CARINA ZERBETTO SEGATO VENDRAMETO

MECANISMOS CENTRAIS ENVOLVIDOS NA HIPERALGESIA INDUZIDA PELOS VÍRUS DENGUE E CHIKUNGUNYA E PROTEÍNA E2 DO VÍRUS CHIKUNGUNYA EM CAMUNDONGOS.

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Orientador: Prof. Dr. Waldiceu Aparecido Verri Júnior

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Londrina, 31 de Julho de 2019.

Dedico este trabalho ao meu filho Bernardo,

que adora os camundongos e as bolachas do laboratório.

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“O sucesso é a soma de pequenos esforços – repetidos dia sim, e no outro também.”
Robert Collier

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RESUMO

As arboviroses Dengue e Febre Chikungunya são duas das viroses mais comuns afetando centenas de países por todo o mundo, seguindo a área endêmica de seus vetores, os mosquitos do gênero *Aedes.* Como não existem medicamentos específicos para ambas as viroses, novas estratégias terapêuticas são necessárias para tratar os principais sintomas como a dor por exemplo. Neste trabalho nós investigamos os mecanismos pelos quais estes vírus induzem dor e buscamos possíveis alvos terapêuticos para este sintoma. O vírus Chikungunya é conhecido por causar uma incapacitante dor articular que pode perdurar durante anos, mesmo após a eliminação completa do vírus, e nós investigamos se a proteína E2 do envelope viral pode estar envolvida nesse processo de indução da dor. Nossos resultados mostraram que o vírus inativado e a proteína E2 recobinante induzem hiperalgesia mecânica e térmica em camundongos, e o tratamento com anticorpos monoclonais anti-rE2 reduziu de forma efetiva a dor induzida pelos estímulos. Também observamos que na ausência ou bloqueio dos canais TRPV1 ocorre redução da hiperalgesia induzida pelo vírus inativado e pela proteína. Por último, encontramos que os neurônios dos gânglios da raiz dorsal apresentaram maior influxo de cálcio em nível basal após a estimulação com o vírus inativado e com a proteína recombinante, indicando ativação neuronal direta através destes estímulos. Pacientes infectados com dengue também apresentam a dor como um dos principais sintomas, então nós investigamos a participação de citocinas e das células da glia na indução da hiperalgesia após a infecção. Nossos dados mostraram que a infecção sistêmica com o vírus dengue induziu hiperalgesia mecânica, aumento na produção das citocinas TNF-α, IL-1β e IL-10 e ativação de astrócitos e micróglias (aumento na expressão de mRNA para GFAP e Iba-1). O tratamento com moduladores destas citocinas e das células da glia confirmou de maneira efetiva a importância destas células e moléculas na hiperalgesia induzida pelo vírus Dengue em camundongos, demonstrando um grande potencial terapêutico que merece investigação futura.

Palavras-chave: Vírus Dengue. Vírus Chikungunya. Hiperalgesia. TRPV1. Células da Glia. Citocinas.

SEGATO-VENDRAMETO, Carina Z. **Mechanisms involved in Dengue and Chikungunya virus-induced mechanical hyperalgesia in mice.** 2019. 97p. PhD Thesis (Doctorade in Experimental Pathology) – State University of Londrina, Londrina, 2019.

ABSTRACT

The arboviruses Dengue and Chikungunya Fever are two of the most common viruses affecting hundreds of countries all over the world, following the endemic zone of its vectors *Aedes* mosquitoes. Since there is no specific treatment for both of them, new therapeutic strategies are required to treat the main symptoms like pain. In this work we investigated the mechanisms by which the virus induce pain and searched for possible therapeutic targets to treat this symptom. Chikungunya virus is known for causing incapacitating joint pain that can least years even after the virus clearance, and we investigated if the E2 envelope protein is involved in inducing pain. Our results showed that Chikungunya inactivated virus and recombinant E2 protein induce mechanical and hyperalgesia in mice, and that treatment with anti-rE2 molecular antibodies reduced effectively the induced hyperalgesia. We also observed that in absence or blockage of TRPV1 channels occurs a reduction of iCHIKV and rE2-induced hyperalgesia. At last, we find out that DRG neurons presented higher calcium influx levels after mice were stimulated with inactivated virus and recombinant protein, indicating neuron activation. Dengue infected patients also present pain as a main symptom, so we investigated the participation of cytokines and glial cells in hyperalgesia induction. Our data showed that systemic infection with DENV induce mechanical hyperalgesia, increased levels of TNF-α, IL-1β and IL-10, and activation of astrocyte and microglia (increased GFAP and Iba-1 mRNA expression). Treatment with cytokines and glia modulators effectively confirmed the importance of those cells and molecules in DENV-induced hyperalgesia in mice, demonstrating a great therapeutic potential that deserves further investigation.

Keywords: Dengue virus. Chikungunya virus. Hyperalgesia. TRPV1. Glial cells. Cytokines.

LISTA DE ABREVIATURAS E SIGLAS

CD	Células dendríticas
CHIKV	Vírus Chikungunya
COX	Ciclooxigenase
DENV	Vírus Dengue
FD	Febre Dengue
FDH	Febre Dengue Hemorrágica
GFAP	Proteína ácida fibrilar glial
GRD	Gânglio da raíz dorsal
IASP	Associação internacional para o estudo da dor
iCHIKV	Vírus Chikungunya inativado
IFN-γ	Interferon gama (γ)
IL	Interleucina
IL-1β	Interleucina 1 beta (β)
IL-1Ra	Antagonista do receptor de Interleucina 1
i.a.	Intra-articular
i.p.	Intraperitoneal
i.t.	Intratecal
LPS	Lipopolissacarídeo
NF-κB	Fator nuclear kappa B
NK	*Natural killer*
OMS	Organização Mundial da Saúde
PGE2	Prostaglandina E2
PKR	Proteína quinase R
PRRs	Receptores de Reconhecimento de Padrão
rE2	E2 recombinante
RIG-I	Receptores semelhantes ao gene induzível pelo ácido retinóico I
RNA	Ácido ribonucléico
SCD	Síndrome do Choque por Dengue
SNC	Sistema nervoso central

Th1 e 2	*T helper* 1 e 2
TLR	Receptores semelhantes a Toll
TNF-α	Fator de necrose tumoral alfa (α)
TRPV1	Receptor de Potencial Transitório Vanilóide tipo 1

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1. INTRODUÇÃO

Arbovírus, do inglês “arthropod-borne virus”, é um termo informal que se refere àqueles vírus que são transmitidos através da picada de artrópodes, incluindo mosquitos, carrapatos e moscas, e que causam doenças geralmente de maneira epidêmica. Este grupo é composto por várias famílias, entre elas a Flaviviridae, que inclui o vírus Dengue, e a Togaviridae, da qual faz parte o vírus Chikungunya. Estas duas doenças arbovirais são transmitidas por mosquitos do gênero *Aedes*, especialmente *Aedes aegypti*, e podem produzir sintomas muito parecidos, principalmente durante a fase aguda (primeiros 7 a 12 dias após a infecção) dificultando o diagnóstico correto, criando problemas para direcionar um tratamento apropriado e, às vezes, levando a casos fatais (DAVID BECKHAM; TYLER, 2015; DAVIS; BECKHAM; TYLER, 2008; WORLD HEALTH ORGANIZATION, 2009).

As primeiras epidemias supostasmente relacionadas ao vírus dengue datam de 1635 nas ilhas Martinica e Guadalupe, e 1699 no Panamá. Porém apenas em 1780 se tem a descrição da primeira epidemia de fato ao vírus, na Filadélfia, Estados Unidos (revisado em MONTOYA et al., 2012).

Já o vírus Chikungunya foi isolado pela primeira vez na Tanzania em 1952 (ROBINSON, 1955) e atraiu atenção mundial durante a primeira epidemia nas ilhas do Oceano Índico. Após atingir vários países da África e Ásia, houve um período de dormência relativa do vírus, e em seguida ele ressurgiu se espalhando para outros países em diferentes continentes (revisado em CAGLIOTI et al., 2013).

Devido ao significativo aumento na ocorrência das arboviroses a nível mundial nas últimas décadas, a busca pelo desenvolvimento de diagnósticos precisos e de vacinas que sejam comuns a vários destes patógenos tem ganhado destaque, podendo ser um grande avanço em termo não apenas científico, mas também econômico (DAVID

BECKHAM; TYLER, 2015; LABEAUD; BASHIR; KING, 2011). No Brasil, os últimos boletins epidemiológicos emitidos pela Secretaria de Vigilância em Saúde mostram que foram notificados mais de 390 mil casos prováveis de dengue no país, chegando a uma taxa de incidência de cerca de 186 casos por 100 mil habitantes, sendo a região Centro-Oeste a mais afetada com 437 casos/100 mil habitantes. Já com relação aos dados de chikungunya, foram notificados quase 11.500 casos prováveis, com uma taxa de incidência de 5,4 casos por 100 mil habitantes no país. As regiões mais afetadas foram Sudeste e Nordeste com taxas de incidência, 6,7 casos/100 mil habitantes (BRASIL. MINISTÉRIO DA SAÚDE, 2020).

1.1 Dengue

A Febre Dengue é uma doença causada por um dos quatro sorotipos do vírus Dengue (DENV), um dos mais prevalentes entre humanos atualmente. Os quatro sorotipos do vírus (DENV-1, DENV-2, DENV-3, e DENV-4) pertencem à família Flaviviridae, são muito parecidos geneticamente e circulam concomitantemente em diferentes regiões do mundo, afetando cerca de 100 países nas zonas tropical e subtropical. Mundialmente, mais de 25 milhões de pessoas são infectadas por ano, incluindo cerca 50 mil hospitalizações com casos graves, principalmente entre crianças, e taxa de mortalidade maior que 5% (GUZMAN et al., 2010; ROTHMAN, 2004; SIMMONS et al., 2012).

A classificação da Organização Mundial da Saúde (OMS) inclui dois tipos principais da doença: a Febre Dengue (FD) e a Febre Dengue Hemorrágica (FDH) ou Síndrome do Choque por Dengue (SCD). A FD é definida como uma doença febril debilitante autolimitante, acompanhada por uma combinação de sintomas inespecíficos incluindo dor de cabeça, dor retro-orbital, mialgia e ocasionalmente, manifestações hemorrágicas (FAGUNDES et al., 2011; KYLE; HARRIS, 2008; NARVAEZ et al., 2011). A definição de caso para FDH inclui a presença de quatro critérios: febre,

manifestações hemorrágicas, trombocitopenia e evidência de extravasamento de plasma (efusão pleural, ascite, hemoconcentração >20% ou hipoproteinemia) (NARVAEZ et al., 2011).

Após a picada do mosquito transmissor, o vírus entra na corrente sanguínea do hospedeiro e a infecção das células ocorre através do reconhecimento do patógeno primeiramente pelas células dendríticas (CDs), através da ligação da proteína E do envelope viral aos receptores celulares, onde alterações conformacionais nessa glicoproteína levam à fusão das membranas do vírus e da célula (NAVARRO-SÁNCHEZ; DESPRÈS; CEDILLO-BARRÓN, 2005).

Uma vez dentro da célula hospedeira, o vírus se replica no citoplasma e é reconhecido pelos receptores de reconhecimento de padrão (Pattern Recognition Receptors - PRRs) que reconhecem RNA viral, sendo principalemente os Toll like receptors (TLR 3, 7 e 8),os receptores semelhantes ao gene induzível pelo ácido retinóico I (RIG-I) e a proteína quinase R (PKR) (AGUIRRE et al., 2012; CLYDE; KYLE; HARRIS, 2006; KOYAMA et al., 2008; TSAI et al., 2009). O reconhecimento das estruturas virais pelos PRRs leva a ativação da cascata intracelular de fosforilação de proteínas, culminando no acionamento de duas principais famílias de fatores de transcrição: o fator de transcrição nuclear NFκ-B e os fatores reguladores de interferons (IFRs). Esses fatores quando ativados promovem a transcrição gênica de citocinas inflamatórias, especialmente interferons (IFNs) do tipo I (α/β), estimuladoras de outras células do sistema imune inato, levando ainda ao amadurecimentos de células dendríticas e ao estabelecimento de uma imunidade antiviral (KOYAMA et al., 2008; NAVARRO-SÁNCHEZ; DESPRÈS; CEDILLO-BARRÓN, 2005; SEVERA; FITZGERALD, 2007).

Os IFNs são citocinas que desempenham um papel importante na resposta imune antiviral, sendo uma das principais moléculas da imunidade inata na defesa contra todos

os tipos de vírus. Deste modo, para infectar os seres humanos de forma eficiente, o DENV deve evitar ou inibir elementos fundamentais do sistema imunológico inato do hospedeiro, como por exemplo a resposta do IFN-I (AGUIRRE et al., 2012; CLYDE; KYLE; HARRIS, 2006; HALLER; KOCHS; WEBER, 2006).

Estudos clínicos tem demonstrado altos níveis de IFNs circulantes em pacientes com FD e FDH, sendo que nesta última esses níveis estão ainda mais aumentados. Esses achados sugerem que altos níveis de IFN-α and IFN-γ em resposta à infecção por DENV desempenham papel protetivo, enquanto baixos níveis de IFN-γ refletem uma resposta antiviral inadequada e está relacionado à formas mais graves (revisado em CLYDE; KYLE; HARRIS, 2006). Desta forma, a resposta imune inata inicial pode determinar a resposta subsequente e o desfecho da doença (UMAREDDY et al., 2008).

Acredita-se que a infecção primária pelo DENV é capaz de estimular uma resposta imune humoral efetiva, duradoura e que garanta proteção contra uma possível reinfecção pelo mesmo sorotipo viral, porém apenas proteção parcial a diferentes sorotipos (ROTHMAN, 2004). No entanto, estudos epidemiológicos sugerem que a produção de anticorpos contra um dos quatro sorotipos do DENV pode aumentar a gravidade da doença em caso de uma infecção secundária por um sorotipo diferente, levando ao desenvolvimento da FDH por facilitarem a entrada do vírus na célula promovendo um aumento da infecção dependente de anticorpos (FLIPSE et al., 2016; GUZMAN et al., 2016; YAMANAKA et al., 2017). Foi demonstrado ainda que existe uma janela de tempo na qual anticorpos neutralizantes que perderam esta habilidade de aumentar a infecção podem ainda fornecer proteção contra a doença letal (BELTRAMELLO et al., 2010), sendo importantes para a eliminação e controle da carga viral circulante.

De maneira geral, a patogênse da Dengue ainda não é completamente compreendida e resulta de inúmeras interações entre o vírus, o hospedeiro e a resposta

imune desenvolvida em resposta à infecção. Desta forma, acredita-se que os riscos do desenvolvimento das formas mais graves da doença são resultantes da combinação de diferentes fatores, entre eles sorotipo e virulência viral, idade, sexo, status do sistema imunológico e fatores nutricionais e genéticos do hospedeiro, que interagem para dar origem à resposta contra a infecção, sendo ela benéfica ou prejudicial para o hospedeiro (CLYDE; KYLE; HARRIS, 2006; GUZMAN et al., 2016).

Embora ainda não existam terapias específicas e eficazes anti-infecção ou imunomodulatórias para prevenir as principais complicações da doença, a manutenção da quantidade apropriada de fluidos e o cuidado com o estado geral do paciente são as principais estratégias de tratamento e podem reduzir a taxa de mortalidade de 20% para menos de 1% (HUNG, 2012; WORLD HEALTH ORGANIZATION, 2009).

Terapias alternativas têm sido utilizadas no intuito de modular a produção de citocinas e toxinas produzidas em resposta ao vírus, especialmente no desenvolvimento dos quadros mais graves de FDH e choque. Tais terapias incluem o uso de anticorpos policlonais, monoclonais e fração Fab de anticorpos anti-FNT-alfa, IL-1, IL-6; inibidores do fator ativador de plaquetas (PAF), pentoxifilina, antioxidantes, n-acetilcisteína, além de inibidores das endorfinas naturais como o naloxone e de antagonistas da bradicinina, também têm sido testados (revisado em SERUFO et al., 2000).

Nos pacientes sem sinais de alerta sugere-se manter as medicações habituais, dando atenção à condição de desidratação e hipovolemia, suspendendo-se diuréticos e vasodilatadores caso seja necessário (BRASIL. MINISTÉRIO DA SAÚDE, 2016).

1.2 Febre do Chikungunya

O vírus Chikungunya (CHIKV) é conhecido por causar a enfermidade chamada de Febre do Chikungunya, caracterizada clinicamente pela ocorrência de poliartrite/poliartralgia aguda e crônica, que é responsável por causar incapacidade e morbidez. A fase aguda

inicial é caracterizada por ocorrência de febre alta, dores de cabeça e outros sintomas como mialgia, fadiga, edema das extremidades, erupções na pele e até mesmo queixas gastrointestinais, que costumam desaparecer em poucos dias (BORGHERINI et al., 2008; HOARAU et al., 2010; PIALOUX et al., 2007; SUHRBIER; JAFFAR-BANDJEE; GASQUE, 2012).

Na maioria dos casos, a doença pode progredir para uma segunda fase caracterizada por uma síndrome reumática crônica, com artralgia grave que pode persistir por semanas, meses ou anos, afetando a vida econômica e social dos portadores (GÉRARDIN et al., 2011; LABEAUD; BASHIR; KING, 2011). Larrieu e colaboradores reportaram em um estudo de caso que mais da metade dos pacientes infectados ainda sofriam os sintomas da doença 2 anos após o diagnóstico (LARRIEU et al., 2010). A dor articular recorrente que perdura por meses ou mesmo anos após a infecção é relatada por cerca de 40% dos infectados, mesmo na ausência do patógeno na corrente sanguínea do hospedeiro (SCHWARTZ; ALBERT, 2010).

A resposta immune contra a infecção por CHIKV parece ser robusta, com forte produção de interferon, de anticorpos específicos e resposta celular, necessários para controlar a replicação e a disseminação do vírus e para resolver a infecção. Contudo, estudos também sugerem um componente patogênico nessa resposta, cujo entendimento ainda é limitado e parece contribuir para o desenvolvimento da fase crônica da doença (OKEOMA, 2016). A infecção leva também a uma resposta imune adaptativa protetiva onde o estabelecimento de uma resposta de anticorpos anti-CHIKV após a infecção primária pode conferir completa proteção contra uma possível reinfecção (LARAS et al., 2005).

Assim como o vírus Dengue, o CHIKV é um vírus RNA de fita simples cujos principais sensores celulares são os Toll like receptors 3, 7 e 8 e os Rig-like receptors

(RLR). Através da ligação com esses receptores celulares, o RNA viral é capaz de ativar células hematopoiéticas, especialmente células dentríticas (SCHWARTZ; ALBERT, 2010) e essa capacidade facilita a disseminação viral para outros órgãos alvos tais como músculos, fígado, rins, coração e cérebro (NG et al., 2009). De modo semelhante a outros vírus, o Chikungunya também parece ter evoluído criando mecanismos que o permitem modular negativamente a produção de IFNs tipo I e de moléculas efetoras estimuladas por essa via de sinalização, reduzindo a eficácia da imunidade inata e facilitando essa disseminação viral (SCHWARTZ; ALBERT, 2010).

Embora o vírus seja eliminado dos tecidos do hospedeiro em um curto espaço de tempo após a infecção, o RNA viral pode permanecer associado a tecidos articulares por cerca de mais 16 semanas, sendo uma das evidências histopatológicas da inflamação articular (HAWMAN et al., 2016). Estudos realizados em macacos observaram reservatório de RNA viral nos tecidos sinoviais e musculares por cerca de 8 semanas, e em órgãos linfóides por cerca de 3 meses após a infecção (LABADIE et al., 2010).

Monócitos e macrófagos são as principais células alvos do CHIKV, tanto em pacientes (HOARAU et al., 2010) quanto em modelos animais (GARDNER et al., 2010), e foi demonstrado recentemente em primatas a importância deles como células ativas em resposta à infecção e também como reservatórios do material genético viral, desempenhando um papel importante na patogênse e na persistência da doença (LABADIE et al., 2010).

A participação das células Natural Killer (NK) na patogênse do CHIKV ainda não é completamente compreendida, mas há evidências de grandes quantidades destas células nos tecidos articulares inflamados de camundongos infectados pelo vírus Chikungunya (GARDNER et al., 2010). Em humanos estas células têm apresentado forte ativação nos

primeiros dias após a infecção, levando a uma resposta mais prolongada dos linfócitos T estando assim relacionadas à cronicidade da doença (HOARAU et al., 2010).

As células T por sua vez são importantes células efetoras durante todos os tipos de infecção viral, sendo que tanto as $TCD4^+$ quanto as $TCD8^+$ são capazes de eliminar células infectadas. As $TCD4^+$ tem ainda um papel essencial no desenvolvimento da inflamação e do edema articular em resposta à infecção por CHIKV, porém não possuem nenhum efeito na replicação e na disseminação viral (TEO et al., 2017).

A resposta do organismo à infecção representa um complexo sistema de vias diferentes que visam erradicar o patógeno e beneficiar o hospedeiro com a resolução da infecção. Porém, muitas destas vias podem causar efeitos adversos no hospedeiro, como por exemplo células NK e Linfócitos T citotóxicos hiperresponsivos, produção excessiva de citocinas pró-inflamatórias e apoptose, resultando em morte celular, lesão e disfunção tecidual (HOARAU et al., 2010). Tem sido observado ainda que osteoblastos infectados pelo CHIKV levam a osteoclastogênese, indicando a importância destas células na destruição óssea e inflamação articular ocasionada pela infecção viral (NORET et al., 2012).

Com relação à produção de citocinas, está bem estabelecido que a ocorrência de febre em pacientes infectados pelo vírus Chikungunya está associada com a síntese de citocinas pró-inflamatórias como IL-1β e o fator de necrose tumoral alfa (TNF-α), que são conhecidas por seu papel indutor de febre e que altas concentrações destas citocinas são comumente encontradas em pacientes que desenvolvem poliartrite em decorrência da infecção viral (NG et al., 2009). Tais achados sugerem potencial papel causativo destas citocinas na dor crônica muscular e articular, porém pouco se sabe sobre os mecanismos envolvidos na dor induzida pela infecção viral.

Semelhante ao discutido acima para o vírus Dengue, não há tratamento antiviral específico contra o Chikungunya, portanto a terapia utilizada é de suporte sintomático, hidratação e repouso. Os anti-inflamatórios não esteroidais (ibuprofeno, naproxeno, diclofenaco, nimesulida, ácido acetilsalicílico) não devem ser utilizados na fase aguda da doença, devido ao risco de complicações renais e sangramento, mas podem ser utilizados em caso de doença reumática crônica (BRASIL. MINISTÉRIO DA SAÚDE, 2017).

1.2.1. Proteína E2 do envelope viral

CHIKV é um pequeno vírus envelopado, com cerca de 70 a 100nm de diâmetro. Seu material genético é composto por RNA de fita simples que codifica quatro proteínas não estruturais (nsP1-4) que são requeridas para a replicação do vírus, e três proteínas estruturais principais, sendo elas as proteínas C (constituinte do capsídeo), E1 e E2 (formando o envelope viral) (CHEVILLON et al., 2008; POWERS, 2010; STRAUSS; STRAUSS, 1994).

As glicoproteínas E1 e E2 estão embutidas na membrana do envelope viral, sendo que semelhantemente aos demais alfavírus, E1 é uma proteína de fusão viral classe II, mediando processos de fusão dependentes de pH com as membranas endossomais, enquanto E2 é responsável por interagir com os receptores de superfície celular. Uma terceira glicoproteína, E3, tem sido associada com vírions maduros em alguns alfavírus (POWERS, 2010; ZIEMIECKI; GAROFF; SIMONS, 1980).

A proteína E2 do vírus Chikungunya tem sido estudada como molécula em potencial para ser utilizada na imunização contra esse vírus. O estudo e foco nesta proteína deve-se ao fato de ser uma das grandes responsáveis pela antigenicidade do vírus e, dessa forma, é coerente que grande parte da resposta inflamatória e dor induzidas pelo vírus estejam relacionadas a ela.

1.3 Dor inflamatória, seus mediadores e mecanismos de ação

Os sinais e sintomas apresentados pelos pacientes infectados pelos vírus Dengue e Chikungunya são clinicamente parecidos, sendo eles a febre de início agudo, dores articulares e musculares, cefaleia, náusea, fadiga e exantema. A principal manifestação clínica que permite a diferenciação da Febre do Chikungunya são as fortes dores nas articulações, comumente acompanhadas de edema, que não ocorrem nos pacientes com Dengue (BRASIL. MINISTÉRIO DA SAÚDE, 2017).

Dentre os sintomas apresentados, a dor juntamente com a febre são as principais queixas de ambos os pacientes, portadores de Dengue ou Chikungunya. Segundo a Associação Internacional para o Estudo da Dor (IASP), a dor pode ser definida como uma "experiência sensorial e emocional desagradável, associada a um dano tecidual real ou potencial" (IASP, 2012).

A dor ocasionada em decorrência de um processo inflamatório, como em resposta a uma infecção viral por exemplo, é resultado da interação entre o tecido danificado pela resposta inflamatória contra o patógeno e os neurônios sensoriais nociceptivos periféricos por meio da participação de mediadores inflamatórios (FERREIRA et al., 2009). Ela é acompanhada pelos fenômenos de alodinia (dor decorrente de um estímulo que normalmente não provoca dor) e hiperalgesia (resposta exacerbada a um estímulo doloroso). Esses termos foram originalmente utilizados para descrever a sensação dolorosa em humanos, contudo, rotineiramente são aplicados também em modelos animais (FLÓREZ, 1993; LOESER; MELZACK, 1999).

A dor é um mecanismo fisiológico protetor essencial, notificando o corpo sobre a presença de estímulos nocivos no ambiente, como a presença de patógenos por exemplo. Após a infecção por esse patógeno, o processo inflamatório que se inicia resulta na produção de citocinas, fatores de crescimento e outros mediadores inflamatórios que

podem se ligar a canais iônicos e ativar diretamente neurônios nociceptivos primários (nociceptores) gerando aumento da sensação dolorosa (ALLAN I. BASBAUM; CLIFFORD J. WOOLF, 1999).

Essa sensação tem início por uma cascata de liberação de citocinas e quimiocinas, os quais desencadeiam uma amplificação dessa resposta promovendo então a produção de prostaglandinas e aminas que agem diretamente sobre seus receptores que estão presentes na membrana dos neurônios nociceptores (MAZZARDO-MARTINS et al., 2010). Desta forma, a produção de citocinas durante a resposta inflamatória têm sido relacionada a eventos celulares e moleculares que resultam em dor inflamatória (VALÉRIO et al., 2009).

Esse sistema capaz de perceber o estímulo e transformar em dor é denominado nociceptivo e possui fibras nervosas características distribuídas em todos os tecidos do corpo. As fibras nociceptivas associadas à resposta inflamatória são chamadas de fibra A-delta e fibra C, e quando são sensibilizadas conseguem transduzir estímulos mecânicos, térmicos ou químicos em impulsos elétricos, que são então transmitidos ao sistema nervoso central (SNC) (revisado por FERREIRA et al., 2009).

Tais fibras sensoriais periféricas que saem dos tecidos se dirigem para o SNC de forma organizada, chegando até o gânglio da raiz dorsal (GRD), onde está localizado o corpo celular de cada neurônio. De cada GRD sai então um prolongamento axônico que se dirige para a medula espinal (nervos espinais) ou para o tronco encefálico (nervos cranianos) (DEVOR, 1999 revisado por FERREIRA et al., 2009) e é dessa forma que o estímulo doloroso é transmitido desde a estimulação periférica no tecido lesionado, passando pelo GRD e chegando à medula espinal, de onde as fibras sensoriais se comunicam com neurônios secundários (ou de segunda ordem). No corno dorsal da medula, a comunicação sináptica entre os neurônios primários e secundários acontece por

meio da liberação de neurotransmissores, como o aminoácido excitatório glutamato ou a substância P, que levarão a ativação dos neurônios subsequentes conduzindo a informação dolorosa até o sistema nervoso central supra-espinal, onde esta é então analisada e interpretada em locais específicos no córtex (revisado por FERREIRA et al., 2009).

É importante frisar que em relação à indução da hiperalgesia inflamatória, a liberação dos mediadores ocorre de forma organizada e sequencial, sendo possível então, ao bloquear um dos eventos dessa sequência, inibir a produção dos mediadores seguintes evitando assim sinais e sintomas do processo inflamatório, inclusive a dor (FERREIRA et al., 2009).

Infecções causadas por bactérias, fungos e vírus são comumente associadas à ocorrência de dor, e até recentemente acreditava-se que isso era devido ao efeito indireto das células do sistema imune e da cascata de mediadores inflamatórios produzidos por elas. Contudo, evidências recentes sugerem que patógenos podem ativar diretamente os nociceptores e causar hiperalgesia mecânica e térmica de maneira independente das células imunes, através de mecanismos moleculares que ainda não foram completamente compreendidos (TRAN et al., 2014).

1.3.1 Células da Glia

As células da glia são células auxiliares que possuem a função de suporte ao funcionamento do sistema nervoso central e representam as células mais abundantes, sendo muito mais numerosas que os próprios neurônios. Dois tipos celulares principais, astrócito e micróglia, têm ganhado evidência por sofrerem alterações estruturais e funcionais quando submetidas à estímulos nocivos (GOSSELIN et al., 2010).

A ativação de TLRs na micróglia e astrócitos criticamente contribui para o desenvolvimento e manutenção da dor inflamatória e neuropática através da síntese e liberação de TNF-α e IL-1β (revisado por JI; CHAMESSIAN; ZHANG, 2016).

1.3.1.1 Micróglia

A micróglia constitui a população celular imune residente do sistema nervoso central de mamíferos. São células semelhantes a macrófagos residentes no sistema nervoso central que são encarregadas da vigilância imunológica e da amostragem do microambiente através de seus numerosos prolongamentos celulares, garantindo assim a manutenção da integridade da barreira hematoencefálica (CARNIGLIA et al., 2017; PARKHURST; GAN, 2010).

Na existência de um estímulo nocivo ou agente patogênico é possível observar um aumento na expressão da proteína Iba-1 (molécula adaptadora de ligação ao cálcio ionizado 1) (ITO et al., 2001), cuja expressão é restrita a micróglia e macrófagos e é superexpressa quando estas células são ativadas (IMAI et al., 1996; MARC R SUTER, YEONG-RAY WEN, 2007). Nessa situação, a micróglia responde rapidamente através da liberação de mediadores pró-inflamatórios tais como TNF-α, IL-1β, IL-12, óxido nítrico, prostaglandinas e quimiocinas que auxiliam no recrutamento de outras células do sistema imune para o local afetado, amplificando assim a resposta inflamatória (revisado por CARNIGLIA et al., 2017).

Uma vez controlado o estímulo nocivo, é necessário que a resposta inflamatória seja resolvida e finalizada, e isso ocorre através de um redirecionamento do fenótipo celular das micróglias para um perfil desativado ou imunomodulador, caracterizado pela liberação de citocinas anti-inflamatórias como o fator de transformação do crescimento beta (TGF-β) e a IL-10 (FRANCO; FERNÁNDEZ-SUÁREZ, 2015). Esses mediadores imunomodulatórios inibem a liberação dos fatores pró-inflamatórios por células imunes

e não imunes e promovem regeneração tecidual, facilitando o retorno à homeostase. Quando esta etapa da resposta é alterada, o dano tecidual excessivo pode levar à morte celular e neurodegenração (CARNIGLIA et al., 2017).

Neste ponto, um grande número de diferentes estímulos sinalizando através de receptores purinérgicos e TLRs desempenham um papel crítico na conversão da micróglia a um estado cronicamente ativado, importante para a amplificação e manutenção da dor de forma crônica (JI; CHAMESSIAN; ZHANG, 2016; PARKHURST; GAN, 2010).

1.3.1.2 Astrócito

Os astrócitos formam uma população celular bastante heterogênea, que desempenha numerosas funções críticas como a liberação e reutilização de neurotransmissores, formação da barreira hematoencefálica, regulação da concentração de íons extracelulares, modulação da transmissão sináptica, controle metabólico entre outras (GOSSELIN et al., 2010; JI; CHAMESSIAN; ZHANG, 2016).

Uma vez que o astrócito reconhece um estímulo externo potencialmente nocivo, é observada uma alteração no fenótipo da célula para o chamado estado de ativação, que resulta em astrócitos morfologicamente reativos. Essa ativação é caraterizada por um aumento na expressão da Proteína ácida fibrilar glial (GFAP), aumento dos prolongamentos celulares, redução no reaproveitamento do glutamato e liberação de inúmeras moléculas neuromodulatórias, como quimiocinas por exemplo, que podem potencializar a transmissão sináptica excitatória na medula espinal (CHEN et al., 2014; GOSSELIN et al., 2010).

Quando comparada à micróglia, a ativação dos astrócitos em condições de dor crônica é bem mais persistente, indicando uma contribuição importante no mecanismo que leva à cronicidade da dor (JI; BERTA; NEDERGAARD, 2013). Inúmeros estudos sugerem uma ampla variedade de estímulos nocivos capazes de iniciar a ativação de

astrócitos na medula espinal, tais como em modelos de dor neuropática, inflamatória ou no câncer, confirmando a importância destas células na fisopatologia da dor aguda e crônica (revisado por GOSSELIN et al., 2010).

1.3.2 Canais TRPV1

A produção de mediadores inflamatórios pelas células do sistema imune, incluindo miróglia, leva ao aumento da expressão de receptores e canais iônicos em inúmeras células na região afetada ou mesmo de forma sistêmica (FERREIRA et al., 2009). O Receptor de Potencial Transitório Vanilóide tipo 1(TRPV1) é um canal catiônico não-seletivo expresso em diversos tecidos e especialmente em neurônios sensoriais, incluindo os do gânglio da raiz dorsal (SZALLASI et al., 2007). Fisiologicamente, ele tem sido relacionado com a transdução de estímulos térmicos em potenciais elétricos de ação pelas células nervosas, podendo ainda pode ser ativado por estímulos químicos, como a capsaicina, (FERREIRA et al., 2009). Está envolvido na detecção de temperaturas nocivas ≥43°C e na indução da hiperalgesia térmica em condições inflamatórias (SZALLASI et al., 2007).

Em resposta a estímulos nocivos que vão desde sinais químicos a variações de temperatura, o canal TRPV1 se abre permitindo influxo de cátions, especialmente Ca^{2+}, causando despolarização elétrica no terminal nervoso, gerando potencial de ação que é propagado até o cérebro e resulta na percepção da dor (CHOI et al., 2016).

Desta forma, tem crescido nos últimos anos o número de estudos que utilizam antagonistas do canal TRPV1 em diferentes modelos, visando conhecer melhor suas vias de atuação para buscar opções terapêuticas para tratamento da dor em diferentes tipos de doenças (revisado por SZALLASI et al., 2007).

2. OBJETIVOS

Avaliar os mecanismos envolvidos na hiperalgesia mecânica induzida pelo vírus Dengue com foco na medula espinal, e avaliar no gânglio da raiz dorsal os mecanismos relacionados com a hiperalgesia mecânica e térmica induzidas pelos vírus Chikungunya e pela proteína E2 do vírus Chikungunya.

3.1 Objetivos Específicos

Avaliar:

- o perfil temporal da produção espinal das citocinas TNFα, IL-1β e IL-10 após a infecção intraperitoneal com DENV;
- o efeito sobre a hiperalgesia mecânica induzida pelo DENV em animais tratados com: etanercepte (receptor solúvel de TNF, TNFRII), anakinra (IL-1ra; antagonista do receptor de IL-1), IL-10 recombinante, minociclina (inibidor de micróglia), α-aminoadipate e fluorocitrato (inibidores de astrócito);
- a ativação espinal de astrócitos e micróglia induzida pela injeção intraperitoneal de DENV através da expressão de GFAP e Iba-1 na medula;
- se a inibição espinal das células da glia (astrócitos e micróglia) reduz a hiperalgesia mecânica induzida pelo DENV;
- o efeito hiperalgésico do CHIKV inativado (iCHIKV) em camundongos, comparando com o efeito da proteína recombinante E2 (rE2);
- o efeito da inibição da proteína recombinante E2 por anticorpos na hiperalgesia induzida pelo iCHIKV e pela rE2;

- se a inibição dos canais TRPV1 através de tratamento intratecal com o antagonista do receptor AMG-9810 ou na sua ausência (animais TRPV1$^{-/-}$) a hiperalgesia induzida pelo iCHIKV e rE2 é reduzida;

- o efeito do estímulo com iCHIKV e rE2 sob o influxo de cálcio e ativação dos neurônios do GRD.

3. ARTIGOS

Esta tese é resultado do trabalho realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer e é composta pelos artigos:

"Targeting glia and cytokines in the spinal cord reduces dengue virus-induced hyperalgesia in mice", de autoria de Carina Z. Segato-Vendrameto, Victor Fattori, Amanda Z. Zucoloto, Tiago H. Zaninelli, Stephanie Badaro-Garcia, Larissa Staurengo-Ferrari, Mariana M. Bertozzi, Ana C. Rossaneis, Telma S. Saraiva, Sergio M. Borghi, Allan H. D. Cataneo, Rubia Casagrande, Juliano Bordignon, Waldiceu A. Verri, Jr., que será submetido à revista Neuropharmacology.

"Chikungunya virus and its envelope protein E2 induce hyperalgesia: inhibition by anti-E2 antibodies and TRPV1 antagonist", de autoria de Carina Z. Segato-Vendrameto, Amanda Z. Zucoloto, Victor Fattori, Larissa Staurengo-Ferrari, Stephanie Badaro-Garcia, Tiago H. Zaninelli, Mariana M. Bertozzi, Telma S. Saraiva; Felipe A. Pinho-Ribeiro, Sergio M. Borghi, Camila Zanluca, Rubia Casagrande, Cláudia N. Duarte dos Santos, Waldiceu A. Verri, Jr., que será submetido à revista Frontiers in Pharmacology.

Cada um dos artigos segue as normas de formatação da revista a que possivelmente será submetido.

3.1 Artigo a ser submetido à Neuropharmacology.

Targeting glia and cytokines in the spinal cord reduces dengue virus-induced hyperalgesia in mice.

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Targeting glia and cytokines in the spinal cord reduces dengue virus-induced hyperalgesia in mice.

Dengue is the most rapidly spreading mosquito-borne viral disease in the world, affecting more than 50 million people annually. The virus transmission among humans is mediated by the bites of *Aedes (Stegomyia)* mosquitoes, specially *Ae. aegypti.* Dengue fever (DF), one of the clinical manifestations of Dengue infection, involves an abrupt febrile illness which can last around 7 days, and is characterized by acute fever accompanied by two or more of the following manifestations: nausea, vomiting, rash, headache, petechiae, positive tourniquet test, leukopenia, retroorbital pain, myalgia, and arthralgia of such great intensity. Since there is no effective antiviral agents specific to treat dengue fever or the severe forms of dengue infection, the current treatment remains largely supportive. It is well known that intense pain, like myalgia and polyarthralgia, is one of the main complains among Dengue infected patients and that there is still few information about the mechanisms involved in hipernociception process. So, the aim of the current study was to investigate the participation of glial cells and inflammatory cytokines in the mechanisms of pain induction in Dengue infection. Infection with Dengue virus increased the intensity of mechanical and thermal hyperalgesia starting at 3rd hour after stimulation maintaining until day 7 post infection. The pro-inflammatory cytokines TNF-α, IL-1β levels were significantly increased after 1h of DENV infection and we also observed increased levels of the anti-inflammatory cytokine IL-10 from 3 to 48 hours after infection. In another set of experiments, we demonstrated that Dengue virus induced mechanical hyperalgesia was significantly reduced in animals TNFR1$^{-/-}$, IL-1R$^{-/-}$ and IL-10$^{-/-}$ and also after treatment with the cytokines inhibitors Etanercept, IL-1ra and with recombinant IL-10. qPCR reveled an increase of GFAP and Iba-1 mRNA expression after infection, indicating glial cells activation. Treatment with glia inhibitors Minocycline, Fluorocitrate and α-Aminoadipate efficiently reduced DENV-induced mechanical hyperalgesia. Our results demonstrated that the activation of glia cells and pro-inflammatory cytokines TNF-α and IL-1β and the ant-inflammatory cytokine IL-10 have an important role in the DENV-induced hyperalgesia and, that the inhibition of these molecules and cells demonstrated a great effect in reducing DENV-induced hyperalgesia, showing a therapeutic potential that deserves more investigation.

Keywords: Dengue virus; hyperalgesia; glia; cytokines.

Introduction

Dengue is the most rapidly spreading mosquito-borne viral disease in the world and according to the World Health Organization (WHO), it is the most important arboviral infection affecting more than 100 countries in the tropical and sub-tropical areas, and has been established in both endemic and epidemic transmission cycles. (GUBLER, 1996; WORLD HEALTH ORGANIZATION, 2009). It is widely cited and currently used an estimative that around 50 million dengue infections occur annually, but new researches have demonstrated that this numbers can be actually higher than 300 million cases per year (SAMIR BHATT et al., 2013; WORLD HEALTH ORGANIZATION, 2009).

Dengue virus (DENV), the causative agent of dengue, is a small, positive-sense single-stranded RNA virus that belongs to the genus Flavivirus, family Flaviviridae (CALISHER, 1988). The virus transmission among humans is mediated by the bites of *Aedes (Stegomyia)* mosquitoes, specially *Ae. aegypti,* but some of the Dengue outbreaks have also been related to other mosquitoes of the genus, like *Ae. albopictus, Ae. polynesiensis* and other anthropophilic *Aedes* species (ABELARDO C. MONCAYO et al., 2004).

The virus is classified into four antigenically distinct but closely related serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) that can be found circulating in several countries in the American continent (WORLD HEALTH ORGANIZATION, 2009). Brazil is considered a hyperendemic area when all four serotypes of DENV can be found (VILLABONA-ARENAS et al., 2014), and in the last 10 years, the country has experienced an increase of more than 20-fold in cases and deaths by dengue infection. In 2018, more than 200.000 probable Dengue cases were registered in the country, with the occurrence of 130 deaths (MINISTÉRIO DA SAÚDE, 2018).

Dengue fever (DF), one of the clinical manifestations of Dengue infection, involves an abrupt febrile illness which can last around 7 days, and is characterized by acute fever accompanied by two or more of the following manifestations: nausea, vomiting, rash, headache, petechiae, positive tourniquet test, leukopenia, retroorbital pain, myalgia, and arthralgia of such great intensity. The severe disease, known as Dengue haemorrhagic fever (DHF) progresses to haemorrhagic manifestations, thrombocytopenia, and hemoconcentration, resulting in plasma leakage caused by an increase of vascular permeability. The most lethal Dengue syndrome is well known as Dengue shock syndrome (DSS) and is characterized by circulatory failure (GUBLER, 1996; GUZMAN et al., 2016).

Since there is no effective antiviral agents specific to treat dengue fever or the severe forms of dengue infection, the current treatment remains largely supportive (MURPHY; WHITEHEAD, 2011; WORLD HEALTH ORGANIZATION, 2009). Regardless of the development and approval in certain countries of a tetravalent dengue vaccine, new research is still necessary to develop treatment strategy to achieve individuals that are unable to receive the vaccine and for unvaccinated infected patients (FRABASILE et al., 2017; HADINEGORO et al., 2015).

Regarding the pathogenesis of DENV infection, it remains poorly understood and involves a complex interaction between viral and host factors, but some studies have already shown that inflammatory response, characterized specially by increased cytokines levels, also plays a fundamental role in the disease pathogenesis (COSTA et al., 2013; GUBLER, 1996; KYLE; HARRIS, 2008; SRIKIATKHACHORN; GREEN, 2009).

Higher levels of pro-inflammatory cytokines, chemokine ligands and interferon (IFN)-γ are commonly reported in patients with severe dengue disease. Nevertheless, there is few information about how this massive cytokine production is induced and

controlled (COSTA et al., 2013). What is well known is that transcription factors such as IRFs and NF-κB work together to trigger the production of type I interferons and inflammatory cytokines leading to the maturation of dendritic cells and the establishment of antiviral immunity (SEVERA; FITZGERALD, 2007). Taken together, the latter findings are pointing that the severity of dengue disease is related to an uncontrolled immune cell activation, exacerbated production of pro-inflammatory mediators and consequent endothelial cell dysfunction due to the virus and several immunopathologic mechanisms (COSTA et al., 2013).

It is well known that intense pain, like myalgia and polyarthralgia, is one of the main complains among Dengue infected patients (GUBLER, 1996; GUZMAN et al., 2016; WORLD HEALTH ORGANIZATION, 2009) and that there is still few information about the mechanisms involved in hipernociception process. So, the aim of the current study was to investigate the participation of inflammatory cytokines and glial cells in the mechanisms of pain induction in Dengue infection.

Methods

General in Vivo experimental procedures

Mice (n = 6 per group per experiment, representative of two separated experiments) were injected intraperitoneally with Dengue virus (DENV 20, 200 and 2000 FFU/200µl saline) or Mock (non-infected control, 200 µL, i.p.) and the mechanical and thermal thresholds were evaluated 1, 3, 5 and 7 h after the stimulus injection and daily during seven days. Samples were collected in different time intervals to determine cytokines levels (TNF-α, IL-1β, and IL-10) and to better investigate the role of these cytokines in DENV-induced hyperalgesia, C57/bl-6, TNFR1$^{-/-}$, IL-1r$^{-/-}$ and IL-10$^{-/-}$ animals were infected and mechanical hyperalgesia were evaluated 1 – 7 hours and daily during 7 days post infection. Wild type mice were also infected and treated intrathecally with etanercept, IL-1r antagonist and recombinant IL-10 and mechanical hyperalgesia were evaluated at the

same time points. In an attempt to determine the molecular signaling in the spinal cord nociception induced by DENV, samples were collected in different time points to access mRNA expression of glial cells activation markers Iba-1 and GFAP. Animals were infected with DENV and 72 hours after the stimulus received intrathecal treatment with one of the glial cells inhibitors at indicated doses: Minocycline (microglia inhibitor, 150 µg, i.t.), Fluorocitrate (astrocyte inhibitor, 0,45 µg, i.t.) or α-Aminoadipate (astrocyte inhibitor, 300 nmol, i.t.). The mechanical threshold was measured during 1, 3, 5 and 7 hours after.

Animals

Male Swiss mice, C57/bl-6 (wild type - WT), TNFR1 deficient (-/-), IL-1r$^{-/-}$ and IL-10$^{-/-}$ (25-30 g), were obtained from the Universidade Estadual de Londrina (Parana, Brazil) to develop the experiments. The animals were housed at 22 ± 2 ºC under a 12-h light/12-h dark cycle with free access to food and water ad libitum and for at least 1 day before testing, they were acclimatized to the laboratory. The Ethics Committee for Animal Research of the Universidade Estadual de Londrina (UEL) approved all experiments performed (process number 13216.2017.97) and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (ZIMMERMANN, 1983) and International Association for the Study of Pain (IASP).

Stimuli and drugs

We used as stimuli a clinical isolate from a non-fatal case of DENV-4 genotype II with hemorrhagic manifestations (DENV-4/ LRV13/422; GenBank: KU513441) (KUCZERA et al., 2016) to perform the experiments. As negative control we used Mock (non-infected control). Drugs were obtained from the following sources: etanercept (soluble Tumor necrosis factor receptor - 10ng) from Wyeth Indústria Farmacêutica Ltda (São Paulo,

Brazil), IL-1ra (Interleukin 1 receptor antagonist - 10ng) from NIBSC (National Institute of Biological Standards and Control, UK), both were dissolved in saline. Minocycline, fluorocitrate, and α-aminoadipate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Mechanical hyperalgesia

The method used to test the mechanical hyperalgesia was previously described (GUERRERO et al., 2006). Concisely, the method consists of inducing a flexion reflex of the tibio-tarsal articulation with a hand-held force transducer (electronic von Frey analgesimeter; Insight, Ribeirão Preto, SP, Brazil) adapted with a non-nociceptive tip probe with area size of 4.15 mm^2. The investigator was previously trained to apply the tip perpendicularly to the central area of the plantar surface, inducing the flexion of the hind limb joints. Results were expressed as the flexion-elicited withdrawal threshold (in grams). The intensity of the hyperalgesia was quantified as the change in pressure applied by subtracting the mean of the 3 values obtained in different times after the stimulus injection from the mean of the three values observed before stimulus injection.

Thermal hyperalgesia

Thermal hyperalgesic responses were determined as described by Hargreaves (HARGREAVES et al., 1988). Briefly, mice were individually confined to plexiglass chambers and a high-intensity projector bulb was positioned beneath the right hind paw to deliver a direct thermal stimulus. The withdrawal latency period of injected paws was determined with an electronic clock circuit. Animals were evaluated at 0 and 7 h and daily during 7 days after stimulus and a cutoff latency of 30 s was used to prevent tissue damage. Results are expressed as Paw-withdrawal latency change (s).

TNFα, IL-1β and IL-10 levels determination

Mice received DENV 200FFU intraperitoneal injection and 1, 3, 5, 24, 48 and 72h after samples of spinal cord were collected to evaluate cytokines levels. TNFα, IL-1β and IL-10 levels were determined as described previously (VERRI et al., 2010) by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience). The results were expressed as picograms (pg) of cytokine per 100 mg of tissue.

Quantitative polymerase chain reaction (qPCR)

Animals were terminally anaesthetized and spinal tissue samples were collected at 1, 3, 5, 24, 48 and 72 h after the i.p. injection of DENV, homogenized in 500 µL of Trizol reagent and centrifuged (12,000 rcf x 15 min x 4ºC), and total RNA was measured with a spectrophotometer. The wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations and extracted using the SV Total RNA Isolation System (Promega) (VERRI et al., 2008). All reactions were performed in triplicate using the following cycling conditions: 50 ºC for 2 min, 95 ºC for 2 min, followed by 40 cycles of 95 ºC for 15s and 60 ºC for 30s. The qPCR was performed in a Light Cycler Nano Instrument (Roche, Mississauga, ON, USA) sequence detection system using the Platinum SYBR Green qPCR Super Mix UDG (Invitrogen, USA). The mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were Gapdh forward: CAT ACC AGG AAA TGA GCT TG, reverse: ATG ACA TCA AGA AGG TGG TG; GFAP forward: GGC GCT CAA TGC TGG CTT CA, reverse: TCT GCC TCC AGC CTC AGG TT; Iba-1 forward: ATG GAG TTT GAT CTG AAT GGA AAT, reverse: TCA GGG CAG CTC GGA GAT AGC TTT. The SYBR green PCR Master Mix was used according to the manufacturer's instructions.

Statistical analysis

The presented results are representative of two independent experiments and are expressed as the mean ± SEM (n=6 per group per experiment). Two-way ANOVA followed by Tukey's test was performed to evaluate the differences between responses. Statistical differences were considered to be significant at $P < 0.05$.

Results

Dengue virus induces mechanical and thermal hyperalgesia in mice.

Infection with Dengue virus increased the intensity of mechanical hyperalgesia starting at 3rd hour after stimulus injection. The highest dose (2000FFU) maintained the increased hyperalgesia until day 7 post infection, differing statistically from the lower doses (Fig. 1A) [column factor F (3, 20) = 589.9; P< 0.0001]. As the highest viral load (2000FFU) presented the best result inducing mechanical hyperalgesia alterations, it was chosen to carry out thermal hyperalgesia and we found out a reduction of resistance to thermal stimulus in the infected group compared to Mock (Fig. 1B) [column factor F (1, 10) = 186,2].

Dengue virus induces spinal cord TNF-α, IL-1β and IL-10 production.

The pro-inflammatory cytokines TNF-α and IL-1β (Fig. 2B) levels were significantly increased after 1h of DENV infection (Fig. 2A) [treatment factor F (6, 34) = 3.529; P = 0.0080] and (Fig. 2B) [treatment factor F (6, 35) = 6.550; P = 0.0001]. IL-1β levels were maintained increased until 72h post infection. It also induced increased levels of the anti-inflammatory cytokine IL-10, from 3 to 48h after stimulation (Fig. 2B) [treatment factor F (6, 35) = 8.912; $P < 0.0001$], evidencing the relevant role of these cytokines during the course of infection.

Role of TNF-α, IL-1β and IL-10 in Dengue virus (DENV)-induced mechanical hyperalgesia in mice.

Dengue virus-induced mechanical hyperalgesia was completely inhibited in the absence

of TNFR and IL-1R compared to WT infected mice (Fig. 4 A and B, respectively), demonstrating an important role of the interaction between these cytokines and receptors to induce pain after DENV infection. On the other hand, IL-10 deficient mice presented no statistical difference with wild type infected mice (Fig. 4 C), showing the importance of IL-10 to control DENV-induced hyperalgesia [columm factor F (4, 25) = 593,6]. DENV-induced mechanical hyperalgesia was also significantly reduced after intra-thecal treatment with Etanercept (Fig. 3D), IL-1ra (Fig. 3E) and with recombinant IL-10 (Fig. 3F), confirming the importance of these three cytokines in the hyperalgesia induction by DENV [column factor F (4, 25) = 121,8; $P < 0.0001$].

Dengue virus induces increase of GFAP and Iba-1 mRNA expression in the spinal cord.

Infection with DENV was capable of induce an increase of mRNA expression of astrocyte marker GFAP (Fig.4A) [treatment factor F (6, 35) = 6.830. $P < 0.0001$] at 3, 48 and 72h after stimulation. Microglia's marker Iba-1 also had increased mRNA expression in the spinal cord after 1, 3, 48 and 72h of infection with the virus (Fig. 4B) [treatment factor F (6, 35) = 23.94; $P < 0.0001$].

Dengue virus-induced mechanical hyperalgesia was reduced by treatment with glia inhibitors Minocycline, α-Aminoadipate and Fluorocitrate.

To identify the cells involved in nociception process after Dengue virus infection, we treated infected animal intrathecally with glial cells inhibitors and measured up the mechanical hyperalgesia 7 hours after injection. The DENV-induced increased hyperalgesia was effectively inhibited after 3 hours of intra-thecal treatment with the glial cell inhibitors Minocycline (microglia inhibitor) (Fig. 5A) [column factor F (2, 15) = 590.3; $P < 0.0001$], α-Aminoadipate (Astrocyte inhibitor) (Fig. 5B) [column factor F (2, 15) = 587.4; $P < 0.0001$] and Fluorocitrate (astrocyte inhibitor) (Fig. 5C) [column factor F (2, 15) = 545.4 $P < 0.0001$].

Discussion

Dengue is currently one of the world's most important tropical diseases and its incidence has increased more than 30 fold in the last few decades together with the geographical expansion of its vector, the Aedes mosquitoes, and with dengue viruses (GUZMAN et al., 2016). Among the main symptoms, pain is a prominent feature of acute dengue as well as a clinical criterion in World Health Organization guidelines in diagnosing dengue, and together with fever, it is the most common clinical findings after dengue infection in humans (WORLD HEALTH ORGANIZATION, 2009). Our results reproduced pain symptom in mice after DENV-4 systemic infection (via intra-peritoneal) with an increase of mechanical and thermal hyperalgesia that lasts at least 7 days, corroborating with other study that showed hipernociception in response to mechanical stimulation after DEN-2 inoculation remaining at high levels during several days (SOUZA et al., 2009).

Infection kinetic studies carried out with a similar inoculum of DENV observed that lethality was preceded by significant changes in cytokine levels, together with other cellular and molecular changes (SOUZA et al., 2009). We evaluate cytokine production after DENV infection and results shown an increased production of IL-1β, TNF-α and IL-10. According to Poole and co-workers, TNF-α and IL-β are released in animals challenged with nociceptive stimuli, and these cytokines are responsible for triggering mechanical hypernociceptive inflammatory pathways, resulting in hypernociception (POOLE, S., CUNHA, F. Q. & FERREIRA, S. H. (1999) IN CYTOKINES AND PAIN, EDS. WATKINS, L. R. & MAIER, 1999). In fact, more recent study have demonstrated that injecting TNF-α and IL-β in mice paw induced mechanical hypernociception, confirming the connection between these inflammatory cytokines and pain (CUNHA et al., 2005).

It was also shown that high levels of cytokines produced by T cells, macrophages/monocytes and endothelial cells (TNF-α, IFN-γ) contributed to endothelial cell damage and hemorrhagic manifestations, been related to the severe form of Dengue (ARIAS et al., 2014). In this sense, the inhibition of cytokine production or action can be an important target for new therapeutic approaches to modulate the inflammatory pain. So, we tested whether knocking out TNFR1, IL-1R and IL-10 cytokine in mice would affect DENV-induced hyperalgesia. We observed a significant alteration in the behavioral pattern of the knocked out animals, demonstrating that these cytokines have a critical role in DENV-induced hyperalgesia. To confirm theses data, we used intra-thecal treatment with TNF-α and IL-1β receptors antagonists and with recombinant IL-10. Our results showed one more time that modulating the action of both, pro-inflammatory and anti-inflammatory cytokines could affect DENV-induced hyperalgesia, indicating a therapeutic potential of these modulation.

Drugs that target downstream common pathways such as TNF-α and IL-1β signaling could be very effective in pain treatment, if the side effects can be tolerated (Suter, 2008). It was already demonstrated that treatment with anti-TNF-α antibodies significantly reduced mortality in a mouse model of lethal dengue virus, emphasizing the role of cytokines in dengue pathogenesis (ATRASHEUSKAYA, A., PETZELBAUER, P., FREDEKING, T.M., IGNATYEV, 2003), confirming that inhibiting these cytokines to treat pain deserves further investigation.

Clinical studies has also demonstrated that patients with DHF has an enhanced level of IL-10 in the serum. It has been related to a shift in the immune response pattern for Th2, playing a negative feedback for pro-inflammatory cytokine and prejudicing the immune response against the virus, associating it to the severe form of Dengue (ABHISHEK et al., 2017; CHATURVEDI et al., 2000). So, it is necessary further

investigation to fully understand this cytokine role in the inflammatory pain after DENV infection.

Knowing that astrocytes and microglial cells can produce and respond to many of the same cytokines employed by immune system cells, and that microglia activation is a long-standing problem for chronic pain, targeting glial cells should be an effective strategy for clinical management of pain. So we questioned if DENV infection could lead to an activation of these glial cells, contributing to the hyperalgesia effect seen before. We checked mRNA expression for GFAP and Iba-1 and figured out an increased level of both of them, indicating astrocyte and microglia activation. It was already demonstrated that DENV2 and DENV4 have the capability to induce reactive oxygen species production and activate microglia, which have been reported as the key components of neuronal damage (OPASAWATCHAI et al., 2019), but there is no data relating this activation to pain in DENV models.

So we treated the infected animals with glial cells inhibitors Minocycline, Fluorocitrate and α-Aminoadipate and demonstrated a significant reduction of DENV-induced hyperalgesia. This results are in agreement with other behavioral studies on glia regulation of pain using fluorocitrate, an anti-metabolic drug which selectively disrupts the Krebs energy cycle of glia by inhibiting the glia-specific enzyme aconitase. According to these studies reviewed by Suter and Wen, fluorocitrate is effective in alleviate pain behavior in animal models of inflammatory pain, neuropathic pain, and postoperative pain. Minocycline in its turn, is a tetracycline antibiotic commonly used as a microglia inhibitor and shows efficacy in several pain models. Drugs such as minocycline, which have already been used clinically for other indications, are certainly easier to get approved for pain treatment than new compounds (MARC R SUTER, YEONG-RAY WEN, 2007).

Our group have already demonstrated that glial cell activation has important role in inflammatory and chronic pain in different experimental settings, and modulate these cells and the mediators produced by them can be a good strategy to treat pain (FATTORI et al., 2017a, 2017b, 2018; ZARPELON et al., 2016).

In conclusion, we find out that Dengue virus infection induce mechanical hyperalgesia in mice enhancing cytokine levels and activating astrocyte and microglia. The inhibition of these molecules and cells demonstrated a great effect in reduce DENV-induced hyperalgesia, showing a therapeutic potential that deserves more investigation.

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Figure Captions

Fig. 1. Dengue virus induces mechanical and thermal hyperalgesia in mice. Animals received intra-peritoneal (i.p.) injection of DENV (20, 200 and 2000 FFU, 100ul) or Mock (control, 100ul). The mechanical (Fig. 1A) hyperalgesia were evaluated 1 – 7 hours after infection and daily during seven days. Thermal hyperalgesia (Fig. 1B) was evaluated 5 hours after infection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; ANOVA followed by Tukey's test.

Fig. 2. Dengue virus induces spinal cord IL-1β, TNF-α and IL-10 production. After 1, 3, 5, 24, 48 and 72 hours of i.p. infection with DENV (2000 FFU), spinal cord samples were collected to levels determination by ELISA (Fig. 2 A to C). Results are presented as means ± S.E.M. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to Mock group; ANOVA followed by Tukey's test.

Fig. 3. Role of TNF-α, IL-1β and IL-10 in Dengue virus-induced hyperalgesia. DENV (2000 FFU) or Mock were injected intraperitoneally in C57/bl-6, TNFR1$^{-/-}$, IL-1r$^{-/-}$ and IL-10$^{-/-}$ animals and mechanical hyperalgesia were evaluated 1 – 7 hours after infection and daily during seven days (Fig. 3 A to C). In the same way, Swiss mice were infected with DENV (2000 FFU) or Mock and 5 minutes later received intra-thecal treatment with Etanercept (10 ng, i.t.), IL-1ra (10ng, i.t.) or recombinant IL-10 (3 ng, i.t.) and mechanical hyperalgesia were also evaluated 1 – 7 hours after infection and daily during seven days (Fig. 3 D to F). The Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; ANOVA followed by Tukey's test.

Fig. 4. Dengue virus induces increase of GFAP and Iba-1 mRNA expression in the spinal cord. Animal were injected i.p. with DENV (2000 FFU) and spinal cord samples were collected 1, 3, 5, 24, 48 and 72 hours to determine GFAP and Iba-1 mRNA

expression analysis by qPCR (Fig. 4 A and B respectively). β-actin was a reference gene to normalize mRNA expression data. Results are presented as means ± S.E.M. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to Mock group; ANOVA followed by Tukey's test.

Fig. 5. Glial inhibitors Minocycline, Fluorocitrate and α-Aminoadipate reduce Dengue virus-induced mechanical hyperalgesia in mice. Animals received intra-peritoneal (i.p.) injection of DENV (2000 FFU) or Mock (control) and 72 hours later received intra-thecal treatment with Minocycline (microglia inhibitor, 150 μg, i.t.), Fluorocitrate (astrocyte inhibitor, 0,45 μg, i.t.) or α-Aminoadipate (astrocyte inhibitor, 300 nmol, i.t.). The mechanical hyperalgesia were evaluated 1 – 7 hours after infection (Fig. 5 A to C). Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; ANOVA followed by Tukey's test.

Figures

Fig. 1.

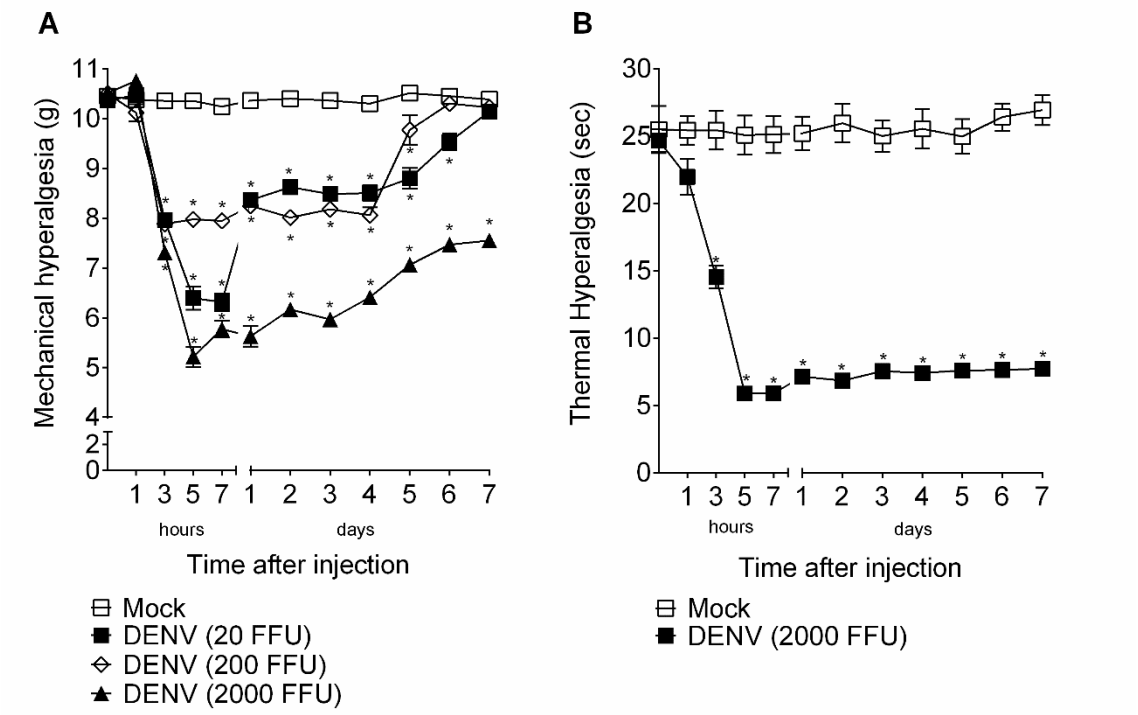


Fig. 2.

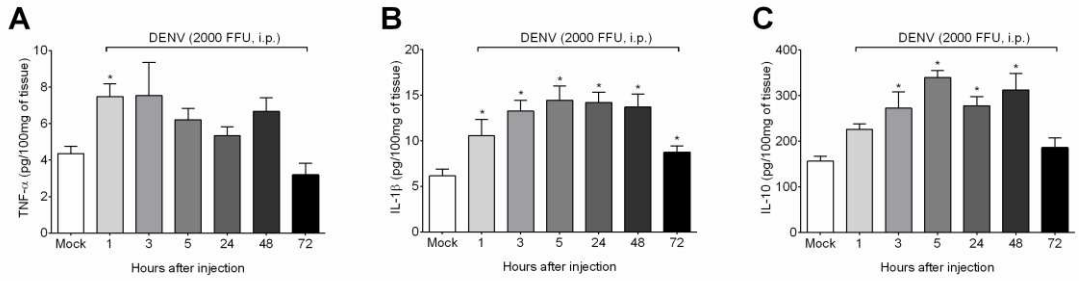


Fig. 3.

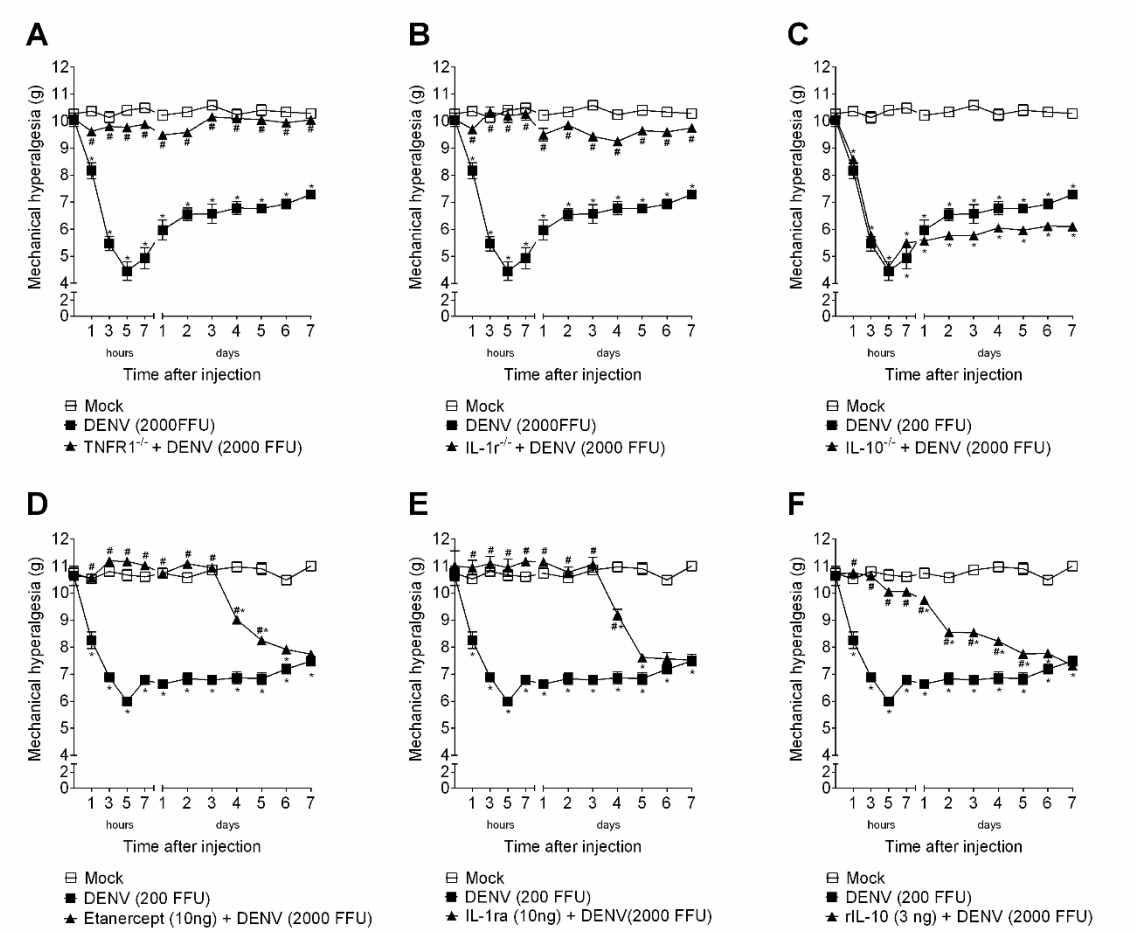

A
B
C
D
E
F
Mechanical hyperalgesia (g)
hours
days
Time after injection
Mock
DENV (2000FFU)
TNFR1-/- + DENV (2000 FFU)
IL-1r-/- + DENV (2000 FFU)
DENV (200 FFU)
IL-10-/- + DENV (2000 FFU)
Etanercept (10ng) + DENV (2000 FFU)
IL-1ra (10ng) + DENV(2000 FFU)
rIL-10 (3 ng) + DENV (2000 FFU)

Fig. 4.

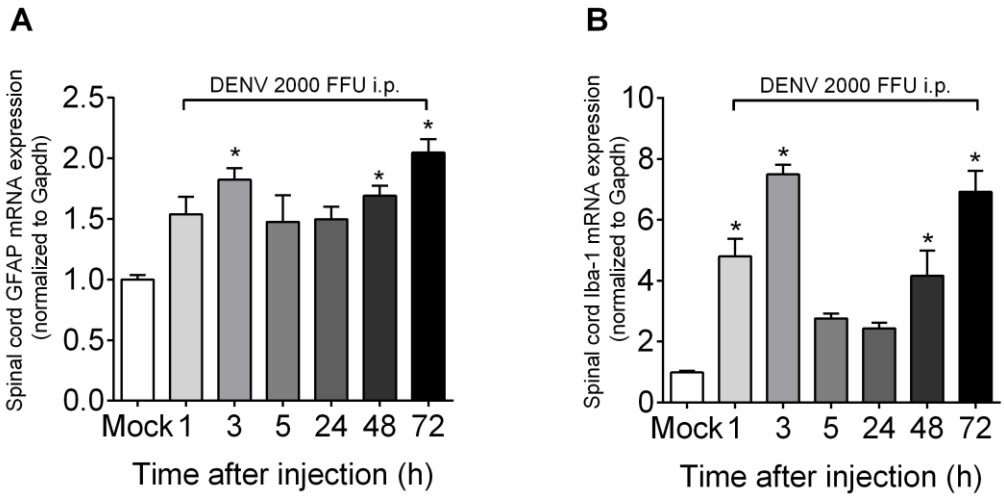
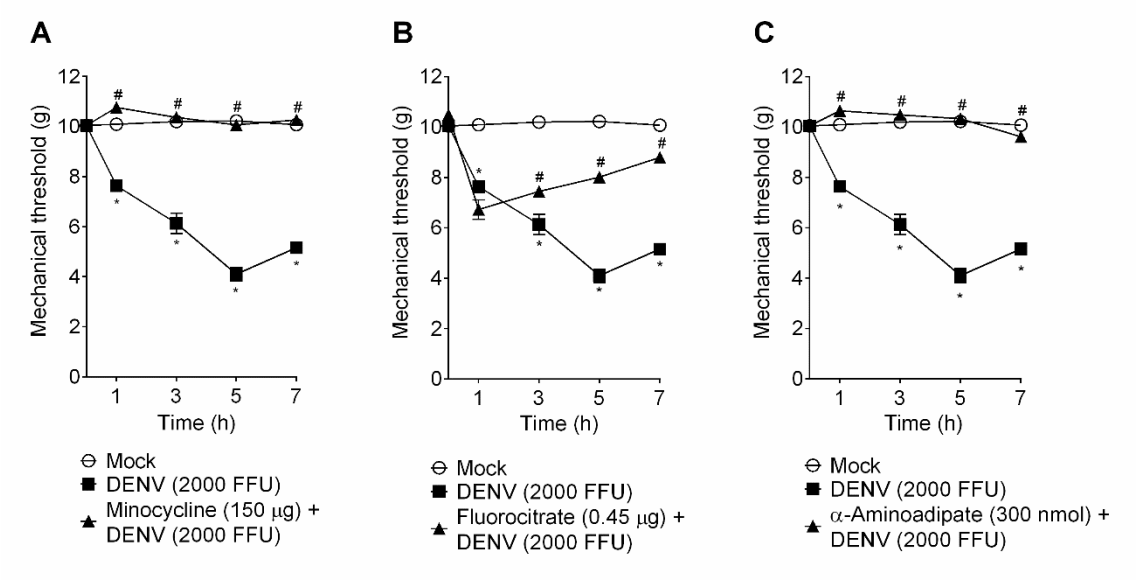

A
B
DENV 2000 FFU i.p.
Spinal cord GFAP mRNA expression (normalized to Gapdh)
Spinal cord Iba-1 mRNA expression (normalized to Gapdh)
Mock 1 3 5 24 48 72
Time after injection (h)

Fig. 5.


A
B
C
Mechanical threshold (g)
Time (h)
Mock
DENV (2000 FFU)
Minocycline (150 μg) + DENV (2000 FFU)
Fluorocitrate (0.45 μg) + DENV (2000 FFU)
α-Aminoadipate (300 nmol) + DENV (2000 FFU)

3.1 Artigo a ser submetido à revista Frontiers in Pharmacology.

Chikungunya virus and its envelope protein E2 induce hyperalgesia: inhibition by anti-E2 antibodies and TRPV1 antagonist.

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Keywords: Chikungunya virus; E2 envelope protein; monoclonal antibody; articular pain; TRPV1.

Abstract

Chikungunya virus (CHIKV) is an arthritogenic alphaviruses, arthropod-borne infectious agent responsible for causing the Chikungunya fever disease. The initial stage of the disease considered acute is characterized as an abrupt clinical onset of crippling joint pains, high fever and rash, being easily confused with dengue. About 90% of the infected patients experience intense polyarthralgia, affecting mainly the extremities but also the large joints as knees and shoulders, leading to loss of daily activities and incapacitation. Chronic disease may be established, with symptoms persisting for months even after clearance of the virus from the blood. Currently, there is no effective antiviral specific for Chikungunya and patient's treatment is purely symptomatic and based on non-salicylate analgesics and non-steroidal anti-inflammatory drugs. Once the envelop proteins are capable of stimulating immune response against CHIKV becoming important therapeutic target, we investigate the participation of recombinant E2 (rE2) protein in hyperalgesia induction, as well as the possibility of treatment with monoclonal antibodies and TRPV1 antagonist to control pain. Inactivated Chikungunya virus and recombinant E2 protein were capable of inducing hypersensitivity to mechanical and thermal stimulation. This effect were efficiently inhibited by treatment with three different types of anti-rE2 monoclonal antibodies. Mechanistically, inactivated CHIKV (iCHIKV) and rE2-induced mechanical and thermal hyperalgesia by triggering DRG neurons calcium influx and activation of TRPV1 channels signaling pathway. These corroborate with the observation

that the absence or blockage of TRPV1 channels reduced the iCHIKV and rE2-induced hyperalgesia. Our results demonstrated the participation of E2 envelop protein in the mechanical and thermal hyperalgesia induction, so its inhibition by anti-E2 monoclonal antibodies and the absence or blockage of TRPV1 channels are options to modulate the hyperalgesic response. Further, DRG neurons from iCHIKV and rE2-stimulated mice also presented a higher baseline level of calcium influx indicating neuron activation. Therefore, the therapeutic potential of E2 monoclonal antibodies to control CHIKV induced articular pain deserves further investigation.

Introduction

Chikungunya virus (CHIKV) is an arthritogenic alphaviruses, arthropod-borne infectious agents responsible for causing the Chikungunya fever disease (CAGLIOTI et al., 2013; HER et al., 2009). First isolated in Tanzania in 1952 (ROBINSON, 1955), CHIKV attracted worldwide attention when it resurged causing a massive outbreak since 2006, notably in Reunion Island, and more recently in the Caribbean, South America and other countries of Europe (BURT et al., 2012; GÉRARDIN et al., 2011; LABEAUD; BASHIR; KING, 2011). Two strains of *Aedes* mosquitoes are capable to begin the cycle of transmission, *A. aegypti* and *A. albopictus* (CAGLIOTI et al., 2013; WEAVER et al., 2012).

After bite of CHIKV-infected female mosquitoe, there is a silent incubation period lasting about 2-4 days (range 1-12 days) which after that not all individuals infected with the virus will develop symptoms (LAM et al., 2001). The ones who develop the symptoms can experience it first in acute stage, which is characterized as an abrupt clinical onset of crippling joint pains, high fever and rash, being easily confused with dengue (BORGHERINI et al., 2008; LAM et al., 2001; ROSS, 1956; STAPLES; BREIMAN; POWERS, 2009). About 90% of the infected patients experience intense myalgia and polyarthralgia, affecting mainly the extremities (ankles, wrists, phalanges) but also the large joints as knees and shoulders, being usually both joints, left and right affected (LAM et al., 2001; PIALOUX et al., 2007; ROBINSON, 1955). In some cases, chronic disease may be established, with symptoms persisting even after clearance of the virus from the blood.

Chikungunya virus genome consists in a single-strand RNA molecule encoding four nonstructural proteins (nsP1-4) that are required for virus replication, and three structural proteins (C, E1, E2) (CAGLIOTI et al., 2013), being E1 a class II viral fusion protein, while E2 mediates receptor attachment (SELVARAJAH et al., 2013). The use of the structural proteins as targets to immunization and therapy against CHIKV infection have received attention over the last few years (BROECKEL et al., 2017; EDELMAN et al., 2000; PARASHAR; CHERIAN, 2014; PARTIDOS et al., 2012; WEAVER et al., 2016). Recent results have been achieved by researchers using recombinant E2 protein to develop different candidate vaccines against CHIKV. Recent study showed that immunization of AG129 mice using a recombinant Modified Vaccinia Ankara (MVA) expressing E2 or E3E26KE1 elicited neutralizing antibodies in all animals and provided 100% protection against lethal disease. They concluded that MVA expressing the glycoprotein E2 of CHIKV represents an immunogenic and effective candidate vaccine against CHIKV infections (VAN DEN DOEL et al., 2014).

The envelope and capsid proteins of CHIKV are involved in the capacity of the virus to elicit both, cellular and humoral immune responses once the E2 protein epitope E2EP3 is identified by $TCD4^{+}$ activated cells inducing IFN-γ production and culminating in joint swelling and inflammation, which was prevented by treatment with the

Fingolimod, an agonist of sphingosine 1-phosphate receptor (HOARAU et al., 2010; TEO et al., 2017). These results show an important participation of E2 protein in joint inflammation induced by CHIKV. On the other hand, concerning humoral immune response, it was verified that E1/E2 and capsid proteins are the main antigens driving the response in both patients with acute and chronic disease (HOARAU et al., 2010). Thus, targeting E2 protein or the effect elicited by this protein could represent a strategy to treat the symptoms as inflammation and to avoid collateral effects as swelling and pain.

Concerning treatment, currently there is no effective antiviral specific for Chikungunya. Treatment is purely symptomatic and based on non-salicylate analgesics and non-steroidal anti-inflammatory drugs (PIALOUX et al., 2007). They usually receive fluids and medicines to relieve symptoms of fever and aching, such as ibuprofen, naproxen, acetaminophen or paracetamol. Studies have reported efficacy in the use of antivirals like interferon-α and ribavirin on chikungunya virus infection and, despite the use of chloroquine has been suggested, trials have failed to confirm its efficacy on CHIKV associated arthralgia (CAGLIOTI et al., 2013; PIALOUX et al., 2007).

In this context, it's well known the efficacy of therapeutic use of human monoclonal antibodies (mAbs) against E2 protein. Warter and colleagues (2011) observed that two mAbs specific for the CHIKV envelope proteins E1 and E2 neutralize CHIKV infection in vitro, showing potential to be therapeutic tools against CHIKV infection (WARTER et al., 2011). Selvarajah and coworkers (2013) have also observed that a human neutralizing mAb targeting the acid-sensitive region in CHIKV E2, when administered therapeutically at 8 and 18 hours after infection, completely protected 100% of mice, confirming its potential to prophylactic and therapeutic protection against viremia also *in vivo* (SELVARAJAH et al., 2013).

Recently, Teo and coworkers (TEO et al., 2017) identified dominant $CD4^+$ T cell epitopes in nsP1 and E2 viral antigens capable of recapitulating joint swelling, vascular leakages, edema, and inflammation and necrosis of the muscles when primary $CD4^+$ T cell pathogenic epitope-specific cell lines were transferred into virus-infected T cell receptor–deficient ($TCR^{-/-}$) mice. Since they found that E2 protein is involved in host inflammatory response against the virus and pain seems to be one of the most critical symptoms involved in this process, and due to the importance of the structural proteins as a potential therapeutic and prophylactic target, we aimed to investigate the participation of the E2 protein in the hyperalgesia induction, as well as the possible mechanisms by which it happens, targeting TRPV1. We also investigate the treatment with monoclonal antibodies capacity to control iCHIKV or rE2-induced pain.

Methods

Experimental protocols

Mice received intra-articular (i.a.) injection of inactivated Chikungunya virus (iCHIKV; 1, 10, 100 and 1000 FFU/10µl saline), recombinant E2 (rE2) protein (30 and 100 ng/10µl saline) or Mock (10 µL). The mechanical hyperalgesia was measured 1, 3, 5 and 7 h after the stimulus injection and daily during seven days. The best virus and protein doses were chosen for the following experiments based on mechanical hyperalgesia. In order to evaluate the possibility of modulating iCHIKV and rE2 protein-induce hyperalgesia, co-treatments with anti-rE2 antibodies (AbM1, AbM2, AbM3, 1 and 5 µg, i.a.) were performed with the inactivated virus or with the protein. Mechanical hyperalgesia was evaluated 1, 3, 5 and 7 h after the co-treatment and daily during seven days. To evaluate the participation of TRPV1 receptor in this model we injected $TRPV1^-$

/- mice with iCHIKV 100 FFU, rE2 0,01 µg or Mock and measured the mechanical hyperalgesia 1, 3, 5 and 7 h and the thermal hyperalgesia 7 h after the stimulus injection. We also treated C57bl6 animals with TRPV1 antagonist AMG-9810 (100 nmol, intrathecal, Cayman Chemical, Ann Arbor, Michigan, USA) in order to confirm the importance of this receptor in the iCHIKV or rE2-induced pain. Based on TRPV1 mechanical hyperalgesia peak, thermal hyperalgesia were evaluated 7 hours after stimulus. Ipsilateral dorsal root ganglia (DRG from L4-L6 spinal cord segments) were also dissected to calcium imaging using confocal microscopy.

Animals

We used male Swiss, C57bl/6 and TRPV1-/- mice (25-30 g) obtained from the Londrina State University (Parana, Brazil) to perform the experiments. The animals were housed at 22 ± 2 ºC under a 12-h light/12-h dark cycle with free access to food and water ad libitum and were acclimatized to the laboratory for at least 1 h before testing. The Ethics Committee for Animal Research of the Universidade Estadual de Londrina (UEL) approved all experiments performed (process number 13216.2017.97) and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (ZIMMERMANN, 1983) and International Association for the Study of Pain (IASP).

Inactivation of Chikungunya virus

CHIKV BR_2015/15010 was isolated from the serum of a patient with chikungunya disease from Northeast Brazil in 2015. The virus was amplified, titrated by foci-forming assay in C6/36 cells (DESPRÈS; FRENKIEL; DEUBEL, 1993) and inactivated using β-propiolactone (0.025%, 72h, 4ºC). The inactivated virus was concentrated by PEG 7%/NaCl 2.3% precipitation and purified using sucrose cushion. A noninfected control (Mock) was prepared in the same manner from β-propiolactone inactivated C6/36 cell-culture supernatant.

Recombinant E2 protein synthesis

The CHIKV recombinant E2 protein (rE2) was expressed by *Drosophila* S2 cells. S2 cells were co-transfected with the plasmids pMt/Bip/V5-HisA (Invitrogen) containing the gene of the E2 protein from CHIKV and pCoBlast. The transfected cells were selected with 25 µg/mL of blasticidin and cultured in SF900II (Invitrogen) medium with 25 mg/mL gentamicin (Gibco). The rE2 protein expression was induced by 700 mM of $CuSO_4$ for 24 h, and the protein was purified from the cell-culture supernatant by immobilized metal *affinity chromatography* (using Ni-NTA agarose resin). A Mock control was prepared from the supernatant of S2 cells induced by 700 mM of $CuSO_4$ and purified following the same protocol. The rE2 expression was confirmed by SDS-PAGE and Western blot using anti-V5 antibodies (Supplementary Figure 1).

Anti-rE2 antibodies synthesis

Hybridomas secreting anti-rE2-CHIKV monoclonal antibodies (MAbs) were obtained by the fusion of mouse myeloma cell line P3X63Ag8.653 (ATCC CRL-1580) and splenocytes from Balb/c mice immunized with the rE2-CHIKV protein following the protocol described previously (ZANLUCA et al., 2014). Three hybridomas were obtained. MAbs were purified on a protein-G column (GE-Healthcare) and quantified

using the Micro BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions.

Mechanical hyperalgesia

Mechanical hyperalgesia was tested in mice as previously reported (GUERRERO et al., 2006). Briefly, the test consists of evoking a tibio-tarsal flexion reflex with a hand-held force transducer (electronic von Frey analgesimeter; Insight, Ribeirão Preto, SP, Brazil) adapted with a non-nociceptive tip probe with area size of 4.15 mm^2. The investigator was trained to apply the tip perpendicularly to the central area of the plantar surface, inducing the flexion of the hind limb joints. The results were expressed as the flexion-elicited withdrawal threshold (in grams). The intensity of the hyperalgesia was quantified as the change in pressure applied by subtracting the mean of the 3 values obtained in different times after the stimulus injection from the mean of the three values observed before stimulus injection.

Thermal hyperalgesia

Thermal hyperalgesic responses were determined as described by Hargreaves (HARGREAVES et al., 1988). Briefly, mice were individually confined to plexiglass chambers and a high-intensity projector bulb was positioned beneath the right hindpaw to deliver a direct thermal stimulus. The withdrawal latency period of injected paws was determined with an electronic clock circuit. Animals were evaluated at 0 and 7 h after stimulus and a cutoff latency of 30 s was used to prevent tissue damage. Results are expressed as Paw-withdrawal latency change (s).

Calcium Imaging

Adult wildtype swiss mice were injected with iCHIKV, rE2 or Mock and euthanized with isoflurane after 7 hours of stimulation. Dorsal root ganglia were dissected into DMEM (Invitrogen, Darmstadt, Germany), dissociated in collagenase A (1 mg/mL)/dispase II (2.4 U/mL) (Roche Applied Sciences 04942078001) in HEPES-buffered saline (Sigma 51558-50ML) (5 mM $CaCl_2$) at 37C for 20 min. The ganglia were then triturated with pipettes with decreasing size and dissociated neurons were incubated with laminin for 45 min at 37ºC, plated and imaged in Confocal Microscope (TCS SP8, Leica Microsystems, Mannheim, Germany). To assess neuron activation, DRG plates were recorded for 2 minutes of initial reading to achieve fluorescence baseline values.

Statistical analysis

The presented results are representative of two independent experiments and are expressed as the mean ± SEM (n = 6 per group per experiment). Two-way ANOVA followed by Bonferroni's test was performed to evaluate the differences between responses. Statistical differences were considered to be significant at $P < 0.05$.

Results

Inactivated Chikungunya virus (iCHIKV) and recombinant E2 (rE2) protein induced mechanical hyperalgesia in mice.

Significant mechanical hyperalgesia was observed starting at the 3rd hour after iCHIKV injection and with highest doses maintained its levels until day 2 (Fig. 1A).

There is no significant difference between the doses of 100 and 1000FFU, therefore, we chose the lower one to perform the next experiments. As the inactivated virus was capable to induce significant hyperalgesia, we tested whether the rE2 protein is involved in this process of inducing articular pain. We found that the highest dose of recombinant protein also induced significant hyperalgesia starting ate the 3rd hour and maintaining until day 3 (Fig. 1B). We chose the dose of 100 ng to perform the next experiments. Experimental groups were compared to Mock as control and this one was compared to saline group but no difference were found (data not shown).

Recombinant E2 protein and inactivated CHIKV-induced mechanical hyperalgesia is inhibited by monoclonal antibodies

We observed that the dose of 5 µg of AbM1 and AbM2, and 1 µg of AbM3 showed efficacy in inhibiting rE2-induced mechanical hyperalgesia significantly (Fig. 2 A, B and C respectively). Once it was demonstrated that the three antibodies were efficient inhibiting the effect of rE2 protein, we demonstrated that they were also capable of inhibiting iCHIKV-induced mechanical hyperalgesia (Fig. D, E and F), which demonstrated the participation of E2 protein in the process and the possibility of modulating pain with antibody treatment. Experimental groups were compared to Mock as control and this one was compared to saline group but no difference were found (data not shown).

Chikungunya inactivated virus and rE2 protein-induced mechanical hyperalgesia is inhibited in the absence or blockage of TRPV1 receptor.

The TRPV1 knockout animals do not presented increased mechanical hyperalgesia in answer to the stimulation with iCHIKV or rE2 (Fig. 3 A and B). We also observed that treating C57bl/6 mice with TRPV1 antagonist AMG-9810 (100 nmol, i.t.) 5 minutes after the stimulation with iCHIKV or rE2 prevented the increased of mechanical and thermal hyperalgesia (Fig. 3 C and D), suggesting the importance of this receptor in Chikungunya virus induced-pain.

Chikungunya inactivated virus and rE2 protein-induced thermal hyperalgesia in mice is inhibited by anti-rE2 monoclonal antibodies and in the absence or blockage of TRPV1 receptor.

After observing the participation of TRPV1 receptors in mechanical hyperalgesia induction, we aimed to evaluate the participation of E2 protein in inducing thermal hyperalgesia. We found out that iCHIKV and rE2 protein were capable of inducing thermal hyperalgesia and our results showed a significant response after 7 hours of stimulation (Fig. 4 A and B). Therefore, we treated animals with anti-rE2 monoclonal antibodies and observed a reduction of the thermal sensitivity after co-injection with rE2 protein (Fig. 4 C, D and E) or inactivated CHIKV (Fig. 4 F, G and H). The TRPV1 knockout animals were also tested for thermal hyperalgesia and it do not present significant increase in answer to the stimulation with iCHIKV nor rE2 (Fig. 4 I and J). We also observed that treating C57bl/6 mice with TRPV1 antagonist AMG-9810 (100 nmol, i.t.) 5 minutes after the stimulation with iCHIKV or rE2 prevented the increased of thermal hyperalgesia (Fig. 4 K and L).

DRG neurons from iCHIKV and rE2-stimulated mice presented a higher baseline level of calcium influx.

DRG neurons from iCHIKV and rE2-stimulated mice presented a higher baseline level of calcium influx than DRG neurons from Mock mice (Fig. 5 A and B). As an increase on calcium influx is considered an indicative of DRG neuron activation (BLAKE et al., 2018; TRAN et al., 2014), our data suggest pain in this model is due to peripheral sensitization after the infection with the virus and its protein.

Discussion

Chikungunya fever is a disease well known by causing severe joint pain in almost the totality of the infected patients, making pain a characteristic symptom of this infection (ROBINSON, 1955). Discomfort in performing the activities of everyday life (e.g., walking, eating and getting dressed) can be present in about 50% of patients (BORGHERINI et al., 2008), so erratic, relapsing, and incapacitating long lasting arthralgia is considered the hallmark of Chikungunya fever (PIALOUX et al., 2007). This scenario proves the economic impact of CHIKV infection, a disease which, considering the globalization of its competent vectors, is at risk of becoming a major public health threat.

The E2 envelop protein has been appointed as one of the viral epitope responsible for eliciting immune response, causing joint inflammation and swelling (HOARAU et al., 2010; TEO et al., 2017). However, it is not known whether pain is associated with E2 protein. That way, in this study we showed that E2 protein is involved in Chikungunya virus-induced joint pain. We found out that after the injection with inactivated virus, the animals presented increased mechanical hyperalgesia, and a similar effect was observed after stimulus with recombinant E2 protein. To our knowledge, this is the first demonstration that this protein induces pain.

Previous studies have demonstrated that persistent arthralgia, often continuous and debilitating, is a frequent concern among patients with chikungunya virus infection, especially among middle-aged and elderly patients, one of the populations most affected in the majority epidemics (BORGHERINI et al., 2008). At the acute stage of CHIKV fever, lost productivity is a frequent concern and more than 70% of affected patients usually took time off from work (STAIKOWSKY et al., 2008). In the same way, the chronic disease is characterized as a severe and persistent arthralgia which can least months or even years affecting the patient's economic and social life (BORGHERINI et al., 2008; GÉRARDIN et al., 2011; LABEAUD; BASHIR; KING, 2011).

The social and economic impact of CHIKV epidemics underlines the exigency for accurate diagnostics and effective anti-viral and analgesic therapeutics, since vaccines are not yet available (GOH et al., 2013). Monoclonal antibodies have been used as standards for laboratory-based diagnoses of arthritogenic alphavirus diseases, including CHIKV, and have been developed in a number of countries (SUHRBIER; JAFFAR-BANDJEE; GASQUE, 2012). The alphavirus E2 protein is thought to be involved in virus attachment to host cell receptors and contains critical binding sites for neutralizing antibodies (KIELIAN; CHANEL-VOS; LIAO, 2010). In this sense, we used treatment with three different types of anti-rE2 monoclonal antibodies to demonstrate that the inactivation of E2 protein have therapeutic potential to treat pain in Chikungunya patients, once it was capable of inhibit mechanical hyperalgesia.

A recent study described the generation and characterization of five monoclonal antibodies specific for the E2 glycoprotein of CHIKV without evidence of cross-reactivity with other alphaviruses, two of which provided complete protection against arthritis in a CHIKV mouse model when administered prior to infection (GOH et al., 2013). As we observed increased pain response after stimulus with E2 protein, we next

wonder whether antibodies against it would be effective in reducing this symptom. We observed that anti-E2 antibodies effectively reduced pain, indicating that this protein might be important to pain in CHIKV infection. To our knowledge, this is the first demonstration of the use of anti-E2 monoclonal antibodies in the treatment of pain after Chikungunya infection.

It is well known that the ion channel transient receptor potential vanilloid 1 (TRPV1) plays an important role in pain processing, being involved in the detection of thermal and mechanical hyperalgesia (BERTA et al., 2017; CHOI et al., 2016). Expressed predominately by primary nociceptive neurons, TRPV1 receptor is a ligand-gated, non-selective cation channel (BARTON et al., 2006; MICHAEL J. CATERINA, TOBIAS A. ROSEN, MAKOTO TOMINAGA, 1999) associated not only with a rise in the intracellular calcium but also with pro-inflammatory gene expression (GOUIN et al., 2017). The lack of data regarding mechanistic details involved in CHIKV-induced joint pain underscores a need to search for specific therapeutic targets to control it. Given we observed CHIKV and rE2-induced thermal hyperalgesia, we decided to investigate the participation of TRPV1 in Chikungunya induced-pain. To test that, mice TRPV1$^{-/-}$ were infected with iCHIKV and rE2. We observed that both iCHIKV and rE2 did not induce mechanical or thermal hyperalgesia effects in TRPV1$^{-/-}$ mice. Similarly, AMG-9810-treated mice also did not present mechanical or thermal hyperalgesia in response to stimulation with iCHIKV and rE2. Strategies targeting TRPV1 are effective at reducing pain in different models (BERTA et al., 2017; GAVVA et al., 2007; LIAO et al., 2017), and TRPV1 antagonist AMG-9810 and others have been shown to be efficient attenuating thermal hyperalgesia induced under inflammatory conditions and increasing the noxious heat threshold (TÉKUS et al., 2010). Therefore, our results suggest that blockage of TRPV1 may be an additional underlying analgesic mechanism against CHIKV-induced pain.

Because we observed a reduction in pain behavior after treatment with TRPV1 antagonists, we next interrogated neuronal activation by performing calcium imaging with DRG neurons dissected from mice infected with iCHIKV and rE2. An increase in calcium influx indicates DRG neuron activation (FATTORI et al., 2018). Therefore, we demonstrated that iCHIKV and rE2 protein produce peripheral sensitization as observed by higher baseline level of calcium influx in DRG neurons when compared to Mock mice. Therefore, that peripheral sensitization might be responsible for the persistent pain observed herein. Other virus such as ZIKV and its envelope protein are capable of inducing a slow increase in intracellular Ca^{2+} concentration mediated by TRPV4, and targeting this receptor results in reduced infectivity in dengue, hepatitis C and Zika viruses (DOÑATE-MACIÁN et al., 2018). Also, Karlas and coworkers verified that a calmodulin inhibitor significantly reduced CHIKV replication in the footpad of pretreated mice, indicating that calcium metabolism may have an important role in the kinetic of infection (KARLAS et al., 2016).

Taking together, our findings suggest that E2 protein is an important component in Chikungunya virus-induced hyperalgesia and targeting it can be a promising alternative to treat joint pain after CHIKV infection, as we first demonstrated using monoclonal antibodies against recombinant E2 protein. The mechanism of hyperalgesia induction by CHIKV seems to be associated with DRG neurons activation mediated by E2 as observed by higher baseline calcium influx in those mice. Moreover, we demonstrated that targeting TRPV1 receptor can be also a promising therapeutic approach in the management of CHIKV-induced pain.

1 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions Statement

CS-V, AZ, VF, LS-F, SB-G, TZ, MM, TS, SB-G and FP-R contributed to the data collection and analysis. CS-V, LS-F, VF, RC and WV contributed to the literature database search, data analysis, and writing of the manuscript. CZ and CD-S contributed producing the inactivated Chikungunya virus, recombinant E2 protein and anti-E2 monoclonal antibodies. WV supervised the study. All authors read and approved the final version of the manuscript.

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Figure Captions

Fig. 1 Inactivated Chikungunya virus (iCHIKV) and recombinant E2 (rE2) protein induced mechanical and thermal hyperalgesia in mice. Animals received intra-articular (i.a.) injection of iCHIKV (1, 10, 100 and 1000 FFU, 10ul), rE2 (30 or 100 ng, 10ul) or Mock (control, 10ul). The mechanical hyperalgesia (A and B) was evaluated 1 – 7 hours after injection and daily during seven days and thermal hyperalgesia (C and D) were measured with the chosen doses at 7 hours after infection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; ∫P<0.05 compared to rE2 group. ANOVA followed by Tukey's test.

Fig. 2 Recombinant E2 (rE2) protein-induced mechanical and thermal hyperalgesia are inhibited by monoclonal antibodies. AbM1 (A), AbM2 (B) and AbM3 (C) (1 or 5 µg, 5 µL) were co-injected (intra-articular) with rE2 (100 ng, 5 µL) or Mock (control, 10 µL). The mechanical hyperalgesia (A to C) was evaluated 1 – 7 hours after injection and daily during seven days and thermal hyperalgesia (D to F) were measured with the chosen doses at 7 hours after injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; #P<0.05 compared to rE2 group. ANOVA followed by Tukey's test.

Fig. 3 Chikungunya inactivated virus-induced mechanical and thermal hyperalgesia are inhibited by monoclonal antibodies. AbM1 (A), AbM2 (B) and AbM3 (C) (1 or 5 µg, 5 µL) were co-injected (intra-articular) with iCHIKV (100 FFU, 5 µL) or Mock (control, 10 µL). The mechanical hyperalgesia (A to C) was evaluated 1 – 7 hours after injection and daily during seven days and thermal hyperalgesia (D to F) were measured with the chosen doses at 7 hours after injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; #P<0.05 compared with iCHIKV group. ANOVA followed by Tukey's test.

Fig. 4 Chikungunya inactivated virus (iCHIKV) and recombinant E2 (rE2) protein-induced mechanical and thermal hyperalgesia are inhibited in absence of TRPV1 channels activity. iCHIKV 100 FFU, rE2 100 ng or Mock were injected (intra-articular) in C57-bl/6 and TRPV1$^{-/-}$ mice and after 5 minutes, C57-bl/6 were treated with TRPV1 antagonist AMG-9810 (100 ng, intra-thecal). The mechanical hyperalgesia was evaluated 1 – 7 hours after injection and the thermal hyperalgesia at 7 hours after injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separated experiments. *P<0.05 compared to Mock group; #P<0.05 compared to stimulus group. ANOVA followed by Tukey's test.

Fig. 5 DRG neurons from iCHIKV and rE2-stimulated mice presented a higher baseline level of calcium influx. Seven hours after intra-articular (i.a.) injection of iCHIKV (100 FFU, 10ul), rE2 (100 ng, 10ul), saline or Mock (control, 10ul), DRGs were dissected for calcium imaging using Fluo-4AM. Panel A displays representative fields of baseline fluorescence of DRG neurons dissected from saline or mock control mice and rE2 or iCHIKV stimulated mice. Panel B shows the mean fluorescence intensity of calcium influx on the baseline (0 second mark). Results are expressed as mean ± SEM, n = 4 DRG plates (each plate is a neuronal culture pooled from 6 mice) per group per experiment, two independent experiments (*p < 0.05 vs. saline and mock; one-way ANOVA followed by Tukey's post-test). 288x220mm (96 x 96 DPI).

Figures

Fig. 1.

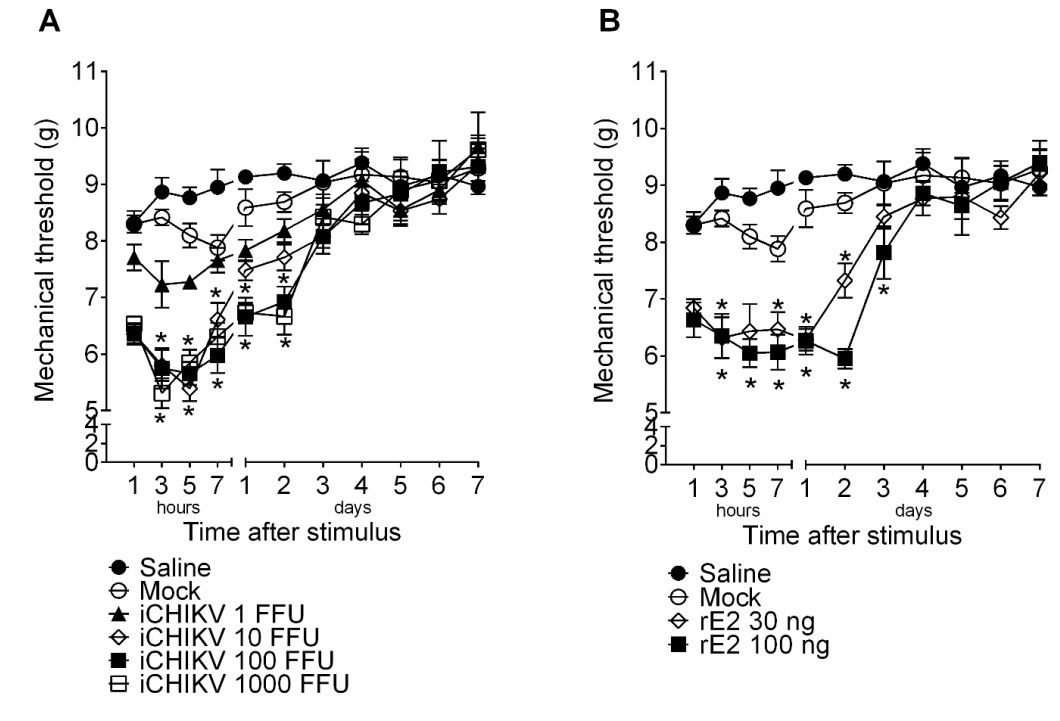


Fig. 2.

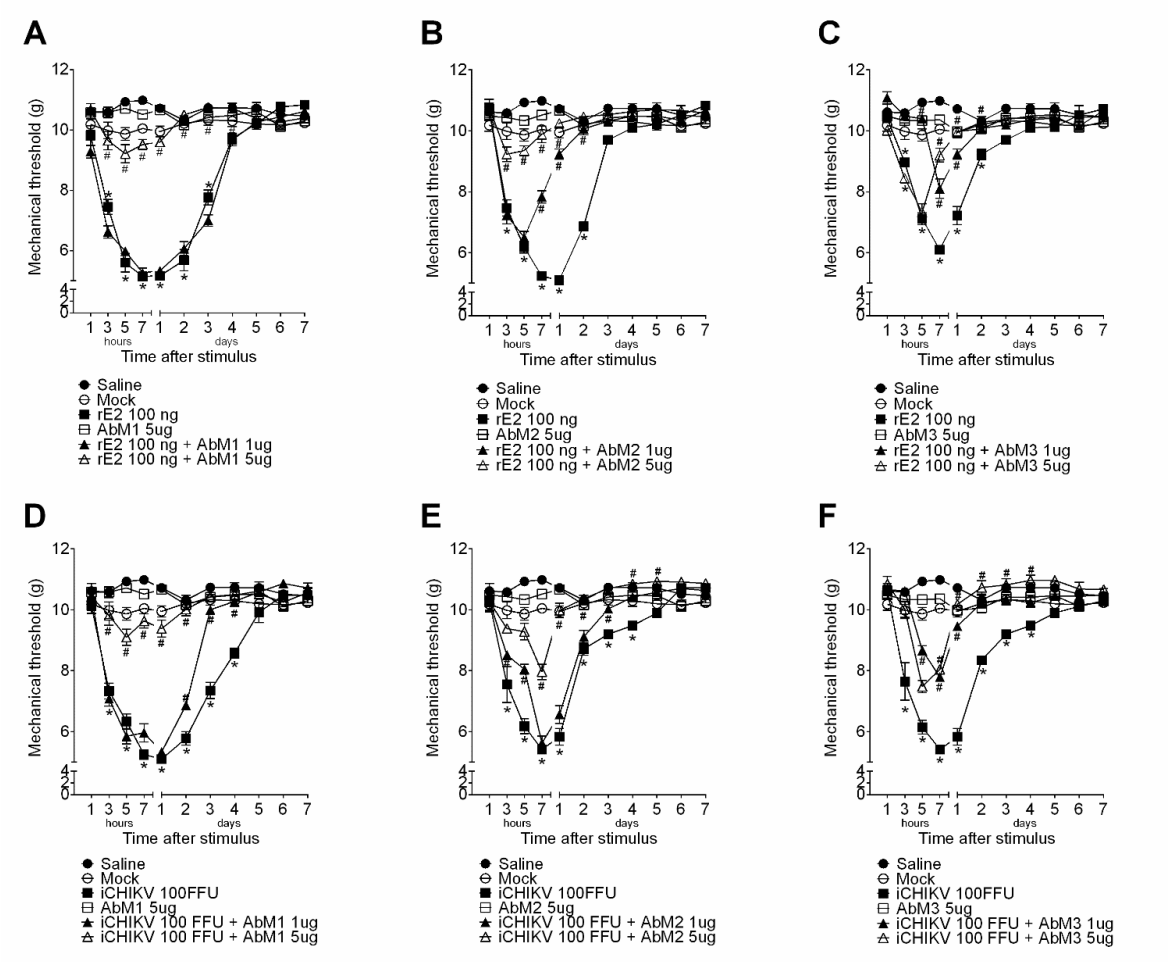

A
B
C
D
E
F
Mechanical threshold (g)
Time after stimulus
hours
days
Saline
Mock
rE2 100 ng
AbM1 5ug
rE2 100 ng + AbM1 1ug
rE2 100 ng + AbM1 5ug
AbM2 5ug
rE2 100 ng + AbM2 1ug
rE2 100 ng + AbM2 5ug
AbM3 5ug
rE2 100 ng + AbM3 1ug
rE2 100 ng + AbM3 5ug
iCHIKV 100FFU
iCHIKV 100 FFU + AbM1 1ug
iCHIKV 100 FFU + AbM1 5ug
iCHIKV 100 FFU + AbM2 1ug
iCHIKV 100 FFU + AbM2 5ug
iCHIKV 100 FFU + AbM3 1ug
iCHIKV 100 FFU + AbM3 5ug

Fig. 3.

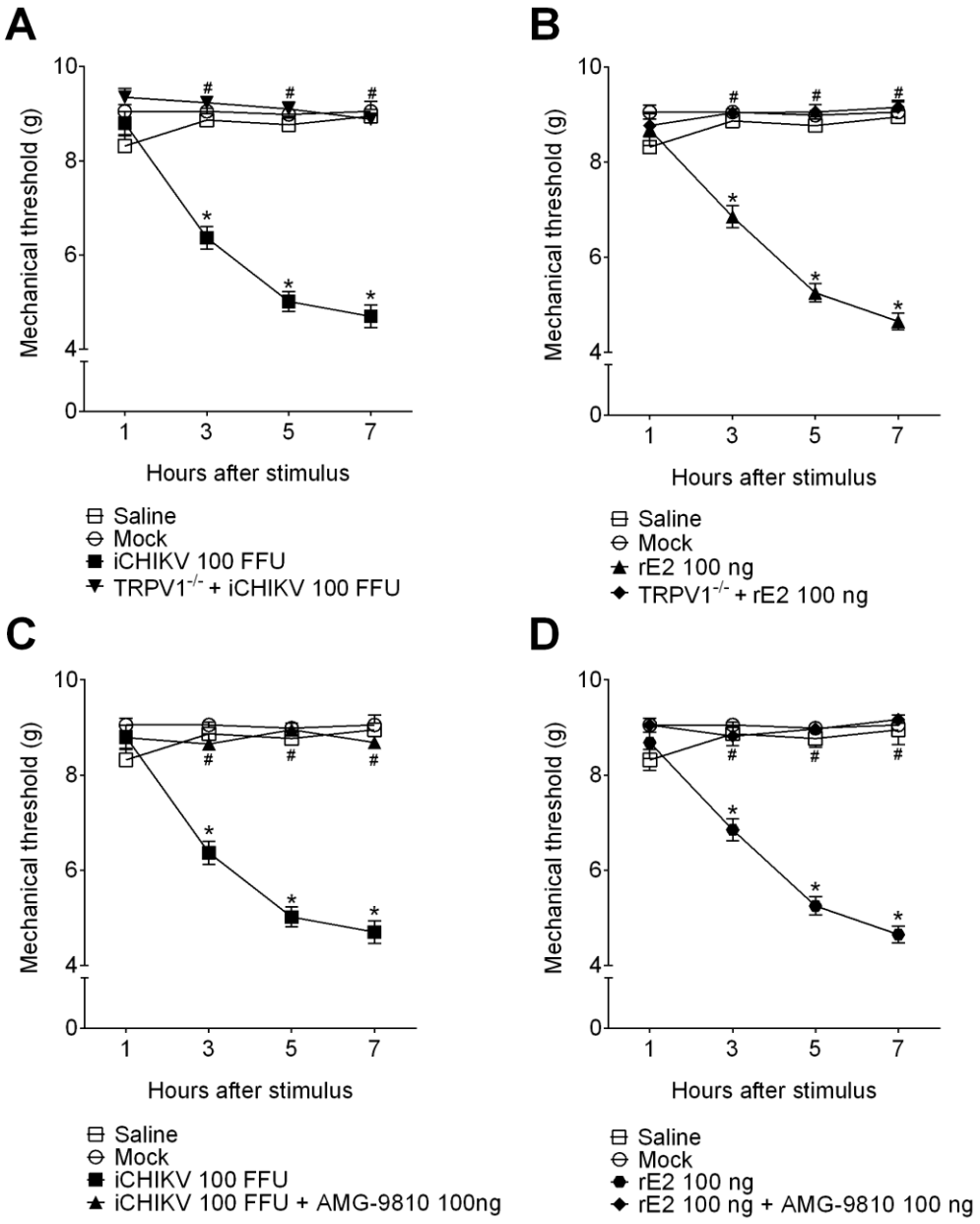

A
B
C
D
Mechanical threshold (g)
Hours after stimulus
Saline
Mock
iCHIKV 100 FFU
TRPV1-/- + iCHIKV 100 FFU
rE2 100 ng
TRPV1-/- + rE2 100 ng
iCHIKV 100 FFU + AMG-9810 100ng
rE2 100 ng + AMG-9810 100 ng

Fig. 4.

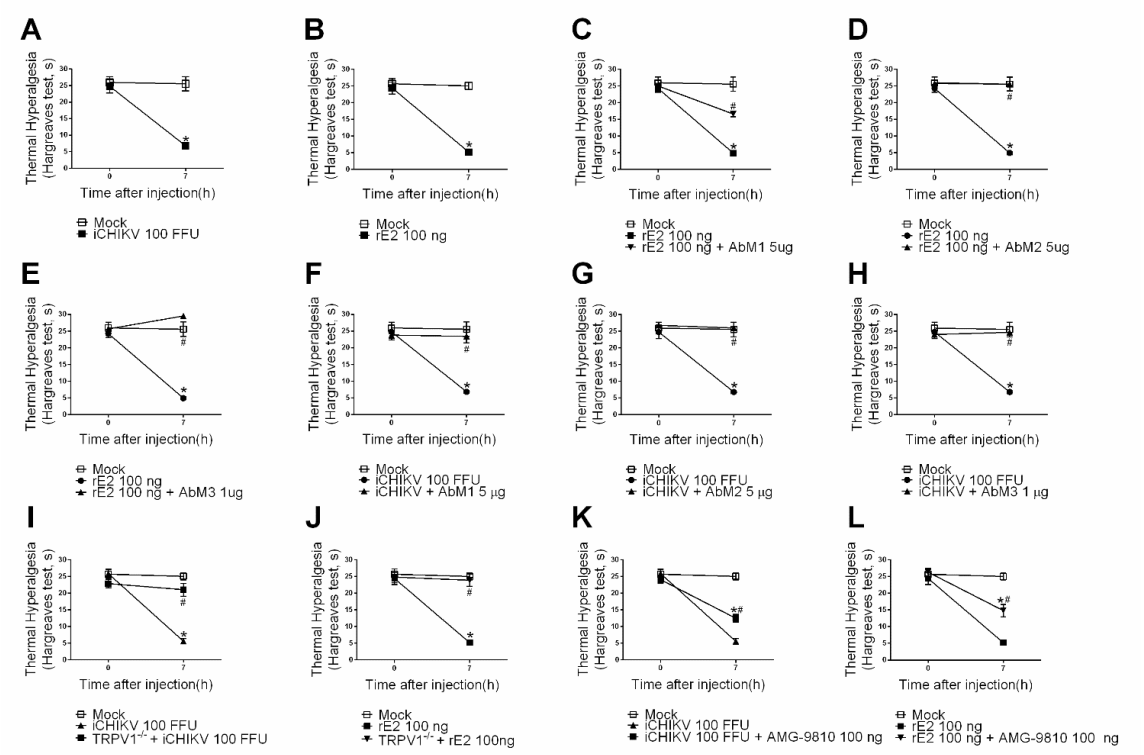
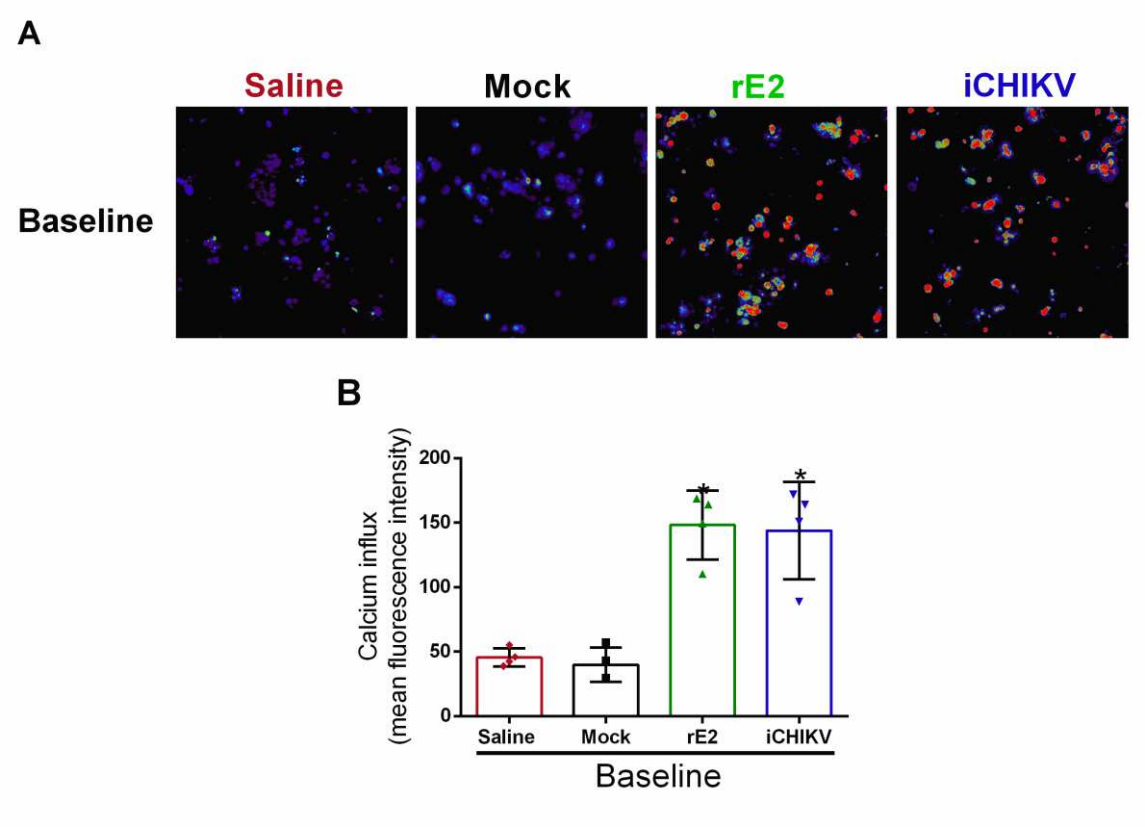
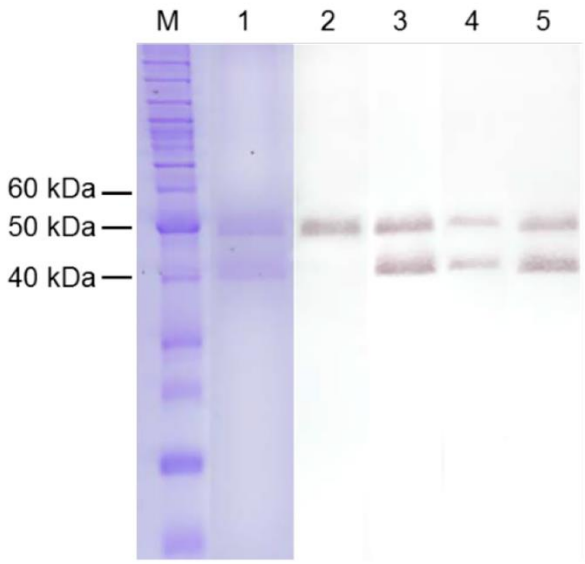

A
B
C
D
E
F
G
H
I
J
K
L
Thermal Hyperalgesia (Hargreaves test, s)
Time after injection(h)
Mock
iCHIKV 100 FFU
rE2 100 ng
rE2 100 ng + AbM1 5ug
rE2 100 ng + AbM2 5ug
rE2 100 ng + AbM3 1ug
iCHIKV + AbM1 5 µg
iCHIKV + AbM2 5 µg
iCHIKV + AbM3 1 µg
TRPV1-/- + iCHIKV 100 FFU
TRPV1-/- + rE2 100ng
iCHIKV 100 FFU + AMG-9810 100 ng
rE2 100 ng + AMG-9810 100 ng

Fig. 5.


A
Saline
Mock
rE2
iCHIKV
Baseline
B
Calcium influx
(mean fluorescence intensity)
200
150
100
50
0
Saline
Mock
rE2
iCHIKV
Baseline
*
*

Supplementary Material

Supplementary Figure



Supplementary Figure 1. Recombinant protein E2 expression confirmed by SDS-PAGE and Western blot. The CHIKV recombinant E2 protein (rE2) was expressed by *Drosophila* S2 cells which were co-transfected with the plasmids pMt/Bip/V5-HisA containing the gene of the E2 protein from CHIKV and pCoBlast. The rE2 protein expression was induced and the protein was purified from the cell-culture supernatant by immobilized metal *affinity chromatography* (using Ni-NTA agarose resin). The rE2 expression was confirmed by SDS-PAGE and Western blot using anti-V5 antibodies.

4. CONCLUSÃO

Diante do aumento de casos de arboviroses, entre elas Dengue e Chikungunya, e da falta de medicamentos específicos e eficazes para o tratamento da dor causada por estas viroses, se faz necessário conhecer melhor os mecanismos envolvidos na patogênese da doença para que possamos buscar drogas capazes de modular tanto a reação inflamatória quanto a hiperalgésica, visando a redução dos principais sintomas com a dor, e buscando evitar a evolução das doenças para formas mais graves.

Os dois trabalhos apresentados nesta tese contribuíram com resultados importantes para a compreensão destes diferentes mecanismos pelos quais os vírus Dengue e Chikungunya são capazes de induzir dor, nos permitindo reconhecer potenciais alvos terapêuticos e repensar o uso de fármacos já conhecidos com foco na inibição da dor causada pelos patógenos. Espera-se que tais resultados sirvam como base para novas pesquisas visando encontrar de fato opções de tratamento que melhorem a qualidade de vida dos pacientes infectados, evitem a progressão da Dengue para a forma hemorrágica e impeçam a cronificação da patologia articular ocasionada pelo Chikungunya.

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APÊNDICE 1

[Ru(bpy)2(NO)SO3](PF6), a Nitric Oxide Donating Ruthenium Complex, Reduces Gout Arthritis in Mice

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$[Ru(bpy)_2(NO)SO_3](PF_6)$, a Nitric Oxide Donating Ruthenium Complex, Reduces Gout Arthritis in Mice

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Monosodium urate crystals (MSU) deposition induces articular inflammation known as gout. This disease is characterized by intense articular inflammation and pain by mechanisms involving the activation of the transcription factor NFκB and inflammasome resulting in the production of cytokines and oxidative stress. Despite evidence that MSU induces iNOS expression, there is no evidence on the effect of nitric oxide (NO) donors in gout. Thus, the present study evaluated the effect of the ruthenium complex donor of NO {$[Ru(bpy)_2(NO)SO_3](PF_6)$} (complex I) in gout arthritis. Complex I inhibited in a dose-dependent manner MSU-induced hypersensitivity to mechanical stimulation, edema and leukocyte recruitment. These effects were corroborated by a decrease of histological inflammation score and recruitment of Lysm-eGFP+ cells. Mechanistically, complex I inhibited MSU-induced mechanical hypersensitivity and joint edema by triggering the cGMP/PKG/ATP-sensitive K (+) channels signaling pathway. Complex I inhibited MSU-induced oxidative stress and pro-inflammatory cytokine production in the knee joint. These data were supported by the observation that complex I inhibited MSU-induced NFκB activation, and IL-1β expression and production. Complex I also inhibited MSU-induced activation of pro-IL-1β processing. Concluding, the present data, to our knowledge, is the first evidence that a NO donating ruthenium complex inhibits MSU-induced articular inflammation and pain. Further, complex I targets the main physiopathological mechanisms of gout arthritis. Therefore, it is envisaged that complex I and other NO donors have therapeutic potential that deserves further investigation.

Keywords: nitric oxide donor, ruthenium, joint pain, oxidative stress, cytokines

INTRODUCTION

Gouty arthritis or gout is the main cause of joint inflammation in men (Vanltallie, 2010). Its prevalence has increased in recent years, especially in developed countries in Europe, United States, and Canada, reaching at least 1% of the adult population (Kuo et al., 2015). Patients are affected by agonizing pain and swelling in the joint, which cause disability and considerably impair the

APÊNDICE 2

The nitric oxide donos GSNO reduces MSU induced gout arthritis in mice

The nitric oxide donor GSNO reduces MSU induced gout arthritis in mice.

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Abstract

Nitric oxide (NO) has a dual role in nociception, which depends on its concentration/dose. Low levels of NO are related to analgesia. In the present study, were investigated the effect of the NO donor S-Nitrosoglutathione (GSNO) in monosodium urate crystals (MSU)-induced gout arthritis. Mice were treated with GSNO (0.1-100 μg/kg, i.p.) and mechanical hyperalgesia and knee edema were assessed at 1–15 h after MSU injection (100 μg/10 μl/ joint). The 15h time point was used for leukocyte recruitment, histology and cytokine levels. In bone-marrow-derived macrophage culture, a system using LPS as priming and MSU as challenge to activate inflammasome was used. GSNO reduced MSU-triggered mechanical hyperalgesia, knee edema, leukocyte recruitment to the synovial fluid, knee joint synovitis, pro-inflammatory cytokine production (TNF-α, IL-1β and IL-33) and did not affect the production of anti-inflammatory cytokine IL-10. In vitro, GSNO inhibited LPS+MSU-induced TNF-α production upon treatment before LPS. GSNO also inhibited IL-1β production upon treatment between LPS and MSU stimuli. These in vitro data suggest that GSNO can inhibit both NF-κB activation and inflammasome activation. Concluding, GSNO consistently inhibited gout arthritis in vivo and cytokine production in vitro, which is a proof-of-concept of the usefulness of NO donors in gout arthritis treatment.

Keywords: Monosodium urate crystals, Gouty arthritis, GSNO, Nitric Oxide donor, NLRP3, Inflammasome, Cytokines, Inflammatory pain.