD11b-Brilliant Violet 605™ (1:200, clone M1/70, [RRID:AB_11126744]), anti-F4/80-APC (1:200, clone BM8, [RRID:AB_893493]), and anti-Ly6G-FITC (1:200, clone 1A8, [RRID:AB_968318]). Cells were centrifuged for 5 min at 300g and the pellet resuspended in 500 µL of washing buffer 2% PFA. FACS data were analysed and plotted using FlowJo software (FlowJo LLC [RRID:SCR_008520]).

2.12. Calcium Imaging

Calcium imaging of DRG neurons was performed as previously described (Chiu *et al.*, 2013). DRGs were dissected into Neurobasal-A medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), dissociated in collagenase A (1 mg/mL)/dispase II (2.4 U/mL) (RocheApplied Sciences, Indianapolis, IN, USA) in HEPES-buffered saline (MilliporeSigma, Burlington, MA, USA) for 70 min at 37°C. After trituration with glass Pasteur pipettes of decreasing size, DRG cells were centrifuged over a 10% BSA gradient, plated on laminin-coated cell culture dishes. DRGs were loaded with 1.2 uM of Fluo-4AM in Neurobasal-A medium, incubated for 30 min 37°C, washout with HBSS and imaged in Confocal Microscope (TCS SP8, Leica Microsystems, Mannheim, Germany). To assess TRPV1 activation, DRG plates were recorded for 6 min, which was divided in: 2 minutes of initial reading (0 second mark, baseline values), following by stimulation with capsaicin for 2 min at 120 seconds mark [1 uM, TRPV1 agonist (Fattori *et al.*, 2016b)] and KCl for 2 min at 240 seconds mark (40 mM, activates all neurons). Calcium flux was analysed by the mean fluorescence measured with the LAS X Software (Leica Microsystems, Mannheim, Germany).

2.13. Calcitonin gene-related peptide (CGRP) release

DRG neurons (5,000 per well) were cultured for one week in Neurobasal-A medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) with half of the medium being replaced with fresh media every two days as described previously (Pinho-Ribeiro *et al.*, 2018). Neurons received vehicle or different concentrations of MaR1 (0.3-3 ng/mL) before stimulus with capsaicin (500 nM) for 1h at 37°C, 5% of CO_2. Supernatant were collected to determine CGRP concentration according to manufacturer's instructions using a CGRP

Enzyme-Linked Immunosorbent (EIA) kit (Cayman Chemical Ann Arbor, MI, USA).

2.14. Statistical analysis

Results are presented as means ± SEM of measurements made on six mice in each group per experiment. Each experiment was conducted twice. Data were analysed using the software GraphPad Prism version 6.01 (La Jolla, CA, USA, [RRID:SCR_002798]). Two-way repeated measure analysis of variance (RmANOVA), followed by Tukey's *post hoc*, was used to analyse data from experiments of multiple time points (mechanical and thermal hyperalgesia). Data from experiments of single time point, it was used one-way ANOVA followed by Tukey's *post hoc*. Statistical differences were considered significant when $p < 0.05$.

2.15. Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a; Alexander *et al.*, 2017b; Alexander *et al.*, 2017c).

3. Results

3.1 MaR1 reduces carrageenan-induced hyperalgesia and neutrophil and macrophage recruitment.

Firstly, we evaluated the efficacy of intrathecal treatment with MaR1 in carrageenan-induced mechanical and thermal hyperalgesia. Treatment with 10 ng of MaR1 reduced carrageenan-induced mechanical (Fig. 1A) and thermal (Fig. 1B) hyperalgesia. To determine leukocyte recruitment, it was used LysM-eGFP mice (Fig. 2A) and staining for GR-1 (neutrophil marker, Fig. 2B) or CD68 (macrophage marker, Fig. 2C). We observed a reduction in carrageenan-induced neutrophil and macrophage recruitment to the hind paw as observed by the reduced intensity of fluorescence of these cellular markers and their respective enzymatic activity (MPO and NAG, respectively) (Fig. 2D and E). For the quantification of these cells, flow cytometry was performed. Treatment with MaR1 reduced both $CD11b^{+}Ly6G^{+}$ neutrophils and $CD11b^{+}F4/80^{+}$ macrophages recruitment to the paw skin (Fig. 2F). Therefore, this dose was chosen for the following experiments involving the intraplantar injection of carrageenan.

3.2. MaR1 inhibits carrageenan-induced spinal cord cytokine production and NF-κB activation.

The efficacy of MaR1 in carrageenan-induced spinal cord cytokine production and NF-κB activation were then investigated. Carrageenan increased spinal cord TNF-α (Fig. 3A) and IL-1β (Fig. 3B) production and NF-κB (Fig. 3C) activation as observed by the reduction in total-p65/phosphorylated-p65 OD ratio. The ratio was obtained by dividing the OD measured from total-p65 by the OD measured from phosphorylated-p65. Therefore, a decrease in the ratio is attributed to higher levels of phosphorylated p65 subunit (Ser536 residue), which indicates the activation of the NF-κB signalling pathway. Importantly, these parameters were reduced after intrathecal treatment with MaR1 (Fig. 3).

3.3. MaR1 reduces CFA-induced mechanical and thermal hyperalgesia

Next, we sought to evaluate the efficacy of MaR1 in a chronic model of pain. For that, it was used intraplantar stimulus with CFA. A single treatment with 10 ng of MaR1 reduced mechanical (Fig. 4A) and thermal (Fig. 4B) hyperalgesia, which lasted for five days after the stimulus. Both doses of 1 and 3 ng reduced mechanical and thermal hyperalgesia starting two days after CFA injection and lasting until the 5^{th} day after the stimulus. The effect of MaR1 at 10 ng initiated at day one after the stimulus, therefore, this dose was chosen for the following experiments involving the intraplantar injection of CFA. To assess the efficacy of MaR1 in already established pain, mice received intrathecal treatment with MaR1 at 10 ng or vehicle, one (Fig. 4C and D) or three days (Fig. 4E and F) after stimulus with CFA. A single treatment with MaR1 one day after the stimulus displayed analgesic effect that lasted for three days (Fig. 4C and D). Treatment with this SPM three days after the stimulus showed a less potent analgesic effect, which lasted for two days (Fig. 4E and F). These set of data show that a single intrathecal administration of MaR1 presents a long-lasting analgesic effect as a pre and post-treatment.

3.4. MaR1 decreases CFA-induced overt pain-like behaviour.

Spontaneous pain is a common symptom of patients with chronic pain (Fattori *et al.*, 2017a). CFA not only induce chronic pain but also overt pain-like behaviour (Calixto-Campos *et al.*, 2015). Thus, the efficacy of MaR1 in CFA-induced overt pain-like behaviour was then evaluated. Mice were treated once with 10 ng of MaR1 (intrathecal) or vehicle (10% ethanol, intrathecal) one day before intraplantar injection of CFA (10 μL/paw). This time point was chosen considering that only the dose of MaR1 at 10 ng reduced CFA-induced hyperalgesia at day one after the stimulus (Fig. 5A and B). Treatment with MaR1 at 10 ng reduced CFA-induced flinches (Fig. 5A) and time spent licking the paw (Fig. 5B).

1143

1144 3.4. MaR1 inhibits neutrophil and macrophage recruitment close to CGRP$^+$ fibres in the hind
1145 paw skin and the release of CGRP by DRG neurons

1146 Once activated, neurons release neuropeptides, such as CGRP, to modulate the
1147 recruitment and activation of immune cells during inflammation (Pinho-Ribeiro *et al.*, 2017).
1148 In the inflammatory site, recruited neutrophils and macrophages have an important role in the
1149 genesis and maintenance of pain. Thus, we next addressed whether treatment with MaR1 could
1150 reduce the recruitment of these cells to the hind paw skin. In the first set of data, we wonder
1151 whether there would be leukocytes close to CGRP$^+$ fibres. We observed a lower number of
1152 leukocytes (CD11b$^+$ cells) close to CGRP$^+$ fibres in the CFA + MaR1 group than the CFA +
1153 vehicle group (Fig. 6A). Given we observed a lower number of leukocytes close to CGRP$^+$
1154 fibres and a reduction in neuronal activation, we next investigated the influence of this SPM on
1155 the release of CGRP by DRG neurons. Previous report indicate MaR1 completely blocks
1156 current in TRPV1$^+$ DRG neurons as observed by whole cell patch-clamp (Serhan *et al.*, 2012).
1157 Herein we show that MaR1 reduced capsaicin-induced CGRP release in DRG neuron culture
1158 in a concentration-dependent manner (Fig. 6B). The concentration of 3 ng/mL of MaR1 was
1159 statistically different when compared to the concentration of 1 ng/mL. To further determine
1160 which cell types were reduced after treatment with MaR1, it was used LysM-eGFP mice
1161 (neutrophil and macrophage marker, Fig. 6C) and staining for GR-1 (neutrophil marker, Fig.
1162 6D) or CD68 (macrophage marker, Fig. 6E). Treatment with MaR1 reduced CFA-induced
1163 neutrophil and macrophage. To have a better quantification and profiling of these cells, flow
1164 cytometry was performed. MaR1 reduced the recruitment of both CD11b$^+$Ly6G$^+$ neutrophils
1165 and CD11b$^+$F4/80$^+$ macrophages (Fig. 7A) and their respective enzymatic activities as observed
1166 by the MPO and NAG activity assays (Fig. 7B and C).

1167

1168 3.5. MaR1 inhibits CFA-induced spinal cord cytokine production and NF-κB activation.

1169 The next step was to evaluate the efficacy of MaR1 in CFA-induced spinal cord cytokine
1170 production and NF-κB activation. CFA induced TNF-α (Fig. 8A) and IL-1β (Fig. 8B)
1171 production, which were reduced after treatment with MaR1. In addition, CFA induced NF-κB
1172 activation (Fig. 8C) as observed by the reduction in total-p65/phosphorylated-p65 OD ratio.
1173 The ratio was obtained by dividing the OD measured from total-p65 by the OD measured from
1174 phosphorylated-p65. Therefore, a decrease in the ratio is attributed to higher levels of
1175 phosphorylated p65 subunit (Ser536 residue) relative to total-p65, which indicates the
1176 activation of the NF-κB signalling pathway. Importantly, a single treatment with 10 ng of MaR1

reduced spinal cord NF-κB activation and pro-inflammatory cytokine production.

3.6. MaR1 decreases CFA-induced activation of astrocytes and microglia

Given the role of glial cells in the development of pain (Scholz *et al.*, 2007; Fattori *et al.*, 2017a), we next assessed the efficacy of MaR1 in CFA-induced spinal cord activation of astrocytes and microglia. Treatment with MaR1 reduced CFA-induced activation of astrocytes (GFAP – astrocytes activation marker) and microglia (IBA-1 – microglia activation marker) as observed by the reduction in both mRNA (Fig. 9A and B) and intensity of fluorescence (Fig. 9C and D).

3.7. MaR1 reduces CFA-induced DRG neurons activation.

Activation of DRG neurons can be observed through an increase in calcium influx (Chiu *et al.*, 2013; Blake *et al.*, 2018). Thus, we wondered whether DRG neurons from CFA-stimulated mice would present a higher response to capsaicin than DRG neurons from mice that received intraplantar saline and whether there would be modulation of this response by MaR1 treatment. DRG neurons from CFA-stimulated mice presented a higher baseline level of calcium influx than DRG neurons from saline mice (Fig. 10A, B, and C). Importantly, MaR1 reduced CFA elevation of baseline calcium levels. As an increase on calcium flux is indicative of DRG neuron activation, these data suggest that MaR1 reduces the activation of DRG neurons in CFA-induced inflammation. In line with the reduction of DRG neurons activation by MaR1, treatment with it reduced the CFA-induced mRNA expression of *Nav1.8* (Fig. 10D) and *Trpv1* (Fig. 10E) in the DRG, which are channels involved in nociceptor sensory neuron sensitization and activation resulting in pain (Chiu *et al.*, 2014). Therefore, MaR1 reduced CFA-induced DRG neurons activation and the expression of markers of nociceptor sensory neurons sensitization.

4. Discussion

The intrathecal treatment with the SPM MaR1 reduces inflammatory pain induced by carrageenan and CFA showing a long-lasting analgesic profile. A single intrathecal pre-treatment with MaR1 at 10 ng reduced CFA-induced hyperalgesia for five days, while a 24h post-treatment reduced CFA-induced hyperalgesia for three days. These data indicate MaR1 presents a potent and long-lasting analgesic effect, which could be useful in the management of pain of inflammatory origin. The analgesic effect of MaR1 is related to the inhibition of inflammation-induced activation of astrocytes and microglia, reduction in NF-κB activation and thereby TNF-α and IL-1β production. In the periphery, MaR1 reduced the recruitment of leukocytes and the number of leukocytes close to $CGRP^+$ fibres. Data also demonstrated the link between intrathecal MaR1 treatment with its peripheral anti-inflammatory and analgesia effects. MaR1 decreased CFA-induced DRG mRNA expression of pain-related channels *Nav1.8* and *Trpv1* and the activation of DRG neurons (as observed by lower baseline levels of calcium influx), which resulted in reduced CGRP release.

The resolution of inflammation relies on temporal changes in the production of lipid mediators (Levy *et al.*, 2001; Bannenberg *et al.*, 2005). A study analysing approximately 300 miRNAs shows that the shift on lipid mediators production impacts on the expression of miRNAs that are hierarchically clustered at different time points towards resolution (Recchiuti *et al.*, 2012). For instance, RvD1 reduces zymosan-induced inflammation by increasing the miR-21, which regulates IL-10 production at later time points (12h and 24h after zymosan when compared to 4h) (Recchiuti *et al.*, 2012). This indicates that SPMs controls specific miRNAs in a time dependent-manner to promote resolution of acute inflammation (Recchiuti *et al.*, 2012). In addition, after stimulus with zymosan, the number of macrophages with a pro-resolving phenotype gradually increases (48 to 72h after stimulus), which also correlates with the resolution of inflammation (Bannenberg *et al.*, 2005). These findings are consistent with the fact that some isolated SPMs also present time-dependent efficacy. For instance, a single intrathecal treatment with RvD1 before the development of tactile allodynia produces an enduring analgesic effect that lasts for 10 days and 30 days in a model of paw incision-induced pain and in a model of skin/muscle incision and retraction surgery-induced pain, respectively (Huang *et al.*, 2011). However, treatment with RvD1 at later time points provides limited analgesia (Huang *et al.*, 2011). A single treatment with RvD2 reduces carrageenan-induced mechanical hyperalgesia for two days (Park *et al.*, 2011). Focusing on MaR1, peripheral treatment with this SPM shows increasing efficacy over time (14 days period) in a model of vincristine-induced neuropathic pain in mice (Serhan *et al.*, 2012). Thus, this time-dependent

effect of SPMs led us to investigate whether the intrathecal treatment with MaR1 could also display a prolonged analgesic profile. In fact, a single pre-treatment with MaR1 provides an analgesic effect for five days while a post-treatment (one day after stimulus) produces analgesia for three days. Altogether, these data indicate MaR1 presents a long-lasting analgesic effect. Interestingly, the lower doses used in this study showed analgesic effect starting on day two and lasting until day five, further indicating time-dependency to the analgesic effect of SPMs.

Peripheral inflammatory pain induces changes in the spinal cord circuit that may lead to central sensitisation (Woolf, 1994; Ma *et al.*, 1996; Pinho-Ribeiro *et al.*, 2017). In fact, the CFA model induces central sensitisation with a stronger activation of astrocyte when compared to microglia (Cao *et al.*, 2014; Zhu *et al.*, 2014; Liao *et al.*, 2017). The phenomenon of central sensitisation has been recognized as the main driver of pathological pain leading to plastic changes in the central nervous system (Woolf, 1983; Scholz *et al.*, 2007). The interaction between glial cells and nociceptor neurons has been linked to these plastic changes that occur in the spinal cord. In rodents, intrathecal treatment with non-selective (e.g. pentoxifylline) or selective glial cell inhibitors (e.g. minocycline for microglia, and fluorocitrate and α-aminoadipate for astrocytes) reduces inflammatory and chronic pain in different experimental settings (Mika *et al.*, 2009; Osikowicz *et al.*, 2009; Pavao-de-Souza *et al.*, 2012; Zarpelon *et al.*, 2016). This suggests that targeting spinal cytokines or glial cells might represent important analgesic approaches for treating chronic pain (Fattori *et al.*, 2017a; Fattori *et al.*, 2017b; Yekkirala *et al.*, 2017). We show that 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. Of interest, MaR1 prevents p65 NF-κB phosphorylation at Ser536 residue (Gu *et al.*, 2016), which is the same residue targeted by the antibody used in the present work. This is important because both cytokines induce neuronal firing, indicating that nociceptor neurons cells respond to these cytokines (Jin *et al.*, 2006; Binshtok *et al.*, 2008). In the spinal cord, TNF-α and IL-1β also contribute to spinal cord plasticity and thereby central sensitisation (Kawasaki *et al.*, 2008). Mechanistically, both cytokines enhance the amplitude of AMPA- and glutamate-induced excitatory currents, while only IL-1β also reduces GABA- and glycine-induced inhibitory transmission in neurons (Kawasaki *et al.*, 2008). In the periphery, these cytokines also contribute to neutrophil recruitment toward tissue and thereby increasing the inflammatory process and pain (Kolaczkowska *et al.*, 2013; Fattori *et al.*, 2016a). The present data indicate a neuronal effect of MaR1, which would ultimately reduce glial cell activation. However, it remains to be determined if MaR1 acts directly in glial cells.

MaR1 blocks TRPV1 with no effect over TRPA1 (Serhan *et al.*, 2012; Park, 2015). This

SPM possesses an IC_{50} of 0.49 ng/mL and completely blocks capsaicin-induced calcium flux with 3 ng/mL (approximately 8.5 nM) in DRG neurons (Serhan *et al.*, 2012). In trigeminal ganglion neurons, MaR1 blocks capsaicin-induced TRPV1 calcium influx in an even lower concentration (0.35 nM) (Park, 2015), indicating that it is a potent TRPV1 inhibitor. Thus, to address whether the effect of MaR1 over TRPV1 activation in our model, DRG neurons from CFA-stimulated mice treated with vehicle or MaR1 were dissected 3 days after the stimulus for calcium imaging and RT-qPCR. A single treatment with MaR1 at 10 ng prevented TRPV1 activation in the DRG neurons (three days after CFA), as observed by lower baseline levels of calcium influx. Given an increase in this parameter is indicative of DRG neuron activation (Chiu *et al.*, 2013; Blake *et al.*, 2018), the lower baseline levels of calcium influx that we found suggests that MaR1 reduces CFA-induced DRG neurons activation. In accordance, previous work shows that MaR1 at 0.35 nM reduces CFA-induced spontaneous excitatory postsynaptic currents (sEPSCs) frequency and amplitude in trigeminal neurons in trigeminal neurons (Park, 2015). Thus, these data from trigeminal neurons indicate MaR1 might reduce spinal cord plastic changes and inhibits central sensitisation *via* both presynaptic and postsynaptic mechanisms (Park, 2015). Other SPMs, such as RvD1, RvD2, and RvE1 also possess similar effect on neuronal firing (Xu *et al.*, 2010; Park *et al.*, 2011). For instance, RvE1 at 1 ng/mL (approximately 2.85 nM) reduces TNF-α-induced sEPSCs frequency and decreases TNF-α-induced potentiation of NMDA-induced currents in spinal cord neurons (Xu *et al.*, 2010). We observed that MaR1 reduced CFA-induced mRNA expression of the channels *Nav1.8* and *Trpv1*. Strategies targeting these channels are effective at reducing pain (Yu *et al.*, 2011; Liao *et al.*, 2017). Therefore, in addition to reducing neuronal activation (present data and others (Serhan *et al.*, 2012; Park, 2015), it is likely that MaR1 also controls TRPV1 expression in DRG neurons during inflammation.

Upon noxious stimuli, nociceptor neurons release neuropeptides such as CGRP and substance P that control the recruitment of immune cells to the inflammatory foci (Pinho-Ribeiro *et al.*, 2017). In fact, ablating or silencing nociceptor sensory neurons modulate sterile and non-sterile inflammation (Roberson *et al.*, 2013; Talbot *et al.*, 2015; Maruyama *et al.*, 2017; Blake *et al.*, 2018). Herein, we show that MaR1 at 3 ng/mL reduced the release of CGRP by DRG neurons, indicating a possible mechanism by which this SPM reduces inflammation and pain. Thus, the MaR1 inhibition of activation and CGRP release by nociceptor neurons might have contributed to the decreased recruitment of neutrophils and macrophages observed in this work. In fact, MaR1 also reduced the number of neutrophils and macrophages proximal to $CGPR^+$ fibres in the paw skin.

Conclusion

We demonstrated that MaR1 displays a long-lasting analgesic effect in a nanogram range dose (10 ng per mouse, intrathecal) in pre- and post-treatment protocols. This analgesic effect is related to the inhibition of astrocyte and microglia activation in the spinal cord. Moreover, MaR1 reduced NF-κB activation and thereby reduced TNF-α and IL-1β production in the spinal cord. In the periphery, MaR1 reduced the number of leukocytes proximal to $CGRP^+$ fibres and at the DRG level, it decreased the mRNA expression of nociceptor neuron sensitisation-related channels *Nav1.8* and *Trpv1*, and reduced DRG neurons activation and CGRP release. The inhibition of CGRP release by nociceptor neurons explains the peripheral effects of MaR1. Given SPMs present a safe pre-clinical profile and efficacy at low doses, they might represent a new family of analgesic drugs useful in the treatment of inflammatory pain of chronic and acute nature.

Authors contribution

FAP-R and LS-F treated the animals and injected the stimuli. VF, SMB and ACR performed behavioural testing. VF, LS-F, ACR, and SMB performed confocal microscopy analysis. VF, FAP-R, LS-F, and ACR performed experiments. VF and LS-F performed *in vitro* experiments. VF, RC, and WAVJ analysed and interpreted data set. VF and WAVJ delineated the study. RC and WAVJ received grants and provided essential reagents. VF wrote the first draft. VF and WAVJ revised and edited the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

Authors declare no conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figures and figure captions

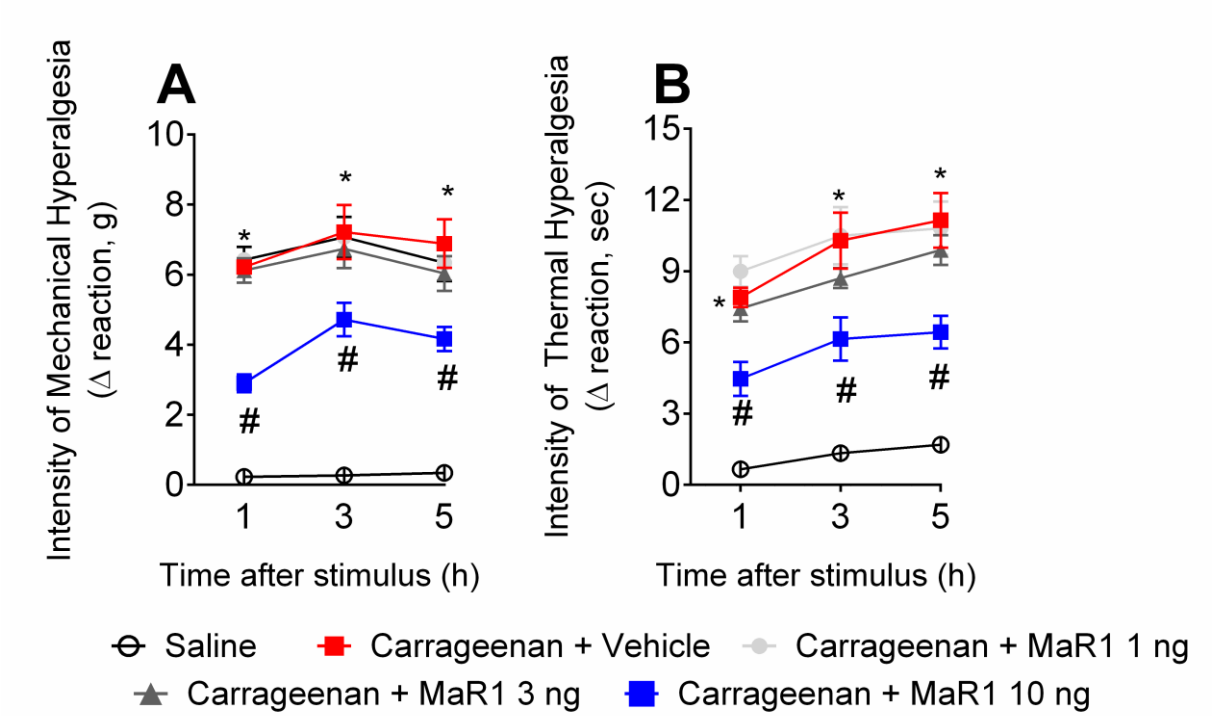
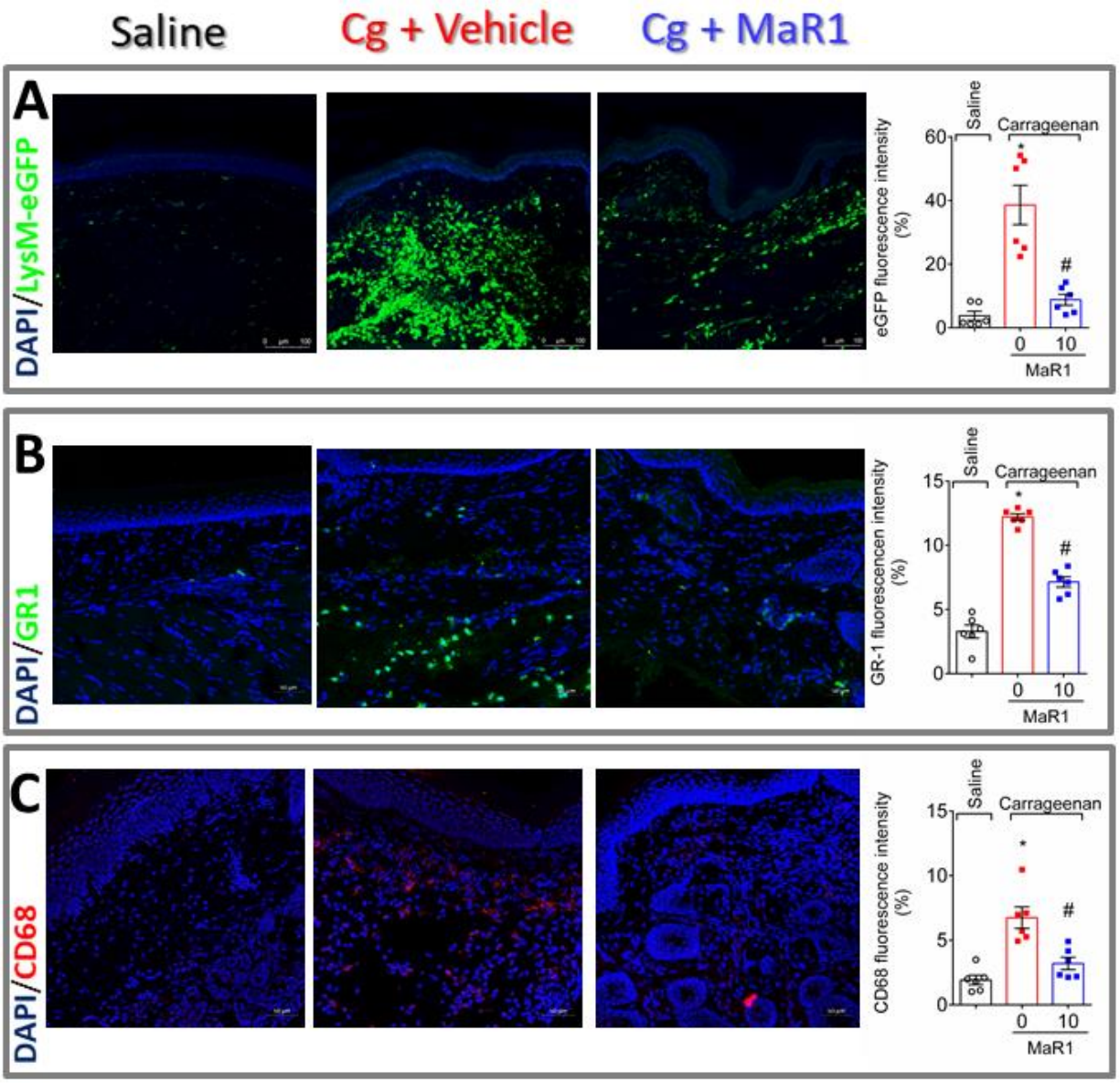


Figure 1. MaR1 reduces carrageenan-induced mechanical and thermal hyperalgesia

Mechanical hyperalgesia (A) and thermal hyperalgesia (B) were evaluated 1, 3, and 5 after intraplantar injection of carrageenan (100 μg/paw). Results from mechanical hyperalgesia are presented as Δ withdrawal threshold (in grams) and for thermal hyperalgesia as Δ withdrawal threshold (in seconds), which was calculated by subtracting the mean measurements at 1-5 h after carrageenan from the zero-time (baseline values) mean measurements. MaR1 reduced carrageenan-induced mechanical (A) and thermal (B) hyperalgesia. Results are representative of two independent experiment and are presented as mean ± SEM of measurements, n = 6 mice per group per experiment (*$p < 0.05$ vs. saline, #$p < 0.05$ vs. 0 mg/kg group; two-way repeated measures ANOVA followed by Tukey's post-test).



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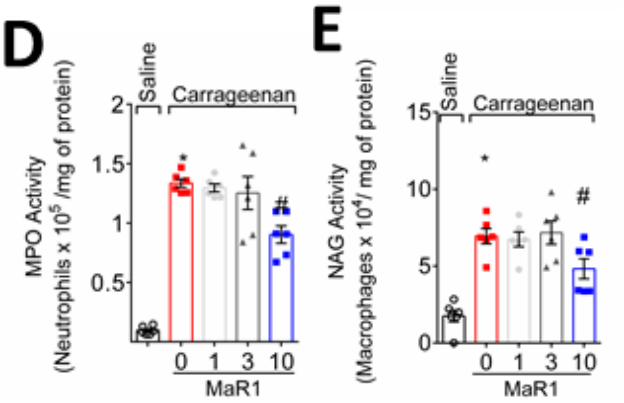

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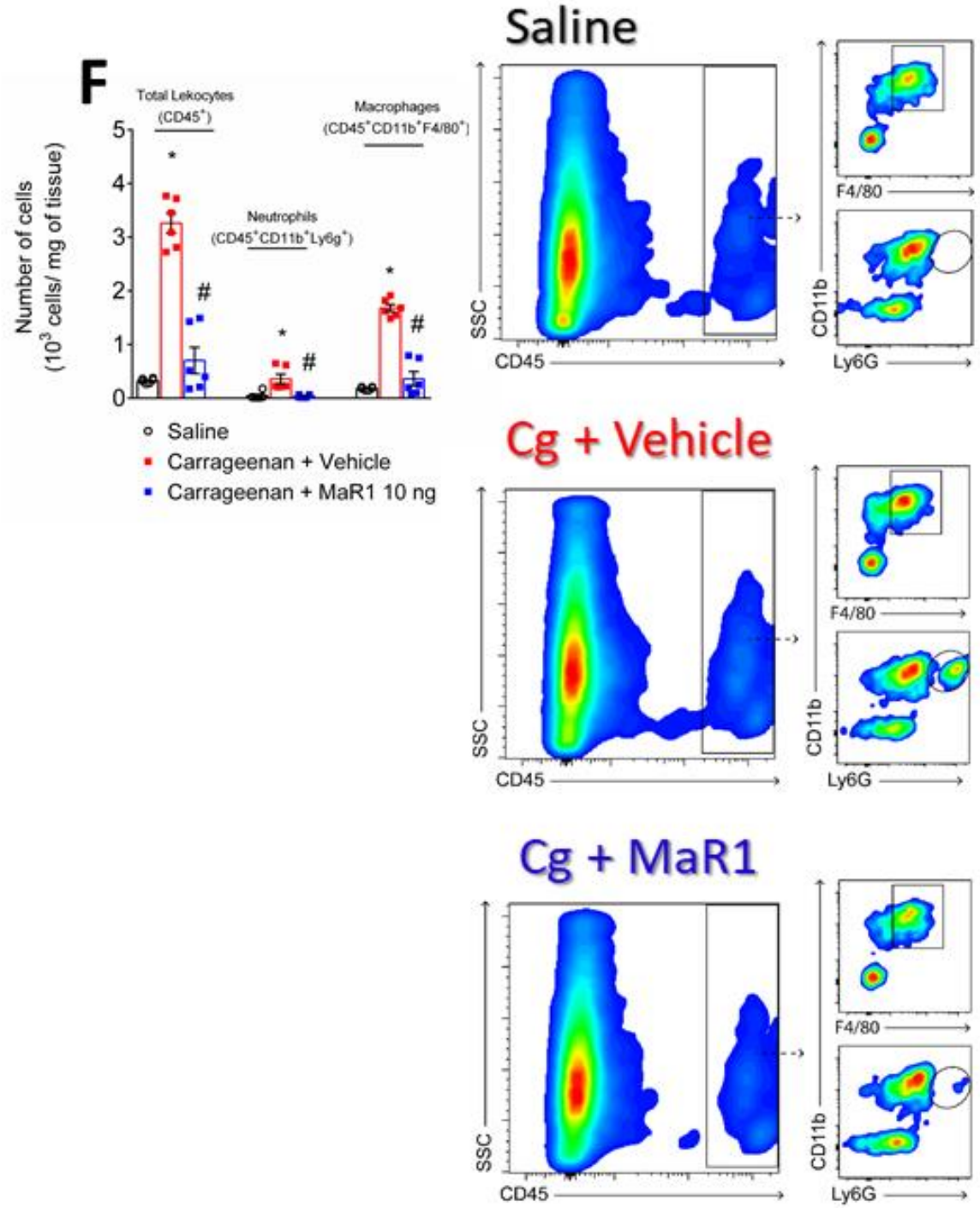


Figure 2. MaR1 inhibits carrageenan-induced neutrophil and macrophage recruitment to the hind paw skin

Hind paw skin of LysM-eGFP (neutrophil and macrophage marker, A) or Swiss (B-F) mice was dissected for determination of neutrophil (GR-1 staining [B], MPO activity [D], and flow cytometry [$CD11b^+Ly6G^+$ cells, F]) and macrophage (CD68 staining [C], NAG activity [E], and flow cytometry [$CD11b^+F4/80^+$ cells, F]) recruitment five hours after carrageenan stimulus. LysM-eGFP mouse, immunofluorescence, enzymatic activity and flow cytometry data show

that MaR1 reduced carrageenan-induced recruitment of neutrophils and macrophages in the paw skin. Results are expressed as mean ± SEM, n = 6 mice per group per experiment, two independent experiments (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group; one-way ANOVA followed by Tukey's post-test.

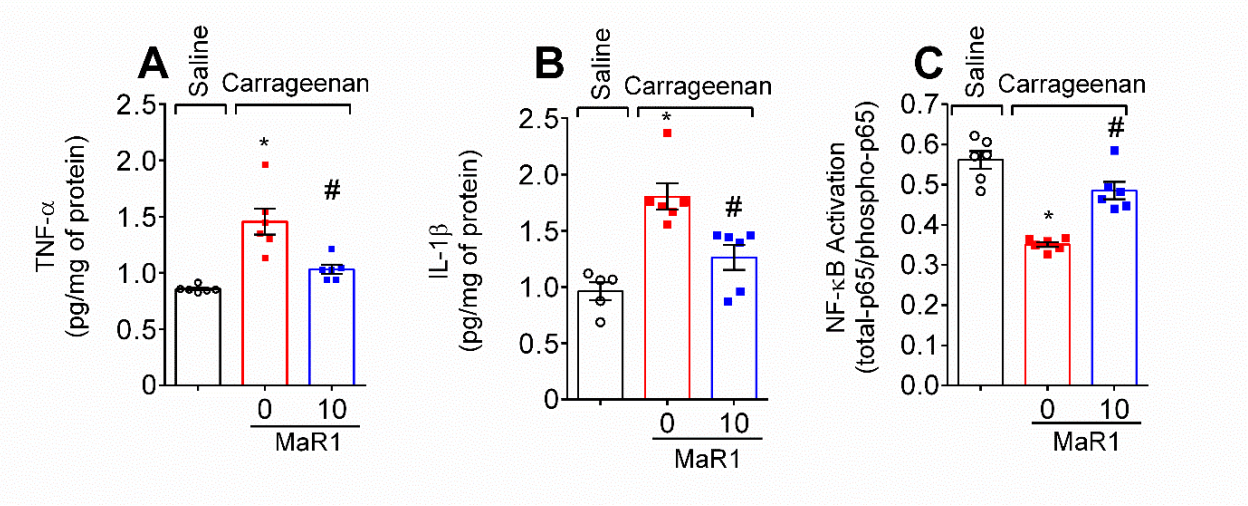
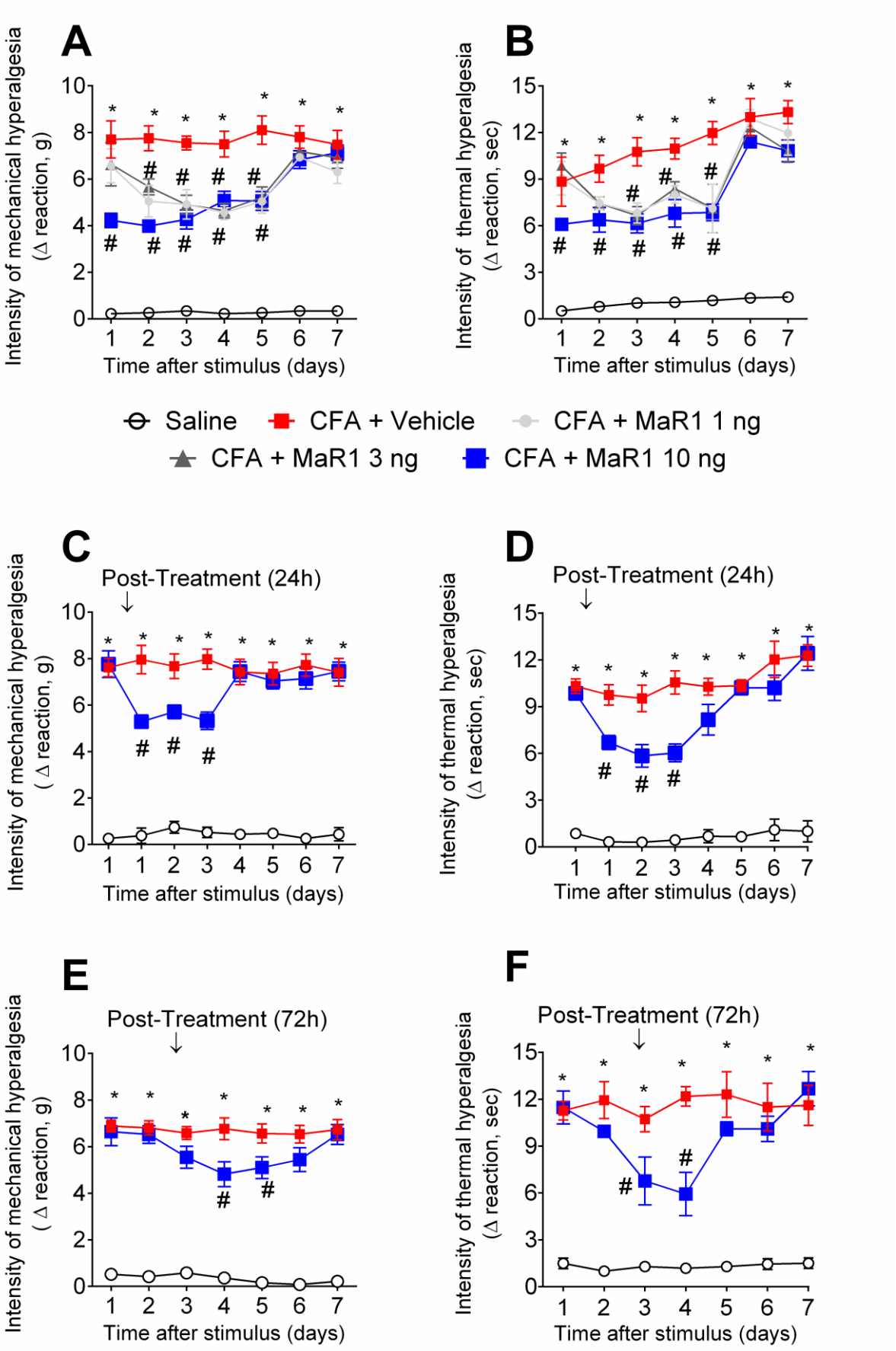


Figure 3. MaR1 inhibits carrageenan-induced spinal cord cytokine production and NF-κB activation

Three hours after intraplantar injection of carrageenan (100 μg/paw), spinal cord was dissected for determination of TNF-α (A), IL-1β (B), and NF-κB activation (C) by ELISA. NF-κB activation was observed as a reduction of total p65/phosphorylated p65 OD ratio. Results are representative of two independent experiment and are presented as mean ± SEM, n = 6 mice per group per experiment (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group; one-way ANOVA followed by Tukey's post-test).


A
B
C
D
E
F
Intensity of mechanical hyperalgesia (Δ reaction, g)
Intensity of thermal hyperalgesia (Δ reaction, sec)
Time after stimulus (days)
Post-Treatment (24h)
Post-Treatment (72h)
Saline
CFA + Vehicle
CFA + MaR1 1 ng
CFA + MaR1 3 ng
CFA + MaR1 10 ng

1564

1565

Figure 4. MaR1 reduces CFA-induced mechanical and thermal hyperalgesia

Mechanical hyperalgesia (A, C, and E) and thermal hyperalgesia (B, D, and F) were evaluated 1-7 days intraplantar injection of CFA (10 µL/paw). Results from mechanical hyperalgesia are presented as Δ withdrawal threshold (in grams) and for thermal hyperalgesia as Δ withdrawal threshold (in seconds), which were calculated by subtracting the mean measurements at 1-7 days after carrageenan from the zero-time (baseline values) mean measurements. Panels A and B show the analgesic effect of MaR1 as a 20 min pre-treatment. Panels C and D show the analgesic effect of MaR1 as a post-treatment one day after CFA. The measurements at the 1st day (C and D) were shown before and after treatment. Panels E and F show the analgesic effect of MaR1 as post-treatment three days after CFA. Results are representative of two independent experiment and are presented as mean ± SEM of measurements, n = 6 mice per group per experiment (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group; two-way repeated measures ANOVA followed by Tukey's post-test).

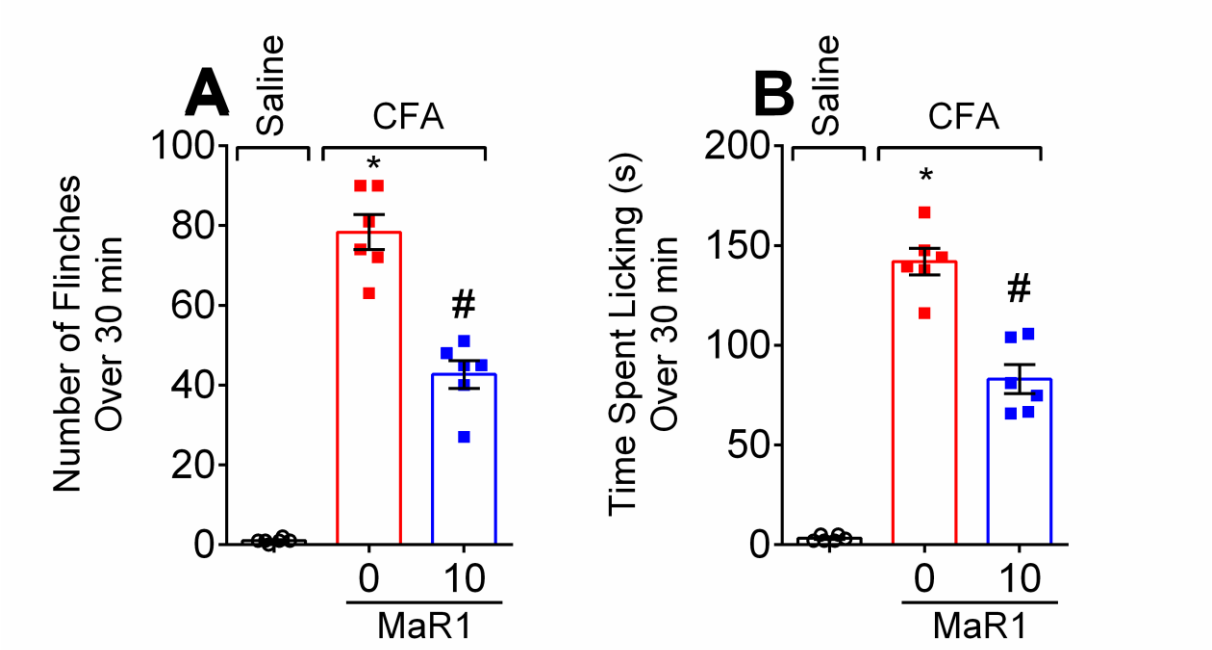
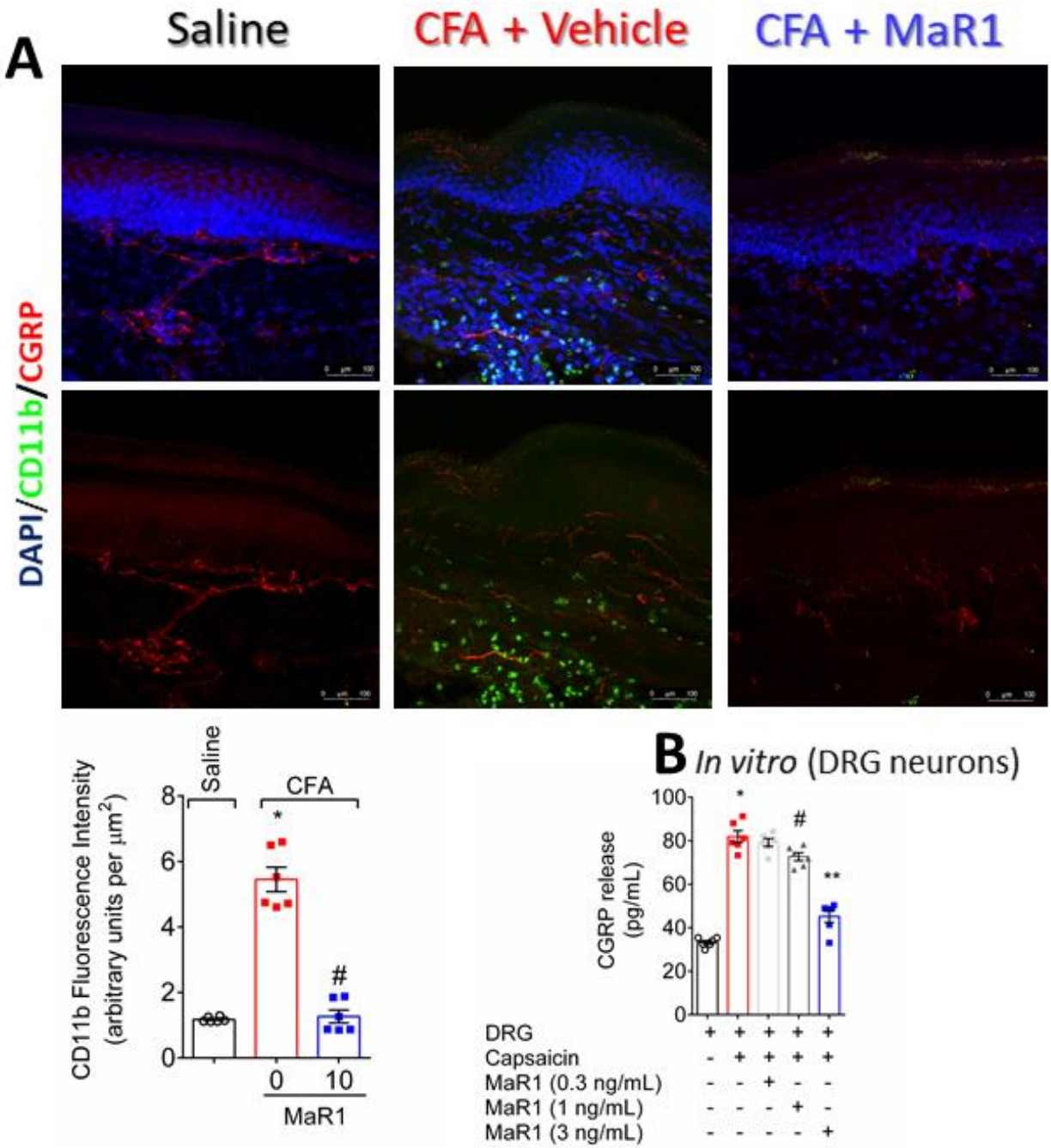


Figure 5. MaR1 decreases CFA-induced overt pain-like behaviour

CFA induced repetitive paw flinches (A) and licking of the paw (B), which were determined over 30 min 1 day after intraplantar injection of CFA (10 µL/paw). Results are representative of two independent experiment and are presented as mean ± SEM, n = 6 mice per group per experiments (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group; one-way ANOVA followed by Tukey's post-test).


A
Saline
CFA + Vehicle
CFA + MaR1
DAPI/CD11b/CGRP
CD11b Fluorescence Intensity (arbitrary units per μm²)
Saline
CFA
0
2
4
6
8
*
#
0
10
MaR1
B In vitro (DRG neurons)
CGRP release (pg/mL)
0
20
40
60
80
100
*
#
**
DRG
Capsaicin
MaR1 (0.3 ng/mL)
MaR1 (1 ng/mL)
MaR1 (3 ng/mL)

1588

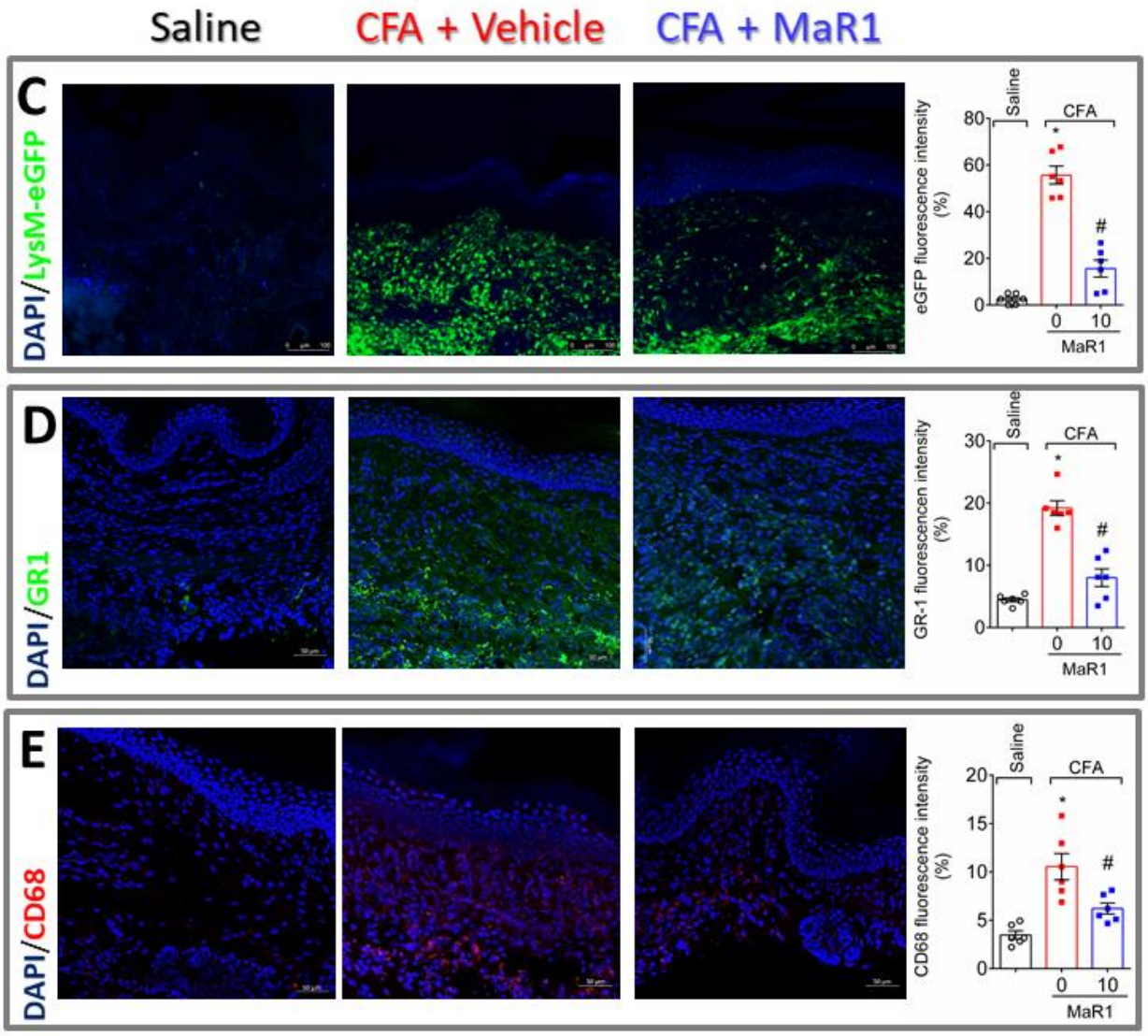
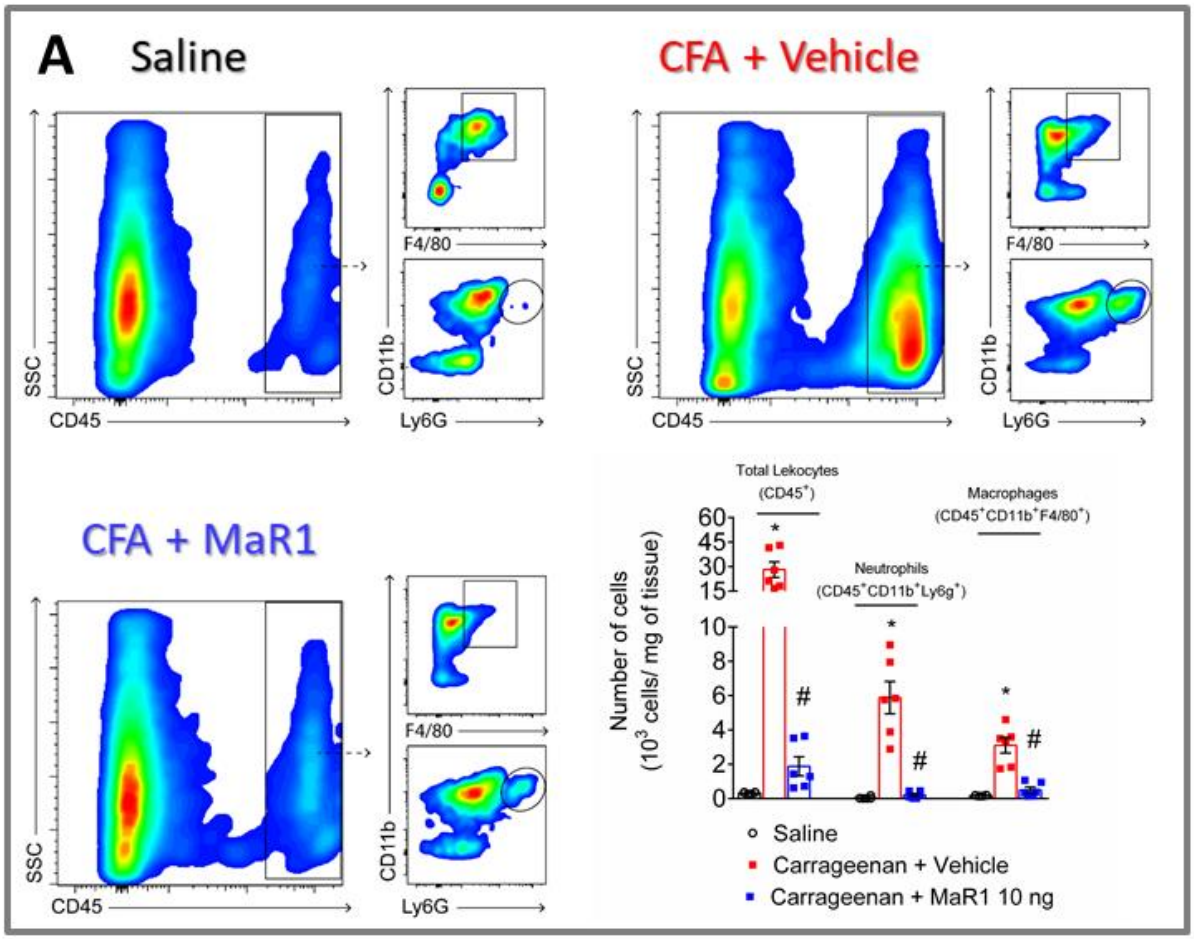


Figure 6. MaR1 reduces the number of leukocytes proximal to CGRP$^+$ fibres and the release of CGRP by DRG neurons.

Hind paw skin was dissected for determination of total leukocytes (CD11b$^+$ cells) close to CGRP$^+$ fibres (A), which showed an increase of total CD11b fluorescence proximal to CGRP$^+$ fibres in the CFA group and reduction by MaR1 treatment. For CGRP release assay (B), naïve DRG neurons received vehicle or different concentrations of MaR1 (0.3, 1, or 3 ng/mL) before stimulus with capsaicin. Supernatant was collected 1h after capsaicin to determine CGRP levels by EIA. Panels C-E analysed with further detail the cellular types recruited to the paw skin during CFA inflammation. LysM-eGFP (C57BL/6 background mice) was used to determine neutrophils and macrophages (C). Staining neutrophils (GR-1, D) and macrophages (CD68, E) markers of hind paw skin samples of Swiss mice also showed that MaR1 reduced CFA-induced recruitment of those cells. Results are expressed as mean ± SEM, n = 6 mice per group per experiment, two independent experiments (*$p < 0.05$ vs. saline, #$p < 0.05$ vs. 0 mg/kg group;

one-way ANOVA followed by Tukey's post-test). Results are expressed as mean ± SEM, n = 6 wells per group per experiment, two independent experiments (*p < 0.05 vs. saline, #p < 0.05 vs. vehicle group; **p < 0.05 vs. 1 ng/mL group; one-way ANOVA followed by Tukey's post-test).



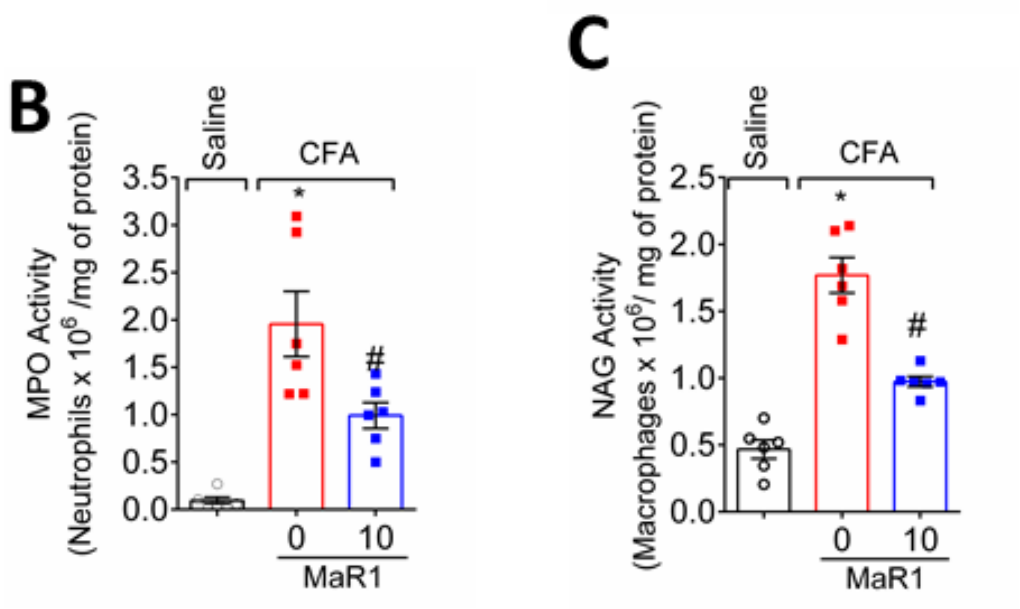


Figure 7. MaR1 inhibits CFA-induced CD11b$^+$Ly6G$^+$ neutrophils and CD11b$^+$F4/80$^+$ macrophage recruitment to the hind paw skin

Hind paw skin was dissected for determination of neutrophil (flow cytometry [A] and MPO activity [B]) and macrophage recruitment (flow cytometry [A] and NAG activity [C]) three days after the stimulus. Results are expressed as mean ± SEM, n = 6 mice per group per experiment, two independent experiments (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group; one-way ANOVA followed by Tukey's post-test).

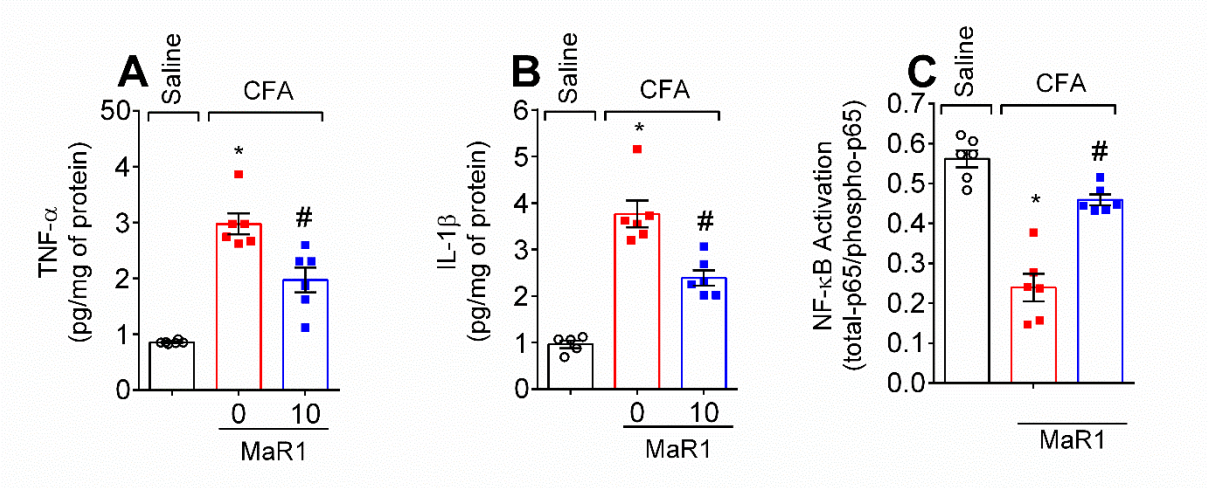


Figure 8. MaR1 inhibits CFA-induced spinal cord cytokine production and NF-κB activation

Three days after intraplantar injection of CFA (10 µL/paw), spinal cord was dissected for determination of TNF-α (A), IL-1β (B), and NF-κB activation (C) by ELISA. NF-κB activation was observed as a reduction of total p65/phosphorylated p65 OD ratio. Results are representative of two independent experiment and are presented as mean ± SEM, n = 6 mice per group per experiments (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group; one-way ANOVA followed by Tukey's post-test).

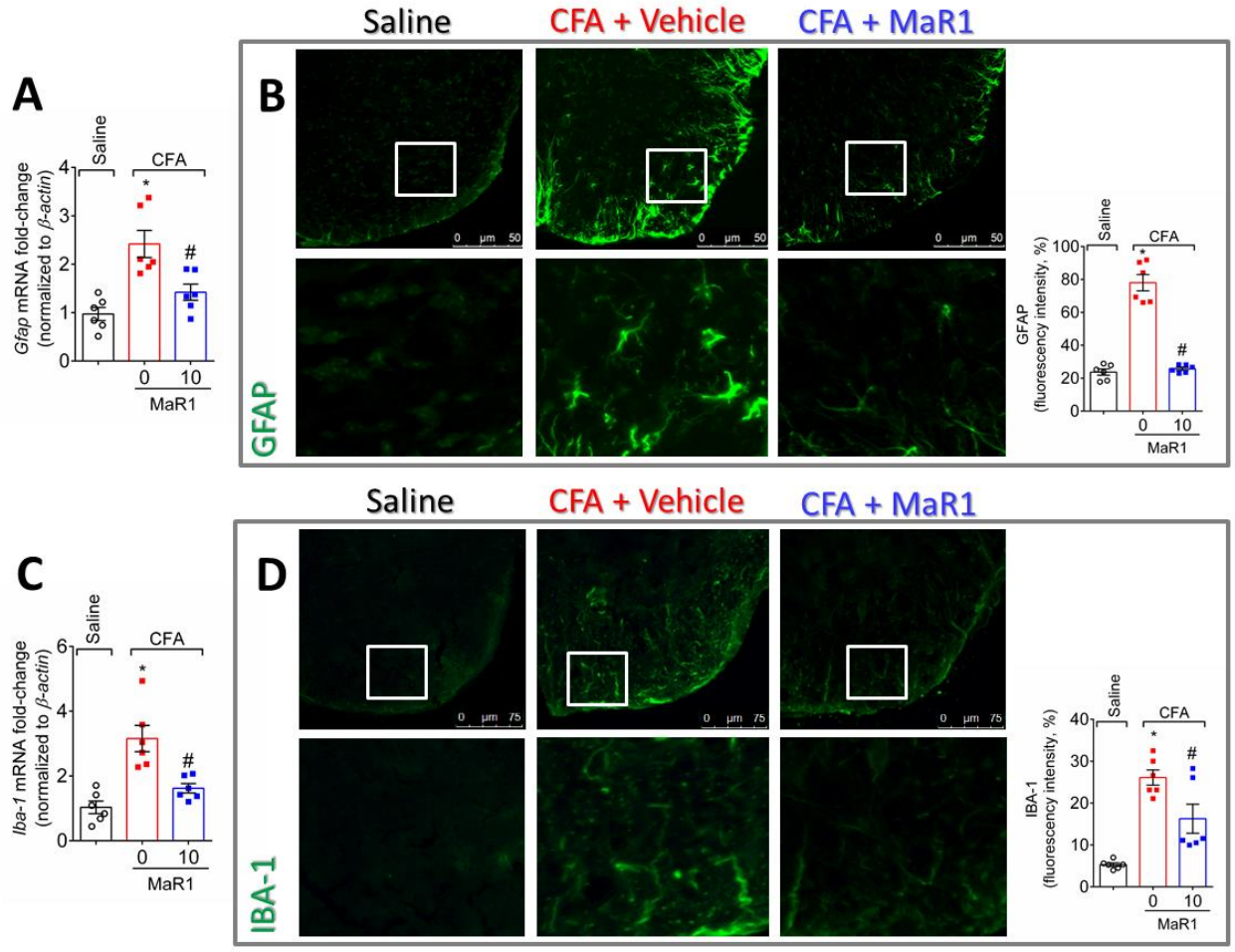


Figure 9. MaR1 decreases CFA-induced astrocyte and microglia activation

Three days after intraplantar injection of CFA (10 µL/paw), spinal cord was dissected for determination of astrocyte and microglia activation by RT-qPCR (A and C) and by immunofluorescence (B and D). GFAP was used as a marker of the activation of astrocytes (A and B) and IBA-1 was used as a marker of microglia activation (C and D). Results are representative of two independent experiment and are presented as mean ± SEM, n = 6 mice per group per experiments (*p < 0.05 vs. saline, #p < 0.05 vs. 0 mg/kg group, **p<0.05 vs 10 mg/kg; one-way ANOVA followed by Tukey's post-test).

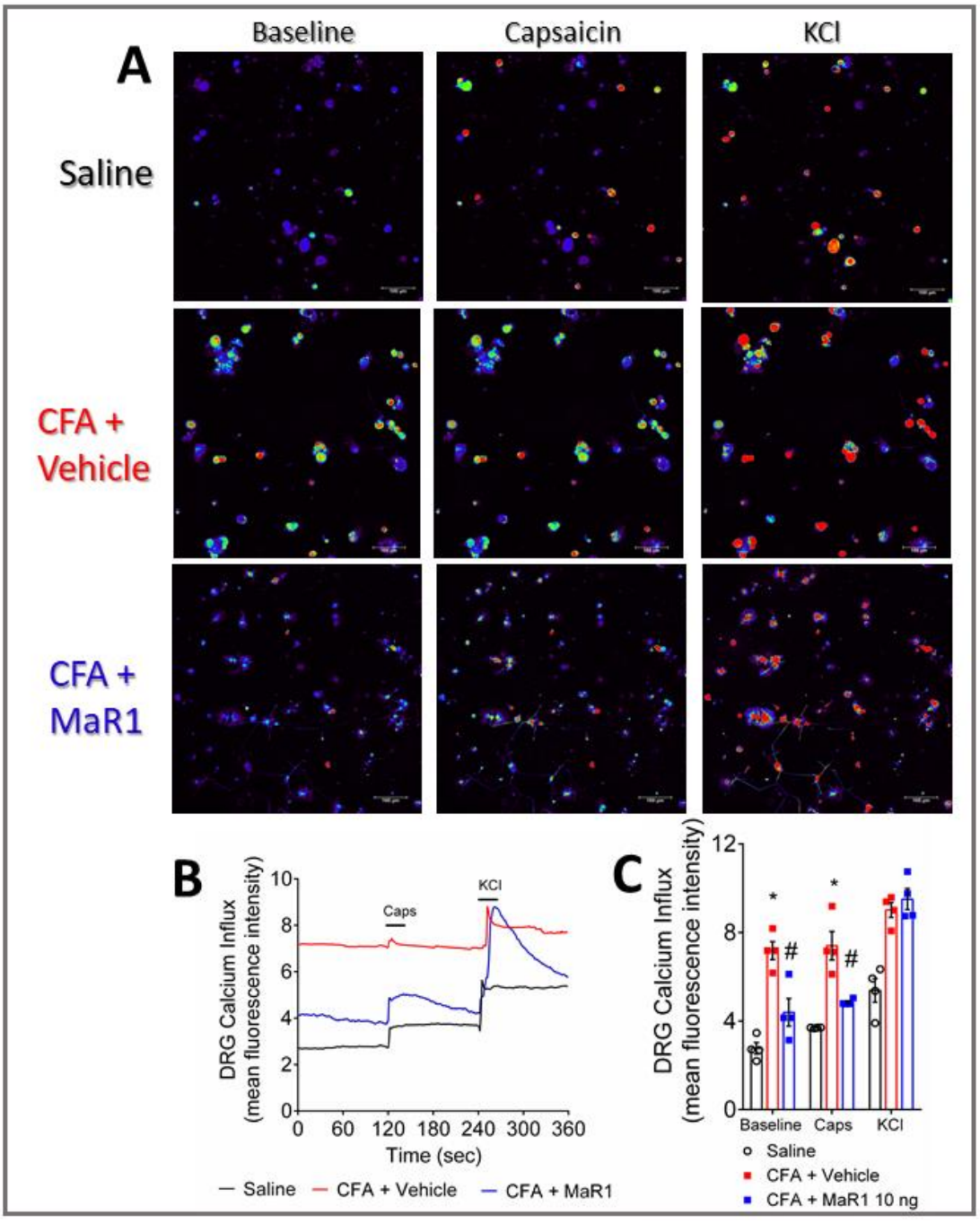


Figure 10. MaR1 reduces CFA-induced activation of DRG neurons

Three days after intraplantar injection of CFA (10 μL/paw), DRGs were dissected for calcium imaging using Fluo-4AM (A-C) and mRNA expression by RT-qPCR (D and E). Panel A displays representative fields of DRG neurons dissected from saline mice, mice stimulated with CFA and treated with vehicle, or stimulated with CFA and treated with MaR1. Panel A: baseline fluorescence (first column), fluorescence after capsaicin (second column), and after KCl control (third column). Panel B displays the fluorescence intensity traces of calcium influx from the representative DRG fields (Panel A) throughout the six min of recording. The representative traces show that the CFA+vehicle DRG neurons presented higher calcium levels in the baseline than saline control and CFA+MaR1 DRG neurons groups. Panel C shows the mean fluorescence intensity of calcium influx on the baseline (0 second mark) and following the stimulus with capsaicin (120 seconds mark, TRPV1 agonist) and KCl (240 seconds mark, activates all neurons). Panels D and E show the DRG neurons RT-qPCR data demonstrating that MaR1 reduced CFA-induced *Nav1.8* (D) and *Trpv1* (E) mRNA expression. Results are expressed as mean ± SEM, n = 4 DRG plates (each plate is a neuronal culture pooled from 6 mice) per group per experiment, and RT-qPCR used n = 6 DRG per group per experiment, two independent experiments (*$p < 0.05$ vs. saline, #$p < 0.05$ vs. 0 mg/kg group; one-way ANOVA followed by Tukey's post-test).

3.2 Non-surgical mouse model of endometriosis-associated pain that responds to clinically active drugs

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Abstract

Endometriosis is an estrogen-dependent inflammatory disease that affects approximately 10% of women. Debilitating pelvic or abdominal pain is one of its major clinical features. Current animal models of endometriosis-associated pain require surgery either to implant tissue or to remove the ovaries. Moreover, existing models do not induce spontaneous pain, which is the primary symptom of patients with chronic pain, including endometriosis. A lack of models that accurately recapitulate the disease phenotype must contribute to the high failure rate of clinical trials for analgesic drugs directed at chronic pain, including those for endometriosis. We set out to establish a murine model of endometriosis-associated pain. Endometriosis was induced non-surgically by injecting a dissociated uterine horn into a recipient mouse. The induced lesions exhibited histological features that resemble human lesions along with an increase in pro-inflammatory cytokines and recruitment of immune cells. We also observed the presence of CGRP-, TRPA1-, and TRPV1-expressing nerve fibers in the lesions. This model induced mechanical allodynia, spontaneous abdominal pain, and changes in thermal selection behavior that indicate discomfort. These behavioral changes were reduced by drugs used clinically for endometriosis, specifically letrozole (aromatase inhibitor) and danazol (androgen). Endometriosis also induced neuronal changes as evidenced by activation of the NF-κB signaling pathway in TRPA1- and TRPV1-expressing DRG neurons. In conclusion, we have established a model of endometriosis-associated pain that responds to clinically active drugs and can, therefore, be used to identify novel therapies.

Introduction

Endometriosis is an estrogen-dependent inflammatory disease that affects 5 to 10% of reproductive-age women in the United States [11]. Histologically, the defining characteristic of endometriosis is the ectopic presence of tissue (lesions) mimicking the appearance of uterine (eutopic) endometrium. These lesions are defined by endometrial-like glands, stroma, and hemosiderin, with blood vessels, nerve fibers, muscle, and immune cells [11]. Retrograde menstruation is hypothesized to be the primary mechanism by which lesions are induced [32; 36]. During retrograde menstruation, damage-associated molecular patterns (DAMPs) can be released. DAMPs activate resident immune cells, including macrophages [26; 57]. Activated immune cells produce pro-inflammatory mediators in an NF-κB-dependent manner, such as TNF-α, IL-1β, and IL-33 that are believed to contribute to pain [26; 57]. In response to these stimuli, nociceptor neurons further increase and maintain inflammation by secreting neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) [36; 57], as well as chemokines such as CCL2 [23]. These mediators may also contribute to the recruitment of more immune cells and lesion growth [23; 36].

One key clinical feature of endometriosis is different forms of debilitating pain, including chronic pelvic pain, dysmenorrhea, dyspareunia, and dyschezia [32; 36]. Of these, pelvic pain represents a major clinical problem since women with that symptom report lower quality of life and mental health [14]. Pain causes affected women to lose, on average, 10.8 hours of work weekly, primarily as a result of reduced effectiveness during working time [39]. Pelvic organ cross-sensitization contributes, moreover, to diverse abdominal pain in disorders of the lower gut, pelvic-urinary, or gynecologic organs leading to significant problems in diagnosing and treating these diseases [9].

Most existing rodent models of endometriosis use surgery to either implant tissue or remove ovaries (ovariectomy) [44]. Ovaries are the main source of estrogen, and because endometriosis is an estrogen-dependent inflammatory disease, hormonal supplementation is required to maintain lesion growth [10; 44; 49]. However, surgical incisions in the skin and deeper tissue increase guarding behavior in animals, indicating pain [29; 33] and can also increase peripheral and central neuronal spontaneous activity, suggesting these procedures influence animal behavior [58; 59]. Unfortunately, no model completely resembles all aspects of endometriosis, and pain is only rarely measured [44]. When pain measurements are made, these rarely reflect the human experience. Usually, the primary focus is on evaluating evoked responses [10; 44; 49]. An evoked response to thermal hyperalgesia is the most frequently used behavioral test in rodent models of endometriosis [10; 44; 49], but sensitivity to heat stimuli is

rarely reported by women with endometriosis. The development of critically needed new endometriosis therapies is most like to be successful if disease models recapitulate the human disease phenotype more accurately.

We have established a novel murine model for endometriosis-associated pain that induces neuronal and behavioral changes consistent with the disease phenotype in women. We validated this model by showing that both evoked and spontaneous pain measures were reduced by letrozole (aromatase inhibitor) and danazol (androgen), demonstrating that this model responds to drugs active in human disease.

Material and methods

Study design

Our objective was to standardize a non-surgical mouse model of endometriosis-associated pain. Our strategy was to interrogate both spontaneous and evoked pain and to perform behavioral analysis of freely moving mice. Block randomization was used to randomize subjects into groups resulting in equal sample sizes.

Mice were treated daily by oral gavage with letrozole 5 mg/kg (cat #PHR1540, MilliporeSigma, Burlington, MA, USA), danazol 35 mg/kg (cat #D8399, MilliporeSigma, Burlington, MA, USA), or vehicle (5% Tween 80, 5% DMSO in PBS) starting at day 29 and ending on day 56. All doses were defined based on previous studies showing efficacy at reducing endometriotic lesions (66, 67). All treatments were performed after behavioral testing. The investigators were blinded to the treatment groups in all testings until the end of the experiment and analysis.

Animals

All procedures were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital (protocol number 16-12-3265). Healthy female C57BL/6 mice (Stock #000664, 8 weeks of age, 20 ± 2g), purchased from Jackson Laboratories (Bar Harbor, ME, USA) were used in this study. Mice were randomly assigned and housed in standard clear plastic cages with no more than 5 mice per cage in a 12:12h light/dark cycle with *ad libitum* water and food. Behavioral testing was performed between 9 a.m. and 5 p.m. in a room maintained at a temperature of 21±1°C. All efforts were made to minimize the number of animals used and their suffering. Euthanasia was performed by CO_2 inhalation.

Induction of Endometriosis

After at least one week of acclimatization, donor mice received a subcutaneous injection of 3 μg/mouse estradiol benzoate to stimulate the growth of the endometrium. Seven days later, the uteri of the donor mice were dissected into a Petri dish containing Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Waltham, MA, USA) and split longitudinally with a pair of scissors. Uterine horns from each donor mouse were minced with scissors and scalpel one at the time, ensuring that the maximal diameter of each fragment was consistently smaller than 1 millimeter (mm) (Supplementary Fig 1). Each dissociated uterine horn was then injected

intraperitoneally using an 18G needle (cat #305185 Thin wall, BD, Franklin Lakes, NJ, USA) into a recipient mouse in 500 µL of HBSS. One donor mouse was used for every two endometriosis mice. Sham mice received an intraperitoneal injection of 500 µL of HBSS.

Histological analysis

Lesions were dissected at 14, 28, 42, and 56 days post-induction (dpi) for histopathological analysis using H&E staining. Paraffin-embedded lesions were cut in 5 µm sections and images were taken in an inverted light microscope using 20 and 40x objectives (Zeiss Axio Observer Z1, Carl Zeiss Microscopy, Thornwood, NY, USA). For immunofluorescence, lesions or paired thoracic and lumbosacral (T10-L3 plus L6-S1) dorsal root ganglia (DRG) were dissected at 56 dpi and maintained in 4% paraformaldehyde (PFA, for 24h) and then to 30% sucrose (for 72h). These ganglia were chosen because of their involvement in abdominal pain and pelvic organ cross-sensitization [12; 13; 35]. Optimum cutting temperature reagent (Tissue-Plus O.C.T., Fisher Healthcare, Thermo Fisher Scientific, Waltham, MA, USA)-embedded lesions were cut in 16 µm or 40 µM (only those involving PGP9.5 staining) sections. OCT-embedded DRGs were cut in 12 µm sections. Images were taken and processed on a confocal microscope using 20x objective (Zeiss LSM 880 laser scanning microscope with Airyscan, Carl Zeiss Microscopy, Thornwood, NY, USA). Primary antibodies used in this study are as follow: anti-CD31 (1:100, clone MEC 7.46, cat #ab7388, Abcam, Cambridge, MA, USA); anti-ERα (1:500, cat #ab3575, Abcam, Cambridge, MA, USA); anti-PGP9.5 (1:50, cat #ab8189, Abcam, Cambridge, MA, USA), anti-CGRP (1:500, cat #C8198, MilliporeSigma, Burlington, MA, USA), anti-TRPA1 (1:250, cat #ACC-037, Alomone Labs, Jerusalem, Israel), anti-TRPV1 (1:250, cat #ACC-030, Alomone Labs, Jerusalem, Israel), anti-CD68 (1:500, cat #ab125212, Abcam, Cambridge, MA, USA); anti-GR1 (1:200, clone RB6-8C5, BioLegend, San Diego, CA, USA); anti-Tryptase (1:100, cat #ab2378, Abcam, Cambridge, MA, USA); anti-CD45 (1:500, cat # ab10558, Abcam, Cambridge, MA, USA); pNF-κB p65 (1:50, cat #sc-166748, Santa Cruz Biotechnology, Dallas, TX, USA). Secondary antibodies used in this study were: goat anti-mouse Alexa 488 (1:4000, cat #A-10680, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA); goat anti-rat 647 (1:500, cat #A-21247, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA); goat anti-rabbit DyLight 488 (1:200, cat #DI-1488, Peterborough, United Kingdom); goat anti-rabbit DyLight 594 (1:200, cat #DI-1594, Peterborough, United Kingdom); goat anti-mouse Alexa 488 (1:2000, cat #A-11001, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

Calcium imaging

Calcium imaging of DRG neurons (T10-L3, L6-S1) was performed as previously described [41]. DRGs were dissected into Neurobasal-A medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), dissociated in collagenase A (1 mg/mL)/dispase II (2.4 U/mL) (RocheApplied Sciences, Indianapolis, IN, USA) in HEPES-buffered saline (MilliporeSigma, Burlington, MA, USA) for 70 min at 37°C. After trituration with glass Pasteur pipettes of decreasing size, DRG cells were centrifuged over a 10% BSA gradient, plated on laminin-coated cell culture dishes. DRGs were loaded with 5 uM of Fluo-4AM in Neurobasal-A medium, incubated for 30 min 37°C, washed with HBSS, and imaged in using an Eclipse Ti-S/L100 inverted microscope (Nikon Instruments, Melville, NY, USA). An ultraviolet light source (Lambda XL lamp, Sutter Instrument) was used for excitation of Fura-2-AM by alternating 340 nm and 380 nm wavelengths. NIS-elements software (Nikon Instruments, Melville, NY, USA) was used to image, process, and analyze 340/380 ratiometric images from DRG neurons. An increase in the 340/380 ratio of 10% or more from baseline levels was considered a positive response to a ligand. To assess TRPV1 or TRPA1 activation, DRG plates were recorded for eight minutes, which was divided into two minutes of initial reading (0-second mark, baseline values), following at the 120 seconds mark by stimulation with capsaicin (500 nM, TRPV1 agonist) or AITC (100 µM, TRPA1 agonist) for four minutes, and finally, beginning at the 360 seconds mark, two minutes of depolarization with KCl (40 mM) to activate all neurons.

Western blot

Lesions or paired thoracic and lumbosacral (T10-L3 plus L6-S1) DRG neurons from sham and endo mice were dissected at 56 dpi. Protein levels were quantified using Pierce™ BCA Protein Assay Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Western blot was performed using 15 µg of protein blotted onto PVDF membranes. Following blocking with 5% dry milk in TBS-T (Tween 0.01%), pH 7.4 we probed with the following primary antibodies in blocking buffer: anti-TRPA1 (1:2000, cat #ACC-037, Alomone Labs, Jerusalem, Israel), anti-TRPV1 (1:2000, cat #ACC-030, Alomone Labs, Jerusalem, Israel), anti-β-actin (1:4000, cat #A3854, MilliporeSigma, Burlington, MA, USA). After washing with TBST, blots were incubated with secondary antibody in blocking buffer. Secondary antibodies used were: goat anti-rabbit (1:8000, cat #1706515, Bio-Rad, Hercules, CA, USA) and goat anti-mouse (1:8000, cat #1706516, Bio-Rad, Hercules, CA, USA). Image J software (NIH,

Bethesda, MD, USA) was used to measure the optical density of the bands.

Behavioral testing

For mechanical and heat hyperalgesia tests, mice were allowed to habituate to the apparatus for at least 2h and during three consecutive days before the beginning of measurements. After habituation, baseline measurements were obtained on two consecutive days prior to the induction of endometriosis. Pain intensity to a mechanical stimulus (mechanical hyperalgesia) in the abdominal region was measured using von Frey filaments. The experimenter was trained, and care was taken not to stimulate the same point consecutively, and the stimulation of the external genitalia was avoided. A jump or paw flinch was considered a withdrawal response [21; 30]. The mechanical threshold was determined by the up and down method starting with 0.4g filament and calculated using the open-source software Up-Down Reader [20].

To measure pain sensitivity to a heat stimulus (heat hyperalgesia), mice were placed on the temperature-controlled (29 °C) glass plate of a Hargreaves apparatus (Model 390G, IITC Life Science, Woodland Hills, CA, USA). A radiant heat source was used to stimulate the paw by gradually increasing the temperature of the plantar surface. The threshold of pain was determined as the latency (in seconds) to evoke a response of paw withdrawal: paw flinches or licking. An exposure limit of 15 s was used to prevent tissue damage.

For the dynamic weight bearing assay, mice were allowed to habituate to the apparatus for 15 min and during three consecutive days before the beginning of measurements. Mice were gently placed in a small Plexiglas chamber ($11.0 \times 19.7 \times 11.0$ cm) with floor sensors containing pressure transducers (Model BIO-DWB-M, Bioseb, France). The system uses software that records the average weight that each paw exerts on the floor (in grams), without any interference of the investigator. For the testing, the mouse was placed in the chamber and allowed to move freely within the apparatus for a period of five minutes.

For the thermal gradient assay, a continuous temperature gradient (7–50 °C) was established along with a metallic base plate where the mice were gently placed. Mice then walked freely while being video-recorded from above (Bioseb, France) as described previously [1]. After an exploration period (30 minutes), individual mice showed a distinct preference, indicating the most comfortable temperature range. Data are presented by the time spent (in seconds) on each zone set at specific temperatures during 60 min and the absolute sum of the squared difference from the mean (*i.e.* sample variance) was used to determine data dispersion. For each treatment, the best-fitted Gaussian curve was also applied to have a better readout of

data dispersion. Both the absolute sum of squares and best-fitted Gaussian curve were based on the last 30 min of the thermal gradient assay as described previously [1]. Each run lasted 1.5 h, and two mice were simultaneously recorded in separate corridors.

For spontaneous abdominal pain measurements, licking of the abdomen, stretching the abdomen (abdominal contortions), and squashing of the lower abdomen against the floor were quantified as previously described [16; 21; 30]. Briefly, direct abdominal licking was quantified during 10 min using bottom-up video recording. We counted the number of times the mouse directly groomed the abdominal region without going for any other region before or after the behavior. For abdominal contortions, mice were placed in individual chambers in a temperature-controlled (29 °C) glass plate and the number of abdominal contortions was quantified for 10 min. Positive responses consist of a contraction of the abdominal muscle together with stretching of hind limbs. For abdominal squashing, the number of times the mice pressed the lower abdominal region against the floor in five minutes was quantified. All behaviors were confirmed in a dark chamber using bottom-up video without the presence of an investigator. In all testing, the investigators were blinded to the treatments.

Cytokine measurements

Lesions were dissected at 56 dpi and homogenized in 500 µL of the appropriate buffer containing protease inhibitors. Since sham mice do not develop lesions, uterine horns from donor mice were used to determine baseline levels of each cytokine. TNF-α, IL-1β, and IL-33 levels were determined by enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, Waltham, MA, USA) accordingly with manufacturer instructions. Protein levels were quantified using Pierce™ BCA Protein Assay Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The results are expressed as picograms (pg) of each cytokine per mg of protein.

Lesion size

Lesions were carefully dissected at 56 dpi and measured using calipers. Lesion size is expressed in millimeters (mm) calculated from the mean of two measurements (width and height).

Statistical analysis

Results are presented as mean ± SEM. Data were analyzed using the software GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA). Two-way repeated measure

analysis of variance (ANOVA), followed by Tukey's *post hoc*, was used to analyze data from experiments of multiple time points (dynamic weight bearing, mechanical, and thermal hyperalgesia). One-way ANOVA followed by Tukey's *post hoc* was used to analyze data from experiments with a single time point. Comparison between two groups was conducted using Student's t-test. For the percentage of mice with visible lesions, statistical analysis was estimated by the Kaplan-Meier method followed by the logrank test. For all analyses, statistical differences were considered significant when $p < 0.05$.

Results

Mouse model of endometriosis-associated pain induces lesions that resemble human lesions

To induce endometriosis-like lesions without surgery, we dissociated the horn of a donor mouse and injected the resulting cells and tissue fragments into the peritoneal space of a recipient animal. We then performed histological analysis using H&E staining to determine whether this model leads to the formation of lesions that resemble those found in women. We observed throughout the course of this model, the presence of lesions with endometrial-like glands, stromal cells, and blood vessels (Fig. 1A). Fifty-six days post-implantation (dpi), we observed visible lesions in 90% of the mice (Fig. 1B). These lesions varied in shape, color, size, and location, which correlates with the variation observed in women (Fig. 1C). Because endometriosis is an estrogen-dependent disease [11], we evaluated estrogen receptor (ER) expression in lesions. By immunofluorescence, we observed the expression of ERα 14 to 56 dpi (Fig. 1E). Blood vessel presence was also confirmed by anti-CD31 staining. Given the role of cytokines in both endometriosis and pain [36; 52], we next evaluated the levels of TNF-α, IL-1β, and IL-33. These cytokines were chosen because they are increased in the lesions of women with endometriosis [36], nociceptor neurons express their receptors [6; 27; 34], and they are directly involved in pain signaling in other models [25; 53; 56; 62]. Since sham mice do not develop lesions (Fig. 1B), uterine horns from donor mice were used to measure baseline levels for each cytokine. We observed an increase in TNF-α, IL1-β, and IL-33 levels in the ectopic lesions when compared to the uterine horn from donor mice (Fig. 1D). Thus, the lesions observed in our model recapitulate key features found in women with endometriosis.

Mouse lesions show the presence of immune cells close to nerve fibers.

Pain is the most debilitating symptom of endometriosis. In endometriotic lesions, the presence of CGRP-, TRPA1-, and TRPV1-expressing fibers [8; 22; 37; 50] and immune cells, such as macrophages, neutrophils, and mast cells [2; 26; 36] are key factors for pain generation. Therefore, we used immunofluorescence to investigate whether nerve fibers and immune cells are present in the lesions. Lesions were double stained using PGP9.5 (neuronal pan-marker) together with CGRP, TRPA1, or TRPV1. We found CGRP-, TRPA1-, and TRPV1-expressing nerve fibers in the lesions (Fig. 2A). We next addressed whether immune cells are positioned near the nerve fibers. Co-staining with CD45 (pan-immune cell marker) and PGP9.5 demonstrated that CD45-positive cells were in proximity to PGP9.5-positive nerve fibers (Fig. 2B). Additional staining showed the presence of macrophages (CD68), mast cells (tryptase), and neutrophils (GR1) (Fig. 2C). These results demonstrate that the nerve fibers and immune

cells, which are a common finding in human lesions, are also found in our mouse endometriotic lesions.

Endometriosis induces NF-κB activation and increases responsiveness in TRPA1- and TRPV1-expressing DRG neurons

Chronic inflammation induces changes in the neuronal pain circuit leading to peripheral sensitization, which is defined by a reduction in the activation threshold and/or an increase in the magnitude of responsiveness at the peripheral ends of nociceptor neurons [18; 42]. To determine whether endometriosis produces neuronal changes consistent with peripheral sensitization, we performed calcium imaging using dorsal root ganglion (DRG) neurons dissected from mice with endometriosis and shams. Activation of DRG neurons can be observed through an increase in calcium influx upon stimulation [17; 41]. Because we observed the presence of TRPA1- and TRPV1-expressing nerve fibers in the lesions, we investigated the activation of these two subpopulations of DRG neurons by using allyl isothiocyanate (AITC, TRPA1 agonist) or capsaicin (Caps, TRPV1 agonist). Both AITC (Fig. 3A-D) and capsaicin (Fig. 3E-H) increased the percentage of responding neurons (Figs. 3A and E) and the amplitude of the response (Figs. 3B and C; 3F and 3G) of DRG neurons from mice with endometriosis compared to sham-treated animals. These changes indicate that endometriosis modified the responsiveness of TRPA1- and TRPV1-expressing DRG neurons. Extending these results, we observed increased TRPA1 and TRPV1 protein levels (Fig. 3D and H) and colocalization with phosphorylated NF-κB p65 subunit (Figs. 3I and J). We conclude that in our model, TRPA1- and TRPV1-expressing DRG neurons increase their responses to chemical irritants, likely as a result of increased expression of cognate ion channels.

Endometriosis-associated pain is sensitive to treatment with drugs known to have clinical activity.

We next determined whether experimental induction of endometriosis lesions induces pain-related behavioral changes in mice. We first assessed this using von Frey filaments (Fig. 4A) looking for mechanical sensitivity of the abdominal wall, dynamic weight bearing (Fig. 4B), and the Hargreaves assay (thermal sensitivity of the paws) (Fig. 4C). These techniques are capable of detecting abdominal pain in different experimental models [21; 30; 31; 60]. We observed an increase in evoked pain only using von Frey filaments (Fig. 4A), as observed by a decrease in the abdominal mechanical threshold. We did not observe any postural change using dynamic weight bearing (Fig. 4B) or any change in thermal hyperalgesia using the Hargreaves

assay (Fig. 4C). Moreover, we did not observe any change on mouse weight throughout the course of the model (Fig. 4D). Based on that result, the von Frey test was the only evoked-pain method used going forward.

Next, we investigated whether our model could predict the efficacy of clinically active drugs, such as letrozole and danazol [45; 47]. We observed that both letrozole and danazol reduced endometriosis-associated pain (Fig. 5A). Letrozole and danazol, also reduced the percentage of mice with visible lesions when compared to the vehicle-treated group (Fig. 5B). However, the remaining lesions were not significantly smaller than control lesions (Fig. 5C). These results show that estrogen-reducing drugs such as letrozole (an aromatase inhibitor) and danazol (an androgen) reduce lesion number and ameliorate endometriosis-associated evoked pain.

Letrozole and danazol normalizes endometriosis-associated changes on thermal selection

We next used a thermal gradient assay to measure the mouse own's determination of general discomfort [1; 51]. This assay uses video recordings to measure behavior in freely moving mice without the presence of a human investigator [1]. Sham mice prefer temperatures 27 to 36ºC with a higher preference for 34ºC while mice with endometriosis exhibited a more dispersed pattern from 21 to 36ºC with no single preferred temperature (Fig. 6A-C). To quantify the data dispersion, variance in location (temperature) was calculated (Fig. 6B). Mice with endometriosis presented a higher variance and this change in normal thermal selection was alleviated by letrozole and danazol with a return to a strong preference for 34-36ºC.

Endometriosis induces spontaneous pain

Clinical pain, in contrast to acute nociceptive pain, often is accompanied by ongoing or intermittent spontaneous pain [15]. However, existing models of endometriosis have not been shown to induce spontaneous pain [10; 44; 49]. Abdominal contortions induced by chemicals (e.g., acetic acid) have been widely used for modeling visceral pain (65, 66), and we wondered whether the disease-based model of endometriosis generates spontaneous abdominal pain. We found that mice with endometriosis lesions exhibit spontaneous pain-like behaviors such as direct abdominal licking (Fig. 7A), abdominal squashing (Fig. 7B, pressuring of the lower abdominal region against the floor), and abdominal contortions (Fig. 7C, contraction of the abdominal muscle together with stretching of hind limbs) at all evaluated time-points. Both letrozole and danazol decreased these behaviors, demonstrating that this model of endometriosis induces spontaneous pain and that this pain can be reduced by clinically active drugs.

Discussion

Even though pain is the major symptom of endometriosis in most patients, investigators developing existing non-surgical models of endometriosis have not reported any attempt to measure spontaneous pain [10; 44; 49]. Indeed, only a minority of studies measure any indication of pain at all. Stimulus-evoked responses at best correlate only with a few aspects of pain, such as withdrawal from a stimulus or learned behavioral avoidance from a potentially painful situation [15]. This evoked type of response relates to only aspects of the symptoms reported by chronic pain patients, who more often report spontaneous ongoing or intermittent pain [15; 55], which the brain processes differently vs. evoked pain [40]. Moreover, most drugs showing analgesic effects in preclinical models that only measure evoked pain have failed in clinical trials [3; 55; 61]; this includes drugs targeting endometriosis [4]. These results together indicate that while preclinical models evaluating evoked pain may have some utility in the discovery of novel targets or mechanisms of action of new drugs, their ability to predict efficacy in humans is low. There is an unmet need for a disease model of chronic spontaneous pain.

Here we describe a non-surgical mouse model of endometriosis that recapitulates many features of the disease phenotype observed in women, especially regarding spontaneous pain. Importantly, both disease and pain phenotypes in the model respond to clinically active drugs. The ability to control the composition and timing of the inoculum may enable studies that contribute to understanding mechanisms of disease development as well as the identification of clinically useful prophylactic treatments. We selected C57BL/6 mice for the model in order to bring the plethora of genome-manipulated strains available in this background to bear on understanding the pathophysiology of endometriosis-associated pain thereby identifying new drug targets. This includes the use of transgenic mice expressing fluorescence and/or luminescent proteins as tissue donors to evaluate and locate lesions in live recipient mice. Due to their small size and large litters, mice are also cost-effective in comparison to other animals. Finally, since this model generates both evoked and spontaneous pain, it is also likely to be useful in predicting the activity of novel drugs to treat existing endometriosis.

We observed an increase in the levels of TNF-α, IL-1β, and IL-33 in the lesion tissue. The presence of cytokines and immune cells contribute to pain generation in endometriosis; TNF-α, IL-1β, and IL-33 are increased in women with endometriosis [36] and increases in either TNF-α or IL-1β both correlate with chronic pelvic pain [36]. These cytokines can act directly on nociceptor neurons [6; 27; 34] thereby initiating pain. In addition, these cytokines act as chemoattractants for immune cells. For instance, in rheumatoid arthritis, TNF-α increases the expression of ST2 (IL-33 receptor) favoring IL-33-induced neutrophil recruitment toward

inflammatory foci [54]. IL-33 also stimulates proliferation and vascularization of surgically-induced endometriosis lesions in mice [38]. Once in the lesion, mast cells, neutrophils, and macrophages produce additional inflammatory mediators that contribute to the maintenance and growth of the lesion [2; 26; 36]. Indeed, reduction of neutrophil recruitment to endometriosis lesions in formyl peptide receptor 1 (FPR1) knockout mice decreases evoked pain, lesion size, mast cell activation, and IL-1β production [19]. The increased cytokine concentrations we observed likely contribute, therefore, to both lesion maintenance and pain generation.

The presence of nerve fibers in lesions is another hallmark of endometriosis [8; 22; 37; 50]. A growing body of evidence demonstrates that immune cells and nociceptor interaction is important for the development of acute and chronic inflammatory diseases [42], including endometriosis [5; 23; 36; 57]. For instance, estradiol stimulates the release of chemokines such as CCL2 by nociceptors. This recruits macrophages toward nerve fibers [23]. Here we demonstrate that immune cells are in close proximity to nerve fibers, which likely contribute to pain both our model and in human disease. Specifically, the main nerve fibers found in women's endometriotic lesions are CGRP-, TRPA1-, and TRPV1-positive fibers [8; 22; 37; 50] and increased expression of TRPV1 in human lesions correlates with chronic pain [43]. Corroborating these clinical data, we show that nerve fibers in mouse endometriotic lesions express both TRPA1 and TRPV1 channels [8; 43]. Furthermore, we observed a pattern of neuronal activation consistent with peripheral sensitization [18; 42] as detected by an increase in NF-κB activation [28; 46] and calcium imaging in DRG neurons [17; 41].

We interrogated pain or general discomfort in our model using both evoked and spontaneous pain behaviors and also assays allowing behavioral choice in freely moving mice (thermal gradient assay) [1]. Among the spontaneous pain behaviors, we detected direct abdominal licking, abdominal squashing, and abdominal contortions. The latter has been widely used for the discovery of novel drugs targeting visceral pain [7; 24]. However, experimental disease models that cause abdominal pain (e.g., colitis, pancreatitis, etc) rarely report abdominal contortions or squashing. Instead, injection of chemical stimuli such as AITC, capsaicin, acetic acid, phenyl-p-benzoquinone, or other chemicals are required to induce spontaneous abdominal pain in mice [48]. Such chemical injections are generally acute and do not adequately reproduce the chronic pain observed in most patients with abdominal pain, which is generally caused by chronic disease [48]. We find that endometriosis induced direct abdominal licking, contortions, and squashing and these behaviors were reduced by letrozole and danazol. Thus, because it is among the first disease models of spontaneous pain, the utility of our model may extend beyond

endometriosis to abdominal and visceral pain more generally.

Conclusion

We have established a model of endometriosis-associated pain that responds to clinically active drugs, such as letrozole (aromatase inhibitor) and danazol (androgen). Importantly, this model recapitulates key features of the disease phenotype in women, such as the presence of endometriotic-like lesions with nerve fibers, blood vessels, immune cells, and pro-inflammatory cytokines. Interrogating spontaneous pain and evoked pain in this model, together with behavioral analysis of freely moving mice, should help facilitate the development of novel therapeutic approaches for endometriosis, including analgesic drugs effective for chronic pain.

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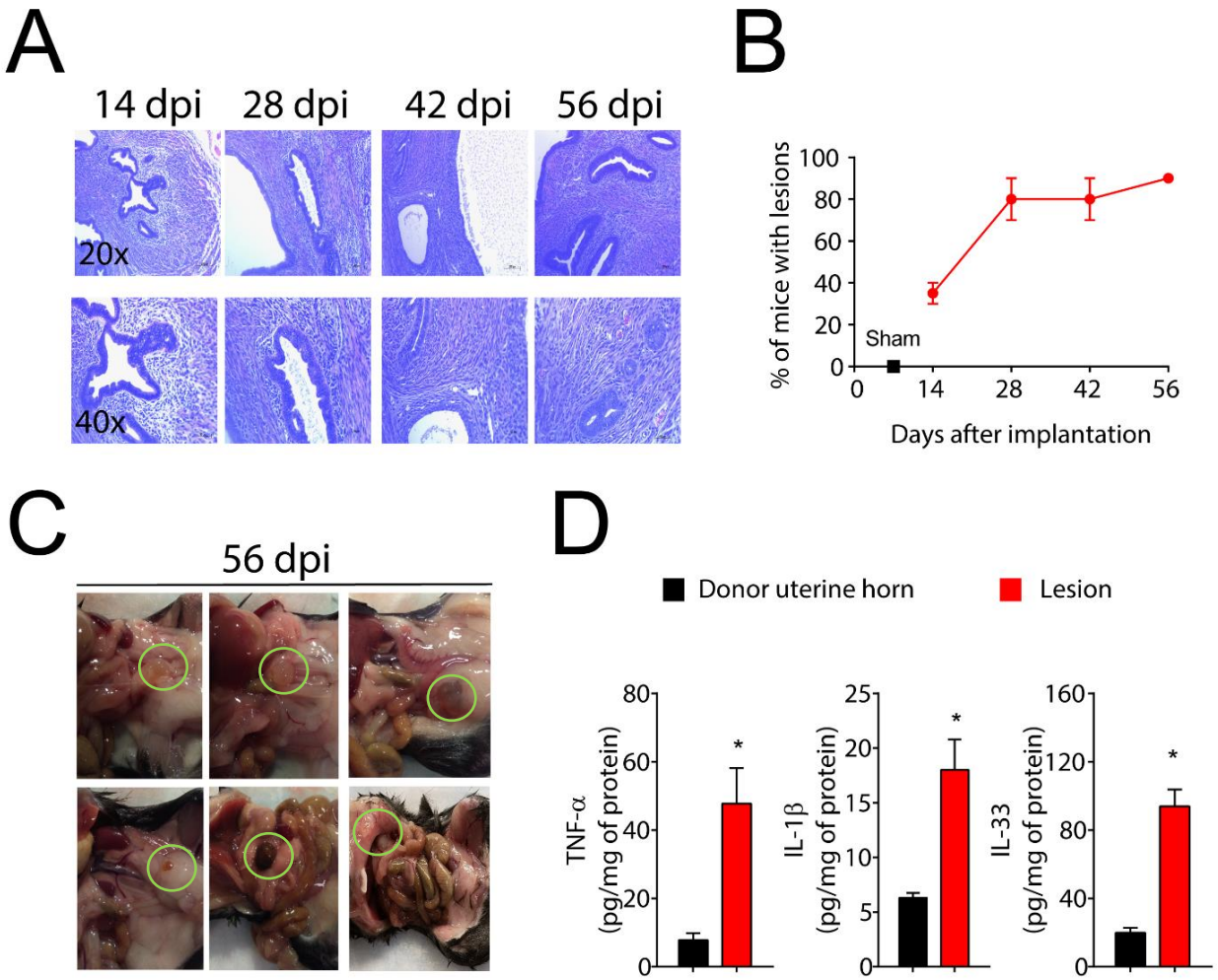
Figures and figure captions



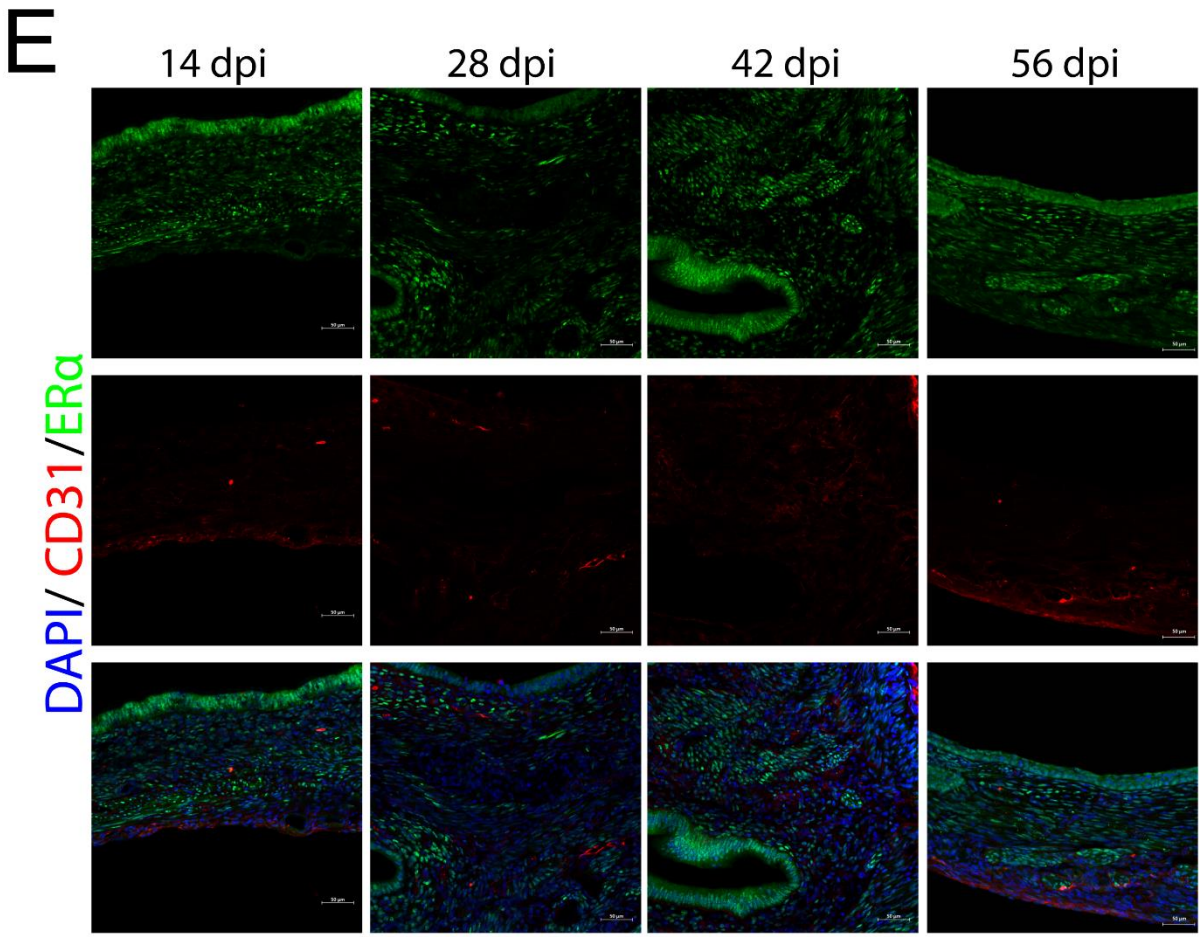


Fig. 1. Mouse model of endometriosis-associated pain induces lesions that resemble human lesions. (A) Endometriotic lesions were dissected for a time-course histopathological analysis by H&E staining. (B) Implantation rate was also quantified in a time-course manner by a simple count of mice with lesions. (C) Representative pictures of the different range of lesions present at 56 dpi. (D) TNF-α, IL-1β, and IL-33 levels in lesion and control tissue as determined at 56 dpi by ELISA. (E) Time-course analysis of ERα expression and blood vessel presence (CD31) in endometriotic lesions as assessed by immunofluorescence. Results are expressed as mean ± SEM, n = 5 or 10 (only panel B) mice per group per experiment, two independent experiments (*$p < 0.05$ vs. control; Student's t-test).

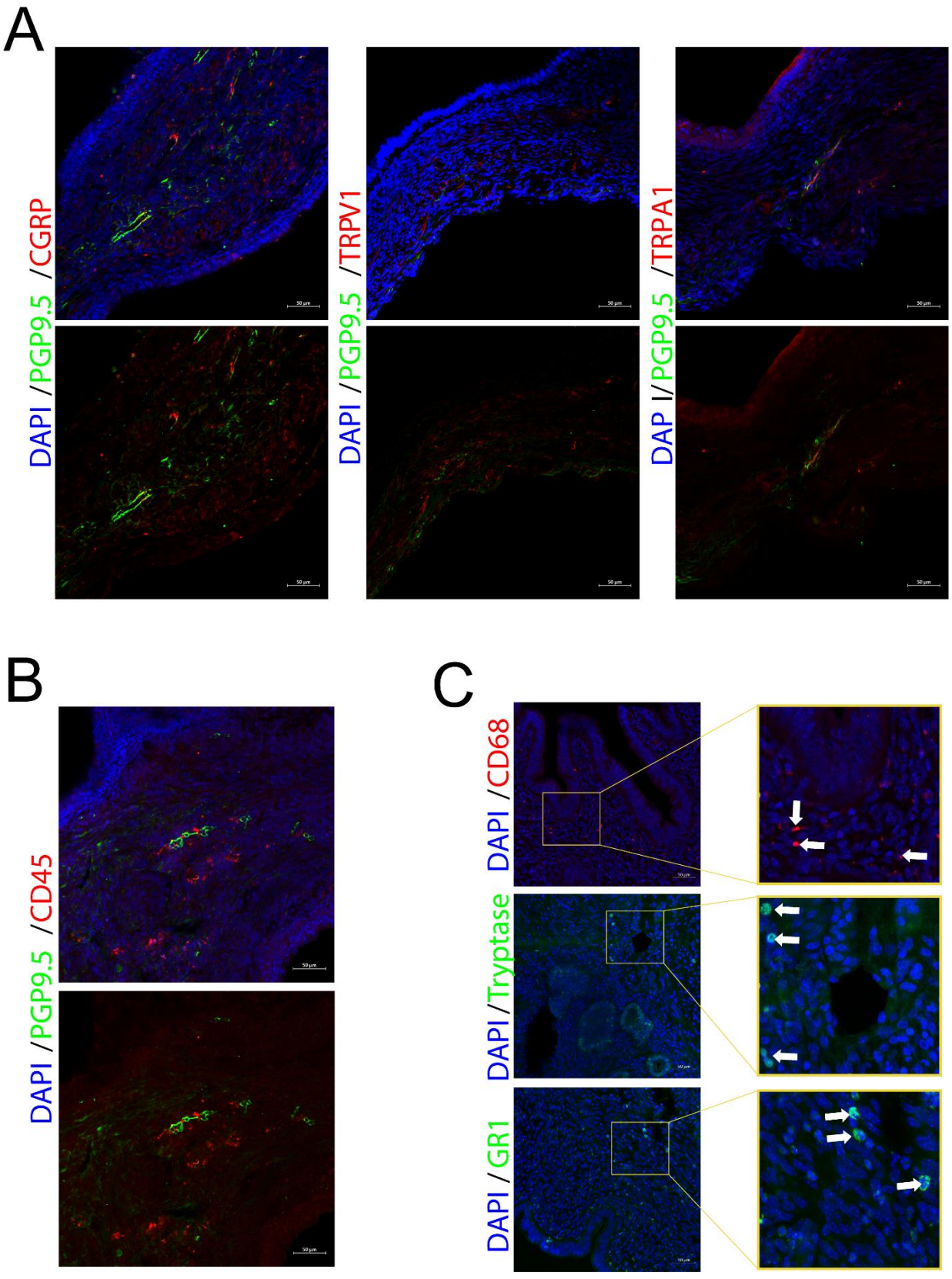
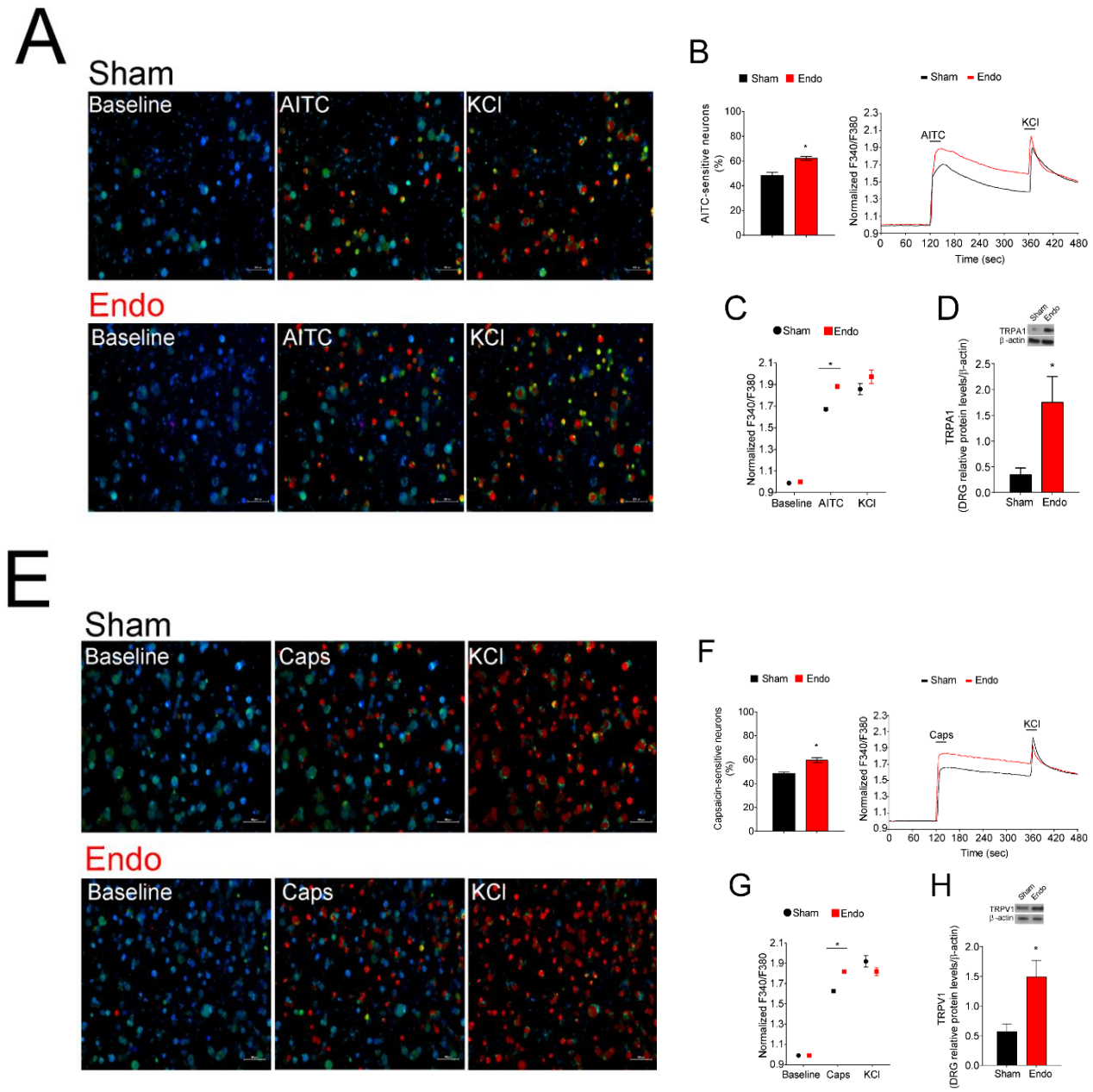


Fig. 2. Mouse lesions show the presence of immune cells close to nerve fibers. (A) Fifty-six dpi, endometriotic lesions were dissected for the determination of CGRP-, TRPA1-, and TRPV1-positive fibers by immunofluorescence. (B) Lesions were dissected for the

determination of immune cells (CD45-positive cells) close to nerve fibers. For panels A and B, PGP9.5 was used as neuronal pan-marker. (C) Macrophage (CD68), mast cell (tryptase), and neutrophil (GR1) recruitment to the lesions were determined by immunofluorescence. Results are expressed as mean ± SEM, n = 5 mice per group per experiment, two independent experiments.



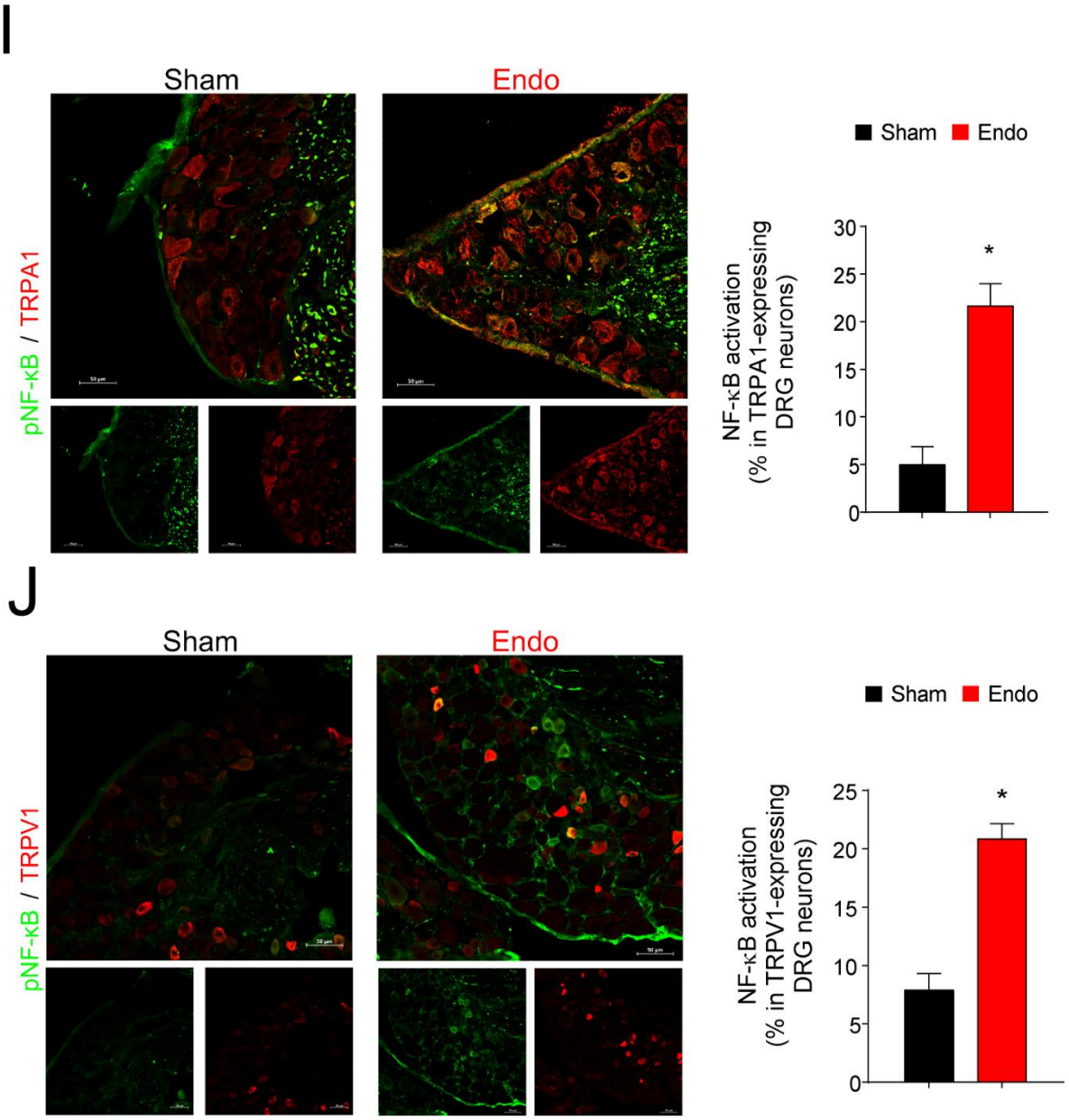


Fig. 3. Endometriosis induces NF-κB activation and increases responsiveness in TRPA1- and TRPV1-expressing DRG neurons. (A-H) Fifty-six dpi, DRG neurons from sham and endometriosis mice were dissected for calcium imaging using Fura-2AM. (B and F) Fluorescence ratio traces representing calcium influx into DRG cells from representative fields (panel A and E) throughout the eight min of recording. (C and G) Mean fluorescence ratio representing intracellular calcium concentration at the baseline (zero-second mark) and following the stimulus with the TRPA1 agonist AITC or the TRPV1 agonist capsaicin (120 seconds mark) and KCl (360 seconds mark, activates all neurons). Results are expressed as mean ± SEM, n = 5 mice (3 DRG plates) per group per experiment, two independent experiments (*$p < 0.05$ vs. sham, Student's t-test [A and E] or two-way ANOVA followed by

Tukey's post hoc [C and G]). (D and H) Fifty-six dpi, DRG neurons from sham and endometriosis mice were dissected for quantification of TRPA1 (D) or TRPV1 (H) protein levels by western blot. Representative bands are displayed next to the graph. Results are expressed as mean ± SEM, n = 4 mice per group per experiment (*$p < 0.05$ vs. sham, Student's t-test). (I and J) Fifty-six dpi, DRG neurons from sham and endo mice were dissected for determination of NF-κB activation in TRPA1- (I) or TRPV1-expressing (J) DRG neurons by immunofluorescence. Results are expressed as mean ± SEM, n = 4 mice per group (*$p < 0.05$ vs. sham, Student's t-test).

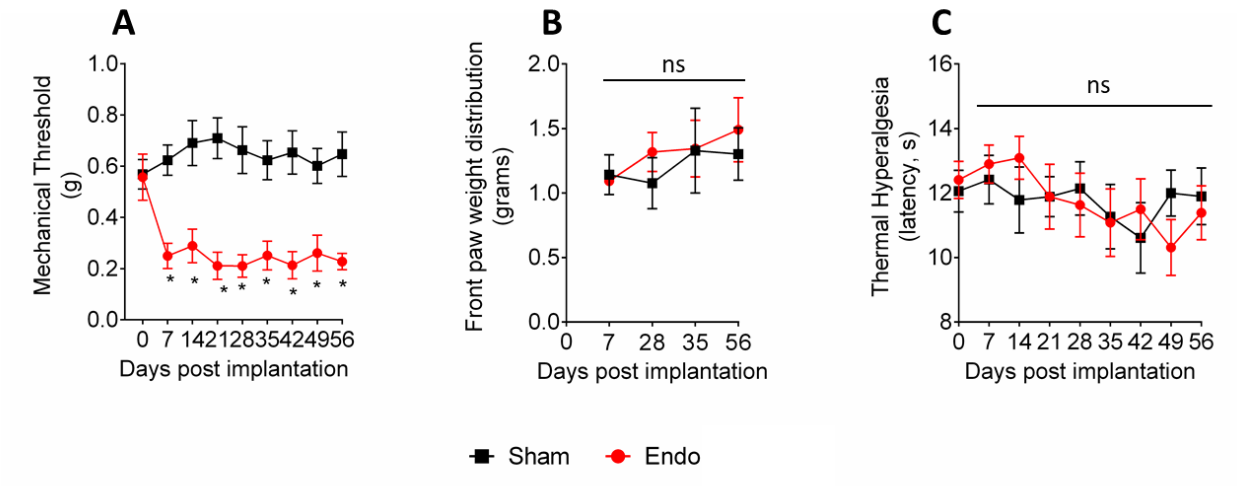


Fig. 4. Endometriosis induces abdominal mechanical pain. (A) Mechanical abdominal pain was determined weekly, 7 to 56 dpi using von Frey filaments. (B) Dynamic weight bearing was used as a non-reflexive method to evaluate abdominal pain. No statistically differences were observed (n.s. = not significant). (C) Thermal hyperalgesia was measured weekly starting 7 to 56 dpi using Hargreaves. No statistically differences were observed (n.s. = not significant). (D) Mouse weight was determined weekly through the course of the model. No statistically significant differences were observed (n.s. = not significant). Results are expressed as mean ± SEM of measurements, n = 10 mice per group per experiment, two independent experiments (*$p < 0.05$ vs. sham, two-way repeated-measures ANOVA followed by Tukey's post hoc).

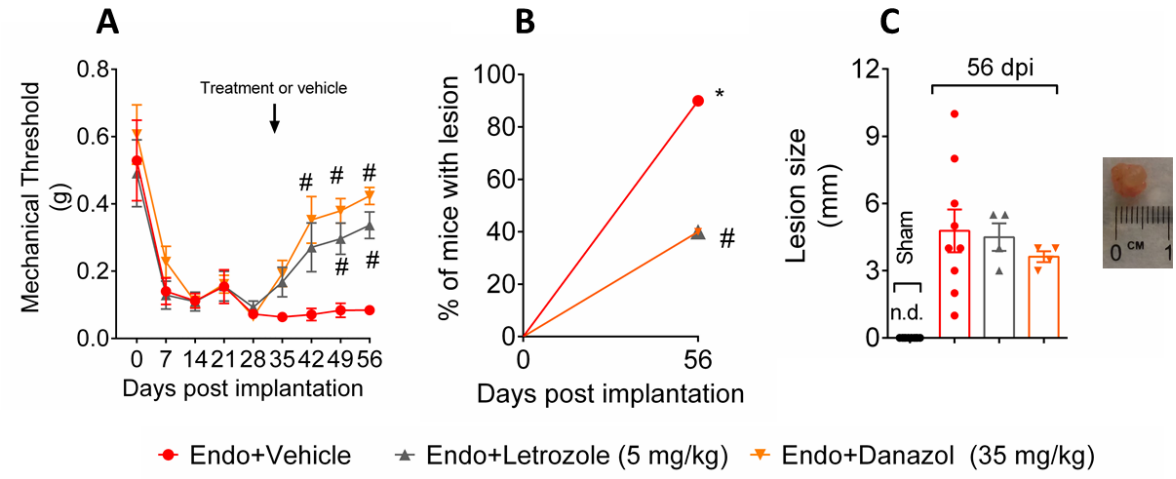
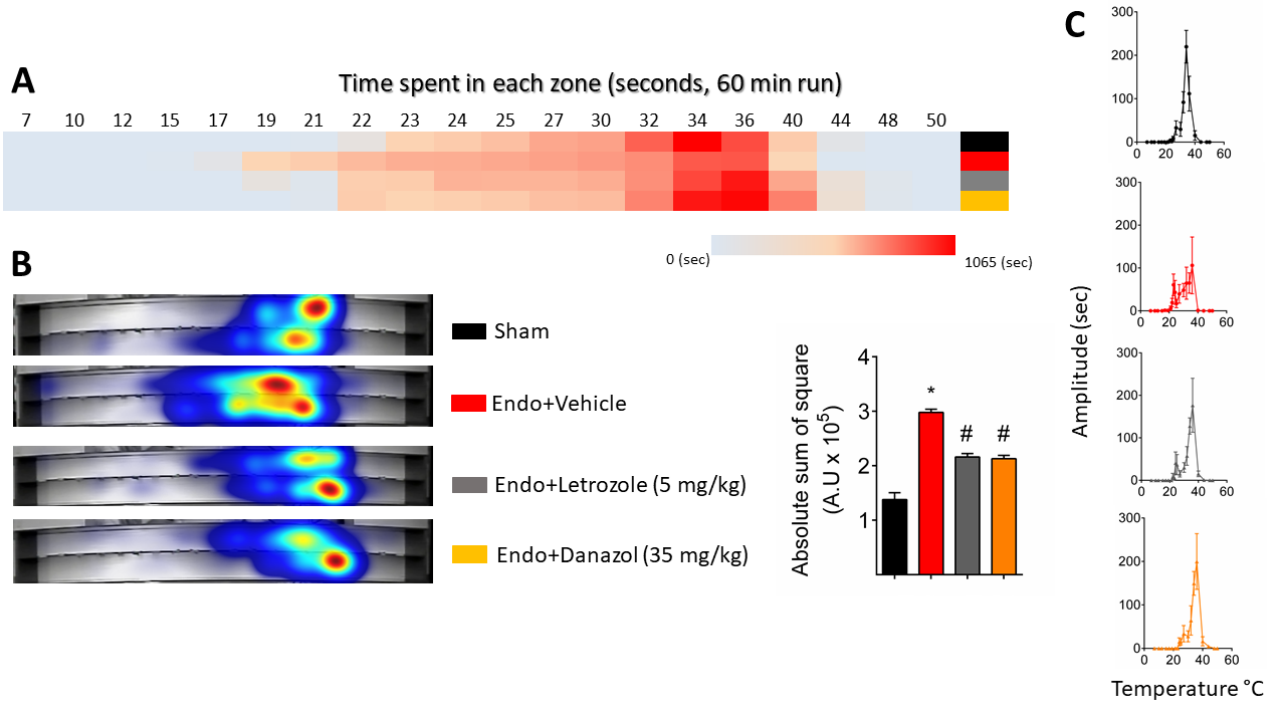


Fig. 5. Endometriosis-associated pain is sensitive to clinically active drugs. (A) Mechanical abdominal pain was determined weekly through 56 dpi using von Frey filaments. Treatment with letrozole, danazol, or vehicle was performed daily starting at 29 dpi and after behavioral testing. Results are expressed as mean ± SEM of measurements, n = 10 mice per group per experiment, two independent experiments (*p < 0.05 vs. sham, #p < 0.0.5 vs endo; two-way repeated-measures ANOVA followed by Tukey's post hoc). (B) The percentage of mice with visible lesions was determined by a simple count of the presence of lesions in mice. Results are expressed as percentage (%) of mice with lesions n = 10 (vehicle-treated group) or 4 (letrozole- and danazol-treated groups) mice per group (*p < 0.05 vs. sham, #p < 0.0.5 vs endo; Kaplan-Meier method followed by the logrank test). (C) Lesion size was determined in the remaining lesions by measuring height and weight. Sham mice do not show any lesion (n.d. = not detectable). A representative picture of a lesion is displayed next to the graph. Results are expressed as mean ± SEM of measurements, n = 10 (vehicle-treated group) or 4 (letrozole- and danazol-treated groups) mice per group (*p < 0.05 vs. sham, #p < 0.0.5 vs endo + vehicle; one-way ANOVA followed by Tukey's post hoc).



2391 **Fig. 6.** Letrozole and danazol normalize endometriosis-associated changes in thermal selection.
2392 (A and B) Heat map of the time spent (in seconds) in each of the zones in the thermal gradient
2393 assay. (B) The absolute sum of square difference from the mean was used to quantify data
2394 dispersion. (C) A best-fit Gaussian curve was applied to confirm differences in data dispersion.
2395 Treatment with letrozole, danazol, or vehicle was performed daily starting at 29 dpi and
2396 occurred after behavioral testing. Results are expressed as mean ± SEM of measurements, n =
2397 6 mice per group per experiment, two independent experiments (*$p < 0.05$ vs. sham, #$p < 0.0.5$
2398 vs endo + vehicle; one-way ANOVA followed by Tukey's post hoc).
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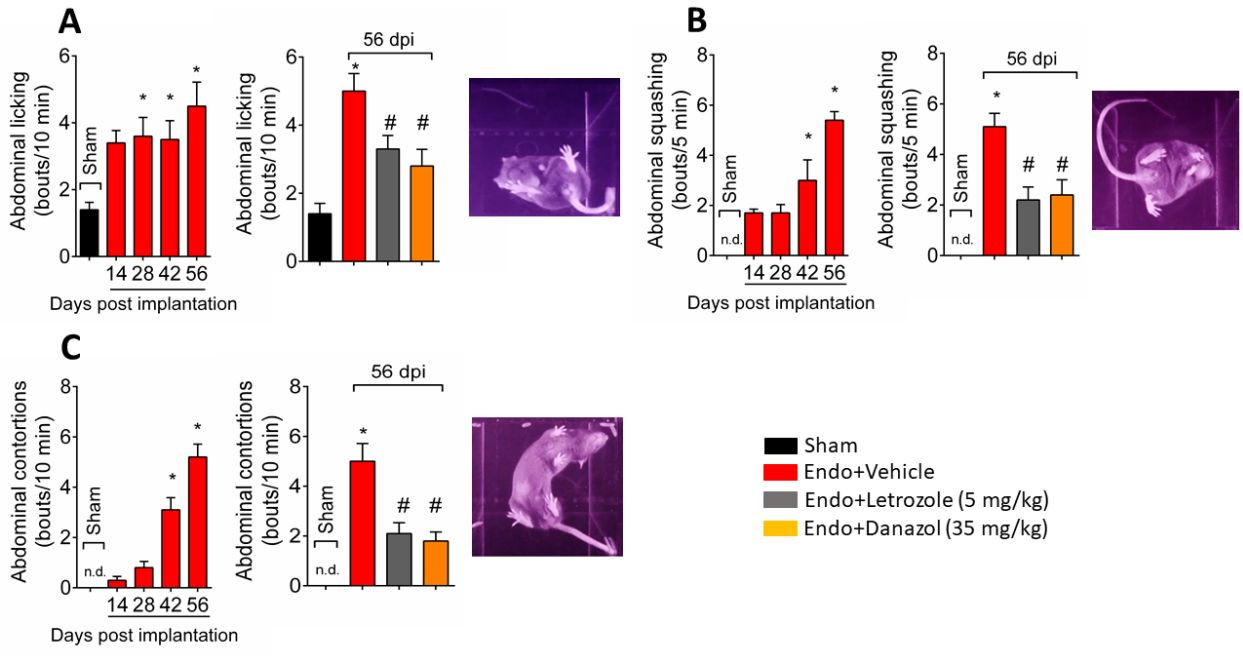

Fig. 7. Endometriosis induces abdominal spontaneous pain. (A) The total number of times that mice directly groomed the abdominal region (without going for any other body region before or after the behavior) was quantified for 10 minutes. (B) For abdominal squashing, the number of times the mice pressed the lower abdominal region against the floor was quantified for five minutes. Sham mice do not display abdominal squashing (n.d. = not detectable). (C) Abdominal contortions were quantified for 10 minutes. A positive response consists of a contraction of the abdominal muscle together with stretching of hind limbs. Sham mice did not display abdominal contortions (n.d. = not detectable). All behaviors were confirmed using a bottom-up video recording on a dark chamber without the presence of an investigator. A representative picture of each behavior is displayed next to the respective graph. Treatment with letrozole, danazol, or vehicle was performed daily starting at 29 dpi and occurred after behavioral testing. Results are expressed as mean ± SEM of measurements, n = 10 mice per group per experiment, two independent experiments (*$p < 0.05$ vs. sham, #$p < 0.0.5$ vs. endo + vehicle; one-way ANOVA followed by Tukey's post hoc).

3.3 Specialized pro-resolving lipid mediators: a new class of non-immunosuppressive and non-opioid analgesic drugs

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Running title: SPMs: a new class of non-immunosuppressive and non-opioid analgesics

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Abstract

We now appreciate that the mechanism of resolution depends on an active and time-dependent biosynthetic shift from pro-inflammatory to pro-resolution mediators, the so-called specialized pro-resolving lipid mediators (SPMs)**.** These SPMs are biosynthesized from the omega-3 fatty acids arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), or docosahexaenoic acid (DHA). Despite effective for a fraction of patients with rheumatic diseases and neuropathic pain, current analgesic therapies such as biological agents, opioids, corticoids, and gabapentinoids cause unwanted side effects, such as immunosuppression, addiction, or induce analgesic tolerance. A growing body of evidence demonstrates that isolated SPMs show efficacy at very low doses and have been successively used as therapeutic drugs to treat pain and infection in experimental models showing no side effects. Moreover, SPMs work as immunoresolvents and some of them present long-lasting analgesic and anti-inflammatory effects (*i.e.* block pain without immunosuppressive effects). In this review, we focus on how SPMs block pain, infection and neuro-immune interactions and, therefore, emerge as a new class of non-immunosuppressive and non-opioid analgesic drugs.

Keywords: analgesia, omega-3, pain treatment, pathological pain, nociceptor, immunoresolvent

1. Introduction

Nociceptive pain works as an early warning device to call our attention to potential danger in the environment and injury. Pain generates adaptive learning intimately linked to negative emotions that ultimately protects the individual (Basbaum *et al.*, 2009; Fattori *et al.*, 2017b). Therefore, to be effective, nociceptive pain must trigger a physiological response that the host cannot ignore. While unpleasant, it is the most important evolutionary conserved protective sign. Individuals diagnosed with congenital insensitivity to pain often harm themselves and have a reduction of lifespan since they cannot differentiate non-noxious from noxious stimuli. Given the importance of the varied emotional shades for the understanding of pain, the taxonomy committee of the International Association for the Study of Pain (IASP) defines it as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." It is now recognized, for example, that mood, cognitive function, memories, state of humor, attention (Bushnell *et al.*, 2013), and the expectations regarding the intensity of the stimulus (Wiech *et al.*, 2014) also shape pain perception.

The contemporary understanding of persistent pathological pain also includes peripheral and spinal cord sensitization of nociceptors and changes in the immune cell phenotypes. While nociceptive pain is triggered by the activation of high threshold nociceptors, in inflammatory pain, a shift from a high threshold to a low threshold type of pain promotes mechanical and thermal hypersensitivity where non-noxious stimuli can now generate pain (Woolf *et al.*, 2000; Verri *et al.*, 2006; Pinho-Ribeiro *et al.*, 2017). That hypersensitivity occurs due to sensitization of peripheral nociceptors, known as hyperalgesia (increased pain from a stimulus that normally provokes pain) or allodynia (pain due to a stimulus that does not normally provoke pain) (Verri *et al.*, 2006). Interestingly, recent evidence demonstrates that peripheral Schwann cells can also initiate pain behaviors induced by a mechanical stimulus in mice (Abdo *et al.*, 2019). Optogenetic activation of Sox10-positive cells (Schwann cell marker) shows that these cells help to sense mechanical stimuli, and their activation is sufficient to induce peripheral neuronal firing (Abdo *et al.*, 2019). During inflammation, however, peripheral innate immune cells such as macrophages, neutrophils, and mast cell release mediators that act on peripheral nerve terminals (Pinho-Ribeiro *et al.*, 2017). Acting on specific receptors, inflammatory mediators such as prostaglandin (PG) E_2, histamine, and cytokines are the main responsible for reducing neuronal threshold and producing peripheral sensitization (Verri *et al.*, 2006; Fattori *et al.*, 2017c; Pinho-Ribeiro *et al.*, 2017). In addition to those mediators, nociceptors can also recognize painful pathogen-derived products such as streptolysin S (Pinho-Ribeiro *et al.*, 2018),

α-hemolysin (Chiu *et al.*, 2013), and β-glucan (Maruyama *et al.*, 2017). Further increasing that list of mediators, recent evidence demonstrates that neurons recognize collagen type 2 immunocomplex (Bersellini Farinotti *et al.*, 2019). Acting on FcγRI and FcγRIIb receptors expressed by mouse TRPV1-positive dorsal root ganglion (DRG) neurons, collagen type 2 immunocomplex increases neuronal excitability and pain (Bersellini Farinotti *et al.*, 2019). Nociceptors and immune cells, therefore, have evolved to communicate with each other to control inflammatory and host responses against pathogens in a complementary, and yet distinct, way. That neuroimmune communication (or axon-axon immune reflex) is initiated after the release of neuropeptides [such as calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and substance P] (Pinho-Ribeiro *et al.*, 2017). Therefore, upon activation, nociceptors shape neurogenic inflammation and the activity of innate and adaptive immune cells in a context-dependent manner (Pinho-Ribeiro *et al.*, 2017). For instance, while in a context of infection by *Staphylococcus aureus,* blocking nociceptors led to increased immune influx and lymphadenopathy (Chiu *et al.*, 2013), in *Streptococcus pyogenes* infection, blocking nociceptors ameliorates disease outcome by increasing neutrophil recruitment and its bactericidal activity (Pinho-Ribeiro *et al.*, 2018). A recent study, however, demonstrated that activation of TRPV1-positive nociceptors in the absence of other inflammatory stimuli is sufficient to initiate an anticipatory immune response (Cohen *et al.*, 2019). CGRP release upon activation of nociceptors with optogenetics increases IL-17-producing TCRγδ and CD4 T cells that ultimately provide type 17 immunity at areas adjacent to the stimulation site and increase host defense in the surrounding tissue (Cohen *et al.*, 2019). This work unraveled a new role for neurons in functional anticipatory innate immunity at sites adjacent to infection.

Chronic inflammation induces neuroplasticity that modify nociceptor phenotype and function in the CNS. Central sensitization is recognized as the main driver of persistent pathological pain, where nociceptors participate in pain processing rather than being only cells conducting nervous impulse. Central sensitization was initially described as an increase in the excitatory synaptic transmission and decrease in the inhibitory synaptic transmission in the dorsal horn of the spinal cord (Woolf, 1983; Woolf *et al.*, 2000; Ji *et al.*, 2001). We now understand, however, that neuronal plasticity in central sensitization is resultant of complex interactions between nociceptors and glial cells (Ji *et al.*, 2016; Pinho-Ribeiro *et al.*, 2017). In the spinal cord, this sensitization, therefore, is mediated by cytokines, chemokines, and growth factors released by resident cells known as microglia, astrocytes, and oligodendrocytes (Scholz *et al.*, 2007; Zarpelon *et al.*, 2016; Fattori *et al.*, 2017b). While neurotransmitters such as glutamate, GABA, and glycine produce synaptic effects at μM range; cytokines, chemokines,

and growth factors released by astrocyte, microglia, and oligodendrocyte do the same at nM concentrations (Coull *et al.*, 2005; Kawasaki *et al.*, 2008; Gao *et al.*, 2009). In a neuropathic pain model, for instance, dorsal horn oligodendrocytes are the primary source of IL-33 that drives the production of IL-1β and TNF-α by microglia and astrocytes (Zarpelon *et al.*, 2016). Production of these pro-inflammatory molecules by microglia occurs in a MAPK p38-dependent mechanism and by astrocytes in a JNK-dependent mechanism (Grace *et al.*, 2014; Fattori *et al.*, 2017b; Zhang *et al.*, 2017). MAPKs can also be activated by IL-1β, TNF-α, and IL-33 resulting in further secretion of important mediators in central sensitization such as IL-1β, TNF-α, IL-6, PGE_2, and BDNF (Grace *et al.*, 2014; Fattori *et al.*, 2017b; Zhang *et al.*, 2017). While clinical data regarding the role of glial cells is less clear (Mogil, 2012; Yekkirala *et al.*, 2017), drugs that inhibit glial activation such as pentoxifylline, α-aminoadipate, fluorocitrate, and minocycline have been successful in pre-clinical stage (Grace *et al.*, 2014; Fattori *et al.*, 2017b; Zhang *et al.*, 2017). Thus, supporting the important role of glial cells for central sensitization and persistent pathological pain in preclinical stage.

1.2 Resolving inflammation

Seminal discoveries from Sergio H. Ferreira and Sir John R. Vane shed light on how aspirin and aspirin-like drugs work and why they block inflammatory pain (Ferreira *et al.*, 1971; Vane, 1971; Ferreira, 1972). Because of these major discoveries, therapeutic approaches to inflammation have mainly focused on suppressing, blocking, or inhibiting pro-inflammatory mediators such as PGE_2 (Moncada *et al.*, 1973; Ferreira *et al.*, 1978). Despite effective, conventional anti-inflammatory drugs present several side effects, are immunosuppressive and highly associated with infectious complications (Orlicka *et al.*, 2013). Similarly, patients with rheumatic diseases often develop resistance to immunobiological drugs, limiting their chronic use (Fattori *et al.*, 2016). Opioid constitute another class of drugs with wide recognition by their potent analgesic effect. However, increasing attention has been paid to opioid abuse and addiction, which are serious public health problems in the US. In fact, direct health care costs can be 8.7 higher in opiate abusers when compared to non-opiate abusers (Kaye *et al.*, 2017). In general, even upon opioid therapy, neuropathic pain, for instance, remains challenging to treat, with only half of the treated population typically report a significant reduction in pain and complete resolution of symptoms is rarely achieved (Schutz *et al.*, 2013).

For many years, inflammation was thought to be resolved after a simple dilution of inflammatory mediators leading to the reestablishment of tissue function. More than 20 years after Ferreira and Vane's discoveries, it was recognized that endogenous specialized lipid

mediators are produced to block neutrophil recruitment and resolve inflammation (Serhan, 1994). It is now clear, therefore, that excessive or uncontrolled inflammation is associated with a unbalance between pro-inflammatory and specialized pro-resolving lipid mediators (SPMs) (Buckley *et al.*, 2014; Serhan, 2017). Using liquid chromatography-mass spectrometry (LC-MS/MS) and cell trafficking in self-limited exudates, Prof. Charles Serhan confirmed and identified those molecules with the ability of resolve inflammation actively, the so-called SPMs (Serhan *et al.*, 2000; Serhan *et al.*, 2002; Bannenberg *et al.*, 2005; Serhan *et al.*, 2009). These SPMs are biosynthesized from the omega-3 fatty acids arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), or docosahexaenoic acid (DHA). Given their structure differences, SPMs were divided into families: the lipoxins (LXs, AA-derived), the resolvins (Rvs, EPA-derived [E-series] or DHA/DPA-derived [D-series]), the protectins (PDs, DHA/DPA-derived), or the maresins (MaRs, DHA/DPA-derived) (Serhan *et al.*, 2015; Chiang *et al.*, 2017; Serhan, 2017). Of interest, another route to produce SPMs is through COX-2 acetylation (Bannenberg *et al.*, 2010; Romano *et al.*, 2015). Aspirin, for instance, acetylates a conserved serine residue in the COX-2 active site changing the enzymatic activity from COX-like to lipoxygenase-like. This acetylation allows the biosynthesis of SPM epimers, the so-called aspirin-triggered (AT) SPMs (Claria *et al.*, 1995; Bannenberg *et al.*, 2010; Romano *et al.*, 2015). To promote their biological effects, SPMs act on G protein-coupled receptors (GPCR). SPMs can be agonists (Xu *et al.*, 2010; Chiang *et al.*, 2012; Chiurchiu *et al.*, 2016; Zhang *et al.*, 2018), inhibitors (Serhan *et al.*, 2009; Bang *et al.*, 2010; Park *et al.*, 2011b; Park, 2015), or allosteric agonists (Pamplona *et al.*, 2012) of specific receptors expressed by immune cells or neurons to modulate host response (Figure 1). In the postcapillary venule, therefore, SPMs limit neutrophil recruitment and stimulate the recruitment of non-phlogistic monocytes. At the inflammatory foci, macrophages then phagocyte apoptotic neutrophils as well as micro-organisms to resolve inflammation and infection (Serhan *et al.*, 2015; Chiang *et al.*, 2017; Serhan, 2017).

The resolution of inflammation is an active and time-dependent mechanism with a biosynthetic shift from pro-inflammatory to pro-resolution mediators, the SPMs (Levy *et al.*, 2001; Bannenberg *et al.*, 2005). For instance, to promote resolution in the zymosan-induced inflammation model, RvD1 increases IL-10 production by inducing miR-21 at later time points 12h and 24h after zymosan when compared to 4h (Recchiuti *et al.*, 2012). Other temporally controlled miRNAs (so-called resolution miRNAs) by RvD1 are miR-146b, miR-208a, and miR-219 (Recchiuti *et al.*, 2012). Consequently, a distinct subpopulation of pro-resolving macrophages ($F4/80^{med}CD11b^{med}$ macrophages) is recruited 48 to 72h after stimulus with

zymosan and correlates with the resolution of inflammation (Bannenberg *et al.*, 2005). Therefore, these works show SPMs resolve inflammation in a time-dependent manner promoting the expression of hierarchically clustered miRNAs (Recchiuti *et al.*, 2012). In accordance, isolated SPMs have demonstrated time-dependent efficacy. In a mouse model of UVB-induced skin inflammation, treatment with LXA_4 72h before stimulus improves the efficacy of that SPM (Martinez *et al.*, 2018). In a pain context, while treatment with RvD1 before the development of tactile allodynia produces an enduring analgesic effect (up to 30 days), treatment with it at later time points provides limited analgesia (Huang *et al.*, 2011). Similarly, a single treatment with MaR1 reduces CFA-induced inflammatory pain for five days by blocking peripheral and central sensitization (Fattori *et al.*, 2019). These set of data show that isolated SPMs demonstrate time-dependent efficacy, which might be useful for the treatment of inflammatory diseases. Therefore, in this review, we focus on how SPMs treat infection and pathological pain. We focus on how SPMs block pain without opioid actions, target neuro-immune interactions and act as immunoresolvents (*i.e.* ability in controlling infection), thus, representing a new class of non-immunosuppressive and non-opioid analgesic drugs.

2. Controlling infections with SPMs

It is widely recognized that some types of infections are intimately linked with an increase in pain sensation. Pathogen-derived products, for instance, can direct activate nociceptors to produce pain (Chiu *et al.*, 2013; Maruyama *et al.*, 2017; Pinho-Ribeiro *et al.*, 2018). In response to that, nociceptors release neuropeptides such as CGRP that shape immune response against these pathogens (Chiu *et al.*, 2013; Maruyama *et al.*, 2017; Pinho-Ribeiro *et al.*, 2018). Bacterial infections are mainly treated with broad-spectrum antibiotic, while pain is treated with conventional analgesic and anti-inflammatory drugs. Specifically, corticosteroids, immunobiological agents, and opioids are linked with immunosuppression and pathogen spread (Orlicka *et al.*, 2013; Cabral *et al.*, 2016; Plein *et al.*, 2018). Opioid abuse is particularly worst in HIV/AIDS. A dysregulation in the pain-processing machinery due to synergistic effects of opioids and HIV on neurons and spinal cord glial cells promotes severe neuropathology and microgliosis in HIV-positive patients that are also opiate abusers when compared to non-abuser HIV-positive patients (Hauser *et al.*, 2012; Liu *et al.*, 2016a). While clinical data is less clear, in a mouse model of *S. pneumonia* infection, for example, the use of morphine is related to a delay in NF-κB-dependent bacterial clearance, which facilitates its spread (Wang *et al.*, 2008). Thus, given opioids reduce the ability of innate cells such as neutrophils and macrophages to

kill bacteria (Plein *et al.*, 2018), their use might increase antibiotic requirement to kill bacteria. Specifically for bacterial infections, antibiotic resistance is a rising problem in all parts of the world. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. Even when effective, β-lactam antibiotics induce bacteriolysis releasing LPS and LTA that can be recognized by immune cells. Recognition of these PAMPs causes post-infectious consequences due to a sustained inflammatory response (Ginsburg, 2002).

Interestingly, the SPMs RvD1, RvD5, and PD1 show a temporal and differential regulation during infections and lower the antibiotic requirements for bacterial clearance, meaning that they work as immunoresolvent rather than immunosuppressant molecules (Chiang *et al.*, 2012; Codagnone *et al.*, 2018). For instance, RvD1 enhances the effect of ciprofloxacin, and RvD1, RvD5, and PD1 increase host responses against *E. coli* to ciprofloxacin and to vancomycin (Chiang *et al.*, 2012). In *Pseudomonas aeruginosa*-induced lung infection, RvD1 synergizes with ciprofloxacin to promote the non-phlogistic phagocytosis, lowering, therefore, bacterial burden (Codagnone *et al.*, 2018). Similarly, treatment with RvD2 (Spite *et al.*, 2009), MaR1 (Hao *et al.*, 2019), or PDX (Xia *et al.*, 2017) prevents sepsis-induced lethality by decreasing both local and systemic bacterial burden. Despite not presenting antimicrobial effect *per se*, RvD2 direct increases *E. coli* phagocytosis by neutrophils while limiting the recruitment of novel neutrophils (Spite *et al.*, 2009). Of interest, recent evidence demonstrates that after electrical stimulation or stimulus with *E. coli*, mouse and human vagus nerve produce SPMs to contribute to host homeostasis (Serhan *et al.*, 2018). Specifically, while human vagus nerve produces RvD5, RvE1, MaR1, and PD1, mouse vagus produces only PDX (Serhan *et al.*, 2018). Electrical stimulation of human vagus increases RvE1, RvD3, RvD4, RvD5, PD1, and MaR1 levels. In mouse, vagus produces RvD4, RvE1, RvE3, and 15-epi-LXA_4 (Serhan *et al.*, 2018). In accordance, vagotomy delays resolution of self-limiting *E. coli* infection by controlling the production of PD conjugate in tissue regeneration (PCTR1) and PD1 (Dalli *et al.*, 2017). These data show the immunoresolvent properties of SPMs setting them up as a new class of drugs to control infection by promoting micro-organism clearance, lowering, therefore, antibiotic requirement to treat infections. Ultimately, these effects might contribute to reducing antibiotic resistance and post-infectious sequelae. Further, these results highlight how neurons can regulate immune responses via SPMs release.

3. Pre-clinical analgesic evidence of SPMs

One of the significant challenges in pain research is to comprehend the mechanisms of

persistent pathological pain and establish efficient therapies to abolish this unpleasant sensation. The available treatments for acute and chronic pain lie on NSAIDs, steroidal anti-inflammatory drugs, gabapentinoids, some antidepressant drugs, and opioids. Despite effective for a fraction of patients with rheumatic diseases and neuropathic pain, current therapies cause unwanted side effects (Lazzaroni *et al.*, 2001; Tziona *et al.*, 2017), immunosuppression (Orlicka *et al.*, 2013; Plein *et al.*, 2018), or induce analgesic tolerance (Cahill *et al.*, 2016) and addiction. On the other hand, isolated SPMs show efficacy at very low doses and have been used to treat pain in experimental models without side effects (Chiang *et al.*, 2017). For instance, while RvE1 shows a potent analgesic effect (dose 1000x lower than morphine) (Xu *et al.*, 2010), MaR1 shows a long-lasting analgesic effect upon a single treatment (up to 5 days) (Fattori *et al.*, 2019). Administration of RvD2 in an animal model of ischemia/reperfusion injury increases tissue levels of other SPMs in a GPR18-sensitive manner (Chiang *et al.*, 2015). These findings shed light on the pharmacological potential of SPMs to control pain and inflammation. Thus, in this section, we discuss preclinical data showing the analgesic properties of SPMs divided based on their precursor molecule *i.e.* AA, DHA, DPA, or EPA. Table 1 summarizes the SPMs we discuss and additional ones. Figure 2 highlights works showing specific signaling pathways that SPMs act to reduce pain.

3.1. AA-derived SPMs

Lipoxins were the first described SPMs (Serhan *et al.*, 1984). This class of SPMs is comprised of lipoxin A_4 (LXA_4), lipoxin B_4 (LXB_4), and AT-LXA_4, a more stable analog. While intrathecal treatment with LXA_4 reduces carrageenan-induced thermal hyperalgesia, the intravenous administration of LXA_4, AT-LXA_4, and LXB_4 reduce edema and thermal hyperalgesia induced by carrageenan in rats. The authors also demonstrated that AT-LXA_4 pretreatment prevents ATP-induced MAPK signaling on astrocytes *in vitro*, indicating direct effect on this glial cell (Svensson *et al.*, 2007). The potential of LXA_4 and LXB_4 was also assessed in a model of cancer-induced bone pain (CIBP), in which treatment with both SPMs reduced mechanical allodynia. Furthermore, AT-LXA_4-treated rats showed higher mechanical threshold and reduced spinal mRNA expression levels of IL-1β and TNF-α (Hu *et al.*, 2012). Similarly, other authors also described that LXA_4 reduces carrageenan-induced hyperalgesia (Abdelmoaty *et al.*, 2013). In addition to analgesic effect, AT-LXA_4 blocks morphine tolerance (Tian *et al.*, 2015). Treatment with AT-LXA_4 decreases chronic morphine-induced thermal hyperalgesia by blocking NALP1-derived IL-1β levels *in vivo* and *in vitro* (Tian *et al.*, 2015). These effects are important considering the current struggling with opioid side effects,

tolerance, and addiction.

Lipoxins are also effective in reducing neuropathic pain. In a model of spinal cord injury (SCI), treatment with LXA_4 decreases mechanical hyperalgesia and microglia activation as observed by reduced mRNA levels of microglia markers (Iba-1 and Py2) and TNF-α. In corroboration, LXA_4 reduces IFN-γ-induced p38 phosphorylation and TNF-α release in microglia primary cell culture, indicating LXA_4 can direct block microglia activation (Martini *et al.*, 2016). Furthermore, in a model of non-comprehensive lumbar disc herniation, LXA_4 reduces mechanical hyperalgesia, decreases pro-inflammatory cytokines IL-1β and TNF-α, and increases anti-inflammatory cytokines IL-10 and TGF-β. In addition, LXA_4 prevents NF-κB, JNK, and ERK phosphorylation, which is one of the possible mechanism of action of LXA_4 (Miao *et al.*, 2015).

LXA_4 prevents mechanical and thermal pain in a model of chronic compression of DRG (CCD) through the reduction of NF-κB activation and NF-κB-dependent cytokines (TNF-α, IL-1β, and IL-6) (Sun *et al.*, 2012). In a chronic constriction injury (CCI)-induced neuropathic pain model in rats, the administration of AT-LXA_4 reduces mechanical hyperalgesia by acting on the JAK2–STAT3–SOCS3 signaling axis (Wang *et al.*, 2014). These data indicate that lipoxins represent potential analgesic molecules.

3.2. DHA- and DPA-derived SPMs

The omega-3 polyunsaturated fatty acid-derived DHA is the precursor of three important SPM families: D-series Rvs, PDs, and MaRs. In this section, we discuss the evidence on these immunoresolvent in pain relief. The D-series Rvs is comprised of six molecules: RvD1, RvD2, RvD3, RvD4, RvD5, and RvD6. Administration of RvD1 blocks pain behaviors evoked by diverse stimuli, such as formalin (Bang *et al.*, 2010; Xu *et al.*, 2010), cinnamaldehyde (Bang *et al.*, 2010), CFA (Bang *et al.*, 2010; Xu *et al.*, 2010; Oehler *et al.*, 2017), carrageenan (Xu *et al.*, 2010), or AITC (Park *et al.*, 2011b). The analgesic effect of RvD1 was also observed by a shortened mechanical hyperalgesia in a model of hind paw chronic post-ischemic pain (CRIP) (Piovezan *et al.*, 2017). The administration of RvD1 before or shortly after skin/muscle incision and retraction surgery diminishes mechanical pain by 60% for ten postoperative days (Huang *et al.*, 2011). Similarly, in a model of chronic post-thoracotomy pain (CPTP) in rats, post-treatment with RvD1 or RvD2 at the surgery or at early time-point alleviates mechanical hyperalgesia with a long-lasting effect (Huang *et al.*, 2011). Collectively, these data demonstrate a potent analgesic effect and safe profile of these SPMs for pain treatment (Wang *et al.*, 2017). In fact, in a model of herniation-induced radicular pain, treatment with RvD1

reduces mechanical hyperalgesia, pro-inflammatory cytokine release, and NF-κB and ERK phosphorylation in the spinal cord and DRG. The levels of analgesic cytokines IL-10 and TGF-β were restored (Liu *et al.*, 2016b). RvD1 administration also reduces chronic pancreatitis-induced visceral pain (Quan-Xin *et al.*, 2012).

Importantly, SPMs direct act on nociceptors. RvD2 is a very potent inhibitor of TRPV1 (IC_{50} = 0.1 nM) and TRPA1 (IC_{50}= 2 nM) in primary sensory neurons, whereas RvD1 activity is selective to TRPA1 (IC_{50} = 9 nM) (Park *et al.*, 2011b). Moreover, RvD1 does not induce the release of endogenous opioid, bind or activate μ opioid receptors. However, blockage of μ opioid receptor activation by endogenous opioids reduces RvD1 analgesia likely because these are two analgesic classes of molecules acting in synergy in the used model (Oehler *et al.*, 2017). Obviously, as the possibility that an SPM binds and activates an opioid receptor was not tested for all SPMs, this was not fully disproved for all SMPs. These data confirm that at least RvD1 analgesia is not dependent on direct activation of opioid receptors. Once again corroborating that SPMs analgesia can contribute to reducing opioid use and that it represents a class of non-opioid analgesics to be further explored. RvD2 inhibits both TRPV1 and TRPA1 channels to reduce formalin-, carrageenan-, capsaicin-, AITC-, and CFA-induced inflammatory pain (Park *et al.*, 2011b) without effects on baseline mechanical threshold or motor functions. Furthermore, RvD2 reverses stimulation-evoked long-term potentiation of C-fiber in the spinal cord (Park *et al.*, 2011b). The analgesic effect of RvD2 was also described in a reserpine-induced fibromyalgia-like model (Klein *et al.*, 2014). Intravenous or intrathecal RvD2 administration reduces mechanical and thermal hyperalgesia. Interestingly, chronic intravenous treatment (during four days) was effective in reducing not only mechanical and thermal hyperalgesia but also depressive behavior (Klein *et al.*, 2014). Together, these data highlight RvD2 as a potent analgesic molecule.

The aspirin-triggered RvD1 (AT-RvD1) or 17(R)-RvD1, a RvD1 analog, is described to have analgesic proprieties as well. Administration of AT-RvD1 in rats (Meesawatsom *et al.*, 2016) and in mice (Abdelmoaty *et al.*, 2013) reduces carrageenan-induced mechanical hyperalgesia and TNF-α spinal cord levels (Abdelmoaty *et al.*, 2013). Furthermore, calcium influx imaging and electrophysiology analysis show that AT-RvD1 specifically suppresses neuronal activation in a TRPV3-dependent manner. A possible effect of AT-RvD1 on TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1 was also disproved (Bang *et al.*, 2012). The authors also demonstrated that AT-RvD1 inhibits CFA-, carrageenan-, and farnesyl pyrophosphate-induced thermal hyperalgesia (Bang *et al.*, 2012). In a reserpine-induced fibromyalgia-like model, AT-RvD1 reduces mechanical and thermal hyperalgesia. Prolonged treatment (during four days)

reduces depressive behavior, and mechanical and thermal hyperalgesia (Klein *et al.*, 2014). Similarly, chronic treatment (20 days) with AT-RvD1 was also effective in the CFA-induced arthritis by reducing TNF-α and IL-1β in the paw tissue (Lima-Garcia *et al.*, 2011). Of note, despite the similar structure of RvD1 and AT-RvD1, these SPMs inhibit different TRP channels as shown in Fig. 1.

While effective in reducing inflammation in a model of arthritogenic K/BxN serum-induced arthritis (Arnardottir *et al.*, 2016) and protecting the host against infections (Norris *et al.*, 2018), RvD6 analgesic effects has not yet been addressed. RvD5, on the other hand, reduces peripheral neuropathic pain induced by chemotherapy (CIPN) and formalin-induced nociceptive behavior. Strikingly, the analgesic effect of RvD5 was sex-selective since this SPM was effective only in male mice (Luo *et al.*, 2019). If this is also true in humans, it represents a significant impact in future RvD5 prescription as an analgesic. The same study also demonstrated that RvD3 and RvD4 at 100 ng (intrathecal) show no analgesic effect in both sexes (Luo *et al.*, 2019).

PDs are another DHA/DPA-derived SPMs family, which is comprised of PD1/Neuroprotectin D1 and PDX, a more stable analog. Equally, these SPMs also control inflammation and pain. The administration of NPD1 reduces formalin- and capsaicin-induced nocifensive behavior and increases the mechanical and thermal threshold in models of TNF-α- and CFA-induced inflammatory pain, respectively (Park *et al.*, 2011a). Furthermore, NPD1 normalizes TNF-α-induced neuronal plasticity *as per* electrophysiology analysis. The analgesic potential of NPD1 also expands to chronic pain scenarios. In a model of CCI-induced neuropathy, peri-sciatic nerve injections of NPD1 normalizes neuronal synaptic plasticity, ameliorates mechanical hyperalgesia, and reduces glial cell activation (Xu *et al.*, 2013b).

MaRs are another DHA/DPA-derived family comprising MaR1 and MaR2. MaR1 activates LGR6 receptor in neutrophils and macrophages to produce immunoresolvent effects (increase in efferocytosis and phagocytosis of *E. coli* and zymosan) (Chiang *et al.*, 2019). LGR6 is expressed by glial cells (microglia, astrocytes, and oligodendrocytes), peripheral immune cells (macrophages, neutrophils, NK cells etc), and neurons. Focusing on pain, MaR1 reduces capsaicin-induced nociceptive behaviors and in corroboration, it inhibits the inward currents evoked by capsaicin but not by AITC, suggesting a selective effect on TRPV1 without activity on TRPA1 ion channel (Serhan *et al.*, 2012). As of clinical relevance, a single intrathecal treatment with MaR1 shows a long-lasting analgesic effect (up to 5 days) in the CFA model of inflammatory pain (Fattori *et al.*, 2019). While in the spinal cord MaR1 decreases astrocytes and microglia activation, NF-κB activation, and spinal pro-inflammatory cytokine release; in

the periphery, it reduces CGRP release by DRG neurons and the number of leukocytes close to CGRP-positive fibers limiting, therefore, inflammation via silencing neurons as well (Fattori *et al.*, 2019). Collectively, these data demonstrate MaR1 analgesic effect is related to the reduction of peripheral and central sensitization. To the date, there is no data published regarding MaR2 analgesic effect. Nevertheless, evidence demonstrates it reduces zymosan-induced inflammation (Deng *et al.*, 2014), and the Verri laboratory has unpublished data demonstrating the analgesic effect of MaR2.

3.3. EPA-derived SPMs

The E-series Rvs are comprised of three molecules: RvE1, RvE2, and RvE3. Administration of RvE1 inhibits LPS-induced microglia activation and TNF-α release *in vitro* in a concentration-dependent manner (Xu *et al.*, 2013a). RvE1 acts on Chem23 receptor expressed by DRG neurons and in the spinal cord and has a potent analgesic effect, as demonstrated in the formalin-, carrageenan-, or CFA-induced inflammatory pain models in mice (Xu *et al.*, 2010; Fonseca *et al.*, 2017). In fact, RvE1 reduces the second phase of formalin-induced overt pain-like behavior with a dose 1000 times lower than morphine (Xu *et al.*, 2010). In the periphery, while reducing neutrophil recruitment and paw edema, in the spinal cord, RvE1 abolishes TNF-α-induced postsynaptic current culminating in the inhibition of ERK signaling pathway, restoring neuronal plasticity (Xu *et al.*, 2010). Another study has shown that RvE1 selectively blocks TRPV1 ion channels activation (IC_{50} = 1 nM) and substance P potentiation of TRPV1 in a receptor-dependent manner (Jo *et al.*, 2016). *In vivo* data show this SPM reduces mechanical and thermal hyperalgesia in CCI- and SLN-induced neuropathic pain. This effect is related to a reduction in microglia and astrocyte activation and, consequently, spinal cord TNF-α production, indicating RvE1 acts both in the periphery and spinal cord (Xu *et al.*, 2013a).

Despite the absence of literature description on the analgesic effects, RvE2 and RvE3 are known as potent anti-inflammatory drugs, indicating that they are promising molecules to be tested as analgesics (Tjonahen *et al.*, 2006; Oh *et al.*, 2012; Isobe *et al.*, 2013; Deyama *et al.*, 2018).

4. Clinical analgesic evidence of SPMs

The identification of RvE1, RvE2, RvE3, RvD1, RvD2, and MaR1 in human synovial fluids and their inverse association with pain score in patients with arthritis, suggest SPMs possibly control pain in humans (Giera *et al.*, 2012). In this section, we discuss clinical evidence

on the analgesic effect of SPMs or omega-3 fatty acid supplementation. Table 2 summarizes the works addressing clinical analgesic evidence of omega-3 supplementation.

Translation of preclinical data to humans faces significant challenges once endogenous SPMs are chemically unstable and often inactivated within tissues near the site of formation (Serhan *et al.*, 2011). One strategy to overcome this problem is the synthesis of SPMs analogs, which present better results in clinical trials due to their enhanced stability with similar bioaction when compared to the endogenous analog. For instance, the addition of a methyl group to carbon 15 of the LXA_4 forms 15(R/S)-methyl-LXA_4, which results on the blockade of dehydrogenation by 15-PGDH (Serhan *et al.*, 1995). Therefore, it is resistant to rapid metabolization (LXA_4 to 15-oxo-LXA_4) and retains the biological actions of native LXA_4 with enhanced anti-inflammatory activity. Topical treatment with 15(R/S)-methyl-LXA_4 shows efficacy and safety in patients with infantile eczema (Wu *et al.*, 2012). The efficacy was determined using the Severity Scale Score (SSS), Eczema Area and Severity Index (EASI), and the Infants' Dermatitis Quality of Life Index (IDQOL) (Wu *et al.*, 2013). Another lipoxin analog, the $BLXA_4$-ME is in clinical trial phase I for the treatment of oral gingivitis, sponsored by The Forsyth Institute (Chandrasekharan *et al.*, 2015). Fifty subjects were treated daily with one μM BLXA4-ME oral rinse. Plaque index (PI), modified gingival index (MGI), bleeding on probing (BOP), and levels of IL-1β in gingival crevicular fluid (GCF) were the parameters analyzed (Chandrasekharan *et al.*, 2015) (ClinicalTrials.gov Identifier: NCT02342691). Also, a 28-day, phase I and II, multicenter, double-blind placebo trial reported efficacy of a RvE1 analog, RX-10045, on patients with chronic dry eye syndrome, a multifactorial disease characterized by inflammation of the ocular surface accompanied by discomfort and visual disturbance (Cholkar *et al.*, 2016) (ClinicalTrials.gov Identifier: NCT00799552). In this study, RX-10045 was administered twice daily in 232 patients with moderate dry eye. Symptoms evaluated included dryness, burning, grittiness, and patients' composition of Worst Symptom Score (Cholkar *et al.*, 2016) (ClinicalTrials.gov Identifier: NCT00799552).

Another approach to study the effects of SPMs in patients is through dietary supplements of omega-3 (n-3) fatty acids (FA). Regular intake of n-3 capsules alters mononuclear cells' (MNCs) membrane composition (Yaqoob *et al.*, 2000), as EPA is incorporated to phospholipids in a dose-dependent manner. This change in membrane composition ultimately reduces the production of PGE_2 by MNCs (Rees *et al.*, 2006) and to affect activation of transcription factors related to expression of inflammatory genes (Calder, 2006). However, this membrane proportion towards EPA and DHA prevalence seems to correlate with n-3 intake. When ingestion is interrupted, the percentage of arachidonic acid

content raises (Yaqoob *et al.*, 2000), which underlines the importance of ongoing supplementation. As most of SPMs derives from EPA and DHA, it seems logical that supplementation with these precursors provides the substrate for the synthesis of MaRs, Rvs, and PDs, and therefore, may play a role in inflammation resolution with possible clinical outcomes (Barden *et al.*, 2016a). Even cellular adaptations and opioid-seeking anxiety induced by morphine were reversed by dietary n-3FA in a murine model of chronic opioid exposure (Hakimian *et al.*, 2017). Studies involving human subjects have also shown positive results. To assess the role of SPM levels and clot remodeling in patients with coronary artery disease (CAD), six individuals taking Lovaza (EPA and DHA capsules) for one year had their SPM profiles compared with healthy volunteers and with CAD patients not taking the capsules (Elajami *et al.*, 2016). Absence of RvD1, RvD2, RvD3, RvD5, and RvE1 was detected on both CAD groups, but not on healthy patients. The group treated with Lovaza had a positive correlation with an increase in AT-RvD3, RvD6, AT-PD1, and RvE2 levels. In addition, it was also demonstrated that these SPMs promote macrophage uptake of blood clots (Elajami *et al.*, 2016). Moreover, it has been suggested that RvD1/LTB_4 ratio can work as a biomarker of non-resolving inflammation in humans. Specifically, low salivary RvD1/LTB_4 ratio correlates with increased carotid artery intima-media thickness (Thul *et al.*, 2017). In corroboration, vulnerable human atherosclerotic plaques from elective endarterectomy show decreased levels of 5-LOX-dependent SPMs (such as LXA_4 and RvD1) when compared to stable human plaques (Fredman *et al.*, 2016). Additionally, administration of RvD1 (Fredman *et al.*, 2016) or EPA supplementation (through an RvE1/Chem23-dependent mechanism) in mice prevent plaque formation and development of atherosclerosis (Laguna-Fernandez *et al.*, 2018). Interestingly, patients with atherosclerosis that were under statin treatment presented higher expression of Chem23 receptor (Laguna-Fernandez *et al.*, 2018). Together, these results suggest an impact of low SPM levels on progression of vascular inflammation, as well as the benefits of n-3FA supplements as a therapeutic approach for treating patients with coronary diseases (Elajami *et al.*, 2016).

Supplementation for three months with n-3FA also increases DHA-derived SPMs and decreases pro-inflammatory mediators such as acute-phase proteins, cytokines, and adhesion molecules in women with low-grade inflammation caused by obesity (Polus *et al.*, 2016). Another study found a positive correlation between SPM levels and changes in cognitive functions of patients with Alzheimer's disease taking n-3FA-rich supplementation for six months (Wang *et al.*, 2015). Furthermore, in another clinical trial involving 74 patients with chronic kidney disease, 8-week intake of n-3FA elevated the production of lipid mediators

along with lower MPO on plasma, which may have a role at limiting inflammation (Barden *et al.*, 2018). Swollen and tender joints of 20 patients with rheumatoid arthritis (RA) showed improvements after n-3FA infusions as adjuvant therapy (Bahadori *et al.*, 2010).

When it comes to pain assessment, although limited, promising results have been published on SPMs and human subjects. One study compared the levels of SPM in 36 patients with arthritis and 36 healthy volunteers, all taking n-3FA orally. Results show an association between synovial fluid RvE2 and reduced pain scores, suggesting a role for SPM in the control of arthritis symptoms (Barden *et al.*, 2016b). Enrolled males and female patients were diagnosed with varied types of arthritis such as RA, psoriatic arthritis, gout, pauci arthritis, B27 spondylarthritis, and other (Barden *et al.*, 2016b). This beneficial effect in a wide range of rheumatic diseases highlight the beneficial effect of SPMs. Forty-five participants were enrolled in a double-blinded, placebo-controlled, randomized study aiming to examine the effect of the combination of verbena extract and n-3 capsules intake, for nine weeks, on subjects with joint discomfort (Caturla *et al.*, 2011). The clinical condition of joints was recorded using two questionnaires validated to dysfunction and pain in participants with osteoarthritis. Briefly, they evaluate pain, stiffness, maximum distance walked, and physical functional disability. Results show a significant improvement of joint status, physical function, and pain relief, with an onset of the effect in the third and fourth weeks (Caturla *et al.*, 2011). In another study published by Kremer et al. (1990), 49 patients with RA consumed two different dosages of n-3FA for 24 weeks, daily (Kremer *et al.*, 1990). Clinical assessment included patient and physician evaluation of pain and global arthritis activity in a 5-point scale (where 0 stands for absent and four very severe pain), as well as grip strength and number of swollen joints. Beneficial clinical outcomes were observed, more commonly on the high-dose group, suggesting a therapeutic alternative for treating RA (Kremer *et al.*, 1990). In accordance, other published reports show evidence of joint pain control and supplementation with n-3FA in women and men (Tulleken *et al.*, 1990; Geusens *et al.*, 1994).

Given the associated increased risk of myocardial infarction, stroke (Chen *et al.*, 2018), kidney injury (Fattori *et al.*, 2017a), infection (Orlicka *et al.*, 2013; Plein *et al.*, 2018); a dual-center, double-blind placebo-controlled randomized study aiming to determine if supplementation with EPA and DHA helps to reduce NSAIDs daily use (Galarraga *et al.*, 2008). In a study with 58 RA patients, a nine month-ingestion of EFA-rich capsules provided a progressive reduction of NSAIDs intake (without worsening disease status) and decrease in pain as per visual analog scale (VAS) was observed when compared to the placebo group (Galarraga *et al.*, 2008). Indeed, a meta-analysis of 17 trials concluded that ‘EPA/DHA

supplementation reduces patient assessed joint pain intensity, morning stiffness, number of painful and/or tender joints, and NSAID consumption' in people affected by RA (Goldberg *et al.*, 2007). Consistent evidence of n-3 efficacy in RA is supported in a systematic review (Miles *et al.*, 2012).

Some studies also report pain improvement in other diseases. Interestingly, dietary interventions with fatty acids ameliorate pain episodes in a trial involving patients with sickle cell disease, whose sickle erythrocytes promote vascular damage, with consequent generation of thrombin and possible thrombotic occlusion (Tomer *et al.*, 2001). In other symptomatic vascular diseases, antithrombotic drugs have similar results (1994; 1996). Along with biochemical measurements, this trial suggests that n-3FA are a promising approaches to control inflammatory pain in subjects suffering from this disease. Up to 50% of type 2 diabetes patients may experience neuropathic symptoms related to lipotoxic stress of nerve cells, resulting in cell death (Tesfaye *et al.*, 2005; Feldman *et al.*, 2017). As DHA shows neuroprotective properties, a study determined the effects of 3 months n-3FA intake of 40 diabetic volunteers (Durán *et al.*, 2019). This study shows a potent reduction in pain-related neuropathy symptoms, positively associated with elevation of DHA levels in participants' plasma (Durán *et al.*, 2019).

While n-3-derived lipids show analgesic effect, n-6 derived ones are correlated with pro-hyperalgesic manifestations (Antonova *et al.*, 2012). As demonstrated by Sibille et al. (2018), osteoarthritis patients with higher n-6/n-3 blood ratio present higher pain/functional limitations, experimental pain sensitivity, and psychosocial distress when compared to the low-ratio group (Sibille *et al.*, 2018). Therefore, lipid mediators derived from n-3FA and n-6FA have opposite regulatory roles in inflammation and pain (Morisseau *et al.*, 2010; Patwardhan *et al.*, 2010; Xu *et al.*, 2010; Wen *et al.*, 2012). In this sense, a randomized trial conducted by Ramsden et al. (2015) aimed to determine whether dietary manipulation of fatty acids would have an impact on 67 patients with chronic headaches (Ramsden *et al.*, 2015). Reduction of n-6 and rise of n-3FA in diet for 12 weeks significantly improved health-related quality of life and significantly reduced pain frequency, intensity, and psychological distress (Ramsden *et al.*, 2015). Similarly, 67 patients with migraine headache received sodium valproate, daily, plus fish oil supplementation (180 mg) for 12 weeks (Tajmirriahi *et al.*, 2012). At the end of the study, a significant reduction of severity, frequency, and duration of migraines was reported (Tajmirriahi *et al.*, 2012).

In summary, clinical trials supporting the analgesic effect of SPMs are limited and usually related to n-3FA intake although the analgesic effect observed by n-3FA ingestion correlates with RvD3 levels (Barden *et al.*, 2016b). Clinical trials focusing on other diseases

are far more advanced in the use of SMPs and modified SPMs with enhanced stability. Thus, a wide field on the therapeutic use of SPMs and their modified analogs can be explored as analgesic drugs.

5. Conclusion and future perspectives

The discovery of SPMs changed the concept of how inflammation ends and opened new avenues for the treatment of inflammatory diseases. SPMs are immunoresolvent molecules (*i.e.* do not present immunosuppressive effects, one of the undesirable side effects of corticosteroids, immunobiological agents, and opioids) and, therefore, harnessing the pharmacology of resolution could provide the basis for reprogramming immune cell and neuronal activities, and host response. At very low doses, SPMs act on specific receptors expressed by immune cells as well as silences nociceptors to limit pain and inflammation in the pre-clinical stage (Figures 1 and 2). Clinically, n-3FA supplementation can be used to increase endogenous SPM levels and prevent inflammation and pain in several diseases. Therefore, SPMs represent a new class of non-immunosuppressive and non-opioid analgesic drugs.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

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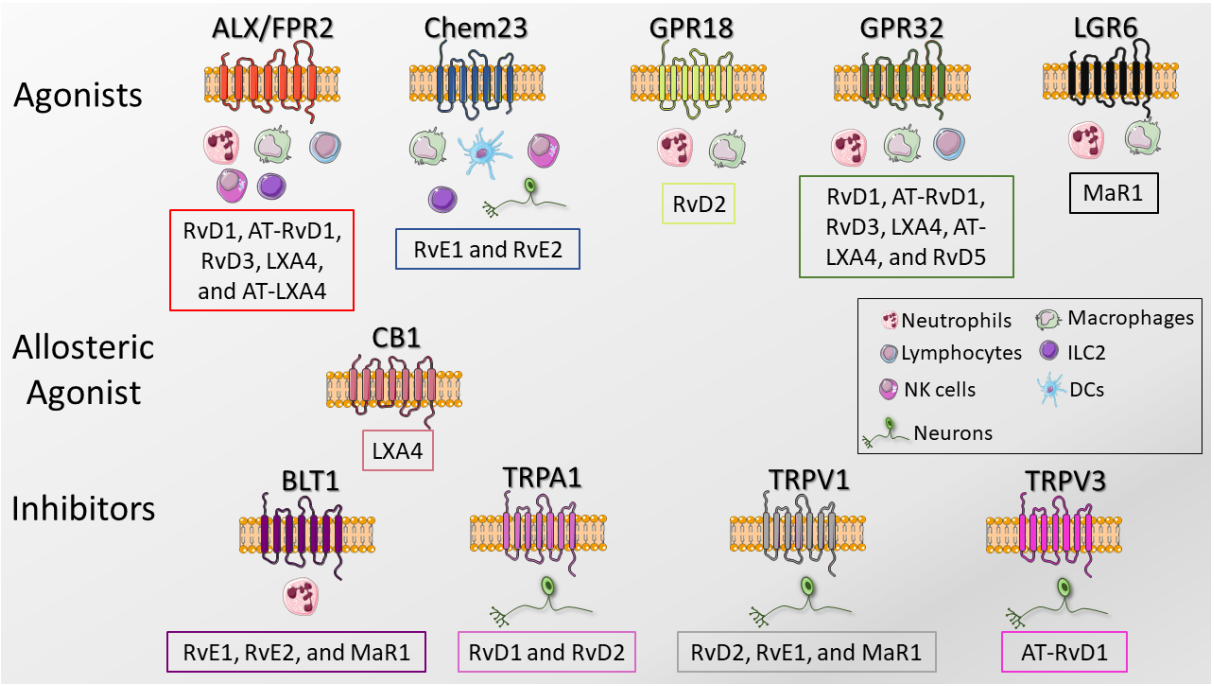

Figure 1. SPMs and their receptors

To produce their biological effects SPMs can be agonists, allosteric agonists, or inhibitors of specific receptors expressed by immune cells or neurons. ALX/FPR2: N-formyl peptide receptor 2; GPR18: G protein-coupled receptor 18; GPR32: G protein-coupled receptor 32; Chem23: Chemerin receptor 23; CB1: Cannabinoid receptor type 1; BLT1: Leukotriene B4 receptor 1; TRPV1: Transient receptor potential cation channel subfamily V member 1; TRPA1: Transient receptor potential cation channel subfamily A member 1; TRPV3: Transient receptor potential cation channel subfamily V member 3; DC: dendritic cells; ILC2: Type 2 innate lymphoid cells; NK cells: Natural killer cells.

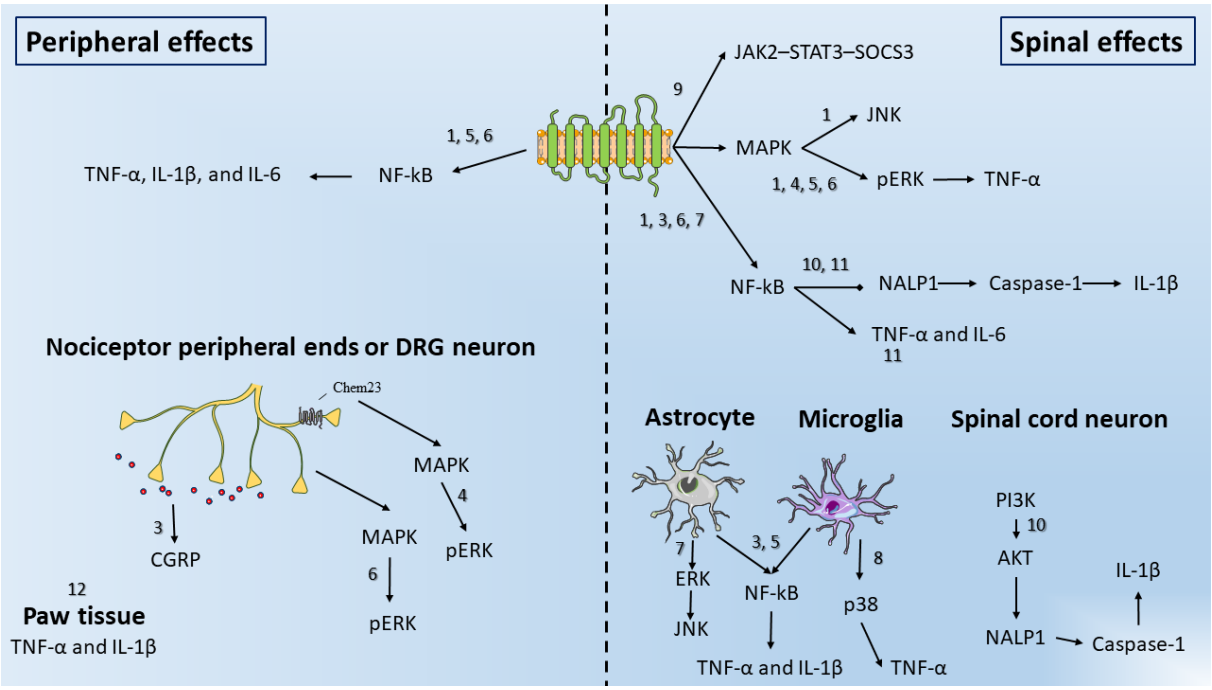

Figure 2. Peripheral and spinal cord effects of SPMs to block pain

Schematic representation of the known signaling pathways targeted by SPM in the periphery or spinal cord. The studies addressing these effects are placed next to each signaling pathway: 1 (Miao *et al.*, 2015), 2 (Abdelmoaty *et al.*, 2013), 3 (Fattori *et al.*, 2019), 4 (Xu *et al.*, 2010), 5 (Sun *et al.*, 2012), 6 (Liu *et al.*, 2016b), 7 (Svensson *et al.*, 2007), 8 (Martini *et al.*, 2016), 9 (Wang *et al.*, 2014), 10 (Tian *et al.*, 2015), 11 (Li *et al.*, 2013), 12 (Lima-Garcia *et al.*, 2011). Chem23: Chemerin receptor 23; CGRP: Calcitonin gene-related peptide; MAPK: Mitogen-activated protein kinase; pERK: Phosphorylated extracellular signal-regulated kinase; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; SOCS3: Suppressor of cytokine signaling 3; JNK: c-Jun N-terminal kinase; NALP1: NLR family pyrin domain containing 1; PI3K: Phosphoinositide 3-kinase; AKT: Protein kinase B.

Table 1 – Pre-clinical analgesic evidence of SPMs

	SPM	Animal	Pain model	Administration rote	Dose	Analgesic and anti-inflammatory actions	Reference
AA	LXA_4	Rat	Carrageenan	i.v.	10 µg/kg	Reduces thermal hyperalgesia and edema	(Svensson *et al.*, 2007)
				i.t.	0.3 nM	Reduces thermal hyperalgesia and edema	
				i.t.	0.3 and 1 ng	Reduces mechanical hyperalgesia	(Abdelmoaty *et al.*, 2013)
			BCIP	i.t.	0.3 nM	Reduces mechanical hyperalgesia	(Hu *et al.*, 2012)
			SCI	i.t.	0.3 nM	Reduces mechanical hyperalgesia, microglia activation, and TNF-α levels.	(Martini *et al.*, 2016)
			Microglia primary culture *(in vitro)*	-	10 nM	Reduces IFN-γ-induced TNF-α release and p38 phosphorylation	
			Radicular pain	i.t.	10 and 100 ng	Reduces IL-1β and TNF-α and increases IL-10 and TGF-β levels. Decreases NF-κB activation, and JNK and ERK phosphorylation	(Miao *et al.*, 2015)
			CCD	i.t.	10 and 100 ng	Reduces mechanical and thermal hyperalgesia, NF-κB activation, TNF-α, IL-1β, and IL-6 at DRG levels	(Sun *et al.*, 2012)
	LXB_4	Rat	Carrageenan	i.v.	10 µg/kg	Reduces thermal hyperalgesia and edema	(Svensson *et al.*, 2007)
			BCIP	i.t.	0.3 nM	Reduces mechanical hyperalgesia	(Hu *et al.*, 2012)
	$AT\text{-}LX_4$	Rat	Carrageenan	i.v.	10 µg/kg	Reduces thermal hyperalgesia and edema	(Svensson *et al.*, 2007)
				i.t.	0.3 nM	Reduces mechanical hyperalgesia	
			Astrocytes primary culture (*in*	-	10 nM	Reduces ATP-induced phosphorylation	

			vitro)			of ERK and JNK	
			CCI	i.t.	200 ng	Reduces mechanical hyperalgesia decreasing signaling through JAK2–STAT3–SOCS3 signaling axis	(Wang *et al.*, 2014)
			CCI	i.v.	40 μg/kg	Reduces mechanical hyperalgesia	
			CCI	i.t.	200 ng	Reduces thermal hyperalgesia, caspase-1 cleavage, and IL-1β maturation.	(Li *et al.*, 2013)
			Chronic morphine injection	i.t.	100 ng	Reduces thermal hyperalgesia, caspase-1 cleavage, and IL-1β maturation by NALP1 inflammasome inhibition.	
			Primary neuron culture (in vitro)	-	100 nM	Reduces consecutive morphine treatment-induced PI3K signaling, AKT phosphorylation, caspase-1 cleavage, and IL-1β maturation through NALP1 inhibition.	(Tian *et al.*, 2015)
DHA / DPA	RvD1	Mouse	Formalin	i.d.	20 ng	Reduces formalin-evoked licking/flicking time of first and second phases	
			CA	i.d.	20 ng	Reduces CA-evoked licking/flicking time of first and second phases	(Bang *et al.*, 2010)
			CFA	i.d. or i.t.	20 ng	Reduces mechanical and thermal hyperalgesia	
			AITC	i.pl.	10 ng	Reduces mustard oil-evoked licking/flicking	(Park *et al.*, 2011b)

					time	
		CFA	i.t.	1 ng	Reduces thermal hyperalgesia	(Xu *et al.*, 2010)
		Carrageenan	i.t.	20 ng	Reduces thermal hyperalgesia	
		CPIP	i.v.	100 ng	Reduces mechanical hyperalgesia	(Piovezan *et al.*, 2017)
		CFA	i.t.	20 ng	Reduces mechanical hyperalgesia	(Oehler *et al.*, 2017)
		SMIR	i.t.	40 ng	Reduces mechanical allodynia and hyperalgesia	(Huang *et al.*, 2011)
		CPTP	i.t.	30 ng	Reduces mechanical hyperalgesia	(Wang *et al.*, 2017)
	Rat	Radicular Pain	i.t.	10 and 100 ng	Reduces mechanical hyperalgesia; Reduces IL-1β and TNF-α and increases IL-10 and TGF-β levels; Decreases NF-κB expression and, ERK phosphorylation.	(Liu *et al.*, 2016)
		Pancreatitis-induced visceral pain	i.t.	100 ng/kg	Reduces mechanical hyperalgesia	(Quan-Xin *et al.*, 2012)
		Formalin	i.t.	10 ng	Reduces second phases formalin-evoked licking/flicking time	
		Capsaicin	i.t.	10 ng	Reduces capsaicin-evoked licking/flicking time	(Park *et al.*, 2011b)
RvD2	Mouse	AITC	i.t.	10 ng	Reduces mustard oil-evoked licking/flicking time	
		CFA	i.t.	10 ng	Reduces mechanical and thermal hyperalgesia	
		Fibromyalgia	i.v.	300 ng	Reduces mechanical and thermal hyperalgesia, and immobility	(Klein *et al.*, 2014)

					time.	
		fPOP	i.t	500 ng	Reduces mechanical and cold allodynia	(Zhang *et al.*, 2018)
			i.v.	500 ng	Reduces mechanical and cold allodynia	
	Rat	CPTP	i.t.	30 ng	Reduces mechanical hyperalgesia	(Wang *et al.*, 2017)
AT-RvD1	Mouse	Carrageenan	i.t.	30 ng	Reduces mechanical hyperalgesia and TNF-α spinal cord levels	(Abdelmoaty *et al.*, 2013)
		Carrageenan FPP	i.d.	30 µM	Reduces mechanical and thermal hyperalgesia	(Bang *et al.*, 2012)
		CFA	i.t.	80 ng	Reduces depression behavior, mechanical and thermal hyperalgesia	(Klein *et al.*, 2014)
		Fibromyalgia	i.v.	300 ng		
	Rat	Carrageenan	i.t.	15 ng	Reduces mechanical hyperalgesia, and C- and Aδ-fibers firing on carrageenan-stimulated animals.	(Meesawatsom *et al.*, 2016)
		CFA-induce arthritis	i.p.	100 ng	Reduces mechanical and thermal hyperalgesia, and IL-1β and TNF-α levels	(Lima-Garcia *et al.*, 2011)
RvD5	Mouse	fPOP	i.t.	500 ng	Reduces mechanical and cold allodynia	(Zhang *et al.*, 2018)
		Formalin	i.t.	10 ng	Reduces phase II licking/flicking time on male mice	(Luo *et al.*, 2019)
		CIPN	i.t.	100 ng	Reduces mechanical hyperalgesia	
PD1/NPD1	Mouse	Formalin	i.t.	10 ng	Reduces phase II formalin-evoked licking/flicking time	(Park *et al.*, 2011a)
		Capsaicin			Reduces licking/flicking time	

			TNF-α			Reduces mechanical and thermal hyperalgesia
			CFA			Reduces mechanical and thermal hyperalgesia
			CCI	peri-sciatic	300 ng	Normalizes neuronal synaptic plasticity, ameliorates mechanical hyperalgesia, and reduces glia activation (Xu *et al.*, 2013b)
			fPOP	i.t.	500 ng	Reduces mechanical and cold allodynia (Zhang *et al.*, 2018)
		Rat	SNI	i.t.	100 ng	Reduces mechanical allodynia and thermal hyperalgesia, spinal levels of GFAP, IBA, pNF-κB, and cytokine production (TNF-α, IL-1β and IL-6) (Gao *et al.*, 2018)
MaR1			Capsaicin	i.pl.	10 ng	Reduces capsaicin-evoked licking/flicking time (Serhan *et al.*, 2012)
		Mouse	TMJIP	*Ex vivo*	0.35 nM	Blocks TRPV1 agonist-evoked increases in spontaneous excitatory postsynaptic and abolishes TMJ inflammation-induced synaptic plasticity in the trigeminal nucleus (Park, 2015)
			fPOP	i.t.	500 ng	Reduces mechanical and cold allodynia (Zhang *et al.*, 2018)
				i.v.	500 ng	Reduces mechanical and cold allodynia
			Carrageenan	i.t.	10 ng	Reduces mechanical and thermal hyperalgesia, (Fattori *et al.*, 2019)

				CFA			decreases astrocytes and microglia and NF-κB activation, and spinal pro-inflammatory cytokine release. In the periphery, it reduces CGRP release by DRG neurons and the number of leukocytes close to CGRP-positive fibers
EPA	RvE1	Mouse	Formalin	i.t.	1 ng	Reduces phase II formalin-evoked licking/flicking time	(Xu *et al.*, 2010)
			Carrageenan		20 ng	Reduces thermal hyperalgesia	
			CFA		10 ng	Reduces thermal hyperalgesia	
			TNF-α		10 ng	Reduces thermal hyperalgesia	
			CCI		100 ng	Reduces mechanical hyperalgesia	
			SNI		100 ng	Reduces mechanical and thermal hyperalgesia	(Xu *et al.*, 2013a)
			Microglia primary culture (*in vitro)*	-	100 ng	Reduces LPS-induced TNF-α release and microglia activation	
		Rat	Carrageenan	i.pl.	285 pM	Reduces mechanical hyperalgesia and edema	(Fonseca *et al.*, 2017)

BCIP: bone cancer-induced pain; SCI: spinal cord injury; CCD: chronic compression of DRG; CCI: chronic constriction injury; CFA: complete Freund's adjuvant; CA: cinnamaldehyde; AITC: allyl isothiocyanate; CPIP: chronic post-ischemic pain; SMIR: skin/muscle incision and retraction; CPTP: chronic post-thoracotomy pain; FPP: farnesyl pyrophosphate; SNI: spared nerve injury; TMJIP: temporomandibular joint inflammatory pain; fPOP: post-operative pain induced by tibial bone fracture; i.v.: intravenous; i.t.: intrathecal; i.d.: intradermal, i.pl.: intraplantar; i.p.: intraperitoneal.

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Table 2 – Clinical evidence on omega-3 fatty acids supplementation and pain-relief outcomes

Disease		n	Treatment	Duration	Outcomes	Reference
Joint discomfort (knee, hip, elbow, hands, or shoulder)		45	370mg of fish-oil powder EPA/DHA (10/8) + 230mg of lemon verbena extract (14% w/w verbascoside) each capsule	9 weeks	Improvement of joint status, physical function, stiffness and pain relief	(Caturla *et al.*, 2011)
Rheumatic diseases	Rheumatoid Arthritis	49	27 mg/kg EPA and 18 mg/kg DHA (low dose) or 54 mg/kg EPA and 36 mg/kg DHA (high dose)	24 weeks	Improvement of pain, grip strength and number of swollen joints mainly on high-dose group	(Kremer *et al.*, 1990)
		90	2.6g/day n-3FA	12 months	Improvement in the patient's global evaluation and the physician's assessment of pain, as well as reduction of anti-rheumatic medication	(Geusens *et al.*, 1994)
		28	6g/day (fish oil)	3 months	Improvement in the number of swollen joints, joint pain index, and Ritchie articular index	(Tulleken *et al.*, 1990)
		58	10g of cod oil (2.2g of n-3FA)/day	9 months	Reduction of NSAID intake without any worsening of their disease activity	(Galarraga et al. 2008)
		15	2.4g/day n-3FA	4 weeks	Plasma SPM in arthritis patients were elevated compared with healthy volunteers, and RvE2 was negatively associated with pain score	(Barden *et al.*, 2016)
	Gout	4	2.4g/day n-3FA	4 weeks		
	Psoratic arthritis	6	2.4g/day n-3FA	4 weeks		
	Pauci arthritis	2	2.4g/day n-3FA	4 weeks		
	B27 spondyloarthritis	2	2.4g/day n-3FA	4 weeks		
	Monoarthritis	2	2.4g/day n-3FA	4 weeks		
Sickle cell syndrome		13	menhaden fish oil (0.25 g/kg/day, containing 0.1 g/kg/day n-3FAs)	12 months	Frequency of pain episodes decreased by approximately half	(Tomer *et al.*, 2001)
Chronic headaches		67	Dietary intervention, reducing n-6 linoleic acid (LA), and increase dietary n-3 EPA and DHA	12 weeks	Reduction on pain frequency, intensity, and psychological distress	(Ramsden *et al.*, 2015)
Diabetic neuropathy		40	1000mg DHA, 200 mg EPA, daily	3 months	Reduction of neuropathic pain symptoms	(Durán *et al.*, 2019)
Migraines		67	400 mg/day sodium valproate with or without	12 weeks	Reduction of migraine headache when combined	(Tajmirriahi *et al.*, 2012)

	combination with EPA 180 mg and DHA120 mg	sodium valproate plus fish oil

EPA: Eicosapentaenoic acid; DHA: docosahexaenoic acid; SPM: Specialized pro-resolving mediator; RvE2: Resolvin E2; NSAID: nonsteroidal anti-inflammatory drug; LA: linoleic acid; n-3FA: n-3 fatty acids

3.4 Maresin-1, A Specialized Pro-Resolving Lipid Mediator, as a Non-Hormonal Alternative for the Treatment of Endometriosis-Associated Pain

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Abstract

Endometriosis is a painful estrogen-dependent inflammatory disease that affects 5 to 10% of reproductive-age women in the United States. For many years, inflammation was thought to be resolved after a simple dissipation of inflammatory mediators. It now recognized that endogenous specialized lipid mediators, so-called specialized pro-resolving lipid mediators (SPMs), are actively produced to block neutrophil recruitment and resolve inflammation. MaR1 is a docosahexaenoic acid (DHA)-derived SPM that acts on LGR6 receptors to promote its immunoresolvent effects. MaR1 selectively blocks the activation of TRPV1 receptors, CGRP release, and promotes a long-lasting analgesic effect. Therefore, in this work, we aimed at addressing whether MaR1 can treat endometriosis using a non-surgical model in mice. Abdominal hyperalgesia was determined using von Frey filaments. Activation of CGPR-positive neurons was determined by immunofluorescence (double staining of phosphorylated p65 NF-κB with CGRP). Herein, we show that MaR1 reduces the activation of a subpopulation of nociceptors that are responsible for producing neuropeptides such as CGRP. We also demonstrated that MaR1 not only reduced pain but also the number of mice with endometriotic lesions, indicating it is a promising drug for the treatment of endometriosis.

Keywords: abdominal pain, pelvic pain, resolution of inflammation, endometriosis treatment

1. Introduction

Endometriosis is a painful estrogen-dependent inflammatory disease that affects 5 to 10% of reproductive-age women in the United States (Bulun, 2009). The ectopic presence of tissue (lesions) mimicking the appearance of uterine (eutopic) endometrium is what defines endometriosis. During retrograde menstruation, damage-associated molecular patterns (DAMPs) activate resident immune cells, including macrophages (Wu *et al.*, 2017; Izumi *et al.*, 2018). Activated immune cells produce pro-inflammatory mediators that activate nociceptors to produce pain. In turn, nociceptor release neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) (McKinnon *et al.*, 2015; Wu *et al.*, 2017), as well as chemokines such as CCL2 (Greaves *et al.*, 2015) to mediate contribute to the recruitment of more immune cells and lesion growth (Greaves *et al.*, 2015; McKinnon *et al.*, 2015). Existing rodent models of endometriosis does not completely resembles the pain aspects of endometriosis (Simitsidellis *et al.*, 2018). While sensitivity to heat stimuli is rarely reported by women with endometriosis, this is the most frequently used behavioral test in rodent models of endometriosis (Tirado-Gonzalez *et al.*, 2010; Bruner-Tran *et al.*, 2018; Simitsidellis *et al.*, 2018). We have recently established a novel murine model that produces evoked and spontaneous pain, which are amenable to the clinically used drugs letrozole (aromatase inhibitor) and danazol (androgen) (accepted at Pain).

For many years, inflammation was thought to be resolved after a simple dissipation of inflammatory mediators. It now recognized that endogenous specialized lipid mediators, so-called specialized pro-resolving lipid mediators (SPMs), are actively produced to block neutrophil recruitment and resolve inflammation (Serhan, 1994; Fattori *et al.*, 2020). MaR1 is a docosahexaenoic acid (DHA)-derived SPM produced by two main routes. Directly by macrophages or transcellular through platelet-neutrophil interactions (Serhan *et al.*, 2012; Abdulnour *et al.*, 2014). MaR1 acts on LGR6 receptors to promote its immunoresolvent effects (Chiang *et al.*, 2019). It has been demonstrated that MaR1 selectively blocks the activation of TRPV1 receptors in mouse neurons and reduces capsaicin-induced nocicefensive behaviors (Serhan *et al.*, 2012). We have recently demonstrated that MaR1 blocks CGRP release and promotes a long-lasting analgesic effect. (Fattori *et al.*, 2019). This effect is related to the blockage of central sensitization and silencing of nociceptors. Therefore, in this work, we aimed at addressing whether MaR1 can work as a disease-modifying drug for the treatment of endometriosis.

2. Material and methods

2.1. Animals

All experiments were performed in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Londrina State University Ethics Committee on Animal Research and Welfare (process number 2669.2019.54). In this study, it was used healthy female C57BL/6 mice (8 weeks of age, 25 ± 1g) were used. Mice were randomly assigned and housed in standard clear plastic cages in light/dark cycle of 12:12h with *ad libitum* water and food. Behavioural testing was performed between 9 a.m. and 5 p.m. in a room maintained at a temperature of 21±1°C. Mice were treated daily with MaR1 at 3, 10, or 30 ng (intreaperitoneally) starting 29 until 56 dpi. The investigators were blinded to the treatments. All efforts were made to minimise the number of animals used and their suffering.

2.2. Induction of Endometriosis

After at least one week of acclimatization, donor mice received a subcutaneous injection of 3 μg/mouse estradiol benzoate to stimulate the growth of the endometrium. Seven days later, the uteri of the donor mice were dissected into a Petri dish containing Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Waltham, MA, USA) and split longitudinally with a pair of scissors. Uterine horns from each donor mouse were minced with scissors and scalpel one at the time, ensuring that the maximal diameter of each fragment was consistently smaller than 1 millimeter (mm). Each dissociated uterine horn was then injected intraperitoneally using an 18G needle (cat #305185 Thin wall, BD, Franklin Lakes, NJ, USA) into a recipient mouse in 500 μL of HBSS. One donor mouse was used for every two endometriosis mice. Sham mice received an intraperitoneal injection of 500 μL of HBSS.

2.3. Behavioral testing

For mechanical hyperalgesia test, mice were allowed to habituate to the apparatus for at least 2h and during three consecutive days before the beginning of measurements. After habituation, baseline measurements were obtained on two consecutive days prior to the induction of endometriosis. Pain intensity to a mechanical stimulus (mechanical hyperalgesia) in the abdominal region was measured using von Frey filaments. The investigator was blinded to the treatment. The experimenter was trained, and care was taken not to stimulate the same point consecutively, and the stimulation of the external genitalia was avoided. A jump or paw

flinch was considered a withdrawal response [21; 30]. The mechanical threshold was determined by the up and down method starting with 0.4g filament and calculated using the open-source software Up-Down Reader [20]. All treatments were performed after behavioral testing.

2.5. Immunofluorescence staining

For immunofluorescence, lesions or paired thoracic and lumbosacral (T10-L3 plus L6-S1) dorsal root ganglia (DRG) were dissected at 56 dpi and maintained in 4% paraformaldehyde (PFA, for 24h) and then to 30% sucrose (for 72h). These ganglia were chosen because of their involvement in abdominal pain and pelvic organ cross-sensitization (Christianson *et al.*, 2006; Malykhina *et al.*, 2006; Christianson *et al.*, 2007). Primary antibodies used in this study were: anti-CGRP (1:500, cat #C8198, MilliporeSigma, Burlington, MA, USA) and phosphorylated NF-κB p65 subunit (1:200, cat #sc-136,548, Santa Cruz Biotechnology, Dallas, TX, USA). Secondary antibodies used: Alexa Fluor 488 (1:1000, cat #A-11008, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 647 (1:500, cat# A-21235, Thermo Fisher Scientific, Waltham, MA, USA). The images and analysis were performed using a Confocal Microscope (TCS SP8, Leica Microsystems, Mannheim, Germany).

2.6. Statistical analysis

Results are presented as means ± SEM of measurements made on six mice in each group per experiment. Each experiment was conducted twice. Data were analysed using the software GraphPad Prism version 6.01 (La Jolla, CA, USA). Two-way repeated measure analysis of variance (ANOVA), followed by Tukey's *post hoc*, was used to analyse data from experiments of multiple time points (mechanical and thermal hyperalgesia). Data from experiments of single time point, it was used one-way ANOVA followed by Tukey's *post hoc*. Statistical differences were considered significant when $p < 0.05$.

3. Results

3.1. MaR1 reduces endometriosis-associated pain, percentage of mice with lesions, and lesion size

First, we addressed whether MaR1 could treat endometriosis. We observed that treatment with MaR1 (30 ng) reduced mechanical hyperalgesia starting at 35 dpi until the end of the experiment (Fig. 1A). Interestingly, MaR1 at 10 ng reduced mechanical hyperalgesia in the last time point. We next wonder whether this SPM could reduce the percentage of mice with lesion. Treatment with MaR1 (30 ng) reduced not only the number of mice with lesions (Fig. 1B), but also lesion size (Fig. 1C). Therefore, this dose was chosen for the next experiments.

3.2. MaR1 blocks activation of CGRP-positive DRG neurons

Once activated, nociceptors release neuropeptides such as CGRP to shape inflammatory response (Pinho-Ribeiro *et al.*, 2017). We have previously demonstrated that MaR1 blocks CGRP release from lumbar mouse DRG neurons (Fattori *et al.*, 2019). Therefore, we next wonder whether MaR1 could reduce the endometriosis-induced activation of CGRP-positive neurons. For that, double staining of phosphorylated p65 NF-κB with CGRP was performed. Treatment with MaR1 not only reduced NF-κB activation in that subpopulation of neurons (Fig. 2A and B) but also reduced NF-κB fluorescence intensity (Fig. 2C).

4. Discussion

Before seen as bystanders, recent studies have demonstrated that nociceptors can sense environmental cues to orchestrate neurogenic inflammation and the activity of innate and adaptive immune cells in a context-dependent manner (Chiu *et al.*, 2012; Pinho-Ribeiro *et al.*, 2017). Nociceptor neurons can detect potentially damaging stimuli, such as pathogens and molecules released from pathogens or injured tissue. Upon these stimuli, nociceptor neurons release neuropeptides such as CGRP and substance P to modulate inflammation and infection. This orchestrated response aimed at protecting and resolving inflammation can be also harmful to the host. For instance, nociceptors have the ability of sensing *Staphylococcus aureus* bacterial infection. Specifically, N-formylated peptides and α-hemolysin (a pore-forming toxin) activates and induces CGRP release by nociceptors and silences immune system (Chiu *et al.*, 2013). In the context of *S. pyogenes* activates neurons through the action of streptolysin S (SLS, a secreted pore-forming toxin) and hijacks pain response to promote bacterial growth and necrotizing fasciitis. Blocking CGPR release by nociceptor neuron using Botulinum toxin serotype A (BoNT/A) improves neutrophil bacterial killing and ameliorates disease (Pinho-Ribeiro *et al.*, 2018). In the context of endometriosis, it has been demonstrated that estrogen activates nociceptors that release CCL2 to promote macrophage migration to endometriotic lesions (Greaves *et al.*, 2015). Released neuropeptides by nociceptors also contribute to lesion formation as well. Blocking substance P interaction with its receptor NK1R reduces the number of viable stromal cells, indicating that substance P provides support for the growth of stromal endometriotic cells (McKinnon *et al.*, 2013). Therefore, molecules with the ability of silencing nociceptors might be promising candidates for endometriosis.

We have previously demonstrated that the SPM MaR1 blocks CGRP release from lumbar mouse DRG neurons (Fattori *et al.*, 2019). Consequently, MaR1 reduces the number of leukocytes close to CGRP-positive fibers to reduce pain. Specifically for endometriosis, in a surgically-induced endometriosis model, administration of eicosapentaenoic acid (EPA, an SPM precursor) reduces the number of lesions in wild-type mice but not in mice lacking in Alox12/15, a key enzyme for Lipoxin A4 formation (Wu *et al.*, 2014). Moreover, treatment with Lipoxin A4 reduced the invasion and migration of endometrial epithelial cells in vitro (Wu *et al.*, 2014). In a cohort study with women diagnosticated with endometriosis show that high omega-3 diet lowers 22% the risk of developing endometriosis (Missmer *et al.*, 2010). Altogether, these data indicate that SPMs show promising effects for the treatment of

endometriosis. In this work, we demonstrated that MaR1 blocks the activation of CGRP-positive DRG neurons and treats endometriosis.

In conclusion, we show that MaR1 reduces the activation of a subpopulation of nociceptors that are responsible for producing neuropeptides such as CGRP. We also demonstrated that MaR1 not only reduced pain but also the number of mice with endometriotic lesions, indicating it is a promising drug for the treatment of endometriosis.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

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Figures

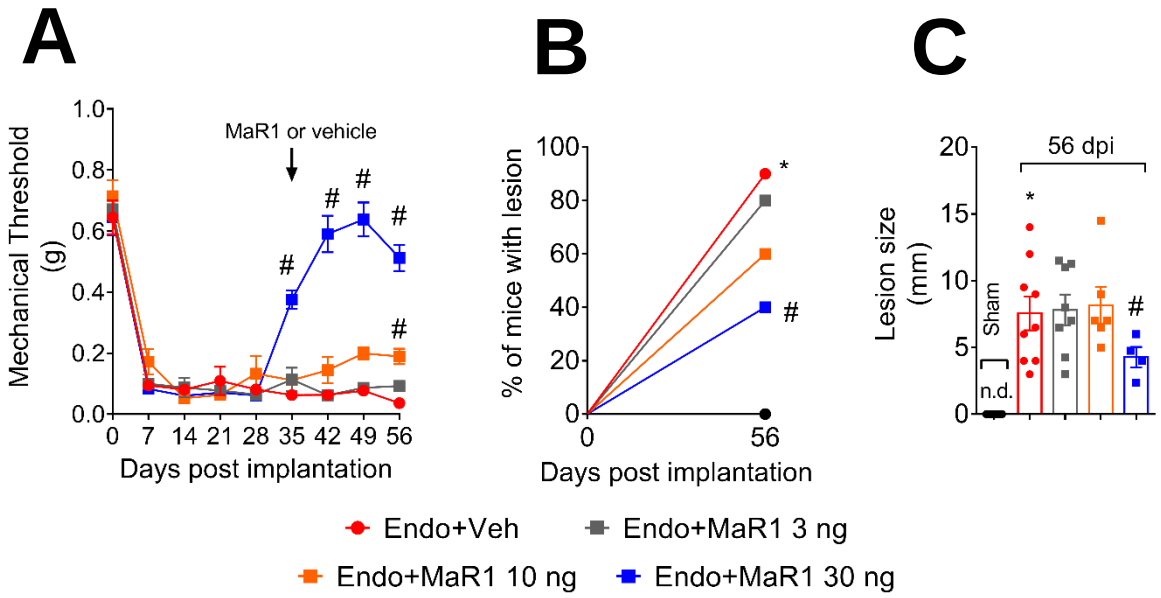

Fig. 1. MaR1 reduces endometriosis-associated pain, percentage of mice with lesions, and lesion size

(A) Mechanical abdominal pain was determined weekly through 56 dpi using von Frey filaments. Treatment with MaR1 was performed daily starting at 29 dpi and after behavioral testing. Results are expressed as mean ± SEM of measurements, n = 10 mice per group per experiment, two independent experiments (*p < 0.05 vs. sham, #p < 0.0.5 vs endo; two-way repeated-measures ANOVA followed by Tukey's *post hoc*). **(B)** The percentage of mice with visible lesions was determined by a simple count of the presence of lesions in mice. Results are expressed as percentage (%) of mice with lesions (*p < 0.05 vs. sham, #p < 0.0.5 vs endo; Kaplan-Meier method followed by the logrank test). **(C)** Lesion size was determined in the remaining lesions by measuring height and weight. Sham mice do not show any lesion (n.d. = not detectable). Results are expressed as mean ± SEM (*p < 0.05 vs. sham, #p < 0.0.5 vs endo + vehicle; one-way ANOVA followed by Tukey's *post hoc*).

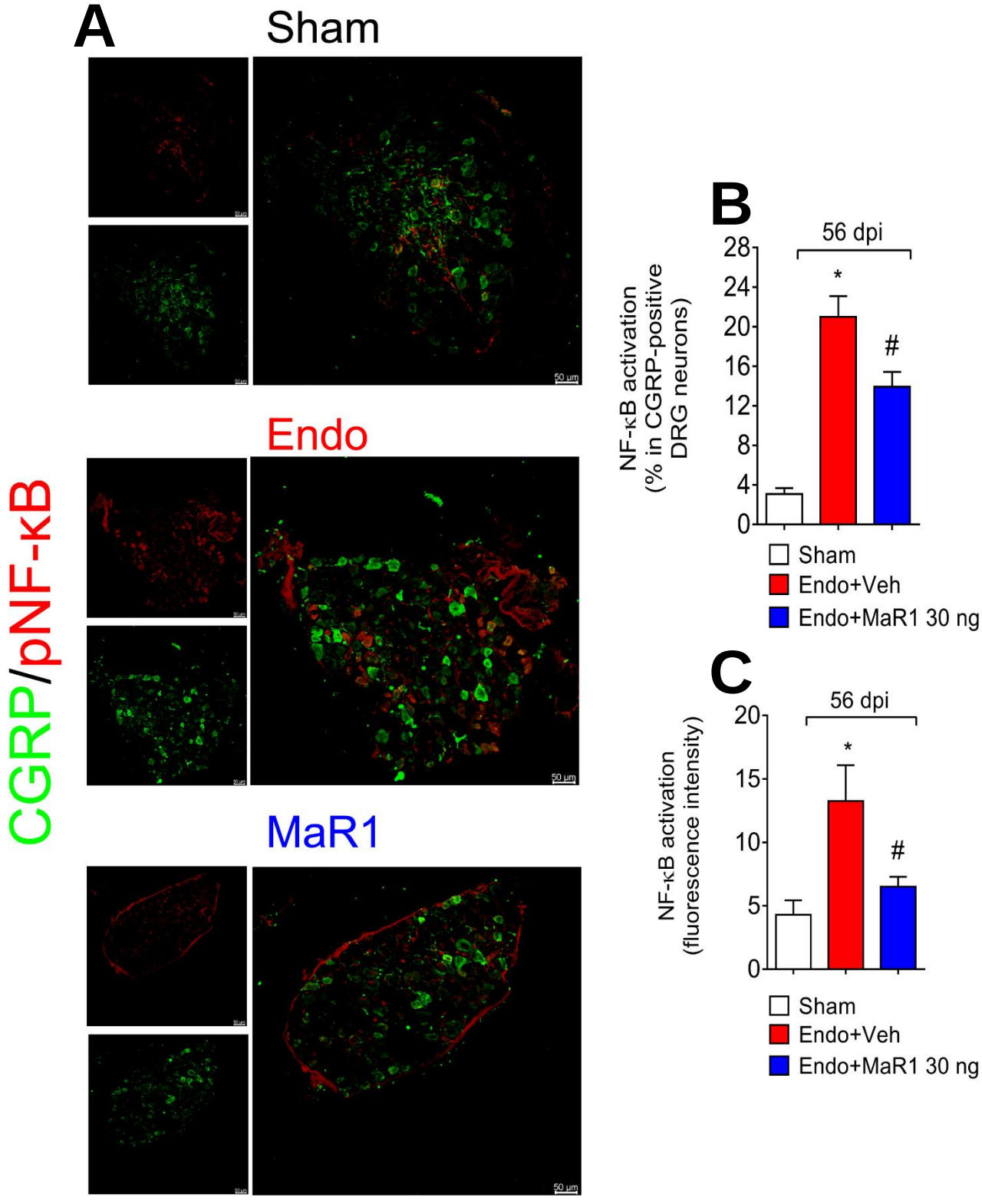

Fig. 2. MaR1 blocks activation of CGRP-positive DRG neurons

Fifty-six dpi DRG neurons from sham, endo (vehicle-treated), and MaR1-treated mice were dissected to determine the activation of CGRP-positive nociceptors. **(A)** Representative pictures of double staining of phosphorylated p65 NF-κB with CGRP from sham, endo (vehicle-treated), and MaR1-treated mice. **(B)** Total number of CGRP-positive neurons stained with

phosphorylated p65 NF-κB. **(C)** phosphorylated p65 NF-κB. Results are expressed as mean ± SEM (*p < 0.05 vs. sham, #p < 0.0.5 vs endo + vehicle; one-way ANOVA followed by Tukey's *post hoc*).

Review

3.5 Capsaicin: Current Understanding of its Mechanisms and Therapy of Pain and Other Pre-Clinical and Clinical Uses

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Abstract: In this review, we discuss the importance of capsaicin to the current understanding of neuronal modulation of pain and explore the mechanisms of capsaicin-induced pain. We will focus on the analgesic effects of capsaicin and its clinical applicability in treating pain. Furthermore, we will draw attention to the rationale for other clinical therapeutic uses and implications of capsaicin in diseases such as obesity, diabetes, cardiovascular conditions, cancer, airway diseases, itch, gastric, and urological disorders.

Keywords: analgesia; capsaicinoids; chili peppers; desensitization; TRPV1

1. Introduction

Capsaicin is a compound found in chili peppers and responsible for their burning and irritant effect. In addition to the sensation of heat, capsaicin produces pain and, for this reason, is an important tool in the study of pain. Although our understanding of pain mechanisms has evolved greatly through the development of new techniques, experimental tools are still extremely necessary and widely used. Among these basic experimental tools for the study of pain mechanisms and development of novel analgesics, we can fairly consider capsaicin as one of the most important sources of knowledge in the pain field. Curiously, many recent studies have confirmed scientifically what was already known by some cultures: capsaicin can also be used to relieve pain [1]. This paradox can also be seen with opioids, which have an established clinical use as analgesics, but also induce hyperalgesia [2]. Therefore, the complexities of capsaicin-triggered responses as well as its therapeutic usefulness highlight the importance of understanding its mechanisms of action not only in pain modulation, but also in other pathological conditions. In this review, we will highlight the importance of capsaicin to the current understanding of neuronal modulation of pain and explore some mechanisms of capsaicin-induced pain. We will focus on the analgesic effects of capsaicin and its clinical applicability in treating pain. Furthermore, we will draw attention to the rationale for other clinical therapeutic uses and implications of capsaicin in diseases such as obesity, diabetes, cardiovascular conditions, cancer, airway diseases, itch, gastric, and urological disorders.

1.1. Discovery, Natural Sources, Role in Plants, Isolation, and Structure of Capsaicin

Chili peppers contain capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a phenolic compound responsible for their characteristic taste and pungency. All plants from Capsicum genus produce varied amounts of capsaicin, except *Capsicum annum*, and all of them have been used as a spice ingredient and consumed by humans for over 6000 years [3,4]. The quantities of capsaicin can represent up to 1% of the mass of the chili peppers and, together with salt, represent the most consumed condiment by humans. Capsaicin is an intriguing molecule since the consumption of chili peppers evokes opposing sensations (pleasant and unpleasant) depending on the individual experience and chili pepper consumption habits. The effects of capsaicin go well beyond the taste and its role in plants' health help us to understand how its use can improve human health [4].

The production of capsaicin among plants from the Capsicum genus was well conserved, likely due to its roles in seed germination and protection from parasites. In fact, capsaicin is not equally distributed in all parts of pepper fruit. Its concentration is higher in the area surrounding the seeds (placental tissue) and this localization is related directly to the role of capsaicin in protecting seed germination [5]. The aversion to eating large amounts of capsaicin keeps rodents and other mammals away and this represents an important mechanism to increase the chances of germination since mammals can grind and digest the seeds making them unable to germinate. Birds, on the other hand, cannot feel this unpleasant taste of peppers [6]. Importantly, pepper seeds resist to birds´ digestive tract, making them the perfect consumers. Capsaicin also protects plants from parasites such as insects and mold, and humans have been using this property to treat infectious diseases and to preserve food [7,8].

Despite the unpleasant sensation that occurs when large quantities of chili peppers are consumed, capsaicin promotes pain relief when used in the right dosage and frequency. These properties caught the attention of researchers long ago and still do nowadays, boosting our knowledge about capsaicin. Capsaicin was first purified in 1876 [9] but its structure started to be described only in 1919 [10]. Currently, the structure and properties of capsaicin are well defined (Figure 1). Capsaicin presents a nonpolar phenolic structure and thus cannot be solubilized in water. The main solvents used to extract and maintain capsaicin properties are nonpolar solvents such as ether, benzene, dimethyl sulfoxide and acetone, but ethanol can also be used as a solvent due to its mixed properties.

Because of its chemical structure, capsaicin can be well absorbed when administered topically or orally, reaching up to 94% of absorption [11]. Following its discovery and characterization, it was observed that capsaicin is actually part of a family of compounds that share similar structural and biologic characteristics.

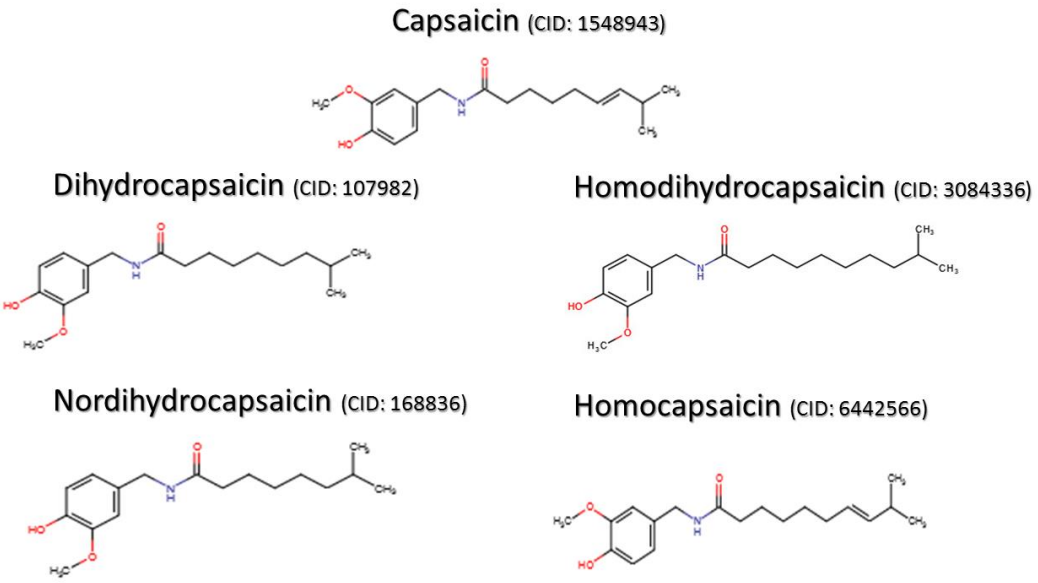

Figure 1. Chemical structure of capsaicin and capsaicinoids. Molecules of capsaicin and capsaicinoids available in PubChem database [12–16]. Compound identifier (CID) number is provided in parentheses. Molecules were drawn using Marvin JS, MarvinSketch in JavaScript.

1.2. Capsaicin-Derived Molecules and Analogs

Plants from Capsicum genus produce many capsaicin-related compounds. Due to their similarity with capsaicin, these molecules can be grouped in a family called capsaicinoids. Capsaicinoids include dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin (Figure 1). All these molecules share structural and activity similarities with capsaicin [17,18], but they are not as abundant as capsaicin that can account for up to 80% of capsaicinoid content of chili peppers. The pungency of all these molecules emphasizes the fact that this activity is defined mainly by the benzene ring region, however, the length of acyl chain can modify it [19]. Besides capsaicinoids, there are other groups of molecules that share similarities with capsaicin such as capsinoids, with reduced pungency, and the extremely potent resiniferoids [20,21]. Importantly, all these capsaicin-related molecules present therapeutic properties to treat pain and other conditions and have been used in research to understand the pathophysiology of pain and diseases. Capsaicin has opened the path to our understanding of pain mechanisms and demonstrated that, although counter-intuitive at first sight, it is possible to treat pain by boosting algesic pathways. Furthermore, the ability of capsaicin to cause activity-induced tolerance to pain demonstrates the complexity of a single pharmacological tool that is able either to trigger or treat pathological pain.

2. Capsaicin and Pain

Capsaicin selectively stimulates nociceptive neurons and has been widely used to study pain-related events. In this topic, we will highlight some aspects of how capsaicin induces pain and its importance to the current understanding of neuronal mechanisms of pain.

2.1. Importance of Capsaicin in Pain Research

Before the discovery of the capsaicin-activated receptor, intradermal injection of capsaicin was used to produce primary and secondary hypersensitivity to noxious and innocuous stimuli in both monkeys and rats [22,23]. Seminal works demonstrated that capsaicin excites nociceptors by increasing the influx of ions, such as calcium, in dorsal root ganglion (DRG) neurons [24,25]. Years later, cloning transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor shed light on the mechanism by which capsaicin induces pain [26]. This work is a landmark in the mechanisms of pain since demonstrated that capsaicin induces pain-like behavior by activation of TRPV1 receptors expressed by nociceptors. At that time, TRPV1 receptors were denominated vanilloid receptor 1 (VR1) [26]. More importantly, this discovery has changed our understanding of pain mechanisms since it demonstrates that a receptor-coupled channel expressed by nociceptors detects environment stimuli resulting in nociceptor depolarization and consequently producing pain. Also, this discovery opened avenues to the development of new drugs since Mendelian disorders in these proteins can produce pain [27]. After that, *in vivo* evidence demonstrated that mice lacking TRPV1 receptors exhibit reduced thermal noxious response and capsaicin-induced paw licking [28]. Whole patch-clamp technique demonstrated that mice lacking TRPV1 receptors present impaired calcium influx in DRG neurons [28]. Therefore, administration of capsaicin in animals was important to elucidate the function of TRPV1 as well as to aid our knowledge about pain processing and modulation. Therefore, the discovery of TRPV1 was essential to validate capsaicin-induced pain models, which can now be used to study neuronal mechanisms of pain, in addition to testing new TRPV1 antagonists and drugs that target the consequences of TRPV1 activation before clinical trials.

2.2. Mechanisms of Capsaicin-Induced Pain

One of the first evidence of a selective action of capsaicin on C-polymodal nociceptors was obtained by the capsaicin-evoked response of C-fibers in the cat saphenous nerve. In addition, injection of capsaicin reduces the thermal threshold in both rats and humans [29]. This seminal work demonstrates that capsaicin selectively acts on C-polymodal nociceptors and the thermodependency of sensory effects on animals and humans [29]. Spinal cord mechanisms of capsaicin-evoked mechanical allodynia depend on G-protein and protein kinases (PKA and PKC) and could be reversed by both G-protein and protein kinase inhibitors. For instance, kinase activity may result in an increase of receptor activity as well as an increase of trafficking and cell-surface expression of molecules [23]. In fact, capsaicin activates PKA and PKC that phosphorylate NMDA receptor subunit NR1 at serine residue 890 and 897, and serine residue 896, respectively, which enhances receptor activity [30,31]. Alongside with this, mitogen-activated protein kinase (MAPK) family has been involved in pain-related states and, indeed, capsaicin administration increases the phosphorylation of p38 MAPK in the periphery and spinal cord dorsal horn [32]. Therefore, inhibition of these kinases has helped to define some of the intracellular mechanisms involved in capsaicin-induced central sensitization. In addition to these kinases, the neuropeptide CGRP is another important component in central sensitization. Capsaicin-induced TRPV1 activation stimulates the release of CGRP in the spinal cord, and intrathecal treatment with CGRP antagonist reduces the development and maintenance of mechanical hyperalgesia and secondary allodynia [33].

Capsaicin-induced pain model was also useful to demonstrate the role of reactive oxygen species (ROS) in central sensitization. Despite their pro-hyperalgesic effect *per se* [34,35], ROS can also be a source of post-translational modification due to their action on redox-sensitive protein residues such as cysteine and serine [36]. In fact, treatment with the ROS scavenger Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) and PBN (*N-tert*-butylnitrone) reduces the activation of neurons in the dorsal horn as observed by the reduction of electrophysiological activity detected by the number of neuronal spikes [37]. As a consequence of that, there is reduction of primary and secondary hyperalgesia, and reduction of neuron responsiveness induced by capsaicin, suggesting a role of ROS in the maintenance of persistent pain [37]. Keratinocytes are in proximity to nociceptors, which may imply a role for these cells in pain. Using Cre-lox technique to promote expression of TRPV1 in keratinocytes demonstrated that capsaicin stimulates TRPV1-expressing keratinocytes inducing c-fos expression in laminae I and II of the ipsilateral spinal cord dorsal horn, which contributes to evoke acute paw-licking nociceptive behavior [38]. This addresses the interaction between keratinocytes and nociceptors in pain-state.

Capsaicin has helped us to understand the mechanisms related to abdominal pain, a condition inherent of patients with irritable bowel syndrome (IBS). Intracolonic injection of capsaicin induces abdominal mechanical hyperalgesia, and pain-related behaviors such as abdominal licking in a morphine-sensitive manner suggesting its nociceptive nature instead of a normal grooming behavior [39]. IBS patients present abdominal mechanical hyperalgesia and allodynia [40]. Nociceptive fibers present in the colon respond to TRPV1 agonist, and, therefore, highlight these receptors as potential targets for abdominal pain [35]. In fact, TRPV1 co-localizes with substance P and calcitonin gene-related peptide (CGRP) in a model of DSS (dextran sulfate sodium)-induced colitis. Substance P and CGRP are two important neuropeptides in pain signaling that together with TRPV1 mediate visceral pain [41]. This is important considering that TRPV1/CGRP pathway is considered an attractive pharmacological approach to treat visceral pain [42]. *In vivo* functional magnetic resonance imaging (fMRI) further corroborates the importance of TRPV1 receptors and demonstrates the activity of supraspinal mechanisms in capsaicin-induced pain. Injection of capsaicin in wild-type (WT) rats activates putative pain neural circuit, such as Papez circuit, and the habenular system; and TRPV1 receptor deficiency reduces the activation in these same brain regions in response to capsaicin [43]. This is important since it points out to the supraspinal modulation of TRPV1 in pain. And additionally to these mechanisms, TRPV1 also modulates the emotional component of visceral pain [44]. Modulation of TRPV1/CGRP pathway is important in arthritis as well [45]. In fact, intra-articular injection of CGRP in normal or

mono-iodoacetate (MIA)-induced arthritis rats reduces the mechanical threshold and increases percentage of sensitized fibers [46], and treatment with CGRP antagonist reduces CGRP- and MIA-induced sensory neuron firing [46], suggesting that peripheral release of CGRP contributes to inflammation and sensitization of joint nociceptors [45].

In the past few years, efforts have been made to identify ligand-receptor and receptor-receptor interactions and their role with pain. Among the first interactions that were shown, we can highlight the capsaicin-TRPV1. In fact, co-administration of capsaicin with QX-314 (a membrane-impermeable sodium channel blocker) facilitates the access of the QX-314 that blocks sodium inward currents in capsaicin-responsive DRG neurons producing analgesia [47]. Nevertheless, in this work, neither the potentially dynamic of TRPV1 permeability to different ions size or charges (unknown at the moment), nor the effect of pore size of the TRPV1 was addressed. TRPV1 receptor was considered a nonselective cation channel with higher affinity for calcium than sodium. TRPV1 agonists such as capsaicin, changes TRPV1 pore size leading to time-dependent discrimination between monovalent and divalent cations over a time frame of seconds that can persist for several minutes [48]. Another striking feature was that phosphorylation of TRPV1 serine 800 residue by PKC allows neurons to discriminate the size of cations by increasing permeability to large cation, and proportionating sensitization of the TRPV1, and enhancement of inward currents [48]. In fact, PKC phosphorylates TRPV1 at serine 800 residues, but not at serine 502, in DRG neurons of rats and contributes to pain in MIA-induced osteoarthritis model [49] (Figure 2). Inhibition of PKC, but not PKA, reduces capsaicin-induced pain-related behavior in MIA-induced osteoarthritis rats [49]. TRPV1 agonists such as N-arachidonoyldopamine (NADA), piperine and resiniferatoxin (RTX) provide distinct pattern of ion selectivity and discrimination [48]. Thus, suggesting that different TRPV1 agonist change the selectivity to inward ions, and the activity of different kinases (such as PKA and PKC) [48,49] could provide different inward ion. Recent data further advanced in this topic by demonstrating that capsaicin binds to TRPV1 pocket as a unique molecule [50].

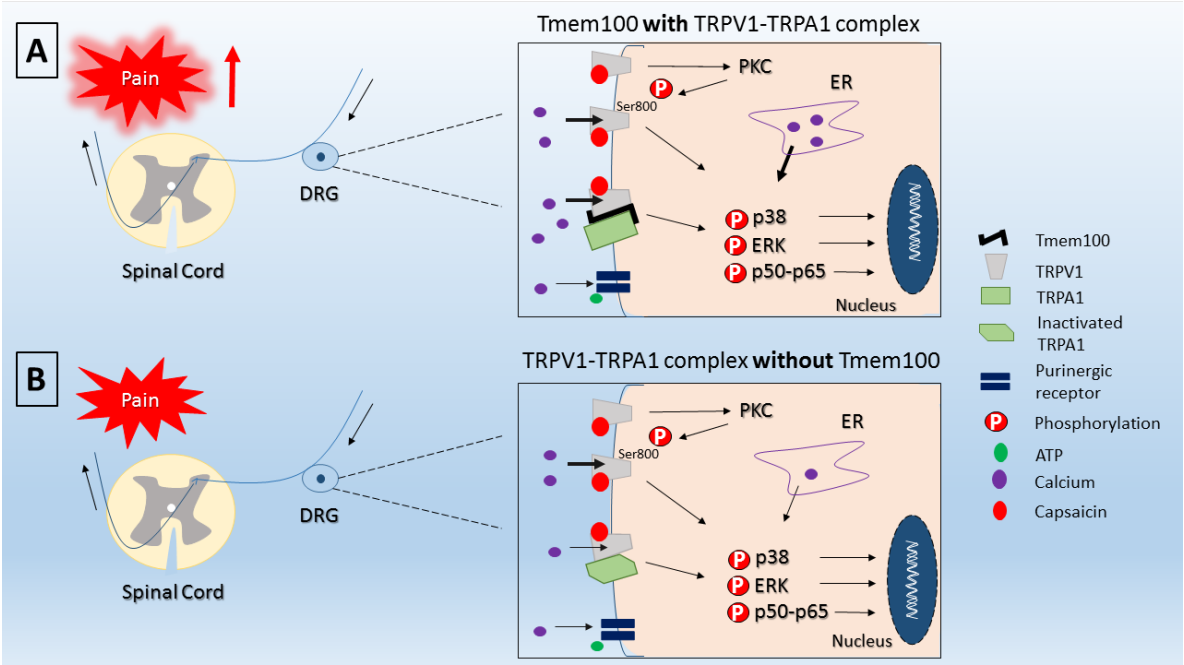

Figure 2. Mechanisms of capsaicin-induced pain. Schematic representation of the phosphorylation at Ser800, which allows TRPV1 discriminating cation influx [50], and participation of Tmem100 in the mechanism of capsaicin-induced pain [49,51,52]. In the presence of Tmem100 (A) activation of TRPV1-TRPA1 complex increases the influx of calcium and contributes to higher perception of pain. On the other hand, without Tmem100 (B) TRPV1-TRPA1 complex produces lower influx of calcium since TRPA1 is found in an inactivated conformation [49,51,52]. Black thin arrow: lower calcium influx; Black thicker arrow: higher calcium influx; DRG: dorsal root ganglion; ER: endoplasmic reticulum; PKC: protein kinase C.

Capsaicin has a very high affinity, sensitivity, and selectivity for TRPV1 and does not activate the homologous TRPV2–TRPV6 receptors [50]. In addition, an elegant work demonstrated how capsaicin binds to TRPV1 and which amino acid residues are involved in this binding. Capsaicin binds to TRPV1 in a "tail-up, head-down configuration" (as coined by the authors). The aliphatic "tail" interacts with the channel through nonspecific van der Waals forces and contributes to binding affinity. Hydrogen bonds between its vanillyl "head" and amide "neck" with residues of glutamic acid E571 and T551 of the channel, respectively, grant specificity for ligand binding [50] (Figure 3). Other interactions with TRPV1, such as Tyr511, Glu570, and Ile569; with the vanillyl "head" allows capsaicin accommodation in this specific pocket (called as vanilloid pocket). On the other hand, RTX (a TRPV1 agonist) molecule is bigger than capsaicin, and possesses a different electron cloud, which does not allow its accommodation in the same vanilloid pocket because this pocket is too shallow for RTX [53]. Therefore, this spatial allocation of both molecules accounts to the distinct agonist pattern and potency explaining the increased potency of RTX compared to capsaicin [53]. In addition to the spatial allocation, structure-activity relationship study demonstrates the functional groups that are essential to these difference. For instance, the amide group is essential for capsaicin activity, while for RTX the five-membered diterpene ring fulfills this role [54]. These studies had an enormous impact because they demonstrated the fundamental pockets to capsaicin or other agonist binding and activation of TRPV1. Therefore, these studies enable future pharmacological approaches based on this knowledge since these agonists can act both as pro-hyperalgesic and anti-hyperalgesic as we will discuss in the next topic.

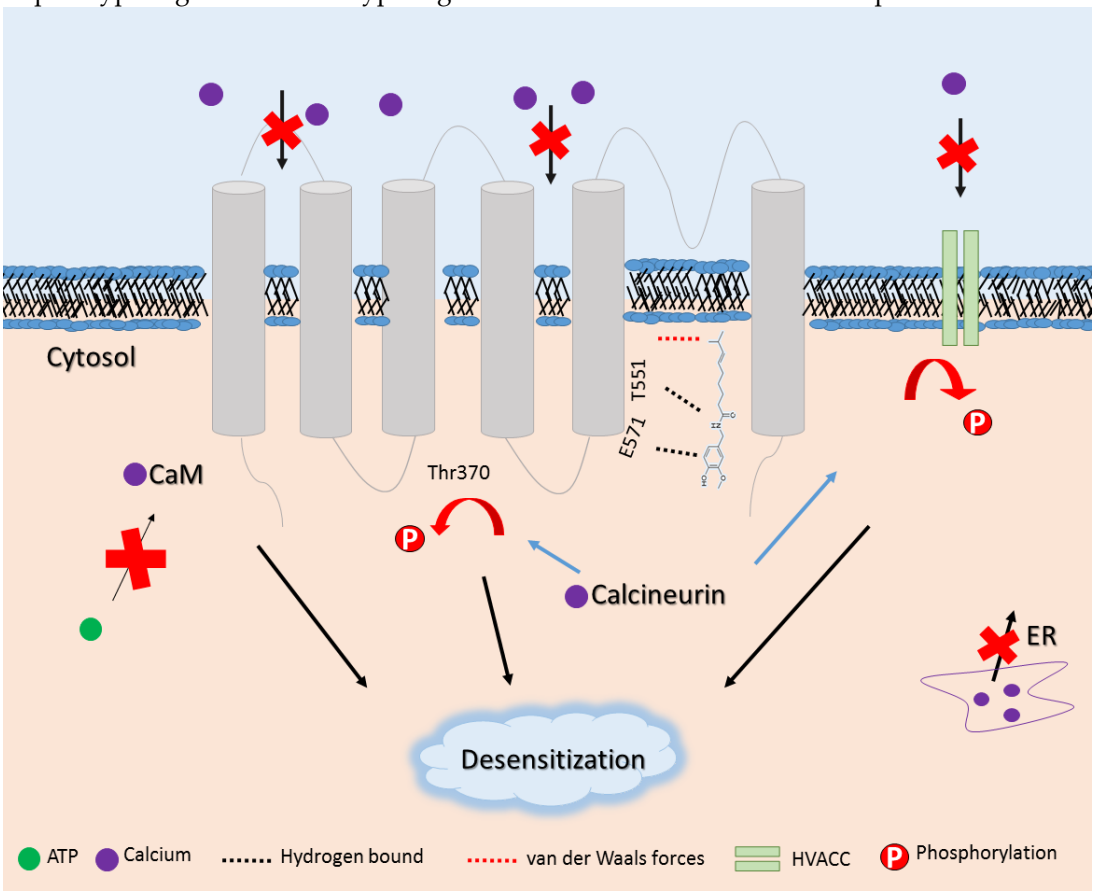

Figure 3. Mechanisms of capsaicin-TRPV1 interaction and desensitization. Capsaicin bounds to TRPV1 in a "'tail-up, head-down configuration" and increases the influx of calcium [50]. A secondary effect due to calcium influx is the activation of calcium-dependent enzymes, such as calcineurin, which dephosphorylates TRPV1 [55,56], downregulates HVACC [57], which culminates in TRPV1 desensitization. Additionally, CaM prevents ATP-induced sensitization of TRPV1 by competing for the same intracellular pocket [55]. CaM: calmodulin; HVACC: high voltage-activated calcium channels; ER: endoplasmic reticulum.

Regarding receptor-receptor interaction, TRPV1-TRPA1 is a well-documented one [58]. This interaction is attributed to the formation of a heterodimer between TRPV1-TRPA1 receptors [59], which is possible due to lipid raft movement and formation of a cluster of receptors in neurons [60]. Recent evidence demonstrated that a trans-membrane receptor called Tmem100 is co-expressed with both TRPV1-TRPA1 complex in DRG neurons and is essential to modulate their activity by acting as an adaptor molecule [51]. Nevertheless, forming TRPV1-TRPA1 complex without Tmem100 is also possible [51,52]. In the TRPV1-TRPA1 complex without Tmem100, TRPV1 inhibits TRPA1 activity since TRPV1-TRPA1 positive DRG neurons present reduction of inward current after mustard oil (TRPA1 agonist) as stimulus, but not to capsaicin. On the other hand, in the presence of Tmem100 TRPV1 increases TRPA1 activity and potentiates pain perception [51] (Figure 2). Additionally, TRPA1-initiated calcium influx promotes PKA activation, thereby sensitizing TRPV1 channels [61].

Therefore, there is a complex interaction of capsaicin and other agonists with TRPV1 that shed light in the complex pathway to understand TRPV1 modulation. TRPV1 crosstalks with other receptors build up an entirely different pharmacology adding up complexity.

2.3. Targeting TRPV1 as a Pharmacological Approach

Currently, capsaicin-induced pain is also used to assess new molecules that target TRPV1 receptor. A whole body of evidence points out to natural product-derived molecules as potential drugs. We recently demonstrated that the flavonoids naringenin [62], vitexin [63], and hesperidin methyl chalcone [64] reduce inflammatory pain by targeting, at least in part, capsaicin-triggered TRPV1 receptors. Other flavonoids also target TRPV1 and reduce pain such as eriodictyol [65] and hesperidin [66], and reduces gastritis such as silymarin [67]. These data corroborate the concept that flavonoids modulate TRPV1. Additionally, other molecules such as α-spinasterol isolated from leaves of the medicinal plant *Vernonia tweedieana* (Baker) produce antinociceptive effect by TRPV1 antagonism [68]. Another well-recognized natural product-derived molecule is curcumin, which has more than 100 different targets, among them TRPV1 [69,70]. Curcumin reduces capsaicin-induced calcium rise and inward current in DRG neurons of both mice and rats [69] by antagonizing TRPV1 receptors [71].

Considering the prevalence of chronic pain and the relevance of TRPV1, the pharmaceutical industry has been focusing its efforts in the development of synthetic drugs targeting TRPV1. These drugs are divided into TRPV1 antagonists and TRPV1 agonists [72], and both groups present considerable disadvantages. For instance, TRPV1 agonists can cause pain and/or erythema before desensitization becomes effective, and TRPV1 antagonists usually present lower efficacy compared to TRPV1 agonists and can cause hyperthermia [72,73].

SB-705498 was one of the first developed TRPV1 antagonists. A single oral administration of 400 mg of SB-705498 reduces capsaicin-evoked flare, alongside with elevation of thermal threshold of the patients [74]. As mentioned, hyperthermia is an important side effect due to TRPV1 antagonist administration. In fact, administration of lower doses (2 and 8 mg) of AMG 517 causes hyperthermia that ranges between 39-40.2°C. On the other hand, repeated administration of this drug for 7 days at a dose of 10 mg reduces hyperthermia, suggesting dose-dependent effect and desensitization [75]. TRPV1 agonists will be discussed in the next section.

3. Mechanisms of Capsaicin-Induced Analgesia

The effects of capsaicin on nociception are not limited to its ability to produce pain. In fact, high or repeated doses of capsaicin induces an initial pain sensation that is followed by analgesia [76]. This loss of sensitivity to painful stimuli was noticed in response to not only thermal, but also mechanical and chemical noxious stimuli [77].

The underlying mechanisms in capsaicin-induced analgesia are being increasingly studied. After exposure to a high or repeated dose of capsaicin, the TRPV1 receptors begin a refractory state commonly termed as desensitization that leads to inhibition of receptor function [78–80] (Figure 3). Capsaicin-induced desensitization involves mechanisms not entirely understood. There is evidence that this process includes depletion of neuropeptides such as substance P in the nerve fibers that express TRPV1

[81,82], and an increase of intracellular calcium levels by inhibition of high voltage-activated (HVA) and low-voltage-activated (T-type) calcium channels [83–85]. A delayed or secondary effect due to calcium influx is the activation of calcium-dependent proteins that leads to desensitization of TRPV1 [55,56]. For instance, a multi-ligand-binding in the cytosolic ankyrin repeat domain (ARD) of TRPV1 allows intracellular ATP binding to specific pockets of TRPV1-ARD and sensitizes this receptor [55]. On the other hand, desensitization of TRPV1 occurs when calmodulin (CaM) binds in a calcium-dependent manner in the same pockets of ATP, since mutation in these pockets eliminates desensitization in the absence of ATP [55]. Specifically, calcineurin, a CaM and calcium-dependent enzyme, dephosphorylates Thr370 residues that were previously phosphorylated by PKA [56]. Additionally, calcineurin downregulates HVA calcium channels limiting calcium influx in DRG neurons [57] (Figure 3). Altogether, these mechanisms lead to desensitization of TRPV1 and account to capsaicin-induced analgesia.

In addition to the mechanism of TRPV1 desensitization, new evidence has emerged showing the efficacy of capsaicin as an analgesic [86]. Capsaicin activates TRPV1, which inhibits Piezo proteins, a family of mammalian cation-selective ion channels that respond to mechanical stretch [86]. Inhibition of Piezo proteins occurs due to calcium-dependent activation of phospholipase Cδ (PLCδ), which depletes phosphoinositides. In fact, injection of phosphoinositides in the cytosol by excised inside-out patch clamp reduces rundown inward current of Piezo channels and reverts inactivation [86]. Therefore, the depletion of these phosphoinositides correlates with inhibition of mechanical-stimulation of Piezo channels through inhibition of inward current [86]. This work uncovers, at least in part, how local capsaicin produces mechanical analgesia.

Capsaicin-induced analgesia is also related to degeneration of sensory fibers [87–90]. The mechanisms through which capsaicin causes cell death are not completely understood. Recent studies indicate that one of the most likely mechanisms is apoptosis via caspase activation. An *in vitro* study demonstrated capsaicin induces DNA fragmentation and reduction of the nucleus in a caspase-dependent manner secondary to cell death of sensory neurons. In addition, the cell death process triggered by capsaicin via TRPV1 is directly related to mitochondrial permeability transition [91]. On the other hand, capsaicin can promote cell death by apoptosis-independent mechanisms such as cell swelling and bleb formation in the membrane. These mechanisms are dependent on extracellular sodium influx via TRPV1, which in turn is controlled by the intracellular concentration of calcium [92]. Capsaicin-induced analgesia is longer in inflammatory conditions than in basal conditions [93,94]. While the intraplantar injection of 10 μg of capsaicin in control mice produced analgesia for 2 days, in groups stimulated with carrageenan or CFA, the same dose of capsaicin produces analgesic effect for 6 and 30 days, respectively [94]. This enhancement of capsaicin-induced analgesia during inflammation is likely related to a facilitated TRPV1 desensitization [93,94] due to TRPV1 expression [40,95].

In addition to peripheral changes, supraspinal mechanisms also modulate capsaicin-induced analgesia. The subdermal injection of capsaicin significantly reduces the jaw-opening reflex and increases the withdrawal threshold to mechanical stimulation in anesthetized rat, and both effects are prevented by microinjection of dopaminergic or opioid antagonist into the nucleus accumbens. The tonic GABAergic inhibition of neurotransmission in the rostral ventromedial medulla (RVM) is also involved in capsaicin-induced analgesia modulation. In agreement, the injection of muscimol (GABA-A receptor agonist), but not naloxone in the RVM prevents capsaicin-induced inhibition of the jaw-opening reflex [96]. This analgesic effect was reversed by intrathecal injection of antagonists of GABA-B and μ-opioid receptors indicating that activation of inhibitory spinal receptors is an important mechanism of capsaicin-induced analgesia [97]. An increase of opioid activity is also observed in the arcuate nucleus of the hypothalamus of rats as assessed by the proopiomelanocortin (POMC) mRNA expression, a precursor of β-endorphin, 20 min after subcutaneous injection of capsaicin [98] (Figure 4).

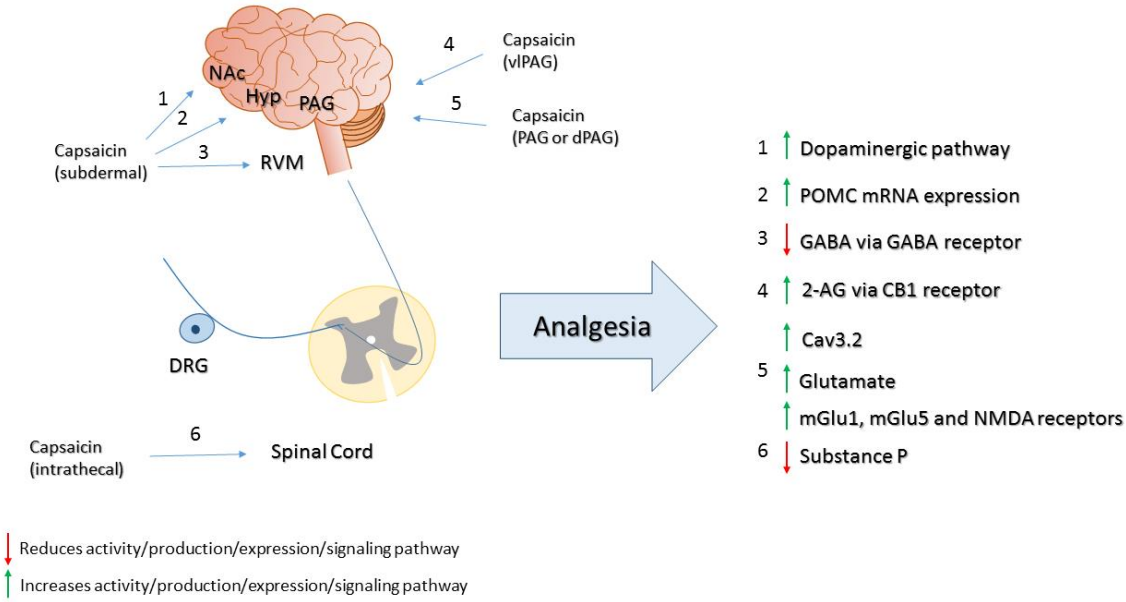

Figure 4. Supraspinal mechanisms of capsaicin-induced analgesia. Subdermal injection of capsaicin produces analgesia by modulating dopaminergic pathway in the NAc (1) [96], opioid pathway in the hippocampus (2) [98], and GABAergic activity in the RVM (3) [96,97]. In addition, vlPAG injection of capsaicin activates endocannabinoid pathway (4) [99], and dPAG by modulating glutamate signaling pathway (5) [100]. Intrathecal injection of capsaicin depletes substance P and also produces analgesia (6) [101–103]. DRG: dorsal root ganglion; NAc: nucleus accumbens; Hyp: hippocampus; RVM: rostral ventromedial medulla; PAG: periaqueductal gray; vlPAG: ventrolateral periaqueductal gray; dPAG: dorsal periaqueductal gray.

Capsaicin also induces analgesia when administered centrally in varied foci. For instance, the intrathecal injection of capsaicin or RTX produces long-term regional analgesia with substance P depletion [101–103]. The analgesic effect via supraspinal TRPV1 following intracerebroventricular injection of capsaicin depends on the activation of Cav3.2 channels since mice lacking this receptor present higher nociceptive response compared to WT mice [104]. The microinjection of capsaicin in the periaqueductal gray (PAG) [79] or its dorsal portion (dPAG) in rats produces antinociception to thermal stimulation and may be preceded by a short period of hyperalgesia [105]. The analgesic effect of capsaicin in the PAG depends on the release of glutamate and local activation of TRPV1, mGlu1, mGlu5 and NMDA receptors [79]. Additionally, there is a decrease of ON-cell and increase of OFF-cell activation in the RVM [105]. In an animal model of diabetic neuropathy, the injection of capsaicin into the ventrolateral PAG (vlPAG) reduces the thermal hyperalgesia [100]. The injection of capsaicin in the vlPAG leads to the activation of inhibitory descending pain mechanisms. The analgesic effect produced by capsaicin injection in vlPAG depends on local TRPV1 activation that culminates in the release of glutamate into RVM and subsequent activation of OFF-cells and activation of inhibitory descending pain pathway [106]. Additionally, the glutamate released act in mGlu5 post-synaptic receptors leading to Gq-protein-coupled PLCβ-DAGLα pathway-dependent formation of the endocannabinoid 2-arachidonolyglycerol (2-AG). In turn, 2-AG activates pre-synaptic CB_1 receptors, leading to retrograde disinhibition of GABA release [99]. In addition, there is co-expression of μ-opioid and TRPV1 receptors in vlPAG. Combined administration of capsaicin and μ-opioid receptor agonist sub-doses at this site produces thermal analgesia in rats with increased glutamate release and inhibition of ON-cell activity in RVM [107]. The injection of capsaicin into the RVM inhibits the overt pain-like response in the inflammatory phase of the formalin test in rats with streptozocin-induced diabetic neuropathy, an effect that may be associated with the up-regulation of TRPV1 receptors in the RVM [108] (Figure 4).

Considering the aforementioned evidence, capsaicin has been used as a support pharmacological agent in pain management. Treatment with capsaicin is effective in different types of painful conditions such as complex regional pain syndromes and neuropathic pain [109,110]; postsurgical neuropathic pain [111,112]; post-herpetic neuralgia [113,114] and painful diabetic peripheral neuropathy [115,116]. There is also report that repeated use of nasal capsaicin prevents cluster headache attacks [117]. In humans, topical capsaicin (0.075%) applied four times a day during 3 weeks causes the degeneration of nerve fibers of the skin and consequently decreases sensitivity to cold and tactile stimuli, but to heat and mechanical stimuli [118].

In patients with post-herpetic neuralgia, topical application of 8% capsaicin patch produced a significant decrease in pain for 12 weeks [119,120]. A patient with post-traumatic neuropathic pain presented 80% reduction of the area of allodynia after the use of 8% capsaicin patch. This effect was observed up to the 18th month after application [112]. Oral treatment with capsaicin candy temporarily relieves pain caused by oral mucositis, a common side effect in cancer patients in chemotherapy or radiotherapy treatment [121].

The repeated topical application of capsaicin can cause intense burning sensation at both low and high doses. However, the pretreatment with local anesthetic avoids the initial discomfort caused by the use of single high dose of capsaicin [110,122]. The association of local anesthetic lidocaine-derived QX-314 with capsaicin applied in a sensory nerve produces long-lasting analgesia in the orofacial area and inhibits the jaw opening reflex induced by stimulation of the tooth pulp in rats [123]. The perisciatic application of lidocaine (2%) or QX-314 (0.2%) associated with capsaicin (0.05%) in rats after plantar incisional surgery decreases the mechanical hypersensitivity 72 hours after incision and delays the onset of mechanical hypersensitivity by the destruction of TRPV1-expressing afferents. Nevertheless, the delay in the onset of mechanical hypersensitivity was also observed in naïve animals as well as signs of neurotoxicity [124]. The topical association of 3.3% tricyclic antidepressant doxepin and 0.025% capsaicin is able to accelerate the development of analgesia in patients with neuropathic pain compared with the separate use of formulations [125].

4. Pre-Clinical and Clinical Uses, and Pharmacological Actions of Capsaicin in Conditions Other Than Pain

4.1. Capsaicin in Weight Reduction and Obesity

Obesity is an escalating public health challenge globally and a major risk factor for various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus and cancer [126,127]. Thus, there is urgent need for new therapeutic strategies to treat obesity. In the past decades, numerous studies have shown capsaicin is effective in promoting weight loss and amelioration of obesity [128–130]. Herein, we will discuss some of the most relevant mechanisms involved in capsaicin's anti-obesity effects.

Obesity is the result of an energy imbalance that develops when energy intake exceeds energy expenditure. Capsaicin can limit energy intake while it contains only negligible amounts of energy itself [131–133]. Thus, great focus has been turned to studying the effect of capsaicin on energy balance. In humans, the addition of capsaicin to the diet enhances anorexigenic sensations, such as satiety and fullness [132,134]. Moreover, capsaicin decreases *ad libitum* food intake and suppress orexigenic sensations, i.e. the desire to eat and hunger, in negative and positive energy balance [131,132,135]. Although the exact mechanism of action of capsaicin is not yet fully understood, several plausible mechanisms have been proposed to explain these effects. An early study in rats demonstrated that adding capsaicin in the diet caused an increase in catecholamine secretion in the adrenal medulla via the activation of the central nervous system (CNS) [136,137]. There is an interaction between sympathetic nervous system (SNS) activity and food intake behavior since food intake decreases when SNS activity increases [138]. Therefore, increased SNS activity by capsaicin ingestion suggests that the reduction in energy intake could be due to the anorexigenic effect of catecholamines [133]. Moreover, the consumption of capsaicin increases the concentration of anorexigenic hormone glucagon-like

peptide 1 and decreases the concentration of orexigenic hormone ghrelin in humans [139]. Accordingly, oral treatment with capsaicin can regulate high fat diet (HFD)-induced alterations in the expression of several anorectic and orexigenic genes and neuropeptides in the hypothalamus and prevent weight gain in mice [140].

Numerous studies have highlighted the role of thermogenesis and increase in energy expenditure (EE) in body weight regulation by capsaicin [130,131,140–143]. Among potential molecular mechanisms involved in this regulatory effect of capsaicin, activation of TRPV1 appears to be critical as EE is greatly attenuated in mice deficient in TRPV1 and in human individuals having a mutated (Val585Ile) TRPV1 [142]. Increased thermogenesis and EE via capsaicin-induced TRPV1 activation is resultant of catecholamine release and subsequent SNS activation of β-adrenoceptors [143,144]. This mechanism is corroborated by studies showing that the administration of β-adrenergic blockers such as propranolol attenuates thermogenesis [144]. The activation of brown adipose tissue (BAT), which is the major site of sympathetically activated non-shivering thermogenesis, via the TRPV1/β-adrenergic axis, has been shown to be central to the thermogenic effect of capsaicin [142,145]. Nevertheless, other effects such as increased fat mobilization (triglyceride oxidation) in white adipose tissue (WAT) and improved energy metabolism in skeletal muscle mediated by TRPV1 activation also seem to be important in increased EE by capsaicin [142,146].

The amount of adipose tissue is tightly regulated and dependent on the differentiation of preadipocytes to adipocytes, a process known as adipogenesis. The modulatory effect of capsaicin on this process has been implicated in the reduction adipose tissue [147,148]. Previous studies have shown that capsaicin reduces the expression of adipocyte differentiation-related proteins PPARγ, C/EBPα, and leptin in a concentration-dependent manner, and the differentiation of 3T3-L1 preadipocytes into adipocytes [149–151]. Similarly, capsaicin also inhibits the differentiation of bone marrow mesenchymal stem cells (BMSCs) into adipocytes [152]. Thus, capsaicin-mediated modulation of adipogenesis is not limited to preadipocytes. The inhibitory effect of capsaicin on this process seems to involve the activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) in conjunction with intracellular ROS release [150]. Activated AMPK blocks anabolic pathways and promotes catabolic pathway. Thus, AMPK activation is also linked to inhibition of cell proliferation and apoptosis [153,154]. In support of this concept, capsaicin targets preadipocyte proliferation by blocking the S-phase of the cell cycle [149]. Capsaicin also reduces the number of BMSCs in S phase and induces cell cycle arrest at G0-G1 [152]. Interestingly, capsaicin induces apoptosis in preadipocytes via the activation of caspase-3, Bax, and Bak, cleavage of PARP, and down-regulation of Bcl-2 [151]. Furthermore, capsaicin induces apoptosis in BMSC via increased production of ROS and reactive nitrogen species (RNS) [152]. Thus, the reduction in preadipocyte/adipocyte population and adipose tissue by capsaicin can also be attributed to the inhibition of proliferation and apoptosis.

In addition to the previously discussed mechanisms of capsaicin's anti-obesity effect, the capsaicin alteration in gut microbial population also seems to be important in preventing HFD-induced weight gain. Oral administration of capsaicin regulated HFD-induced alterations in the abundance of certain bacterial groups in the cecum of Swiss mice, e.g. *Bacterioidetes*, *Firmicutes*, *A. muciniphila*, and *Enterobacteriaceae* [145]. Gut microflora is important in the regulation of host metabolism and energy harvest and may contribute to the development of obesity [155]. In fact, dysbiosis in gut microflora is commonly observed in obese humans and animals [156–158]. Therefore, the beneficial alteration in gut microbial population may also be beneficial in HFD-induced obesity.

It is noteworthy that, despite abundant evidence supporting the beneficial role of capsaicin in weight management, some studies have reported no or minimal effects of capsaicin on weight loss in humans [159,160]. Other studies have suggested that the magnitude of capsaicin´s effects on weight loss in humans is actually quite small [131,160]. For instance, 10 kcal negative energy balance, which is the predicted for hedonically acceptable capsaicin doses, in an average weight, middle-aged man would produce an ultimate weight loss of 0.5 kg over 6.5 years [131]. This is important considering that the long-term sustainability is uncertain due to factors such as desensitization upon long-term intake, side effects, and pungency of capsaicin [131,160]. Nevertheless, on a population scale, modest sustained

weight loss can be predicted to generate substantial health and economic benefits [161]. Furthermore, it is likely that the analgesic therapy using capsaicin would not reduce the life quality of patients as observed with tricyclic antidepressants, which increase weight gain [162]. Indirectly, the reduction of weight gain will diminish co-morbidities such as knee pain.

4.2. Capsaicin in Glucose Homeostasis and Diabetes

In addition to the effects of capsaicin on body metabolism [130,146], this pungent compound may also have beneficial effects on glucose and insulin homeostasis and diabetes. Dietary and supplementation with capsaicin display an impact on glucose and insulin levels in humans [163–165]. Regular consumption of capsaicin-containing chili attenuates postprandial hyperinsulinemia in healthy adults [163] and supplementation with it improves postprandial hyperglycemia and hyperinsulinemia in women with gestational diabetes mellitus (DM) [165]. Further, a crossover study performed on healthy male volunteers revealed that capsaicin lowers glucose and increases insulin levels shortly after oral administration in an oral glucose tolerance test [164]. Importantly, this study not only determined that capsaicin could be detected in the blood as early as 10 minutes after ingestion and levels maintained for up to 90 minutes, but also that capsaicin levels correlates with the lower glucose levels and maintenance of the insulin levels [164].

Animal studies have reported similar beneficial effects of capsaicin administration on glucose and insulin homeostasis [166–168]. Additionally, these studies have also shed light on the mechanisms that may be involved in these effects. For instance, capsaicin may inhibit glucose tolerance by inhibiting adipose tissue inflammatory responses in obesity [169,170]. *In vitro*, capsaicin suppresses IL-6 and MCP-1 gene expression and protein release from adipose tissue and adipocytes of obese mice [169]. Further, dietary capsaicin markedly reduces adipose tissue macrophages and levels of inflammatory adipocytokines (TNF-α, MCP-1, IL-6, and leptin) and normalizes fasting glucose levels in obese mice [170]. Obesity-related inflammatory proteins can block insulin signaling [171,172]; therefore, capsaicin may reduce glucose tolerance by suppressing their production in obese mice.

Similarly to many of the other actions described for capsaicin (reviewed herein), there is evidence that the modulation of blood glucose levels and insulin secretion by capsaicin is TRPV1-dependent. Capsaicin induces the secretion of insulin and antihyperglycemic hormone glucagon like peptide-1 in the ileum of WT but not TRPV1$^{-/-}$ mice [173]. Moreover, improved glucose tolerance, insulin levels, and blood glucose profiles by chronic dietary capsaicin are absent in TRPV1$^{-/-}$ mice [173]. In support of this concept, TRPV1 is functionally expressed in islet β-cells, neurons, rat pancreas, and rat β-cell lines RIN and INS1, and capsaicin can modulate insulin secretion by these cells via TRPV1 [167,174–176]. In rats, for instance, capsaicin dose-dependently increases insulin secretion and plasma insulin concentrations in TRPV1 expressing islet β-cells and this effect is inhibited by the TRPV1 inhibitor capsazepine [176].

Recent advances in research have revealed that TRPV1 receptors play a central role in the development and progression of type 1 and 2 diabetes [175,177]. In fact, the ablation TRPV1 expressing sensory nerves by capsaicin has been shown to modulate disease development and/or progression [174,175]. Sensory nerves innervating the pancreas are considered major players in the development of pancreatitis and islet inflammation and destruction [174]. Capsaicin-induced permanent elimination of TRPV1-expressing pancreatic sensory neurons reduces islet infiltration, insulin resistance, and β-cell stress in neonatal diabetes-prone non-obese diabetic (NOD) mice [174]. Therefore, capsaicin-induced depletion of TRPV1-expressing neurons prevents the development of diabetes in mice that are genetically predisposed to type 1 diabetes [174]. Similarly, in Zucker diabetic fatty (ZDF) rats, which are used to study various aspects of human type 2 diabetes, the selective elimination of TRPV1 expressing sensory fibers in the islets of Langerhans by capsaicin prevents plasma glucose levels increase and glucose tolerance, and enhances insulin secretion [175]. Interestingly, capsaicin also protects mice from the development of type 1 diabetes via TRPV1 by a mechanism related to gut-mediated immune tolerance. Oral administration of capsaicin attenuates the proliferation and activation of autoreactive T cells in pancreatic lymph nodes (PLNs), protecting mice from diabetes development [177]. The engagement of TRPV1 enhances a discreet population of CD11b^{+}/F4/80^{+} macrophages in PLNs, which

is essential for capsaicin-mediated attenuation of T-cell proliferation in an IL-10-dependent manner [177]. Therefore, capsaicin/TRPV1 signaling can limit glucose levels increase and diabetes development.

4.3. Capsaicin in Cardiovascular Conditions

There is evidence that capsaicin has potential beneficial effects on the cardiovascular system [178–180]. The cardiovascular system is rich in capsaicin-sensitive sensory nerves that play a major role in regulating cardiovascular function through the release of neurotransmitters such as CGRP and substance P [180,181]. CGRP is considered to be one of the most powerful vasodilators and plays an important role in regulating blood pressure under both physiological and pathophysiological conditions [182–184]. Capsaicin stimulates the release of CGRP through the activation of TRPV1 and therefore decreases blood pressure [180,185]. However, the protective effects of endogenous CGRP rely on the intact function of capsaicin-sensitive sensory nerves since high dose of capsaicin pretreatment, which selectively depletes transmitters in capsaicin-sensitive sensory nerves, could abolish the protective effects of CGRP or even enhance hypertension [186–188]. Although blood pressure regulation by capsaicin-stimulated CGRP release is more widely described, dietary capsaicin has also been shown to reduce blood pressure in hypertensive rats and delay the onset of stroke in stroke-prone spontaneously hypertensive rats (SHRsp) by increasing the phosphorylation of PKA and endothelial nitric oxide synthase (eNOS) via TRPV1 activation [189,190]. It is noteworthy to mention that CGRP antagonists, such as Olecegepant (BIBN4096BS), BI44370A, Telcagepant (MK-0970), and MK-3207 do not alter basal blood pressure despite the role of CGRP in regulating blood pressure [191].

In addition to the regulatory effects on blood pressure, other cardioprotective effects have also been described for capsaicin. Long-term activation of TRPV1 by capsaicin decreases lipid storage and atherosclerotic lesions in aortic sinus and thoracoabdominal aorta of mice [192]. Additionally, activation of TRPV1 by capsaicin impedes foam cell formation by inducing autophagy in oxidized low-density lipoprotein (oxLDL)-treated vascular smooth muscle cells and ultimately slows down the process of atherosclerosis [193]. Moreover, it is likely that the antioxidant property of capsaicin also contributes to their protective effects on cardiovascular system. The oxidation of LDL is an initiating factor for the development and progression of atherosclerosis [194]. *In vitro*, capsaicin increases the resistance of LDL to oxidation by delaying the initiation of oxidation and/or slowing the rate of oxidation [195]. In HFD rats, capsaicin treatment reduces lipid peroxide levels in the serum [196,197]. Moreover, it has been reported that regular consumption of chili for 4 weeks increases the resistance of serum lipoproteins to oxidation in adult men and women [198]. These reports further support the potential clinical value of capsaicin on the prevention of cardiovascular diseases, such as atherosclerosis and coronary heart disease.

Capsaicin has been shown to inhibit platelet aggregation [199,200], which may also provide protection against cardiovascular diseases [201]. Capsaicin's anti-aggregating effect on platelets is attributed to the alteration in the fluidity of platelet membrane [202,203]. The anti-aggregating effect of capsaicin on platelets seems to be TRPV1-independent since a selective competitive TRPV1 inhibitor A-993610 does not affect the ability of capsaicin to inhibit platelet aggregation [200]. However, there is conflicting data showing TRPV1-dependent pro-aggregating effect of capsaicin, via serotonin release, and adenosine diphosphate- and thrombin-induced platelet activation [204]. Therefore, further investigation is needed to verify the anti-haemostatic property of capsaicin and the mechanisms involved.

4.4. Capsaicin in Cancer

Despite several advances in therapies, cancer is still a major cause of morbidity and mortality worldwide [205]. In the past decades, the anticancer activity of capsaicin has been broadly investigated for a variety of cancer types. Capsaicin has been shown to possess chemopreentive and chemotherapeutic effects [206,207], and *in vivo* studies support the antitumorigenic activity of capsaicin [207,208]. In contrast, there is conflicting evidence that capsaicin may also act as carcinogenic or co-carcinogenic [209], thus capsaicin might play a role in either preventing or causing cancer.

The exact cellular mechanisms involved in capsaicin's anticancer effects are still not completely understood, however, numerous studies have attributed it to apoptosis, cell-cycle arrest, and anti-angiogenic effects [207,210,211]. Many types of cancer disrupt apoptotic pathways and/or enhance anti-apoptotic ones, and the loss of apoptotic signaling is highly associated with malignancy [212]. Capsaicin can induce apoptosis in over 40 different types of cancer cell lines [213,214]. Some of the mechanisms that have been described are activation of cAMP-activated protein kinase [215] in human osteosarcoma cells and PPARγ-induced apoptosis in HT-29 human colon, endoplasmic reticulum stress in human nasopharyngeal carcinoma and pancreatic cancer cells, down-regulation of STAT3 target genes Bcl2 and survivin in multiple myeloma cells, among others [213]. Interestingly, in many types of cancers, capsaicin exhibits pro-apoptotic activity, which seems to be related to TRPV1 or TRPV6 activation. The activation of these receptors by capsaicin induces calcium-mediated mitochondrial damage and subsequent cytochrome c release [216,217].

Cell cycle and growth arrest are important defense mechanisms against cancer and targets for cancer prevention and therapy [218], and capsaicin has been shown to modulate both. In human bladder cancer cell line 5637, capsaicin induces G0/G1 phase arrest by inhibiting cyclin-dependent kinases (CDK) 2, CDK4 and CDK6 [210]. Similarly, capsaicin reduces in a concentration-dependent manner cyclin D1 in colon cancer cell lines [213,219]. In breast cancer cells, on the other hand, capsaicin induces cell-cycle arrest by modulating the epithelial growth factor receptor/HER2 pathway and p27 expression in estrogen receptor-positive and -negative cells [220]. Taken together, these data show that capsaicin may halt growth and division of cancer cells by targeting cell cycle regulators. Nevertheless, it is important to mention that several other mechanisms of capsaicin-induced cell-cycle arrest have also been described for capsaicin [213].

Angiogenesis is an essential factor for the progression of most types of cancer. It has been demonstrated that capsaicin has anti-angiogenic properties both *in vitro* and *in vivo* by interfering with angiogenic signaling pathways [221]. Treatment of endothelial cells with capsaicin suppresses VEGF-induced proliferation, migration and tube formation in mice via down-regulation of p38 MAPK, protein kinase B (PKB or AKT) and focal adhesion kinase (FAK) activation [221]. Further, capsaicin increases the degradation of hypoxia inducible factor 1α in non-small cell lung cancer, which is a key transcription factor for VEGF transcription [222]. Collectively, these studies highlight the anticancer potential of capsaicin by regulating several mechanisms that are commonly altered in cancer cells and are important for tumor growth.

Despite the mounting evidence supporting a chemo-preventive role for capsaicin in cancer cell culture and animal models, a consensus about whether capsaicin prevents or promotes cancer has not yet been reached [223]. Several animal studies have shown that capsaicin is potentially carcinogenic. For instance, approximately 60% of rats fed a semisynthetic diet containing 10% chilies develop neoplastic changes in the liver [224]. Also, mice fed 0.03% capsaicin in a semisynthetic diet over their lifetime develop benign polypoid adenomas of the cecum [225]. Moreover, studies report that capsaicin may also have co-carcinogenic potential. Topical application of capsaicin on the dorsal skin of mice with 9, 10-dimethylbenz(a)anthracene (DMBA)/12-Otetradecanoylphorbol-13-acetate (a known skin tumor inducer) significantly accelerated tumor formation and growth and induced more and larger skin tumors. Mechanistic study revealed that pre-treatment with capsaicin elevated cyclooxygenase-2 and iNOS and up-regulated the phosphorylation of nuclear factor-kappa B (NF-κB), ERK, and p38, indicating that inflammation, ERK and p38 collectively play a crucial role in cancer-promoting effect of capsaicin in carcinogen-induced skin cancer in mice [226]. Chili extract and hot chili pepper containing

capsaicin promoted the development of stomach tumors initiated by methyl-acetoxy methylnitrosamine in mice and increased the incidence of *N*-methyl-*N*-nitrosoguanidine–induced gastric cancer in rats, respectively [227,228]. Furthermore, capsaicin (125 mg/kg)-induced systemic denervation of sensory neurons results in significant increase of lung and cardiac metastases in adult mice injected orthotopically with syngeneic 4T1 mammary carcinoma cells [229]. In line with these findings, many epidemiologic studies indicate that consumption of hot peppers, containing capsaicin, might be associated with an increased risk of cancer, especially gallbladder or gastric cancer [230,231]. However, many of these epidemiologic studies present considerable limitations.

4.5. Capsaicin in Airway Diseases

Nociceptors play important role in airway diseases such as allergic rhinitis and asthma [232,233], which are accompanied by intense inflammatory infiltrate [232–234]. Nociceptors also play an active role in the regulation of immune response since they can recognize and respond to danger and environment stimuli [235,236]. Therefore, the inhibition of their activity in airway diseases may be beneficial to the host [232,233]. In fact, injection of capsaicin in mice exposed to ovalbumin exacerbates airway inflammation by increasing the number of leukocytes in the broncho alveolar lavage fluid (BALF) [232]. Further corroborating this concept, the ablation of the nociceptor by using the Nav1.8-Cre/DTA mice strain [232] or using interference RNA for TRPV1 [233] reduce these same parameters in allergic rhinitis and asthma models, suggesting an endogenous role for TRPV1. In this sense, QX-314 silences nociceptors, which leads to the reduction of the number of infiltrating leukocytes in the BALF, IL-5 production, and improvement of airway inflammation [232]. IL-5 is one of the main cytokines in asthma. In a cascade of events, IL-5 activates in a calcium-dependent manner capsaicin-responsive nodose ganglia and Nav1.8-positive nociceptors, which in turn release vasoactive intestinal polypeptide (VIP). VIP activates innate lymphoid cells 2 (ILC2) and culminates in airway inflammatory exacerbation [232].

Non-allergic rhinitis (NAR) or idiopathic rhinitis (IR) may be described as chronic nasal symptoms, such as obstruction and rhinorrhea that occur in relation to non-allergic, non-infectious triggers such as change in the weather, exposure to caustic odors or cigarette smoke, and barometric pressure differences [237]. Intranasal application of capsaicin has beneficial effects in this type of rhinitis, although this application is initially irritating to the applied area, it can eventually desensitize the sensory neural fibers and reduce nasal hyper-responsiveness [238]. The desensitization of sensory nerves with capsaicin has been shown to provide symptom relief for up to 9 months. Patients treated with intranasal capsaicin reported significantly reduced visual analog scale scores for overall nasal symptoms, rhinorrhea, and nasal blockage [239]. In agreement with previous reports, in a placebo-controlled study with of 24 patients with non-allergic non-infectious perennial rhinitis, the group treated with 0.15 mg capsaicin spray solution over 2 weeks showed significant and long-term reduction in the visual analogue scale scores. However, no significant difference was observed in the concentrations of leukotriene C4, D4 or E4, prostaglandin D2, and tryptase when compared to placebo group [240]. On the other hand, the same dose and treatment protocol used in the previous work showed no significant therapeutic effect in patients with perennial allergic rhinitis due to house dust mite [241], suggesting that the application of capsaicin would be benefit only in non-allergic-related rhinitis. A recent study has shown that NAR/IR is associated with an increased expression of TRPV1 in the nasal mucosa and substance P levels in nasal secretions. Mechanistic studies revealed that capsaicin exerts its therapeutic action by ablating TRPV1-substance P nociceptive signaling pathway in the nasal mucosa [242].

The role of capsaicin as a therapeutic agent was not addressed in the work of Talbot et al. [232], therefore, the question whether prolonged administration of capsaicin could produce similar results to those in NAR still remains. In spite of that, this study has shed some light on the role of nociceptors in airway diseases, which highlight these cells as key players in the physiopathology of several diseases. Additionally, this study highlights QX-314 as a solid candidate for the treatment of diseases that TRPV1

plays a role since QX-314 requires an opener (endogenous or exogenous activator of TRPV1) to access nociceptors and inhibit them [47,123,232].

4.6. Capsaicin in Itch

Itch (pruritus) elicits scratching response, whereas pain causes withdrawal responses. Both itch and pain are detected by primary sensory neurons in DRG and trigeminal ganglion, and therefore, share transduction machinery involving TRPV1, TRPA1, and Toll-like receptors (TLRs) [243]. Despite these similarities, whole population analysis of nociceptors reveals the presence of three distinct populations, which are further divided into seven subgroups. These subgroups are differentiated by the expression of neuronal receptors or ion channels [244]. For instance, DRG neurons of the group VI co-express B-type natriuretic polypeptide b (Nppb) receptor and IL-31ra, which implies these DRG neurons as mediators of itch sensation [244]. This also reveals the highly complex machinery of peripheral nociceptors and uncovers novel receptors as targets for pain or itch relief. In fact, nociceptors play an important role in pruritic diseases [245,246] since silencing nociceptors with QX-314 reduces non-histaminergic and histaminergic itch [245]. Both non-histaminergic and histaminergic itch activate TRPV1 and TRPA1 channels and allow QX-314 entry in DRG neurons [245]. In addition, ablation of nociceptors reduces skin inflammation and psoriatic plaque formation [246]. These set of data highlight specific subsets of nociceptors as important players in itch.

Supporting the role of TRPV1-expressing neurons in itch, treatment with dermal patch of 0.025% of capsaicin reduces itch in psoriatic patients [247,248], although in one of these studies, 18 of 44 patients refer burning, stinging, itching, and redness of the skin [248]. In two other studies, treatment with 8% capsaicin patch reduces itch intensity and frequency in three patients with nostalgia paresthetica [249], and in 7 patients with neuropathic pruritus [250]. Also, in these studies, the majority of the patients referred erythema and moderate pain, pointing out to an important common side effect due to dermal capsaicin treatment. Of note, capsaicin 0.1% reduces allyl isothiocyanate (AITC)-evoked scratching in mice [245]. Regardless of the above mentioned efficacy capsaicin in itch, robust data and further clinical trials are needed to confirm the beneficial properties of capsaicin. In addition, the side effects mentioned can be a drawback to the use of capsaicin in itch.

4.7. Capsaicin in Gastric Disorders

Sensory neurons are responsible for maintenance of gastric integrity [251]. Therefore, the gastroprotective effects of capsaicin lie in the modulation of the sensory neurons, since chemical ablation of these neurons mitigates capsaicin protective effects [252–254]. Daily treatment with 400 μg of capsaicin, three times a day, reduces ethanol- and indomethacin-induced gastric mucosal damage in healthy human subjects [255]. In terms of animal models, treatment with capsaicin also reduces indomethacin-induced microbleeding [255]. Corroborating, intragastric administration of capsaicin in rats and dogs attenuates aspirin-, indomethacin- and ethanol-induced gastric damage [251], and enhances gastric protection by stimulating capsaicin-sensitive sensory neurons. This effect was demonstrated using ^{51}Cr-EDTA clearance technique, which evaluates epithelial integrity by mucosal blood-to-lumen permeability [254]. The gastroprotective mechanism of capsaicin is due to the activation of TRPV1 at gastric sensory neurons which stimulates the release of CGRP and NO [251,255] since co-treatment of capsaicin and L-nitro-arginine methyl ester (L-NAME, a NOS inhibitor) reduces capsaicin effectiveness in mice [256].

Helicobacter pylori (H. pylori) is one of the main causative agents of gastric ulcer, and its presence correlates with use of NSAIDs [257]. Capsaicin reduces *H. pylori*-induced gastric ulcer by reducing IL-8 production. In addition, capsaicin also reduces *H. pylori*-induced NF-κB activity evaluated by luciferase activity for p65 subunit and nuclear translocation by confocal immunofluorescence in gastric epithelial cells [258]. Moreover, it is noteworthy that capsaicin per se possess bactericidal activity and inhibits *H. pylori* growth *in vitro* which may contribute to its protective effect [259]. Thus, the medical premise that consumption of chili peppers may be prejudicial to the host is not entirely true. In fact, epidemiologic studies with 103 patients with peptic ulcer in China [260], and 190 in India [261] suggest that

consumption of chili peppers is inversely proportional to the incidence of peptic ulcer pointing out to the gastroprotective effects of capsaicin.

4.8. Capsaicin in Urological Disorders

Capsaicin has been studied as an alternative therapy for the relief of the symptoms of neurogenic bladder, a urological disorder that seriously affects the quality of life of patients [262]. Neurogenic bladder is often present in patients with multiple sclerosis, spinal cord injury, and other neurological pathologies. Neurogenic detrusor overactive (NDO) and detrusor hyperreflexia are dysfunctions that characterize neurogenic bladder and lead to urgency and increase in urinary frequency, and in many cases, incontinence [262,263]. Overactive bladder is a clinical condition that resembles neurogenic bladder [264], however, its etiology is not associated with neurological or urogenital diseases [265,266].

The first possibility of clinical use of capsaicin in the treatment of urinary tract disorders was demonstrated using intravesical injection of a 100 mL of 1 mM (30 mg) solution of capsaicin (dissolved in alcohol and saline) in patients with multiple sclerosis that presented bladder detrusor hyperreflexia. The same dose has been used successfully in several studies of patients with neurogenic detrusor over activity after spinal cord injury or neurogenic bladder [267–270]. The use of alcohol as a solvent can cause irritation and become a limiting factor in the use of capsaicin, causing pelvic pain in more than 50% of patients as reviewed before [271]. The efficacy of an alternative dilution of capsaicin in a glucidic solution to treat patients with neurogenic detrusor over activity was also demonstrated. However, this dilution has not been able to avoid pain reported by treatment with capsaicin [272].

Capsaicin also seems to have a protective effect against bladder disorders. An animal study demonstrated that the pretreatment with capsaicin (125 mg/kg, s.c.) was able to prevent spinal cord injury-induced hyperreflexia of the detrusor in rats. A boost treatment 4-5 days after spinal injury maintained the effect of capsaicin [273].

The effect of capsaicin or RTX is related to the action on TRPV1 receptors in the urinary tract, not only in sensory fibers that innervate these structures, but also in urothelial cells [274,275]. *In vitro* studies with bladder urothelial cells from non-neurogenic overactive bladder patients showed that expression and activation of TRPV1, as well as capsaicin-sensitivity are increased in comparison with healthy volunteers [276,277]. Capsaicin targeting of TRPV1 receptors in the C-fibers leads to the activation followed by desensitization, being responsible for the beneficial effect of capsaicin on the bladder activity, but also by the initial pain sensation due to their use [262].

The use of both capsaicin and RTX is still not a routine clinical practice and can become an alternative treatment for patients who do not respond to conventional therapy with oral antimuscarinics, especially those with neurogenic bladder. However, both molecules present the disadvantage of repeated intravesical applications and the initial discomfort that may discourage the patient adherence to treatment [262].

5. Clinically Available Capsaicin Pharmaceutical Formulations

Among the therapeutic uses of capsaicin in the clinic, the most common is for the management of pain. Low-concentration creams, lotions, and patches containing capsaicin (0.025-0.1% wt/wt) intended for daily topical application have been available in most countries since the early 1980s. These topical formulations are usually self-administered medications and often without the requirement of a prescription [278]. Clinical studies have revealed that three to five topical skin applications per day for periods of two to six weeks have modest beneficial effects against various pain syndromes, including post-herpetic neuralgia, diabetic neuropathy, and chronic musculoskeletal pain [279,280]. Another topical capsaicin formulation available is the high concentration patch containing 8% capsaicin, which is widely used to treat post-herpetic neuralgia, HIV neuropathy, and other conditions with neuropathic pain symptoms [281,282]. The capsaicin 8% patch rapidly delivers capsaicin into the skin while minimizing unwanted systemic effects, and it is already approved for treatment of neuropathic pain in Europe and USA (only post-herpetic neuralgia) [116]. Robust clinical data demonstrate the efficacy of 8% patch in the treatment of neuropathic pain [116,283,284]. Of note, in a study with patients with

neuropathic pain in Scotland [283], and another involving 629 patients of 22 countries and regions [284], suggest that the 8% patch presents similar efficacy to pregabalin, no differences in time to response between treatments, and therefore, represents a promising alternative for the treatment of neuropathic pain [283,284]. The administration of this formulation requires a single application for 30 or 60 minutes under the supervision of a health-care professional, which reduces potential variability in administration and a lack of patient compliance, in addition to avoiding environmental exposure of patients to capsaicin [278,281,282].

Pharmaceutical formulations for per oral administration of capsaicin are available in the form of capsules containing chili peppers [140]. The therapeutic dose for per oral administration of capsaicin has not been established, however, the generally recommended daily dose stated on labels of commercially available capsules is 1350-4000 mg of capsicum with 0.25% capsaicin. This range of dose has been shown to increase energy expenditure, fat oxidation, thermogenesis, and decrease appetite in humans [130], although both lower (0.4-2 mg) and higher (135-150 mg) doses are also effective in promoting these effects [135,160,285]. Other pharmaceutical formulations containing capsaicin are capsicum nasal sprays and homeopathic preparation of *Capsicum annum* and Eucalyptol nasal sprays. These formulations have been used to treat nonallergic rhinitis and the symptoms associated with this condition [286,287]. Although a therapeutic dose has not been established yet, a previous study has shown that 4 μg/puff of capsicum, three times a day for three consecutive days, is efficacious for non-allergic, non-infectious perennial rhinitis [287].

6. Conclusions and Future Perspectives

Capsaicin and food-containing capsaicin have been together with humans over thousands of years, but only more recently that our understanding of how capsaicin affects our organism has significantly advanced. Capsaicin has been essential to our understanding of physiological and pathological processes as well as the relevance of TRPV1 channels. Figure 5 summarizes the pharmacological actions of capsaicin reviewed herein. Capsaicin importance is corroborated by the varied pharmaceutical formulations available and clinical applications, such as the capsaicin 8% patch to treat neuropathic pain. Despite being an old molecule, capsaicin is still a hot topic in scientific community and presents a wide horizon of potential therapeutic uses. Therefore, new pharmaceutical formulations, development of new analogs, or targeting the capsaicin-activated receptor TRPV1 are promising pharmacological approaches in the following years.

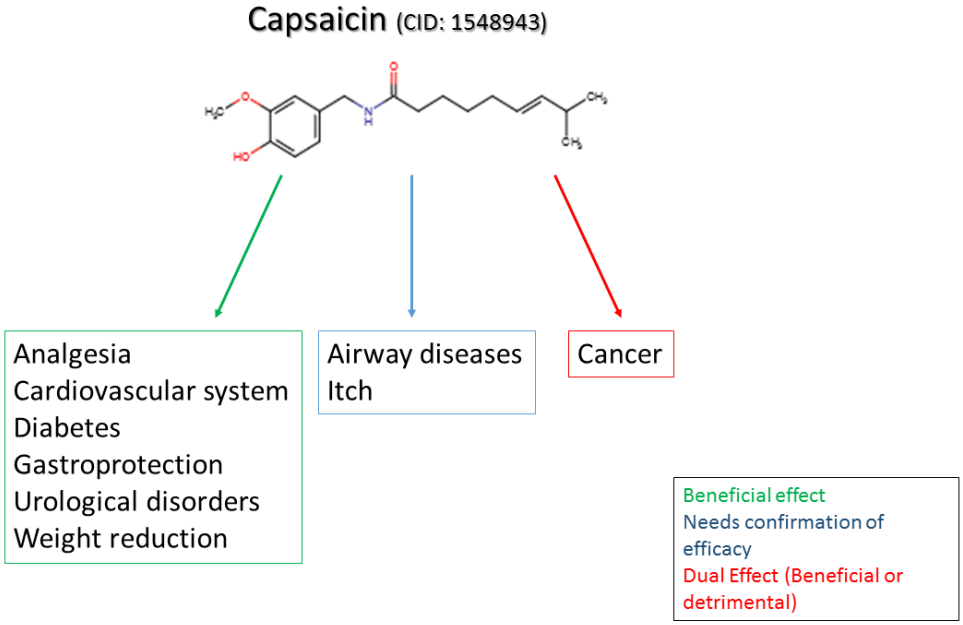

Figure 5. Summary of the current knowledge on capsaicin activities-related to diseases. Green arrow indicates the diseases in which capsaicin presents beneficial effects, and therefore, could be useful as a treatment. Blue arrow indicates diseases in which the effect of capsaicin is still controversial and the therapeutic effect of capsaicin and TRPV1 agonists and antagonists need further investigation. Red arrow indicates that capsaicin might play a role in either preventing or causing cancer.

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4 CONSIDERAÇÕES FINAIS

Em conclusão, demonstramos que a MaR1 apresenta efeito analgésico duradouro (5 após um único tratamento) no modelo de dor induzida por CFA que está relacionado a capacidade desse SPM em bloquear a ativação de nociceptores TRPV1-positivos e a liberação do neuropeptídeo CGRP. Ademais, padronizamos um modelo não-cirúrgico de dor associada à endometriose que é sensível a fármacos clinicamente usados, como letrozol e danazol. Em termos de novas moléculas, o tratamento com MaR1 reduzi não apenas a dor abdominal, mas também a porcentagem de camundongos com lesões e o tamanho das lesões. Parte desse efeito está relacionado com a habilidade da MaR1 em reduzir a ativação de nociceptores CGRP-positivos. Esses dados indicam que a MaR1 é uma molécula promissora para o tratamento de doenças inflamatórias, como a endometriose. A figura 3 resume os efeitos do SPM MaR1 nos modelos propostos nessa tese.

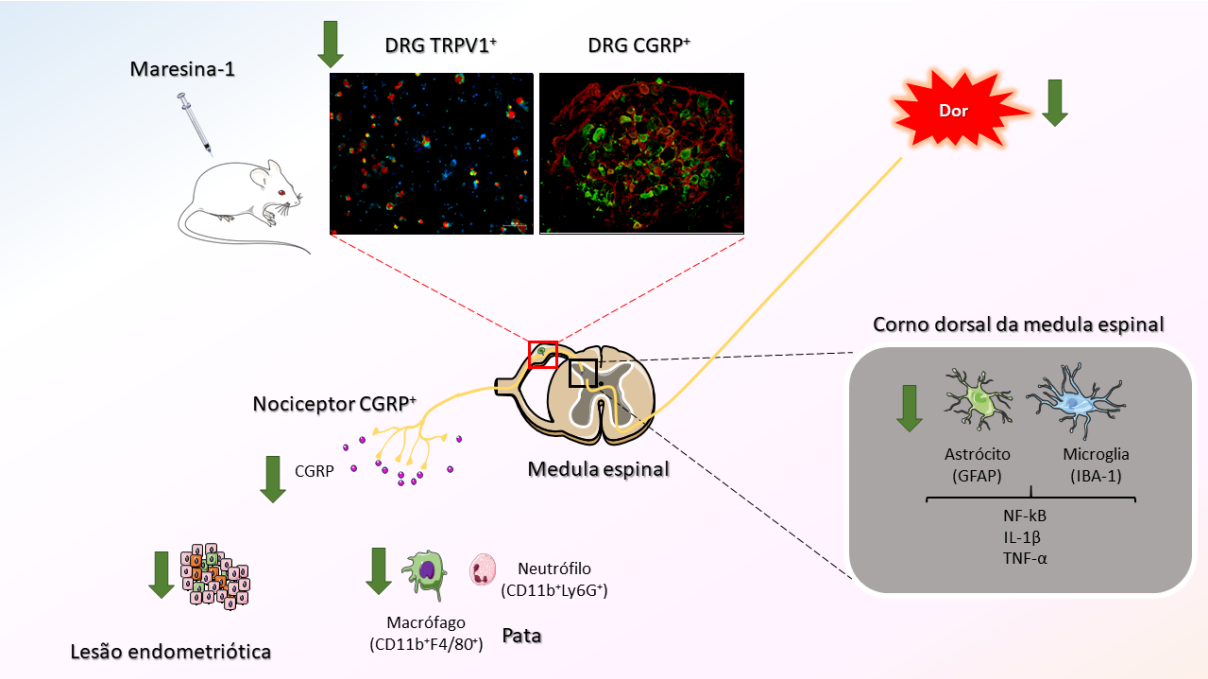

Figura 3. Mecanismo de ação analgésico e anti-inflamatório proposto para MaR1. No modelo de dor induzida por CFA, enquanto que na medula espinal esse SPM reduz a sensibilização central como observada pela redução da ativação de astrócitos e micróglia; na periferia, é possível observar bloqueio na liberação do neuropeptídeo CGRP e redução de leucócitos próximos às fibras CGRP-positivas. No modelo da endometriose, além da dor abdominal, a MaR1 reduz a porcentagem de camundongos com lesões e o tamanho das lesões endometrióticas. Fonte: próprio autor.

Durante o período de doutoramento, foram publicados um total de 45 artigos científicos e 1 capítulo de livro (Apêndice A). Os artigos científicos foram subdivididos em “Publicações Como Primeiro Autor” e “Publicações Como Co-Author” de acordo com o ano. É importante ressaltar que houve a publicação de pelo menos um artigo por ano como primeiro autor durante o período de doutoramento. Dentre os artigos adicionados no “Apêndice A”, vale destacar a publicação de artigos como primeiro autor em periódicos de alto impacto como *PNAS*, *Pharmacological Research* e *Expert Opinion in Therapeutic Targets*.

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APÊNDICE A

APÊNDICE A – Publicações de artigos científicos durante o período de doutorado que não fazem parte da tese (2016-2019)

Durante o período de doutoramento (2016-2019), foram publicados um total de 45 artigos científicos e 1 capítulo de livro. Os artigos científicos foram subdivididos em "Publicações Como Primeiro Autor" e "Publicações Como Co-Author" de acordo com o ano. Dentre os artigos adicionados nessa seção, vale destacar a publicação de artigos como primeiro autor em periódicos de alto impacto como *PNAS, Pharmacological Research* e *Expert Opinion in Therapeutic Targets*.

A.1 Publicações de Artigos Científicos Como Primeiro Autor

A.1.1 Ano de 2016

A.1.1.1 Neutrophils and arthritis: role in disease and pharmacological perspectives. ***Fattori V****, Amaral FA, Verri WA Jr. Pharmacol Res. 2016 Oct;112:84-98. doi: 10.1016/j.phrs.2016.01.027. Epub 2016 Jan 27. Review.*

A.1.2 Ano de 2017

A.1.2.1 Differential regulation of oxidative stress and cytokine production by endothelin ETA and ETB receptors in superoxide anion-induced inflammation and pain in mice. ***Fattori V****, Serafim KG, Zarpelon AC, Borghi SM, Pinho-Ribeiro FA, Alves-Filho JC, Cunha TM, Cunha FQ, Casagrande R, Verri WA Jr.* ***J Drug Target****. 2017 Mar;25(3):264-274. doi: 10.1080/1061186X.2016.1245308. Epub 2016 Oct 24.*

A.1.2.2 Vinpocetine reduces diclofenac-induced acute kidney injury through inhibition of oxidative stress, apoptosis, cytokine production, and NF-κB activation in mice. ***Fattori V****, Borghi SM, Guazelli CFS, Giroldo AC, Crespigio J, Bussmann AJC, Coelho-Silva L, Ludwig NG, Mazzuco TL, Casagrande R, Verri WA Jr.* ***Pharmacol Res****. 2017 Jun;120:10-22. doi: 10.1016/j.phrs.2016.12.039. Epub 2017 Mar 14.*

A.1.2.3 Targeting IL-33/ST2 signaling: regulation of immune function and analgesia.

***Fattori V**, Hohmann MSN, Rossaneis AC, Manchope MF, Alves-Filho JC, Cunha TM, Cunha FQ, Verri WA Jr. **Expert Opin Ther Targets**. 2017 Dec;21(12):1141-1152. doi: 10.1080/14728222.2017.1398734. Epub 2017 Nov 5. Review.*

*A.1.2.4 IL-33/ST2 signaling boosts inflammation and pain. **Fattori V**, Borghi SM, Verri WA Jr. **Proc Natl Acad Sci U S A**. 2017 Nov 21;114(47):E10034-E10035. doi: 10.1073/pnas.1716120114. Epub 2017 Nov 6*

A.1.3 Ano de 2018

A.1.3.1 Budlein A, a sesquiterpene lactone from Viguiera robusta*, alleviates pain and inflammation in a model of acute gout arthritis in mice. **Fattori V**, Zarpelon AC, Staurengo-Ferrari L, Borghi SM, Zaninelli TH, Da Costa FB, Alves-Filho JC, Cunha TM, Cunha FQ, Casagrande R, Arakawa NS, Verri WA Jr. **Front Pharmacol**. 2018 Sep 25;9:1076. doi: 10.3389/fphar.2018.01076. eCollection 2018.*

A.2 PUBLICAÇÕES DE ARTIGOS CIENTÍFICOS COMO CO-AUTOR

A.2.1 Ano de 2016

*A.2.1.1 Light-emitting diodes at 940nm attenuate colitis-induced inflammatory process in mice. Belém MO, de Andrade GMM, Carlos TM, Guazelli CFS, **Fattori V**, Toginho Filho DO, Dias IFL, Verri WA Jr, Araújo EJA. **J Photochem Photobiol B**. 2016 Sep;162:367-373. doi: 10.1016/j.jphotobiol.2016.07.008. Epub 2016 Jul 9.*

*A.2.1.2 Quercetin inhibits peripheral and spinal cord nociceptive mechanisms to reduce intense acute swimming-induced muscle pain in mice. Borghi SM, Pinho-Ribeiro FA, **Fattori V**, Bussmann AJ, Vignoli JA, Camilios-Neto D, Casagrande R, Verri WA Jr. **PLoS One**. 2016 Sep 1;11(9):e0162267. doi: 10.1371/journal.pone.0162267. eCollection 2016.*

*A.2.1.3 Naringenin reduces inflammatory pain in mice. Pinho-Ribeiro FA, Zarpelon AC, **Fattori V**, Manchope MF, Mizokami SS, Casagrande R, Verri WA Jr. **Neuropharmacology**. 2016 Jun;105:508-519. doi:*

10.1016/j.neuropharm.2016.02.019. Epub 2016 Feb 18.

*A.2.1.4 Pyrrolidine dithiocarbamate inhibits superoxide anion-induced pain and inflammation in the paw skin and spinal cord by targeting NF-κB and oxidative stress. Pinho-Ribeiro FA, **Fattori V**, Zarpelon AC, Borghi SM, Staurengo-Ferrari L, Carvalho TT, Alves-Filho JC, Cunha FQ, Cunha TM, Casagrande R, Verri WA Jr. **Inflammopharmacology**. 2016 Jun;24(2-3):97-107. doi: 10.1007/s10787-016-0266-3. Epub 2016 May 9.*

A.2.2 Ano de 2017

*A.2.2.1 Leishmania (L). amazonensis induces hyperalgesia in balb/c mice: contribution of endogenous spinal cord TNFα and NFκB activation. Borghi SM, **Fattori V**, Ruiz-Miyazawa KW, Miranda-Sapla MM, Casagrande R, Pinge-Filho P, Pavanelli WR, Verri WA Jr. **Chem Biol Interact**. 2017 Apr 25;268:1-12. doi: 10.1016/j.cbi.2017.02.009. Epub 2017 Feb 17.*

*A.2.2.2 Tempol, a superoxide dismutase mimetic agent, inhibits superoxide anion-induced inflammatory pain in mice. Bernardy CCF, Zarpelon AC, Pinho-Ribeiro FA, Calixto-Campos C, Carvalho TT, **Fattori V**, Borghi SM, Casagrande R, Verri WA Jr. **Biomed Res Int**. 2017;2017:9584819. doi: 10.1155/2017/9584819. Epub 2017 May 14.*

*A.2.2.3 Diosmin reduces chronic constriction injury-induced neuropathic pain in mice. Bertozzi MM, Rossaneis AC, **Fattori V**, Longhi-Balbinot DT, Freitas A, Cunha FQ, Alves-Filho JC, Cunha TM, Casagrande R, Verri WA Jr. **Chem Biol Interact**. 2017 Aug 1;273:180-189. doi: 10.1016/j.cbi.2017.06.014. Epub 2017 Jun 15.*

*A.2.2.4 Leishmania infection: painful or painless? Borghi SM, **Fattori V**, Conchon-Costa I, Pinge-Filho P, Pavanelli WR, Verri WA Jr. **Parasitol Res**. 2017 Feb;116(2):465-475. doi: 10.1007/s00436-016-5340-7. Epub 2016 Dec 9. Review.*

A.2.2.5 trans*-Chalcone, a flavonoid precursor, inhibits UV-induced skin inflammation and oxidative stress in mice by targeting NADPH oxidase and cytokine production.*

*Martinez RM, Pinho-Ribeiro FA, Steffen VS, Caviglione CV, **Fattori V**, Bussmann AJC, Bottura C, Fonseca MJV, Vignoli JA, Baracat MM, Georgetti SR, Verri WA, Casagrande R. **Photochem Photobiol Sci**. 2017 Jul 1;16(7):1162-1173. doi: 10.1039/c6pp00442c. Epub 2017 Jun 8.*

A.2.2.6 The nitroxyl donor angeli's salt ameliorates Staphylococcus aureus*-induced septic arthritis in mice. Staurengo-Ferrari L, Ruiz-Miyazawa KW, Pinho-Ribeiro FA, Domiciano TP, **Fattori V**, Mizokami SS, Pelayo JS, Bordignon J, Figueiredo F, Casagrande R, Miranda KM, Verri WA Jr. **Free Radic Biol Med**. 2017 Jul;108:487-499. doi: 10.1016/j.freeradbiomed.2017.04.016. Epub 2017 Apr 15.*

*A.2.2.7 The sesquiterpene lactone, budlein a, inhibits antigen-induced arthritis in mice: role of NF-κB and cytokines. Zarpelon AC, **Fattori V**, Souto FO, Pinto LG, Pinho-Ribeiro FA, Ruiz-Miyazawa KW, Turato WM, Cunha TM, Da Costa FB, Cunha FQ, Casagrande R, Arakawa NS, Verri WA Jr. **Inflammation**. 2017 Dec;40(6):2020-2032. doi: 10.1007/s10753-017-0642-1.*

A.2.3 Ano de 2018

*A.2.3.1 The flavonoid quercetin inhibits titanium dioxide (TiO2)-induced chronic arthritis in mice.Borghi SM, Mizokami SS, Pinho-Ribeiro FA, **Fattori V**, Crespigio J, Clemente-Napimoga JT, Napimoga MH, Pitol DL, Issa JPM, Fukada SY, Casagrande R, Verri WA Jr. **J Nutr Biochem**. 2018 Mar;53:81-95. doi: 10.1016/j.jnutbio.2017.10.010. Epub 2017 Nov 5.*

A.2.3.2 Interleukin-33 receptor (st2) deficiency improves the outcome of Staphylococcus aureus*-induced septic arthritis. Staurengo-Ferrari L, Trevelin SC, **Fattori V**, Nascimento DC, de Lima KA, Pelayo JS, Figueiredo F, Casagrande R, Fukada SY, Teixeira MM, Cunha TM, Liew FY, Oliveira RD, Louzada-Junior P, Cunha FQ, Alves-Filho JC, Verri WA. Front Immunol. 2018 May 16;9:962. doi: 10.3389/fimmu.2018.00962. eCollection 2018.*

A.2.3.3 Pyrrolidine dithiocarbamate inhibits mouse acute kidney injury induced by diclofenac by targeting oxidative damage, cytokines and NF-κB activity. Borghi SM,

***Fattori V**, Ruiz-Miyazawa KW, Bertozzi MM, Lourenco-Gonzalez Y, Tatakihara RI, Bussmann AJC, Mazzuco TL, Casagrande R, Verri WA Jr. **Life Sci.** 2018 Sep 1;208:221-231. doi: 10.1016/j.lfs.2018.07.038. Epub 2018.*

*A.2.3.4 Vinpocetine ameliorates acetic acid-induced colitis by inhibiting NF-κB activation in mice. Colombo BB, **Fattori V**, Guazelli CFS, Zaninelli TH, Carvalho TT, Ferraz CR, Bussmann AJC, Ruiz-Miyazawa KW, Baracat MM, Casagrande R, Verri WA Jr. **Inflammation**. 2018 Aug;41(4):1276-1289. doi: 10.1007/s10753-018-0776-9.*

*A.2.3.5 Mice lacking interleukin-18 gene display behavioral changes in animal models of psychiatric disorders: Possible involvement of immunological mechanisms. Lisboa SF, Issy AC, Biojone C, Montezuma K, **Fattori V**, Del-Bel EA, Guimarães FS, Cunha FQ, Verri WA, Joca SRL. **J Neuroimmunol**. 2018 Jan 15;314:58-66. doi: 10.1016/j.jneuroim.2017.11.008. Epub 2017 Nov 10.*

*A.2.3.6 Naringenin mitigates titanium dioxide (TiO2)-induced chronic arthritis in mice: role of oxidative stress, cytokines, and NFκB. Manchope MF, Artero NA, **Fattori V**, Mizokami SS, Pitol DL, Issa JPM, Fukada SY, Cunha TM, Alves-Filho JC, Cunha FQ, Casagrande R, Verri WA Jr. **Inflamm Res**. 2018 Dec;67(11-12):997-1012. doi: 10.1007/s00011-018-1195-y. Epub 2018 Oct 28.*

*A.2.3.7 Lipoxin A4 inhibits UV radiation-induced skin inflammation and oxidative stress in mice. Martinez RM, **Fattori V**, Saito P, Melo CBP, Borghi SM, Pinto IC, Bussmann AJC, Baracat MM, Georgetti SR, Verri WA Jr, Casagrande R. **J Dermatol Sci.** 2018 Apr 27. pii: S0923-1811(18)30201-9. doi: 10.1016/j.jdermsci.2018.04.014.*

*A.2.3.8 Bisphenol A exposure impairs epididymal development during the peripubertal period of rats: inflammatory profile and tissue changes. Ogo FM, de Lion Siervo GEM, Staurengo-Ferrari L, de Oliveira Mendes L, Luchetta NR, Vieira HR, **Fattori V**, Verri WA Jr, Scarano WR, Fernandes GSA. **Basic Clin Pharmacol Toxicol.** 2018 Feb;122(2):262-270. doi: 10.1111/bcpt.12894. Epub 2017 Sep 19.*

A.2.3.9 Hesperidin methylchalcone suppresses experimental gout arthritis in mice by inhibiting NF-κB activation. Ruiz-Miyazawa KW, Pinho-Ribeiro FA, Borghi SM,

*Staurengo-Ferrari L, **Fattori V**, Amaral FA, Teixeira MM, Alves-Filho JC, Cunha TM, Cunha FQ, Casagrande R, Verri WA Jr. **J Agric Food Chem**. 2018 Jun 27;66(25):6269-6280. doi: 10.1021/acs.jafc.8b00959. Epub 2018 Jun 13.*

*A.2.3.10 15d-PGJ2-loaded nanocapsules ameliorate experimental gout arthritis by reducing pain and inflammation in a PPAR-gamma-sensitive manner in mice. Ruiz-Miyazawa KW, Staurengo-Ferrari L, Pinho-Ribeiro FA, **Fattori V**, Zaninelli TH, Badaro-Garcia S, Borghi SM, Andrade KC, Clemente-Napimoga JT, Alves-Filho JC, Cunha TM, Fraceto LF, Cunha FQ, Napimoga MH, Casagrande R, Verri WA Jr. **Sci Rep.** 2018 Sep 18;8(1):13979. doi: 10.1038/s41598-018-32334-0.*

A.2.3.11 Trans*-Chalcone attenuates pain and inflammation in experimental acute gout arthritis in mice. Staurengo-Ferrari L, Ruiz-Miyazawa KW, Pinho-Ribeiro FA, **Fattori V**, Zaninelli TH, Badaro-Garcia S, Borghi SM, Carvalho TT, Alves-Filho JC, Cunha TM, Cunha FQ, Casagrande R, Verri WA Jr. **Front Pharmacol**. 2018 Oct 2;9:1123. doi: 10.3389/fphar.2018.01123. eCollection 2018.*

*A.2.3.12 The citrus flavanone naringenin reduces gout-induced joint pain and inflammation in mice by inhibiting the activation of NFκB and macrophage release of IL-1β. Kenji W. Ruiz-Miyazawa, Sergio M. Borghi, Felipe A. Pinho-Ribeiro, Larissa Staurengo-Ferrari, **Fattori V**, Glaura S.A. Fernandes, Antonio M. Casella, Jose C. Alves-Filho,Thiago M. Cunha, Fernando Q. Cunha, Rubia Casagrande, Waldiceu A. Verri Jr. **J Functional Foods.** (48)106-116*

*A.2.3.13 The lipid mediator Resolvin D1 reduces the skin inflammation and oxidative stress induced by UV irradiation in hairless mice. Saito P, Melo CPB, Martinez RM, **Fattori V**, Cezar TLC, Pinto IC, Bussmann AJC, Vignoli JA, Georgetti SR, Baracat MM, Verri WA Jr, Casagrande R. **Front Pharmacol**. 2018 Oct 31;9:1242. doi: 10.3389/fphar.2018.01242. eCollection 2018.*

A.2.4 Ano de 2019

A.2.4.1 Kaurenoic acid extracted from Sphagneticola trilobata *reduces acetaminophen-induced hepatotoxicity through inhibition of oxidative stress and pro-*

*inflammatory cytokine production in mice. Marcondes-Alves L, **Fattori V**, Borghi SM, Lourenco-Gonzalez Y, Bussmann AJC, Hirooka EY, Casagrande R, Verri WA Jr, Arakawa NS. **Nat Prod Res**. 2019 Mar;33(6):921-924. doi: 10.1080/14786419.2017.1416372. Epub 2017 Dec 20.*

A.2.4.2 Contribution of spinal cord glial cells to L. amazonensis *experimental infection-induced pain in BALB/c mice. Borghi SM, **Fattori V**, Pinho-Ribeiro FA, Domiciano TP, Miranda-Sapla MM, Zaninelli TH, Casagrande R, Pinge-Filho P, Pavanelli WR, Alves-Filho JC, Cunha FQ, Cunha TM, Verri WA Jr. **J Neuroinflammation**. 2019 May 28;16(1):113. doi: 10.1186/s12974-019-1496-2.*

*A.2.4.3 Contribution of spinal cord oligodendrocytes to neuroinflammatory diseases and pain. Borghi SM, **Fattori V**, Hohmann MSN, Verri WA. **Curr Med Chem.** 2019;26(31):5781-5810. doi: 10.2174/0929867325666180522112441. Review.*

*A.2.4.4 The granulopoietic cytokine granulocyte colony-stimulating factor (G-CSF) induces pain: analgesia by rutin. Carvalho TT, Mizokami SS, Ferraz CR, Manchope MF, Borghi SM, **Fattori V**, Calixto-Campos C, Camilios-Neto D, Casagrande R, Verri WA Jr. **Inflammopharmacology**. 2019 Dec;27(6):1285-1296. doi: 10.1007/s10787-019-00591-8. Epub 2019 Apr 3.*

*A.2.4.5 Treatment with maresin 1, a docosahexaenoic acid-derived pro-resolution lipid, protects skin from inflammation and oxidative stress caused by UVB irradiation. Cezar TLC, Martinez RM, Rocha CD, Melo CPB, Vale DL, Borghi SM, **Fattori V**, Vignoli JA, Camilios-Neto D, Baracat MM, Georgetti SR, Verri WA Jr, Casagrande R. **Sci Rep**. 2019 Feb 28;9(1):3062. doi: 10.1038/s41598-019-39584-6.*

*A.2.4.6 Repurposing of the nootropic drug vinpocetine as an analgesic and anti-inflammatory agent: evidence in a mouse model of superoxide anion-triggered inflammation. Lourenco-Gonzalez Y, **Fattori V**, Domiciano TP, Rossaneis AC, Borghi SM, Zaninelli TH, Bernardy CCF, Alves-Filho JC, Cunha TM, Cunha FQ, Casagrande R, Verri WA Jr. **Mediators Inflamm**. 2019 Mar 31;2019:6481812. doi:*

10.1155/2019/6481812. eCollection 2019.

A.2.4.7 Metabolic syndrome aggravates cardiovascular, oxidative and inflammatory dysfunction during the acute phase of Trypanosoma cruzi *infection in mice. Lucchetti BFC, Boaretto N, Lopes FNC, Malvezi AD, Lovo-Martins MI, Tatakihara VLH,* ***Fattori V****, Pereira RS, Verri WA Jr, de Almeida Araujo EJ, Pinge-Filho P, Martins-Pinge MC.* ***Sci Rep.*** *2019 Dec 11;9(1):18885. doi: 10.1038/s41598-019-55363-9.*

A.2.4.8 [Ru(bpy)2(NO)SO3](PF6), a nitric oxide donating ruthenium complex, reduces gout arthritis in mice. Rossaneis AC, Longhi-Balbinot DT, Bertozzi MM, ***Fattori V****, Segato-Vendrameto CZ, Badaro-Garcia S, Zaninelli TH, Staurengo-Ferrari L, Borghi SM, Carvalho TT, Bussmann AJC, Gouveia FS Jr, Lopes LGF, Casagrande R, Verri WA Jr.* ***Front Pharmacol****. 2019 Mar 12;10:229. doi: 10.3389/fphar.2019.00229. eCollection 2019.*

A.2.4.9 Probucol ameliorates complete freund's adjuvant-induced hyperalgesia by targeting peripheral and spinal cord inflammation. Zucoloto AZ, Manchope MF, Borghi SM, Dos Santos TS, ***Fattori V****, Badaro-Garcia S, Camilios-Neto D, Casagrande R, Verri WA Jr.* ***Inflammation****. 2019 Aug;42(4):1474-1490. doi: 10.1007/s10753-019-01011-3.*

A.3 Publicações de Capítulo de Livro Como Primeiro Autor

A.3.1 Ano de 2017

A.3.1 Neuroimmune regulation of pain and inflammation: targeting glial cells and nociceptor sensory neurons interaction. ***Victor Fattori****, Sergio M. Borghi, Ana C. Rossaneis, Mariana M. Bertozzi, Thiago M. Cunha and Waldiceu A. Verri.* ***Frontiers in CNS Drug Discovery****, Volume: 3, Chapter 4, Pp. 146-200 (55).*

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ANEXO I

ANEXO I – Comprovante publicação artigo científico no periódico *British Journal of Pharmacology*

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RESEARCH PAPER

The specialised pro-resolving lipid mediator maresin 1 reduces inflammatory pain with a long-lasting analgesic effect

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ANEXO II

ANEXO II – Comprovante de aceite do artigo científico no periódico *Pain* (doi 10.1097/j.pain.0000000000001832)

Research Paper

PAIN

VIDEO

Nonsurgical mouse model of endometriosis-associated pain that responds to clinically active drugs

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Abstract

Endometriosis is an estrogen-dependent inflammatory disease that affects approximately 10% of women. Debilitating pelvic or abdominal pain is one of its major clinical features. Current animal models of endometriosis-associated pain require surgery either to implant tissue or to remove the ovaries. Moreover, existing models do not induce spontaneous pain, which is the primary symptom of patients with chronic pain, including endometriosis. A lack of models that accurately recapitulate the disease phenotype must contribute to the high failure rate of clinical trials for analgesic drugs directed at chronic pain, including those for endometriosis. We set out to establish a murine model of endometriosis-associated pain. Endometriosis was induced nonsurgically by injecting a dissociated uterine horn into a recipient mouse. The induced lesions exhibited histological features that resemble human lesions along with an increase in proinflammatory cytokines and recruitment of immune cells. We also observed the presence of calcitonin gene–related peptide–, TRPA1-, and TRPV1-expressing nerve fibers in the lesions. This model induced mechanical allodynia, spontaneous abdominal pain, and changes in thermal selection behavior that indicate discomfort. These behavioral changes were reduced by drugs used clinically for endometriosis, specifically letrozole (aromatase inhibitor) and danazol (androgen). Endometriosis also induced neuronal changes as evidenced by activation of the NF-κB signaling pathway in TRPA1- and TRPV1-expressing dorsal root ganglion neurons. In conclusion, we have established a model of endometriosis-associated pain that responds to clinically active drugs and can, therefore, be used to identify novel therapies.

Keywords: Visceral pain, Abdominal pain, Women's health, Nonevoked pain

ANEXO III

ANEXO III – Comprovante *Editor's Choice* e capa pelo periódico *Pain* (number PAIN-D-19-01010R1)

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The article you submitted to *PAIN* has been chosen as an Editor's Choice article and will be featured in the journal and on its website. We would like you to consider submitting an audio or video abstract as well, as it will surely increase the article's visibility and profile.

If you are interested in submitting an audio or video abstract, please let us know at painj@iasp-pain.org, complete and sign the attached documents, and plan to submit within 2 weeks of receipt of this invitation. If we do not receive a response as to your intentions within 3 days, we will proceed with publication of the article.

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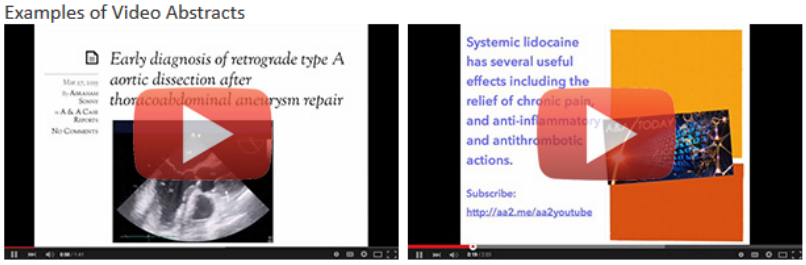
Examples of Video Abstracts



(These videos are from *Anesthesia & Analgesia and appear on aa2day.org and on YouTube.*)

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How to Submit an Audio or Video Abstract

- Using a service such as Dropbox or Hightail, send a link to painj@iasp-pain.org. The subject line of your email should include your manuscript's ID number.
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I do hope you will take advantage of this opportunity to bring greater attention to your work.

Please let us know if you have any questions.

Kind regards,

Francis J Keefe, PhD
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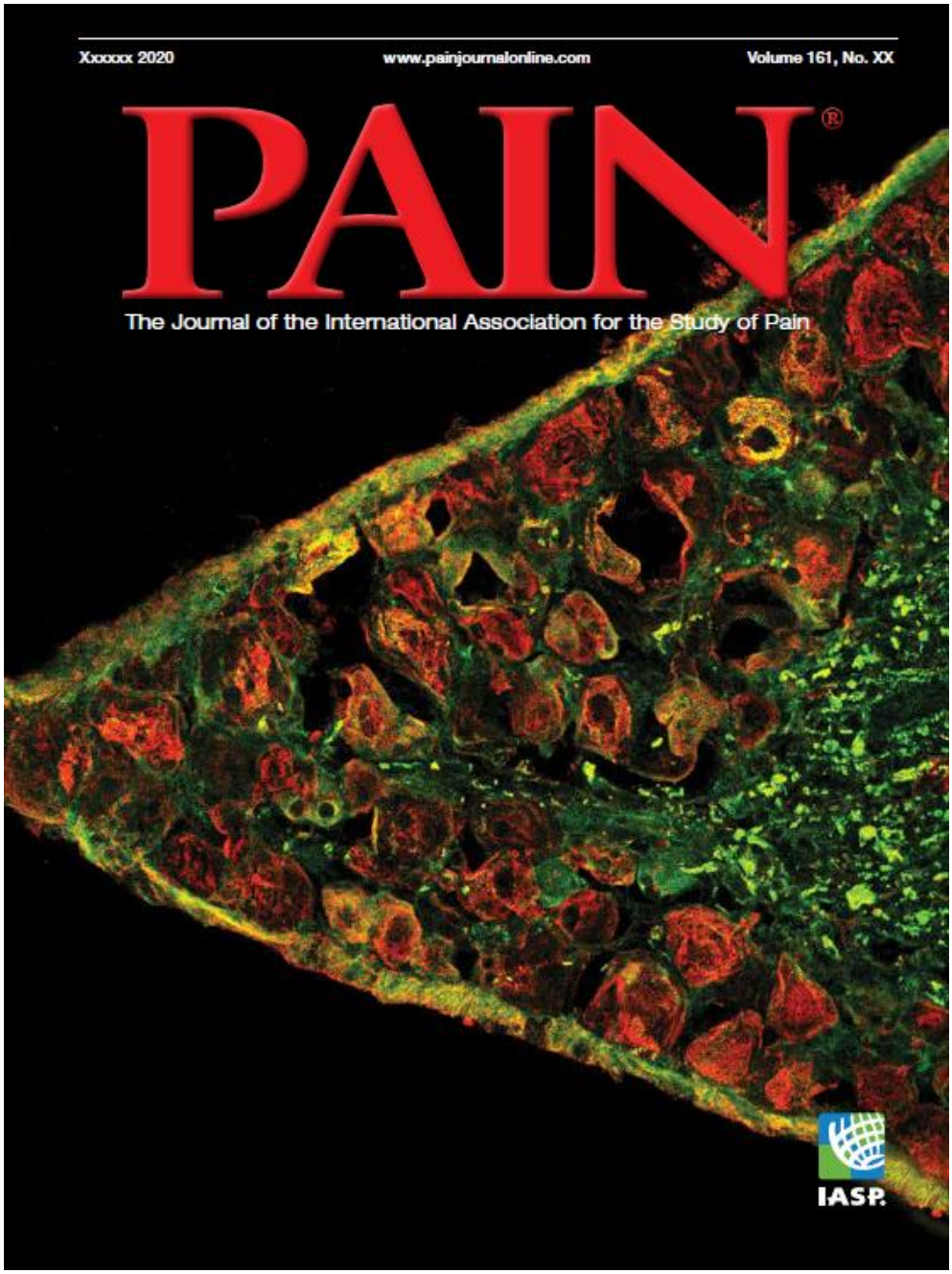
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ANEXO IV

ANEXO IV – Comprovante publicação artigo científico no periódico *Pharmacological Research*

Pharmacological Research
Volume 151, January 2020, 104549

Review

Specialized pro-resolving lipid mediators: A new class of non-immunosuppressive and non-opioid analgesic drugs

Victor Fattori [a], Tiago H. Zaninelli [a], Fernanda S. Rasquel-Oliveira [a], Rubia Casagrande [b], Waldiceu A. Verri Jr [a]

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https://doi.org/10.1016/j.phrs.2019.104549

ANEXO V

ANEXO V – Comprovante publicação artigo científico no periódico *Molecules*

Open Access Review

Capsaicin: Current Understanding of Its Mechanisms and Therapy of Pain and Other Pre-Clinical and Clinical Uses

by Victor Fattori †, Miriam S. N. Hohmann †, Ana C. Rossaneis, Felipe A. Pinho-Ribeiro and Waldiceu A. Verri *

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