Pontifícia Universidade Católica do Rio Grande do Sul

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Programa de Pós-Graduação em Biologia Celular e Molecular

GABRIELA LUCAS DA SILVA

Estudo do papel dos receptores purinérgicos P2X7 em processos inflamatórios cutâneos em modelos *in vitro* e *in vivo*

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Orientadora: Profa Dra Fernanda Bueno Morrone

Co-orientador: Dr Rafael Fernandes Zanin

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Aprovada em:____de_____de_____.

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Àquela que me deu a vida...

Àquela que dedicou parte de sua vida a mim...

Àquela que nunca mediu esforços para me fazer feliz...

Àquela que me ensinou que o conhecimento é nosso maior bem...

Àquela que foi e sempre será meu maior exemplo...

Dedico este trabalho a minha mãe, **Marina Leivas Lucas**, e não poderia ser diferente!

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"Creio que a **imaginação** pode mais que o **conhecimento**. Que o **mito** pode mais que a **história**. Que os **sonhos** podem mais que os **fatos**. Que a **esperança** sempre vence a **experiência**. Que só o **riso** cura a **tristeza**. E creio que o **amor** pode mais que a morte.

Robert Fulghum

RESUMO

A dermatite de contato irritante (ICD, *irritant contact dermatitis*) é uma reação inflamatória local não alérgica da pele que ocorre independentemente da participação de células T. É ocasionada pela exposição a substâncias químicas de baixo peso molecular e acredita-se que seja iniciada por dano ou ativação de células epidermais que desencadeiam a ativação do sistema imune inato. O nucleotídeo ATP é uma molécula energética que desempenha importantes funções como mensageiro extracelular. Em tecidos saudáveis, o ATP está localizado quase exclusivamente no meio intracelular, entretanto quando ocorre injúria tecidual, grandes quantidades de ATP são liberadas para o meio extracelular. No meio extracelular, o ATP pode ser hidrolisado por nucleotidases gerando ADP e AMP. As nucleotidases controlam a disponibilidade do ATP para os receptores purinérgicos. Muitas observações experimentais têm indicado que o ATP atuando via receptores purinérgicos desempenha um papel preponderante em processos inflamatórios na pele. Vários modelos *in vitro* e *in vivo* têm sido utilizados para o estudo da ICD, entretanto a fisiopatologia da doença ainda é pouco conhecida. Este estudo teve como objetivo avaliar o envolvimento do receptor purinérgico P2X7 (P2X7R) na ICD utilizando abordagens *in vivo* e *in vitro.* O óleo de cróton (CrO) é um irritante químico que tem mostrado exercer seus efeitos inflamatórios independente de células T, portanto nós utilizamos o modelo de dermatite de contato irritante induzida por CrO em camundongos para investigar o envolvimento do receptor P2X7 nos mecanismos imunopatológicos da ICD. Através da utilização de diversas abordagens farmacológicas *in vitro* e *in vivo* e do uso de camundongos com deleção gênica para o receptor P2X7, nós primeiramente mostramos evidências de que a ativação do P2X7R por ATP em macrófagos e células dendríticas (DCs) está relacionada com o recrutamento de neutrófilos induzido por CrO. Além disso, foi demonstrado que o CrO decresce a hidrólise de ATP e ADP no soro de camundongos e provoca a necrose de queratinócitos em cultura, ambos os efeitos foram relacionados com a ativação do receptor P2X7. Portanto, a partir dos dados obtidos neste estudo, nós sugerimos que o óleo de croton exerce seus efeitos tóxicos promovendo a morte celular de queratinócitos, diminuindo a atividade de ectonucleotidades e aumentando os níveis extracelulares de ATP. O ATP extracelular, via ativação de P2X7R em DCs e macrófagos, provoca a liberação de IL-1β que é parcialmente responsável pelo recrutamento de neutrófilos observado na dermatite de contato irritante induzida por óleo de cróton. Digno de nota, o tratamento com o antagonista seletivo do receptor P2X7, A438079, diminuiu o edema e outros efeitos pró-inflamatórios induzidos pelo óleo de cróton, apontando o receptor P2X7 como um alvo farmacológico importante para o desenvolvimento de novas terapias para o tratamento da ICD.

Palavras-chave: dermatite de contato irritante, ectonucleotidases, receptor P2X7, ATP, IL-β.

ABSTRACT

The irritant contact dermatitis (ICD) is a non-allergic inflammatory reaction of the skin that occurs independently of the T cells participation. ICD is caused by exposure to low molecular weight chemicals and it is initiated by damage or activation of epidermal cells which trigger the activation of the innate immune system. The purine nucleotide adenosine triphosphate (ATP) is the universal energy molecule and appears as is an important extracellular messenger. In healthy tissues, ATP is almost exclusively localized intracellularly, whereas in pathological conditions as inflammation, the tissue injury leads to the release of large amounts of ATP to the extracellular medium. In the extracellular medium, ATP can be hydrolyzed by nucleotidases to their breakdown products ADP and AMP. These nucleotidases control the availability of nucleotides for purinergic receptors. Currently, experimental observations indicate that extracellular ATP acting via purinergic receptors plays a relevant role on skin inflammation. Several models *in vitro* and *in vivo* had been used to study the mechanisms of ICD. However, this pathology is still poor understood. This study aimed to evaluate the involvement of the purinergic receptor P2X7 in ICD using *in vivo* and *in vitro* approaches. The croton oil (CrO) is a chemical irritant that has been shown to exert its effects independent of inflammatory T cells, therefore we used the mice model of irritant contact dermatitis induced by CrO to investigate the involvement of P2X7 receptor in the immunes mechanisms of ICD. We use several pharmacological approaches, *in vitro* and *in vivo* tests and mice with gene deletion to P2X7 receptor and showed evidences that the P2X7 receptor activation by ATP in macrophages and dendritic cells (DCs) is connected with neutrophil recruitment induced by CrO. Furthermore, we demonstrated that CrO decreased the hydrolysis of ATP and ADP in the serum of mice and caused necrosis of keratinocytes in culture, both effects related to activation of P2X7. Taken together, the data obtained in this study suggested that CrO exerts its toxic effects by promoting keratinocytes necrosis and decreasing the activity of ectonucleotidades leading to an increase of ATP extracellular levels. Then, extracellular ATP promotes the P2X7 receptor activation in DCs and macrophages causing the release of IL-1β, which is partially responsible for the neutrophils recruitment observed in irritant contact dermatitis induced by croton oil. Noteworthy, the treatment with the selective P2X7 receptor antagonist A438079 decreased the edema and others proinflammatory effects induced by croton oil, pointing the P2X7 receptor as an important pharmacological target for the treatment of ICD.

Key words: irritant contact dermatitis, nucleotidases, P2X7 receptor, ATP, IL-1β.

LISTA DE ABREVIATURAS

ADP – adenosina 5’ - difosfato

AMP – adenosina 5’ – monofosfato

APC – célula apresentadora de antígeno

ASC – proteína associada a apoptose contendo um domínio CARD

ATP – adenosina 5’ – trifosfato

CARD – domínio terminal que recruta caspase

CD39 – ecto-apirase

CD73 – ecto-5’nucleotidase

CLA – antígenos linfocitários cutâneos

DAMP – padrão molecular associado a dano

DC – célula dendrítica

DNCB – 2,4-dinitroclorobenzeno

DNFB – 2,4-dinitrofluorbenzeno

ICD – dermatite de contato irritante

ACD – dermatite de contato alérgica

E-NPP – ecto-nucleotídeo pirofosfatase/fosfodiesterase

IL-1 – interleucina 1

IL-1β – interleucina 1 beta

IL-1R – receptor para interleucina 1

IL-1Ra – antagonista do receptor para interleucina 1

IL-6 – interleucina 6

IL-8 – interleucina 8

IL-10 – interleucina 10

IL-18 – interleucina 18

LC – células de langerhans

LRR – domínio C-terminal rico em leucinas

NBD – domínio central ligado a nucleotídeos

NK – células *natural killer*

NLR – receptores de ligação a nucleotídeos

NLRP – receptores de ligação a nucleotídeos contendo o domínio terminal pirina

NTPDase – nucleosídeo trifosfato difosfoidrolase

OCD – dermatite de contato ocupacional

P2X7R – receptor purinérgico P2X7

PAMP – padrão molecular associado a patógeno

pDC – célula dendrítica plasmocitóide

PYD – domínio pirina

T_H – células T *helper*

T_{CM} – células T de memória provenientes da circulação

T_{RM} – células T de memória residentes

TNFα – fator de necrose tumoral α

TPA – 13-acetato de 12-O-tetradecanoilforbolester

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1 INTRODUÇÃO

1.1 A Pele

A pele é um órgão de revestimento complexo e heterogêneo cuja função principal é proteger o organismo de agressões externas (Leonardi, 2008; Ribeiro, 2010). Recobrindo aproximadamente 2 m^2 da superfície corpórea e representando 15 % do peso corporal, ela atua como uma linha de defesa contra patógenos e insultos químicos e físicos (Nestle et al., 2009). Para exercer suas funções, a pele se constitui de duas camadas de estrutura e propriedades distintas: epiderme e derme (Figura 1), dispostas e inter-relacionadas de modo a se adequar de maneira harmônica ao desempenho de suas funções (Koster and Roop, 2004).

A epiderme é constituída por diferentes tipos celulares, incluindo melanócitos, queratinócitos, células de Langerhans (LCs, *langerhans cells*), células T intraepiteliais, células de Merckel e, contém quatro subcamadas principais: 1) camada córnea, que atua como barreira; 2) camada granulosa, onde os queratinócitos presentes produzem queratina, sintetizam citocinas e quimiocinas e sofrem apoptose, diferenciando-se em corneócitos; 3) camada espinhosa, na qual se inicia o processo de diferenciação; 4) camada basal, camada mais profunda da epiderme responsável pela proliferação celular, sendo resistente ao processo apoptótico (Fuchs and Byrne, 1994; Fuchs and Raghavan, 2002).

Separando a epiderme da derme subjacente, encontra-se a membrana basal, estrutura altamente organizada, constituída por uma malha de proteínas derivadas tanto dos queratinócitos epidérmicos quanto dos fibroblastos dérmicos, além de vários tipos de colágeno, lamininas, subunidades de integrinas e proteoglicanos. Embora a epiderme

apresente uma histologia simples, a derme é anatomicamente mais complexa, sendo constituída por uma grande variedade de tipos celulares. Ela contém muitas células imunes especializadas, incluindo células dendríticas (DCs, *dendritic cells*), células T *helper* CD4+ (T_H) e células *natural killer* (NKs). Além destas, estão presentes mastócitos, macrófagos, células dendríticas dérmicas e fibroblastos (Nestle et al., 2009; Spellberg, 2000; Strid et al., 2009).

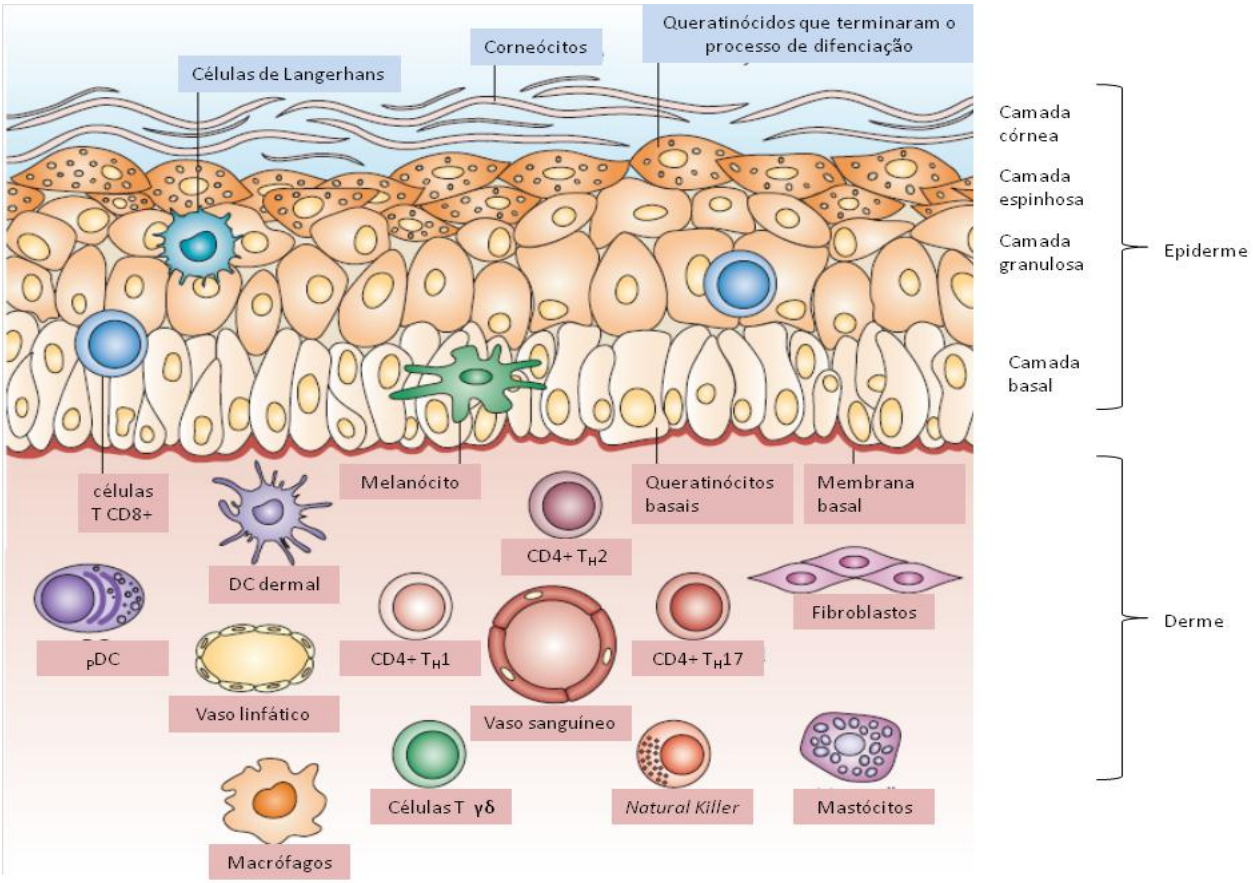

Figura 1. Tipos celulares presentes nas diferentes camadas da pele. A epiderme contém a camada basal, a camada espinhosa, a camada granulosa e a camada córnea. A camada basal é a responsável pelo processo de renovação da pele, nesta camada os queratinócitos estão em constante proliferação, sofrem diferenciação e migram para as camadas superiores. Células especializadas da epiderme incluem os melanócitos (produtores de melanina), células de Langerhans, e algumas células T do tipo CD8+. A derme contém muitos tipos celulares especializados, como células dendríticas mielóides (DCs) e plasmocitóides (pDCs), células *natural killers*, subtipos de células T (T_H1, T_H2 e T_H17, γδ T), macrófagos e mastócitos. (Adaptado de Nestle et al., 2009, *Nature Immunology*).

A interação coordenada entre os diferentes tipos celulares presentes nas camadas da pele permite que este órgão responda a estímulos nocivos (Nestle et al., 2009), desencadeando uma resposta de proteção ao organismo, denominada resposta inflamatória, cuja finalidade é erradicar o agente agressor, evitando a sua disseminação a outras regiões do organismo, promovendo o reparo tecidual e restabelecendo a homeostasia do tecido (Medzhitov, 2008). Portanto, a pele participa ativamente na defesa do hospedeiro sendo considerado um importante órgão do sistema imune (Grone, 2002; Spellberg, 2000).

1.1.1 Queratinócitos

Os queratinócitos são as principais células epidermais e secretam constitutivamente ou induzem à secreção de uma grande variedade de mediadores, como peptídeos antimicrobianos, quimiocinas e citocinas (IL-1, IL-6, IL-10, IL-18 e TNFα) (Albanesi et al., 2005). A secreção de citocinas por queratinócitos influencia sua proliferação e diferenciação; afeta algumas funções do sistema imune, como a migração e diferenciação de células inflamatórias; e pode desencadear a produção de outras citocinas (Grone, 2002).

Além das funções anteriormente citadas, os queratinócitos podem induzir respostas funcionais em células T de memória e desempenhar funções de células apresentadoras de antígenos. Portanto, os queratinócitos são considerados células pró-inflamatórias, estrategicamente posicionadas, que atuam como iniciadores da resposta inflamatória cutânea (Nestle et al., 2009).

1.1.2 Células de Langerhans (LCs)

Segundo Nestle et al. (2009), pesquisas recentes a cerca do papel das LCs têm revelado resultados inesperados e a confirmação da real função destas células na homeostase do tecido cutâneo e em situações patológicas ainda é pouco esclarecido. LCs são células dendríticas residentes na camada basal epiderme (Doan et al., 2008). As LCs estão entre as primeiras DCs a entrar em contato com antígenos na pele, portanto já foram consideradas células dendríticas clássicas que atuam como células apresentadoras de antígenos (Baer, 1983), essenciais para a resposta a antígenos na pele (Silberberg-Sinakin et al., 1980; Silberberg-Sinakin and Thorbecke, 1980). Estudos *in vitro* têm mostrado que as LCs podem processar antígenos lipídicos e/ou microbianos e apresentá-los a células T efetoras (Hunger et al., 2004). Esta observação levou os pesquisadores a pensar que LCs poderiam ser responsáveis pela indução da hipersensibilidade de contato, uma reação inflamatória que ocorre após a primeira exposição a um antígeno e requer a ativação de células T. Entretanto, a depleção de LCs em modelos animais resultou em aumento da hipersensibilidade, apontando para a possibilidade das células de Langerhans funcionarem como inibidoras e não como indutoras de reações de hipersensibilidade (Kaplan et al., 2008).

1.1.3 Células dendríticas dermais e macrófagos

Existe uma grande variedade de células dendríticas e macrófagos na derme e cada uma delas desempenha respostas imunológicas altamente diferenciadas (Nestle et al., 2009).

Células dendríticas dérmicas são as DCs residentes na derme. Fukunaga et al. (2008) demonstraram que são as DCs dermais e não as LCs que desempenham o maior

papel na resposta imune cutânea. DCs dermais podem ser ativadas por patógenos ou por sinais de injúria tecidual e participam da resposta inflamatória cutânea através da secreção de mediadores inflamatórios e recrutamento de neutrófilos ao local. Em alguns casos, os mediadores liberados por estas células são benéficos e contribuem para erradicação de agentes infecciosos e/ou reparo tecidual, mas em outros podem desencadear uma resposta tecidual patológica com inflamação persistente (Chen and Nunez, 2010; Fukunaga et al., 2008; Nestle et al., 2009; Toebak et al., 2009).

Os macrófagos da pele são predominantemente células residentes que exercem funções homeostáticas removendo células apoptóticas, mas também servem como células imunes sentinelas, pois expressam uma grande variedade de receptores que são capazes de reconhecer substâncias estranhas ou células anormais. Sob condições infecciosas ou inflamatórias, outros macrófagos, além dos residentes podem ser recrutados para o tecido com o objetivo de combater infecções, iniciar o reparo tecidual e resolver a inflamação. Entretanto, devido às suas propriedades citotóxicas e pró-inflamatórias, os macrófagos podem gerar dano tecidual e contribuir para o desenvolvimento de processos inflamatórios crônicos (Gordon and Martinez, 2010; Nestle et al., 2009).

1.1.4 Células T residentes

As células T residentes na pele desempenham um papel fundamental na imunologia cutânea. Em condições normais, a pele contém mais de 2×10^{10} células T residentes, o que representa mais que duas vezes o número de células T no sangue. Células T epidermais estão principalmente distribuídas na camada basal, frequentemente próximas às células de Langerhans. Na derme, as células T estão

situadas preferencialmente na junção dermo-epidérmica ou em apêncices cutâneos subjacentes. Células T CD4+ e CD8+ estão presentes em números praticamente iguais e são, em sua maioria, células T de memória que expressam antígenos associados a linfócitos (CLA, *lymphocyte-associated antigen*) (Nestle et al., 2009).

Estudos recentes têm mostrado que células T de memória residentes (T_{RM}) na pele não recirculam e são potentes efetoras da resposta imune cutânea a antígenos e a agentes infecciosos, funcionando como uma linha de defesa rápida e independente de células T de memória centrais provenientes da circulação (T_{CM}) (Clark et al., 2012; Jiang et al., 2012). Entretanto, embora as células T sejam importantes efetoras de respostas imunes cutâneas, sua ativação excessiva está correlacionada a algumas patologias cutâneas, como psoríase e dermatite de contato (Coenraads and Goncalo, 2007).

1.2 Dermatite de contato

A dermatite de contato é uma doença inflamatória cutânea induzida pelo contato repetido com substâncias químicas de baixo peso molecular, chamadas de xenobióticos ou haptenos (Nosbaum et al., 2009). A dermatite de contato pode ser subdividida em dois tipos: dermatite de contato alérgica (ACD, *alergic contact dermatitis*) e dermatite de contato irritante (ICD, *irritant contact dermatitis*). Como ambas as patologias apresentam sinais clínicos semelhantes, torna-se difícil à distinção entre elas. Entretanto, ICD e ACD podem ser claramente diferenciados com base em seus mecanismos imunológicos (Ku et al., 2009). A ICD tem sido considerada uma inflamação que envolve apenas células inflamatórias da imunidade inata, enquanto que

a ACD é considerada uma inflamação dependente de células T que envolve mecanismos típicos da imunidade adquirida (Coenraads and Goncalo, 2007; Slodownik et al., 2008).

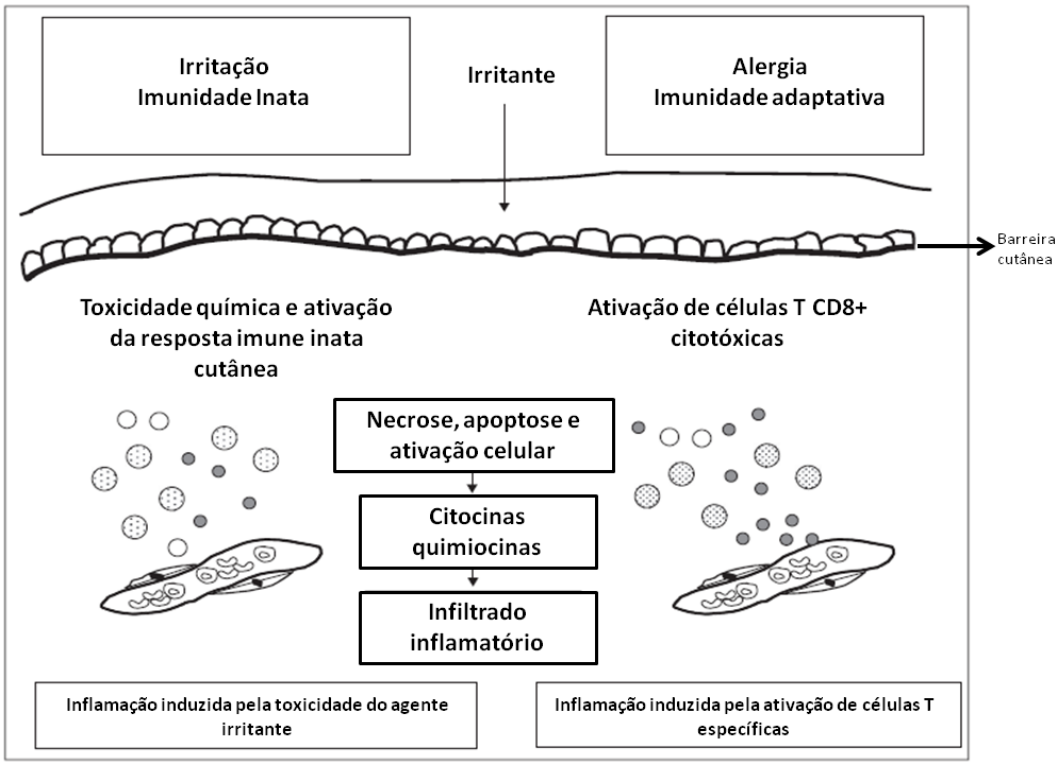

Figura 2. Mecanismos imunológicos envolvidos na dermatite de contato irritante e na dermatite de contato alérgica. Os estágios iniciais das duas patologias se diferem, enquanto em ICD a própria toxicidade da substância química sobre as células da pele desencadeia a resposta inflamatória, em ACD esta reação é desencadeada por ativação de células T específicas. Os estágios seguintes são semelhantes envolvendo necrose, apoptose, liberação de citocinas e quimiocinas e infiltrado inflamatório, explicando porque estes dois tipos de dermatites são difíceis de serem diferenciados clinicamente. (Fonte: Nosbaum et al. 2009; *European Journal of Dermatology*).

1.2.1 Dermatite de contato alérgica (ACD)

A dermatite de contato alérgica tem sido considerada uma reação de hipersensibilidade do tipo IV (Nosbaum et al., 2009; Zhang and Tinkle, 2000). As

reações de hipersensibilidade do tipo IV resultam da inflamação mediada por células T e não envolvem anticorpos, pois as respostas inflamatórias são provocadas pela maneira como as células T encontram e respondem ao antígeno (Doan et al., 2008). Este tipo de reação ocorre em duas fases: uma fase sensibilização e uma fase de indução. Na fase de sensibilização, os haptenos penetram na epiderme e são captados por DCs que migram para os linfonodos de drenagem, onde apresentam os haptenos conjugados a peptídeos às células T CD8+ efetoras e as células T CD4+. Precursores de células T expandem-se clonalmente nos linfonodos, recirculam pelo sangue e migram de volta à pele. Quando o mesmo hapteno é aplicado sobre a pele, ele é captado por células epidérmicas, como queratinócitos e DCs, que apresentam os haptenos conjugados a peptídeos à células T específicas. A ativação destas células T induz a apoptose de queratinócitos e a produção de citocinas e quimiocinas pelas células cutâneas, levando ao recrutamento de leucócitos do sangue para a pele (Kaplan et al., 2012; Vocanson et al., 2005).

1.2.2 Dermatite de contato irritante (ICD)

A dermatite de contato irritante é o tipo mais comum de dermatite e foi por muito tempo negligenciada. Nos últimos anos, o entendimento dos processos patogênicos da doença tem despertado novamente a atenção dos pesquisadores (Slodownik et al., 2008), entretanto as alternativas de prevenção e tratamento ainda são escassas (Saary et al., 2005).

A ICD é uma reação inflamatória local não alérgica da pele que ocorre independentemente da participação de células T (Nosbaum et al., 2009). Acredita-se que este tipo de dermatite seja iniciado por dano ou ativação de células epidermais induzido por exposição aguda ou crônica a agentes químicos (Han et al., 2007). Após a exposição

a agentes químicos com potencial irritante, células epidermais liberam citocinas pró-inflamatórias, quimiocinas e outros mediadores que levam a vasodilatação, infiltração de leucócitos, edema e eritema (Han et al., 2007; Kupper, 1990), mecanismos típicos de uma reação inflamatória estéril (Chen and Nunez, 2010). Portanto, neste tipo de dermatite, as propriedades tóxicas da substância química são responsáveis pela injúria tecidual que leva a uma resposta inflamatória inata característica (Gibbs, 2009). Os queratinócitos possuem um papel essencial na iniciação e no desenvolvimento da ICD. Eles produzem mediadores que recrutam outras células aos locais da lesão gerando uma cascata de produção de mediadores inflamatórios por diversos tipos celulares que levam às modificações histológicas e clínicas manifestadas em pacientes com ICD (Nosbaum et al., 2009). Estudos com camundongos deficientes em certos tipos celulares demonstram que macrófagos, células dendríticas, mastócitos, células NK e células endoteliais também contribuem para o desenvolvimento da doença (Vocanson et al., 2007).

1.2.2.1 Modelos de dermatite de contato irritante

Desde 1980, a Comissão Européia tem realizado esforços para reduzir o número de animais em estudos que visam avaliar o potencial irritante de substâncias químicas, isto resultou em um grande estímulo para o desenvolvimento de técnicas *in vitro* para avaliação da toxicidade de químicos. Estas técnicas consistem principalmente em testes em tecidos, dosagem de biomarcadores de dano e testes de viabilidade celular em diferentes linhagens (Gibbs, 2009). Segundo Welss et al. (2004) a medida da viabilidade celular através do ensaio do MTT pode ser uma alternativa relativamente confiável para avaliar o potencial irritante de substâncias químicas, pois há uma correlação entre o

potencial irritante e a redução da viabilidade celular. Entretanto, somente a medida da citotoxicidade pode não ser suficiente para distinguir irritantes de não-irritantes, apontando para a necessidade de que sejam medidos também biomarcadores de irritação. Os biomarcadores descritos são principalmente citocinas, como IL-1, IL-6, IL-8, IL-10 ou metabólitos do ácido araquidônico (Welss et al., 2004a).

Quase todas as substâncias são capazes de desencadear ICD, entretanto o potencial irritante de uma substância é determinado de acordo com suas propriedades físicas e químicas. O tamanho molecular, o estado de ionização e a solubilidade em óleo é que determinam o poder de penetração da substância e, portanto, seu potencial de irritação *in vivo* (Slodownik et al., 2008). Uma variedade de irritantes, incluindo 2,4-dinitroclorobenzeno (DNCB), 2,4-dinitrofluorbenzeno (DNFB), 13-acetato de 12-O-tetradecanoilforbolester (TPA) e óleo de cróton tem mostrado induzir ICD em modelos animais (Han et al., 2007).

O óleo de cróton tem sido descrito como um irritante que induz infiltrado inflamatório e edema em animais (Ku et al., 2009). Zhang et al. (2000) demonstraram que a aplicação de óleo de cróton na orelha de camundongos desencadeia uma resposta inflamatória similar em camundongos atímicos e em camundongos normais, sugerindo que a atividade irritante exercida por este agente ocorre independentemente da participação de células T e é mediada por células do sistema imune inato. Portanto, o modelo de edema de orelha induzido por óleo de cróton em camundongos pode ser considerado um modelo de dermatite de contato irritante (Zhang and Tinkle, 2000). Este modelo tem sido utilizado por muitos autores para avaliar o potencial anti-inflamatório de novas substâncias e/ou derivados de plantas (Bracht et al., 2011; Veras et al., 2013; Ye et al., 2012). Segundo Colorado et al. (1991), a aplicação de uma solução a 2% de óleo de cróton na orelha de camundongos induz um processo inflamatório agudo e a

efetividade anti-inflamatória de drogas pode ser avaliada, após a eutanásia dos animais, por comparação entre a orelha tratada com óleo de cróton e a orelha não tratada. O efeito anti-inflamatório pode ser medido pesando as orelhas em balança analítica ou medindo a espessura das orelhas utilizando um paquímetro (Colorado et al., 1991; Tubaro et al., 1986a).

1.3 Resposta inflamatória

A inflamação evoluiu como um mecanismo homeostático complexo que permite ao corpo detectar e reagir contra organismos estranhos (Medzhitov, 2008). O sistema imune inato usa um número limitado de receptores de reconhecimento de padrões (PRRs) para reconhecer padrões moleculares associados a patógenos (PAMPs) – características estruturais conservadas nos microorganismos, mas não no hospedeiro. Os PRRs são divididos em categorias e estão presentes como proteínas extracelulares ou como proteínas associadas a membranas nas células fagocitárias (Doan et al., 2008). Os receptores semelhantes a toll (TLRs) pertencem a uma categoria de PRRs que tem sido bastante estudada nos últimos anos, eles medeiam o reconhecimento de diversos patógenos, pois reconhecem uma grande variedade de PAMPs, como componentes da parede celular de bactérias e fungos; lipoproteínas bacterianas; e ácidos nucléicos virais e bacterianos (Barton and Kagan, 2009). A ativação destes receptores leva à quimiotaxia de leucócitos, geração de espécies reativas de oxigênio, fagocitose, secreção de citocinas e muitas outras respostas efetoras da inflamação (Iribarren and Wang, 2011; Lin et al., 2011).

Embora a teoria de que o sistema imune reaja exclusivamente ou principalmente a moléculas não-próprias seja geralmente aceita e amplamente validada por achados clínicos e experimentais, ela é incapaz de explicar uma série de observações (Di Virgilio, 2005). Esta teoria não explica porque não há reação imune contra proteínas que são sintetizadas no final da vida e, portanto, não são expostas aos linfócitos durante a maturação do sistema imune ou, porque, alguns tecidos transplantados sofrem menos rejeição do que outros (Medzhitov, 2008).

Com o passar dos anos, tem-se observado que o corpo humano não reage somente a moléculas estranhas (não-próprias), mas também a moléculas passíveis de causar danos aos tecidos (Di Virgilio, 2005; Medzhitov, 2008). Estas moléculas foram denominadas sinalizadoras de perigo. Os sinais de perigo foram postulados primeiramente por Matzinger (1994) como parte de um modelo de imunidade que sugere que o sistema imune responde a substâncias que causam dano, ao invés de reagir apenas aquelas que são estranhas. Em outras palavras, o corpo reage ao "perigo" e não ao "estranho" (Di Virgilio, 2005; Matzinger, 1994).

Os sinais de perigo consistem em moléculas ou estruturas moleculares, liberadas ou produzidas por células em condições de estresse ou sofrendo processos anormais de morte celular. Estes sinais são reconhecidos por células apresentadoras de antígenos (APCs, *antigen-presenting cells*), que se tornam ativadas e geram sinais co-estimulatórios e, assim, iniciam as respostas imunes e a inflamação (Iyer et al., 2009). Portanto, quando algum dano tecidual ocorre, as APCs podem reagir diretamente através do reconhecimento de sinais de perigo (la Sala et al., 2003). Esta teoria explica como diferentes agentes físicos e químicos são capazes de induzir a liberação de mediadores endógenos, que por sua vez, são responsáveis por alertar o sistema imune e

desencadear uma resposta inflamatória mesmo na ausência de infecção (Chen and Nunez, 2010; Medzhitov, 2008).

A inflamação induzida pelo contato a agentes químicos ocorre na ausência de microorganismos e tem sido chamada de inflamação estéril. Assim como a inflamação induzida por microorganismos, a inflamação estéril é marcada por recrutamento de neutrófilos e macrófagos e pela produção de citocinas e quimiocinas, como TNFα e IL-1β. A inflamação estéril ocorre em resposta à morte celular, dano ou mau funcionamento do tecido. Nestas situações, moléculas que em situações normais estariam delimitadas pelas membranas celulares são liberadas para o meio extracelular e servem como sinalizadores de perigo, denominados padrões moleculares associados a danos (DAMPs, *damage-associated molecular patterns*) (Chen and Nunez, 2010).

Embora qualquer substância que, em condições normais, seja encontrada somente dentro das células, possa a princípio atuar como DAMP, somente poucos candidatos foram identificados. Os nucleotídeos, como ATP, UTP ou ADP, os quais são normalmente armazenados no citosol, são liberados de uma variedade de células sob condições de estresse (Gallucci and Matzinger, 2001). No meio extracelular, estes nucleotídeos são reconhecidos por receptores purinérgicos específicos e atuam como indutores da resposta inflamatória servindo como moléculas sinalizadoras de perigo para as células apresentadoras de antígenos (Di Virgilio, 2005; Eltzschig et al., 2012).

1.4 Sinalização purinérgica e dermatite de contato

A molécula 5’-trifosfato de adenosina (ATP) foi descoberta há 80 anos. Sabe-se que desde muito cedo na evolução, o ATP foi identificado como um substrato

energético, configurando o metabolismo de todas as formas de vida. Atualmente, evidências crescentes mostram que o ATP não é somente uma fonte de energia para a célula, mas também uma importante molécula sinalizadora, tanto em condições fisiológicas como patológicas (Burnstock, 2006a, b).

O papel do ATP como molécula de sinalização intercelular e a sinalização purinérgica foram inicialmente sugeridos em 1970, quando o ATP foi identificado como transmissor no sistema nervoso autônomo (Burnstoc.G et al., 1970). Em 1972, o conceito de nervos purinérgicos e transmissão purinérgica foi reformulado e, após alguma resistência, é amplamente aceito na atualidade (Burnstock, 1972), sendo a transmissão purinérgica a forma primordial de sinalização química intercelular (Burnstock and Verkhratsky, 2010).

O ATP está presente na célula de forma livre no citosol e também armazenado em vesículas. Pode ser liberado para o meio extracelular através de diferentes mecanismos, como: exocitose mediada por Ca^{2+}, transportadores de membrana ou, difusão através de canais de alta permeabilidade. Assim que é liberado para o meio extracelular, o ATP é degradado em seus derivados ADP, AMP e adenosina por ectonucleotidases que representam importantes componentes da sinalização purinérgica (Gallucci and Matzinger, 2001; Robson et al., 2006). O metabolismo extracelular do ATP está representado na figura 3.

Dentre as ectonucleotidases destacam-se a família das nucleosídeo trifosfato difosfoidrolases (NTPDases), a 5’ ecto-nucleotidase e a ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPP) as quais possuem ampla distribuição nos tecidos e são enzimas capazes de alterar os níveis de ATP, ADP, AMP e adenosina. As NTPDases são descritas como a família de enzimas de mamíferos que catalizam a hidrólise de ATP, ADP e AMP e possuem diferentes preferência pelos substratos. A

cascata de hidrólise iniciada pelas NTPDases pode ser terminada pela ecto-5'nucleotidase (CD73) com a hidrólise de nucleotídeos monofosfatados a adenosina (Fields and Burnstock, 2006; Robson et al., 2006; Zimmermann, 2000, 2001).

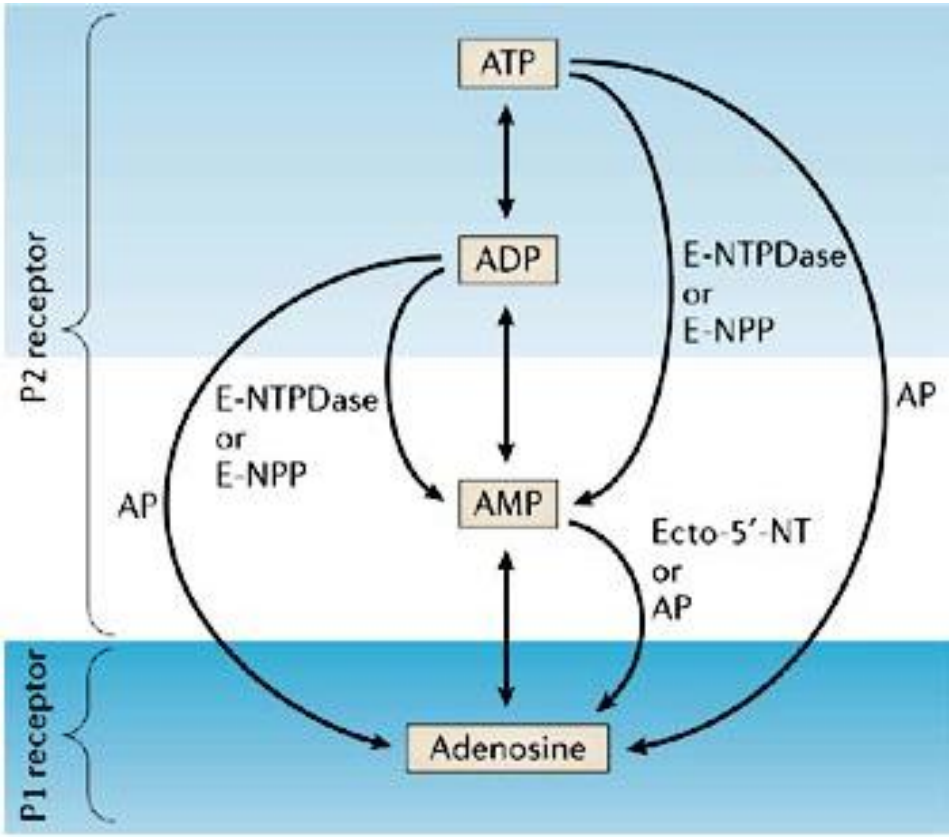

Figura 3. Representação esquemática do metabolismo extracelular do ATP. O metabolismo do ATP extracelular é regulado por ectonucleotidades, incluindo NTPDases, E-NPPs. A degradação de AMP a adenosina é realizada pela 5'ectonucleotidase (ecto-5'-NT). (Fonte: Fields & Burnstock, 2006; *Nature Reviews Neuroscience*).

Até o momento foram clonados e funcionalmente caracterizados oito membros da família das NTPDases. As NTPDases, classificadas de 1 a 8, no passado foram designadas com vários nomes aqui descritos entre parênteses: NTPDase 1 (CD39, ATPDase, ecto-apirase), NTPDase 2 (CD39L1, ecto-ATPase), NTPDase 3 (CD39L3,

HB6), NTPDase 4 (UDPase, LALP70), NTPDase 5 (CD39L4, ER-UDPase, PCPH), NTPDase 6 (CD39L2), NTPDase 7 (LALP1) e NTPDase 8 (Zimmermann, 2001).

A NTPDase 1 (CD39) hidrolisa ATP e ADP com aproximadamente a mesma velocidade (Heine et al., 1999). Mizumoto et al. (2002) demonstraram que camundongos *knockout* para NTPDase 1 (CD39) desenvolvem forte dermatite de contato quando expostos ao óleo de cróton. Os camundongos *knockout* para CD39 foram incapazes de metabolizar o ATP liberado em resposta a irritantes químicos, causando um acúmulo de ATP no espaço extracelular e, como consequência, uma resposta inflamatória exacerbada. Estes resultados sugerem que a dermatite de contato induzida por agentes químicos possui um mecanismo patogênico mediado por nucleotídeos e um mecanismo protetor dependente de CD39. O reconhecimento destes mecanismos pode levar ao desenvolvimento de novas estratégias terapêuticas para o tratamento da dermatite de contato (Mizumoto et al., 2002a).

Além da hipótese de Mizumoto (2002), um grande número de observações experimentais tem indicado que o ATP desempenha um papel preponderante na inflamação da pele, atuando como um amplificador da resposta imune através da geração de outros mediadores inflamatórios que recrutam leucócitos para o local da lesão (Seiffert et al., 2006). Foi demonstrado que o ATP afeta a expressão de quimiocinas (Pastore et al., 2007) e tem efeitos estimulatórios na expressão e liberação de IL-6, via receptores purinérgicos em cultura de queratinócitos normais (NHEKs) (Inoue et al., 2007).

Como resultado de um único evento de liberação de ATP por diferentes tipos celulares, muitas classes de receptores (algumas vezes receptores de ações opostas) são ativadas em células efetoras, podendo gerar uma extensiva rede de cascatas de sinalização intracelular responsáveis pelos seus efeitos tardios. Uma vez liberado, o

ATP atua como molécula de sinalização celular ativando uma família de receptores denominados receptores purinérgicos (Burnstock and Verkhratsky, 2010).

Os receptores purinérgicos são classificados em receptores P1, seletivos para adenosina, ou receptores P2, seletivos para ATP, ADP, UDP. Os receptores P1 são acoplados à proteína G; os receptores P2 se subdividem em P2X - que são acoplados a canais iônicos e P2Y - pertencentes à família de receptores acoplados à proteína G (la Sala et al., 2003). Em 1990, os receptores para purinas e pirimidinas foram clonados e caracterizados (Surprenant and North, 2009). Atualmente, sabe-se que os receptores P1 estão subdivididos em quatro tipos (A1, A2a, A2b, A3), os receptores P2X em sete subtipos (1-7) e, os receptores P2Y em oito subtipos (1,2,4,6,11,12,13,14) (Burnstock, 2007; Gao et al., 2013).

Nos últimos anos, têm aumentado o número de estudos demonstrando que a sinalização purinérgica desempenha importante função regulatória em várias doenças inflamatórias (Eltzschig et al., 2012). Os receptores purinérgicos são expressos em diversas células do sistema imune cutâneo, como queratinócitos, células de langerhans, macrófagos e células dendríticas; fazendo destes receptores importantes alvos para o tratamento e/ou prevenção de doenças inflamatórias cutâneas (Burnstock et al., 2012). Weber et al. (2010) demonstrou que a ativação do receptor purinérgico P2X7 é essencial para a fase de indução da dermatite de contato alérgica em camundongos (Weber et al., 2010), abrindo novas perspectivas para o tratamento da ACD com antagonistas deste receptor.

1.4.1 Receptor P2X7

Os receptores P2X7 são receptores ionotrópicos e sua ativação é dependente da concentração de ATP no meio extracelular que deve ser maior que 100 μM (Carroll et al., 2009). A ativação do receptor causa a abertura reversível de um poro na membrana plasmática da célula, promovendo um aumento da permeabilidade a solutos hidrofílicos de peso molecular até 900 Da e causando uma perturbação na homeostase iônica da célula (Ferrari et al., 2006; Tran et al., 2010). Foi observado que o aumento da concentração de ATP extracelular não provoca a dessensibilização do receptor, entretanto pode levar a morte celular. Por outro lado, o aumento da hidrólise de ATP via ectonucleotidades promove o fechamento do poro e o reestabelecimento da homeostase iônica (Di Virgilio et al., 1998).

O receptor P2X7 é considerado um importante regulador da inflamação e da imunidade. Vários antagonistas de P2X7R têm sido identificados a fim de avaliar as funções deste receptor. Recentemente, foi publicado um estudo com a caracterização completa de um antagonista do receptor P2X7, AZ11645373, que mostrou alta afinidade pelo receptor de humanos, porém apresentou baixa afinidade por receptores de ratos (Stokes et al., 2006). Outros antagonistas seletivos de P2X7R e com afinidade comparável em humanos, ratos e camundongos têm sido desenvolvidos e caracterizados (Carroll et al., 2007; Donnelly-Roberts and Jarvis, 2007).

Uma das principais consequências da ativação do receptor P2X7 é a liberação e o processamento de IL-1β via inflamossomo. A IL-1β pertence a uma família de três citocinas: IL-1α, IL-1β e IL-1Ra (Arend et al., 2008), que atuam através de interações com o receptor para IL-1 (IL-1R) (Chen and Nunez, 2010). IL-1β é uma citocina que funciona como um mediador chave na defesa do hospedeiro (Ferrari et al., 2006) e é um

potente mediador inflamatório, já que desencadeia funções biológicas como o recrutamento de neutrófilos e monócitos, a ativação de DCs e indução da liberação de mediadores pró-inflamatórios adicionais (Chen and Nunez, 2010; Nestle et al., 2009).

Produtos bacterianos (ex. LPS) e mediadores inflamatórios endógenos causam a síntese de IL-1β na forma de uma pró-citocina inativa (pró-IL-1β) que permanece dispersa no citosol até que um segundo estímulo dirija o processamento e a liberação de sua forma ativa. Portanto, são necessários dois estímulos para que a liberação de IL-1β aconteça, um primeiro que ativa a transcrição gênica de pró-IL-1β e um segundo que promove seu processamento e liberação (Ferrari et al., 2006). O ATP via P2X7R tem sido descrito como um sensor de perigo (DAMP) que dirige a maturação e a liberação de IL-1β via inflamossomo (Ferrari et al., 1997).

Os inflamossomos pertencem a uma subfamília de receptores PRRs denominada de receptores de ligação a nucleotídeos contendo domínio com seqüência repetida de resíduos do aminoácido leucina (NLRs). NLRs possuem uma estrutura tripartida consistindo de um domínio C-terminal rico em leucinas (LRRs, *leucine-rich repeats*), um domínio central ligado a nucleotídeos (NBD, *nucleotide-binding domain*), e um domínio efetor N-terminal. As subfamílias de NLRs diferem neste domínio efetor N-terminal que é requerido para a transdução de sinal. A maioria dos NLRs possuem um domínio terminal que recruta caspase (CARD, *caspase recruitment domain*) ou um domínio pirina (PYD, *pyrin domain*). Os NLRPs possuem o domínio terminal pirina e alguns deles podem promover a conversão de caspase-1 inativa na forma ativa (Bauernfeind et al., 2011).

Caspases são consideradas enzimas efetoras ou iniciadoras da apoptose. Entretanto, a caspase-1 pertence a uma subclasse de caspases, conhecidas como caspases inflamatórias, as quais clivam precursores inativos de citocinas e as secretam

em sua forma ativa. Alguns dos substratos processados via caspase-1 são os precursores de IL-1β e IL-18 (Dinarello, 2009). É assumido que os inflamossomos se formam através de uma ligação direta ou indireta que recruta pro-caspase-1 via interações CARD. O inflamossomo NLRP3 não pode ativar caspase-1 diretamente, antes disso ele recruta a proteína associada a apoptose contendo um domínio CARD (ASC) através de seu domínio pirina (Bauernfeind et al., 2011).

NLRP3 é o membro da família dos NLRs mais estudado e tem recebido uma atenção especial nos últimos anos devido as suas funções em respostas inflamatórias estéreis. A ligação do ATP extracelular ao receptor purinérgico P2X7 leva à ativação de panexina-1 e oligomerização de NLRP3 via efluxo de K^+ (Bauernfeind et al., 2011; Schroder et al., 2010). Portanto, na presença de um primeiro sinal que promova o aumento da transcrição gênica de pró-IL-1β, o ATP pode funcionar com um segundo sinal que ativa NLRP3 promovendo ativação de caspase-1 com consequente maturação e liberação de IL-1β ativa (Di Virgilio, 2007). Esse processo está esquematizado na figura 5.

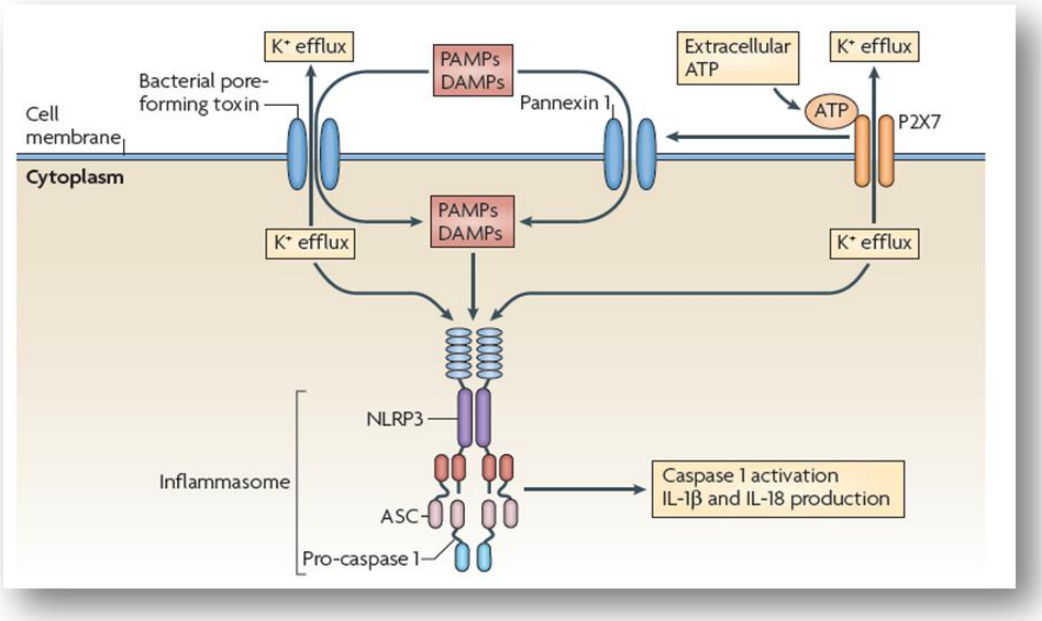

Figura 4. Representação esquemática da ativação do inflamossomo NALP3 via receptor P2X7. O ATP extracelular se liga ao receptor P2X7 e desencadeia o efluxo de K+ e a ativação de panexina-1. A ativação de panexina-1 leva a ativação do inflamossomo, com consequente ativação de caspase-1 e clivagem de pró-IL-1β em IL-1β ativa. (Fonte: Schroder et al., 2010; *Science*)

2 OBJETIVOS

2.1 Objetivo geral

Considerando que: (1) a dermatite de contato irritante é uma patologia cutânea de alta prevalência cujos mecanismos são pouco esclarecidos, e que (2) o envolvimento do receptor P2X7 tem sido descrito em diversas patologias inflamatórias; o presente estudo tem por objetivo avaliar o envolvimento do receptor P2X7 na dermatite de contato irritante utilizando modelos *in vivo* e in *vitro*.

2.2 Objetivos específicos

- Avaliar os efeitos do bloqueio farmacológico e da deleção gênica do receptor P2X7 na dermatite de contato induzida por óleo de cróton em camundongos.

- Determinar os efeitos do antagonista do receptor P2X7 (A438079), da deleção gênica do receptor P2X7, da ecto-enzima apirase e do inibidor de caspase-1 (N-1330) sobre a migração de células inflamatórias para o tecido, sobre a liberação local e sistêmica IL-1β e sobre o edema induzidos pela aplicação tópica de óleo de cróton em camundongos.

- Determinar o efeito da depleção de macrófagos e células dendríticas sobre o edema, sobre a migração de neutrófilos e sobre a liberação local de IL-1β, induzida por óleo de cróton em camundongos.

- Determinar o envolvimento do receptor P2X7 sobre a atividade liberadora de IL-1β induzida por ATP e óleo de cróton por macrófagos e células dendríticas, utilizando cultura de células isoladas da pele de camundongos.

- Analisar os efeitos do óleo de cróton, do antagonista do receptor P2X7 (A438079) e da ecto-enzima apirase sobre a hidrólise de nucleotídeos no soro de camundongos.

- Avaliar o efeito do óleo de cróton e do antagonista do receptor P2X7 (A438079) sobre a viabilidade celular de queratinócitos humanos (linhagem celular HaCaT).

3. ARTIGOS CIENTÍFICOS

CAPÍTULO I

ARTIGO CIENTÍFICO

P2X7 receptor is required for neutrophil accumulation in a mouse model of irritant contact dermatitis

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P2X7 receptor is required to neutrophil accumulation in a mouse model of irritant contact dermatitis

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ABSTRACT

Irritant contact dermatitis (ICD) is an inflammatory reaction caused by chemical toxicity on the skin. The P2X7 receptor (P2X7R) is a key mediator of cytokine release, which recruits immune cells to sites of inflammation. We investigated the role of P2X7R in croton oil (CrO)-induced ICD using *in vitro* and *in vivo* approaches. ICD was induced *in vivo* by CrO application on the mouse ear and *in vitro* by incubation of murine macrophages and dendritic cells (DCs) with CrO and ATP. Infiltrating cells were identified by flow cytometry, histology and myeloperoxidase (MPO) determination. Effects of the ATP scavenger apyrase were assessed to investigate further the role of P2X7R in ICD. Animals were also treated with N-1330, a caspase-1 inhibitor, or with clodronate, which induces macrophage apoptosis. CrO application induced severe inflammatory Gr1+ cell infiltration and increased MPO levels in the mouse ear. Selective P2X7R antagonism with A438079 or genetic P2X7R deletion reduced the neutrophil infiltration. Clodronate administration significantly reduced Gr1+ cell infiltration and local IL-1β levels. *In vitro* experiments confirmed that A438079 or apyrase treatment prevented the increase in IL-1β that was evoked by macrophage and DC incubation with CrO and ATP. These data support a key role for P2X7 in ICD-mediated inflammation via modulation of inflammatory cells. It is tempting to suggest that P2X7R inhibition might be an alternative ICD treatment.

Key words: irritant contact dermatitis – neutrophils – P2X7R

1 INTRODUCTION

The skin is the primary defense between the body and the environment (Nestle et al., 2009). Contact dermatitis is a common inflammatory skin disease that involves activation of the innate and adaptive immune systems. Contact dermatitis comprises both irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) (Saint-Mezard et al., 2004). ICD is defined as a locally arising reaction that appears after chemical irritant exposure. The chemical agents are directly responsible for cutaneous inflammation because of their inherent toxic properties, which cause tissue injury (Nosbaum et al., 2009). This inflammatory response activates innate immune system cells, such as macrophages, DCs and neutrophils (Gibbs, 2009; Nosbaum et al., 2009; Tubaro et al., 1986a). Conversely, ACD is a delayed-type hypersensitivity response, which is triggered by specific T-cell activation and proliferation (Krasteva et al., 1999). Croton oil (CrO) is a chemical irritant that causes topical inflammation when applied to mouse skin (McDonald et al., 2010; Mizumoto et al., 2002b). CrO application induces marked oedema and cell migration with massive neutrophil migration, which are features of an ICD model (Tubaro et al., 1986a; Tubaro et al., 1986b).

The P2X7 receptor (P2X7R) is an ATP-gated cation channel that is expressed on inflammatory cells (Tran et al., 2010). This receptor reportedly controls diverse pro-inflammatory cellular signalling based on its ability to initiate post-translational cytokine processing, such as that of IL-1β (Di Virgilio, 2007; Ferrari et al., 2006). P2X7R is activated by ATP (Bours et al., 2011; Georgiou et al., 2005) and is an important stimulator of the NLRP3 inflammasome (Di Virgilio, 2007). Interference of leucocyte migration is an effective approach to treat skin inflammation (Duthey et al.,

2010). NLRP3 is a signalling pathway that drives proteolytic activation of caspase-1 and IL-1β release (Qu et al., 2009), which recruits leucocytes to sites of infection and/or injury leading to host tissue damage (Iyer et al., 2009). Different cell types produce IL-1β, and it is not known which of these cell types mediate the inflammatory response during the ICD effector phase.

We used a CrO-induced ICD model to investigate the nature of the inflammatory cells and the role of P2X7R in this process. We verified that P2X7R activation is required for neutrophil accumulation and cytokine production in this experimental paradigm. Therefore, we suggest that P2X7R inhibition might represent a potential therapeutic alternative for ICD treatment.

2 MATERIAL AND METHODS

Drugs

A438079 was obtained from Tocris (Ellisville, MO, USA). CrO, apyrase, dexamethasone and clodronate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-1330 (Ac-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Bachem Americas, Inc. (Torrance, CA, USA).

Animals

Male Swiss mice, or C57BL/6 WT and C57BL/6 P2X7$^{-/-}$ (6–8 weeks, 25–30 g) were used in this study. Swiss and C57BL/6 mice were obtained from the Federal University of Pelotas (UFPEL; Pelotas, RS, Brazil), and P2X7$^{-/-}$ mice were donated by Dr. Robson Coutinho-Silva, Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil). The P2X7$^{-/-}$ mice were generated using the method developed by Dr.

James Mobley (PGRD; Pfizer Inc., Groton, CT, USA). The P2X7$^{-/-}$ receptor-deficient mice used in this study were inbred to C5BL/6.

The animals were maintained at controlled temperature (22 ± 1°C) and humidity (60–70%) with a 12-h light–dark cycle (lights on 7:00 AM). Food and water were available *ad libitum*. Animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. All of the tests were performed between 7:00 AM and 7:00 PM. The experimental procedures reported in this manuscript followed the 'Principles of Laboratory Animal Care' from the National Institutes of Health and were approved by the Institutional Animal Ethics Committee (protocol number: 10/00206).

Irritant contact dermatitis model

Swiss mice received a topical application of 1% CrO on the right ear and vehicle (acetone) on the left ear according to the method described by Mizumoto et al. (2002). Briefly, 6 h after CrO application, the animals were euthanized, and the ears were collected for analysis.

In a separate experiment, P2X7 receptor involvement was assessed using animals that had genetic deletion of this receptor. C57BL/6 mice were used as controls for this series of experiments. ICD was induced as described previously. All of the experiments were performed with a minimum of five animals per group and were repeated at least three times.

Ear oedema measurement

The animals were euthanized 6 h after CrO application. A 6-mm-diameter disc from the right and the left ears was removed with a circular metal punch and was

weighed on an analytical balance.CrO-induced swelling was assessed as the weight difference (mg) between the right (inflamed) and the left (vehicle-treated) ears.

Pharmacological treatments

To characterize the role of P2X7R in the CrO-induced inflammatory response, *Swiss* mice were treated with the P2X7R antagonist A438079 (80 µmol/kg, i.p.) or the ATP scavenger apyrase (0.2 U/ear, s.c.) 2 h after CrO application. A separate group was treated with the positive control dexamethasone (0.5 mg/kg, s.c.). To evaluate whether IL-1β was involved in the processes, *Swiss* mice were also treated with the caspase-1 inhibitor N-1330 (6.25 µmol/kg) according to Mathiak et al. (2000). (Mathiak et al., 2000)

Depletion of phagocytic cells with clodronate

Liposomally encapsulated clodronate (dichloromethylene diphosphonate) is a macrophage and DC apoptosis inducer (Jordan et al., 2003). To verify the role of these cells in the inflammatory process, clodronate (dichloromethylene diphosphonic acid; Sigma–Aldrich) or sterile PBS-containing liposomes were instilled subcutaneously (locally – in the ears) and intravenously (25 mg/kg) 24 h before CrO application.

Myeloperoxidase activity

Neutrophil recruitment to the ears was quantified by tissue myeloperoxidase (MPO) activity according to the method described by Pereira et al. (2011) with minor modifications. The ear tissue was homogenized in 5% (w/v) ethylenediamine tetra acetic acid (EDTA)/NaCl buffer (pH 4.7) and centrifuged at 6500 rpm for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethylammonium bromide buffer

(pH 5.4), and the samples were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were recentrifuged under the same conditions mentioned previously (4400 g, 15 min, 4°C), and 25 µl supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM Na_3PO_4 and 0.3 mM hydrogen peroxide. Absorbance was measured at 650 nm, and the results are expressed as optical density per milligram of tissue (Pereira et al., 2011).

Determination of IL-1β levels in ear tissue

Ear tissues were homogenized in phosphate-buffered saline (PBS) containing 0.4 M NaCl, 0.1 M phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 0.05% Tween-20, 0.5% bovine serum albumin and 2 µg/ml aprotinin A. Samples were centrifuged at 5000 rpm for 10 min at 4°C, and the supernatants were used for the assay. IL-1β levels were measured using an enzyme-linked immunosorbent assay kit according to supplier recommendations (DuoSet Kit; R&D Systems, Minneapolis, MN, USA). The results were expressed as pg/mg tissue.

Flow cytometry

Skin cells were obtained from mouse ears according to McLachlan et al. (2009). Fc receptors were blocked by resuspending cells in culture supernatant containing 24G2 antibody (ATCC) plus 1% mouse serum and 1% rat serum (FcBlock). Samples were later stained for surface markers as described below. Macrophages were stained with anti-CD11b (APC – clone M1/70), neutrophils were stained with anti-Gr1 (PE – clone RB6-8C5) and dendritic cells were stained with anti-CD11c (PE-Cy7 – clone HL3). All of the antibodies were purchased from BD Biosciences (San Jose, CA, USA). Cells were analysed using FACSCantoII (Becton Dickinson, Franklin Lakes, NJ, USA) and

BD FACSDiva software, and FACS data were analysed with Flowjo software (version 7.6.5; Tree Star, Inc, Ashland, OR, USA) (McLachlan et al., 2009).

Histological analysis

The collected ears were fixed in buffered formalin solution (10%) for 24 h, and the samples were subsequently paraffin embedded. Slices that were 5 µm thick were obtained and stained with haematoxylin and eosin. A pathologist who was blinded to the treatment reviewed each specimen for leucocyte infiltration.

Murine macrophage and dendritic cell culture

In accordance with Inaba et al., C57BL/6 murine DCs were grown from bone marrow with granulocyte–macrophage colonystimulating factor (GM-CSF) and IL-4 (both BD Biosciences). On culture day 6, the cells were separated into adherent (macrophages) and non-adherent (dendritic cells). Cells were incubated with 25 µM A438079 and Apyrase (2 U) for 30 min followed by stimulation with 1% CrO or nothing (0 h). After 3 h, all of the cells were incubated with 2 mM ATP for 12 h, and IL-1β expression was analysed in the supernatant with an ELISA kit (DuoSet Kit; R&D Systems, Minneapolis, MN, USA) (Inaba et al., 1992).

Statistical analysis

Results are expressed as the mean ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. P-values < 0.05 were considered to be significant. All of the tests were performed using GraphPad® 5 Software (version 5.0; Graphpad Software Inc., San Diego, CA, USA).

3 RESULTS

P2X7R mediates neutrophil recruitment in ICD

To investigate whether neutrophil recruitment in our ICD model was dependent on P2X7R, we verified the histological sections. The analysis demonstrated that CrO-induced neutrophil infiltration in the ear tissue compared with the control, whereas apyrase, A438079 and dexamethasone reduced the neutrophil infiltration, which was also accompanied by decreased ear oedema (Fig. 1a–c). In addition, we measured MPO levels in the Swiss mouse ears in the presence or absence of P2X7R-specific antagonist, apyrase or dexamethasone after CrO application. CrO administration increased MPO levels in the ears (Fig. 1d), which was significantly reduced by P2X7R antagonist A438079 (75 ± 6%) and dexamethasone (72 ± 5%) treatment. IL-1β is a primary cause of inflammation, and growing evidence suggests that P2X7R is a key mediator of IL-1β release (Ferrari et al., 2006). CrO application markedly increased IL-1β levels in the ears of the Swiss mice (Fig. 1e). The increased IL-1b levels were reduced 37 _ 9%, 45 ± 5% and 81 ± 4% by apyrase, A438079 or dexamethasone treatment, respectively, after CrO administration (Fig. 1d).

These data were further confirmed by flow cytometry. Figure S1a demonstrates that CrO application results in increased Gr1+ cell recruitment into the ear, which was diminished after apyrase and A438079 treatments. Additionally, we observed an increase in the percentage of DCs (CD11c+ cells) and macrophages (CD11b+ cells) that were present after CrO administration, which were also reduced after apyrase and A438079 treatments.

To confirm the role of P2X7R in ICD in vivo, we assessed CrO-mediated neutrophil infiltration in C57BL/6 WT and $P2X7^{-/-}$ mice. Histological analysis

demonstrated that P2X7$^{-/-}$ mice exhibited reduced neutrophil influx and had a partial reduction in ear oedema after CrO application (Fig. 2a–c). These results were further confirmed by flow cytometry (Fig. S1b). In addition, as shown in Fig. 2d, CrO application was associated with neutrophil accumulation in C57BL/6 WT mice, as indicated by a significant increase in MPO activity in the skin. CrO-induced MPO activity was significantly decreased by 35% in P2X7$^{-/-}$ mice compared with WT mice, suggesting the involvement of P2X7R in CrO-mediated neutrophil recruitment. Similar to the first experiments with the Swiss mice, IL-1β levels were elevated after CrO exposure in C57BL/6 WT mice (Fig. 2d). In contrast, CrO-mediated IL-1β induction in P2X7$^{-/-}$ mice was significantly reduced (28 ± 6%) compared with WT (Fig. 2e). Taken together, these data indicate that the P2X7 receptor is required for CrO-mediated neutrophil recruitment and suggest that IL-1β release may be associated with this process.

Neutrophil recruitment is significantly decreased after clodronate liposome administration

It has been demonstrated that other innate immune system cells are necessary for neutrophil recruitment (Inaba et al., 1992; Lee et al., 2010; Tubaro et al., 1986b). To address this issue, we used liposome-encapsulated clodronate to deplete APCs. Flow cytometry (Fig. S1c) demonstrated that clodronate treatment primarily reduced CD11b- and CD11c-expressing cells. Furthermore, CrO did not increase MPO levels (Fig. 3c), IL-1β levels (Fig. 3d) or the Gr1+ cell population (Fig. S1c) in mice that were pretreated with clodronate, thereby suggesting a correlation between neutrophil recruitment and CrO-mediated IL-1β release by DCs and macrophages. Additionally, histology corroborated with reduced neutrophil infiltration after clodronate treatment (Fig. 3a–c).

Moreover, clodronate pretreatment significantly decreased CrO-mediated ear oedema (Fig. 3b).We hypothesized that CrO treatment triggered ATP release, thus activating DCs and macrophages following P2X7 receptor stimulation and IL-1β secretion. To evaluate this hypothesis, we performed an in vitro experiment with macrophages and DCs that had been differentiated from mouse bone marrow. The cells were pretreated with A438079 or apyrase or were left untreated. The cells were subsequently stimulated with CrO or vehicle for 3 h followed by incubation with 2 mM ATP. After 12 h, IL-1β release was measured by ELISA. Figure S2 demonstrates that CrO or ATP incubation evoked IL-1β release from macrophages and DCs, which was significantly reduced by pretreatment with A438079 or apyrase, suggesting that extracellular ATP-mediated P2X7R activation is important for IL-1β secretion by macrophages and DCs. Taken together, the results described previously indicate that MPs and DCs play a key role in CrO-induced ICD and suggest that P2X7R activation in MPs and DCs may be involved.

Reduced IL-1β secretion impairs neutrophil accumulation

P2X7R is a key player in IL-1b processing and release (Ferrari et al., 2006). The IL-1b release following ATP-mediated P2X7 receptor stimulation occurs via the NLRP3 inflammasome (Di Virgilio, 2007). NLRP3 activates caspase-1, an enzyme that cleaves inactive pro-IL1-β to active IL1-β (Lee et al., 2010). To gain further insights in our experimental paradigm, we treated Swiss mice with the irreversible caspase-1 inhibitor N-1330 (Ac-Tyr-Val-Ala-Asp-chloromethylketone). Interestingly, N-1330 treatment significantly decreased neutrophil accumulation as assessed by histology (Fig. 4a,c). In addition, ear oedema was decreased in N-1330-treated animals compared with the control group. Furthermore, MPO levels (27 ± 4%) and IL-1β production (36 ± 11%) were also reduced by N-1330 treatment (Fig. 4d,e). Flow cytometry data (Fig.

S1d) were also consistent with these results. In addition, CD11b+ and CD11c+ cells were also reduced. Taken together, these results reinforce the possible association between IL-1β-mediated neutrophil recruitment and ear oedema.

4 DISCUSSION

Little is known about the involvement of purinergic signalling in skin inflammation. Recently, Weber et al. (2010) reported a functional link in ACD between ATP, P2X7R and the NLRP3 inflammasome to regulate IL-1β release. In this study, we investigated the role of P2X7R in CrO-mediated neutrophil recruitment in ICD. We demonstrated that P2X7R is implicated in neutrophil recruitment and ear oedema. As was reported by Weber et al., we verified that $P2X7^{-/-}$ mice displayed a partial reduction in CrO-induced ear oedema (Fig. 2b) (Weber et al., 2010). Interestingly, ear oedema was markedly reduced in mice that had been treated with the selective P2X7 receptor antagonist A438079. It is important to mention that genetically modified animals (including P2X7 KO mice) might exhibit compensatory changes in signal transduction; however, knockout strategies are still useful tools to confirm the functional data that is obtained using pharmacological approaches (Kim et al., 2010).

In this study, we demonstrated that CrO application increased neutrophil accumulation, which was confirmed by MPO levels, Gr1+ staining and histology of mouse ears. Remarkably, in P2X7_/_ mice (Fig. 2), treatment with the selective P2X7R antagonist A438079 or administration of the ATP-degrading enzyme apyrase markedly reduced this effect (Fig. 1). Previous data demonstrated the involvement of P2X7R in tissue injury-mediated leucocyte infiltration (Chessell et al., 2005; Martins et al., 2012;

Moncao-Ribeiro et al., 2011). Therefore, the results obtained herein reinforced the relationship between P2X7R activation and neutrophil accumulation in our ICD model.

Next, we reported that clodronate liposome administration subcutaneously and intravenously eliminated CD11b+ cells (macrophages) and partially eliminated CD11c+ cells (DCs; Fig. S1c). Elimination of these cells occurred concurrently with diminished neutrophil infiltration, similar to the effect observed in P2X7R knockout animals (Fig. 1a,c and d). These findings demonstrate a key role of DCs and macrophages in initiating the ICD inflammatory process. Of note, IL-1β levels were comparable with the control after clodronate liposome treatment, indicating that IL-1β is produced mainly via immune cells, as was described by Chen et al. (2010). (Chen and Nunez, 2010).

P2X7R is implicated in IL-1β processing and release, which supports leucocyte migration to inflamed tissue (Ferrari et al., 2006). In this study, we demonstrate that CrO increased IL-1β levels in the ear of the treated mice, and this effect was significantly reduced by A438079 or apyrase treatment (Fig. 1b). In addition, CrO induced IL-1β accumulation was significantly decreased in P2X7$^{-/-}$ mice compared with C57BL/6 WT mice (Fig. 2d), suggesting IL-1β involvement following P2X7R activation in the CrO-ICD model. Extracellular ATP activates the caspase-1/Nlrp3 inflammasome complex via P2X7 receptor signalling, which generates inflammatory cytokines such as IL-1β (Bauernfeind et al., 2011; Di Virgilio, 2007; Georgiou et al., 2005; Iyer et al., 2009). In this study, we demonstrated that simultaneous treatment with CrO and ATP induced the release of significant amounts of IL-1β via P2X7R in macrophages and DCs (Fig. 3e).

Croton oil induced keratinocyte necrosis (data not shown), suggesting that ATP is released during CrO exposure. We further demonstrated that caspase-1 inhibition reduced IL-1β formation (Fig. 4d) and consequently reduced neutrophil accumulation

(Fig. 4a–c). Mc Donald et al. (2010) demonstrated that ATP initiates the inflammatory response that causes neutrophil recruitment. Thus, we suggest that P2X7R-mediated IL-1β secretion is related to tissue neutrophil recruitment in the ICD model, and we also suggest participation of other pathways in addition to P2X7R and IL-1β-mediated neutrophil recruitment (Bertelsen et al., 2011; Duthey et al., 2010).

In summary, our compelling evidence indicates the importance of P2X7R and APCs (macrophages and DCs) in promoting neutrophil accumulation and ear oedema in ICD, which may be correlated in part, to enhanced IL-1β levels. Therefore, it is tempting to suggest that P2X7R inhibition represents a potential therapeutic alternative for ICD treatment.

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Author Contributions

G. Silva, N. Sperotto, T. Borges, C. Takya, and Rafael Zanin performed research and interpreted the data. C. Bonorino and R. Coutinho-Silva contributed essential reagents or tools. T. Cristina, R. Coutinho-Silva, M. M. Campos, R. Zanin and F.B. Morrone assisted with the data analysis. G. Silva wrote the draft of the manuscript. M.M Campos, R. Zanin, F.B. Morrone were responsible for study design, writing and manuscript correction.

Ethical approval

The experimental procedures reported in this manuscript followed the Principles of Laboratory Animal Care from the National Institutes of Health (NIH) and were approved by the Institutional Animal Ethics Committee (protocol number: 10/00206).

Conflict of interests

The authors have declared no conflicting interests.

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FIGURE LEGENDS

Figure 1

A438079 and apyrase treatment decreased the croton oil (CrO) chemical irritant-mediated inflammatory response. Irritant contact dermatitis (ICD) was elicited as described in Materials and methods. **(a)** Ear sections were stained with haematoxylin and eosin (10x) 4 h after topical 1% CrO application in the presence or absence of apyrase (0.2 U/ear, s.c.), A438079 (80 µmol/kg, i.p.) and dexamethasone (0.5 mg/kg, s.c.). **(b)** Swiss mice were treated with 1% CrO, acetone (vehicle), apyrase, A438079 and dexamethasone. Oedema (ear weight) was measured 6 h after CrO application. **(c)** Histological polymorphonuclear (PMN) cell quantification. **(d)** Myeloperoxidase (MPO) activity and **(e)** IL1β secretion in the ears of the Swiss mice that received 1% CrO topically in acetone or acetone alone (control) in the presence or absence of apyrase (0.2 U/ear, s.c.), A438079 (80 µmol/kg, i.p.) and dexamethasone (0.5 mg/kg, s.c.). Data represent the mean ± SEM of five animals. Significantly different from the control *P < 0.05; **P < 0.01; ***P < 0.001 significantly different from CrO treatment. #P < 0.05 significantly different from vehicle treatment. All of the parameters were measured 6 h after the application of CrO.

Figure 2

P2X7R genetic deletion decreased the croton oil (CrO) chemical irritant evoked inflammatory response. Irritant contact dermatitis (ICD) was elicited as described in the Materials and methods section. **(a)** Ear sections from WT and $P2X7^{-/-}$ mice were stained with haematoxylin and eosin (109) 4 h after topical 1% CrO application. **(b)** $P2X7^{-/-}$ and WT mice were treated with 1% CrO and acetone (vehicle) on the ear. Oedema (ear

weight) was measured 6 h after CrO application**. (c)** Polymorphonuclear (PMN) cell quantification from histology. **(d)** Myeloperoxidase (MPO) activity **(e)** IL1β secretion in $P2X7^{-/-}$ and C57BL/6 WT mouse ears that received topical 1% CrO application. Data represent the mean ± SEM of five animals. **P < 0.01 and ***P < 0.001 significantly different from WT and $P2X7^{-/-}$ and #P < 0.001 significantly different compared with CrO.

Figure 3

Depletion of macrophages and dendritic cells (DCs) decreased croton oil (CrO)-mediated inflammation. Irritant contact dermatitis (ICD) was elicited as described in the Materials and methods section. Clodronate was administered 24 h before ICD induction, and ears were collected 4 h later. **(a)** Ear sections were stained with haematoxylin and eosin (109) 4 h after topical 1% CrO application in the presence or absence of Clodronate (25 mg/kg). **(b)** *Swiss* mice were treated with 1% CrO, acetone (vehicle) and clodronate. Oedema was measured 6 h after CrO application. **(c)** Polymorphonuclear (PMN) cell quantification from histology. **(d)** Clodronate application (25 mg/kg) on myeloperoxidase (MPO) activity and (e) IL1β secretion. Data represent the mean ± SEM of three experiments. **P < 0.01;***P < 0.001 denote significance compared with control values; ##P < 0.001; #P < 0.01 denote significance compared with control values.

Figure 4

Caspase-1 inhibition decreased croton oil (CrO)-mediated neutrophil accumulation. Irritant contact dermatitis (ICD) was elicited as described in the Materials and methods section. N-1330 (6.25 lmol/kg), an irreversible inhibitor of caspase-1, was administered

30 min prior to ICD induction, and ears were collected 4 h later. **(a)** Ear sections were stained with haematoxylin and eosin (10x) 4 h after topical 1% CrO application in the presence or absence of N-1330 (6.25 μmol/kg). **(b)** *Swiss* mice were treated with 1% CrO, acetone (vehicle) and N-1330. Oedema (ear weight) was measured 6 h after CrO application. **(c)** Polymorphonuclear (PMN) cells were quantified from histology. **(d)** Myeloperoxidase (MPO) activity and **(e)** IL1β secretion. *P < 0.05; **P < 001 denotes the significance levels compared with control values; #P < 0.001 denotes the significance levels compared with vehicle values.

Supplementary Figures

Supplementary Figure 1

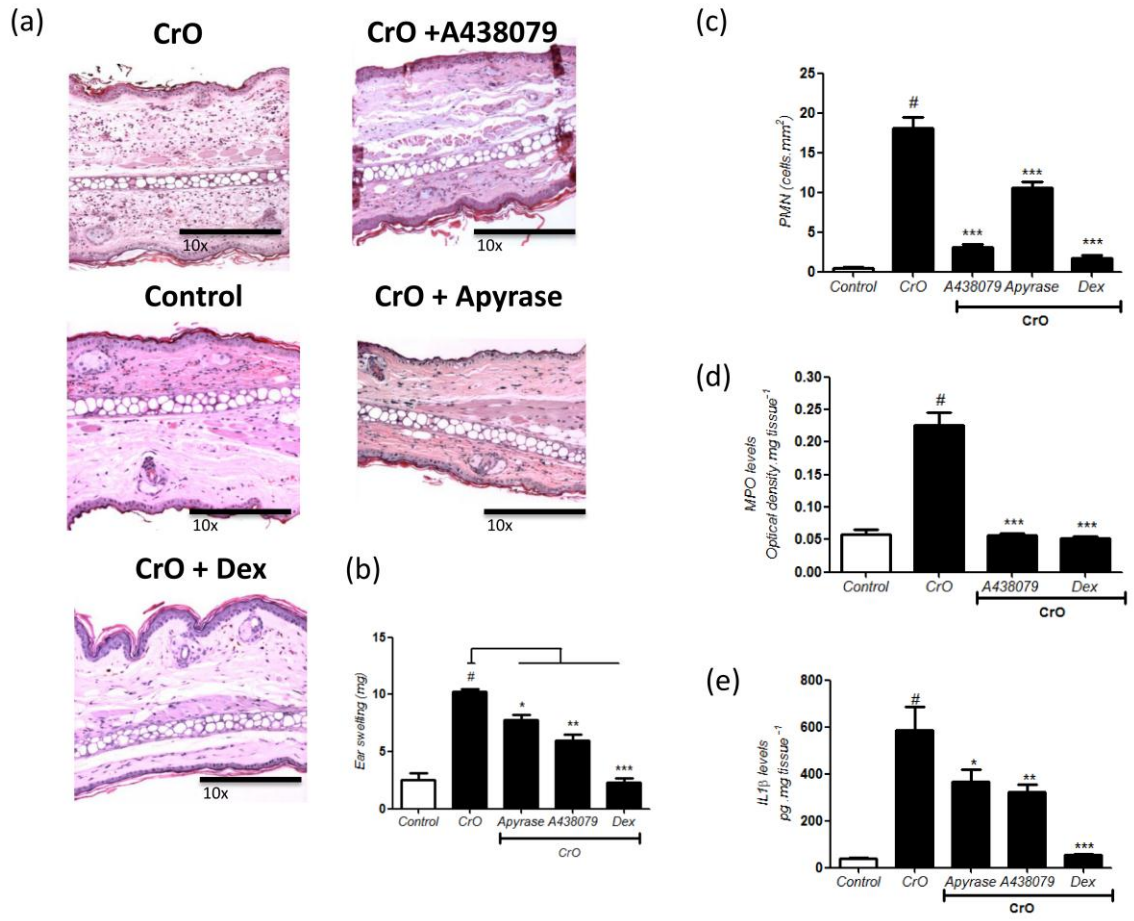
(a) Dot plot with percentages of Gr1+, CD11b+, and CD11c+ cells in ear tissue from Swiss mice that received topical 1% CrO application in presence or absence of A438079 (80 μmol/kg, i.p.) or apyrase (0,2 U/ear, s.c.). Figures represent at least three independent experiments with pooled ears from 3 mice per experiment. Flow cytometry analyses were performed 4 hours after croton oil application. **(b)** Dot plots with the percentage of Gr1+, CD11b+ and CD11c+ cells in ear tissue from C57/B6 and P2X7-/- mice that received topical 1% CrO application. **(c)** Dot plots with the percentage of Gr1+, CD11b+, and CD11c+ cells in ear tissues. Figures are representative of at least three independent experiments with pooled ears from 3 mice per experiment. Flow cytometry analyses were performed 4 hours after croton oil application. **(d)** Dot plots with the percentage of Gr1+, CD11b+, and CD11c+ cells in ear tissues from Swiss mice that received topical 1% CrO application in the presence or absence of N-1330 (6.25 μmol/kg). Figures are representative of at least three independent experiments with

pooled ears from 3 mice per experiment. Flow cytometry analyses were performed 4 hours after croton oil application.

Supplementary Figure 2

IL1-β was measured from murine DC and macrophage culture supernatants that had been primed with Croton oil (1%) CrO 3 hours after ATP treatment (2 mM) in the presence or absence of A438079 (25 μM) or apyrase (2 U/mL). Data represent the mean ± SEM of three experiments. **P<0.01 denotes the significance levels compared with WT + CrO.

Figure 1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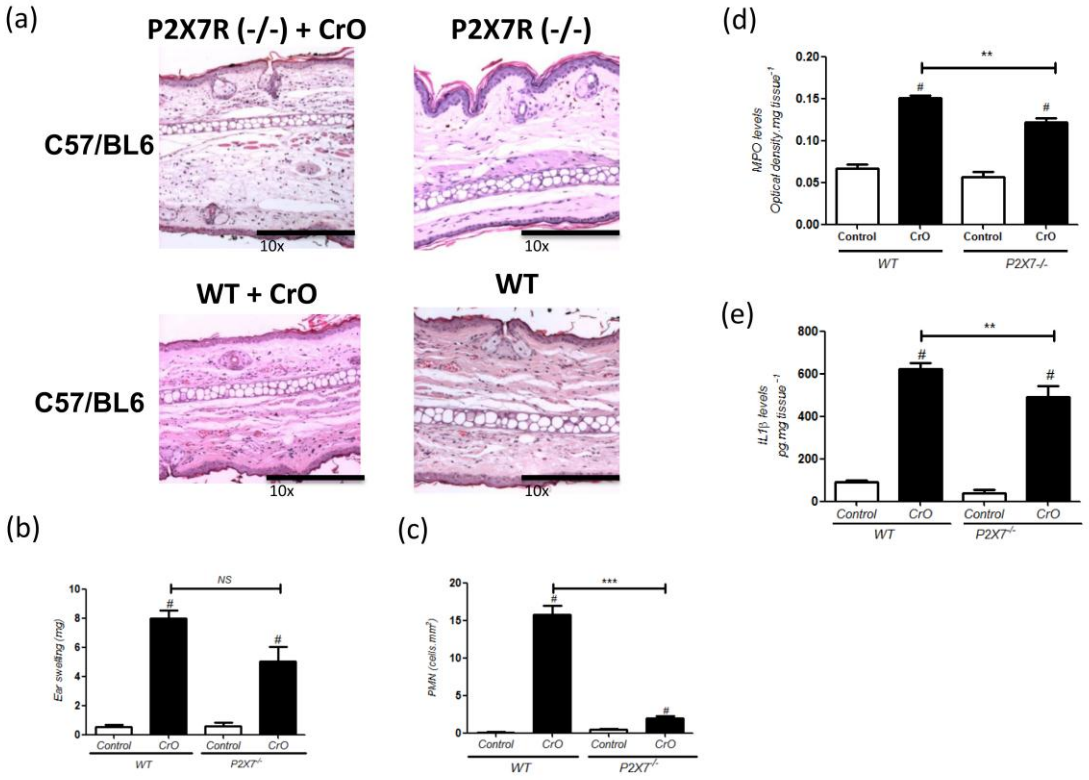
CrO
CrO +A438079
Control
CrO + Apyrase
CrO + Dex
10x
(b)
Ear swelling (mg)
Control
CrO
Apyrase
A438079
Dex
(c)
PMN (cells.mm²)
(d)
MPO levels
Optical density.mg tissue⁻¹
(e)
IL1β levels
pg .mg tissue⁻¹

Figure 2


(a)
P2X7R (-/-) + CrO
P2X7R (-/-)
C57/BL6
10x
10x
WT + CrO
WT
C57/BL6
10x
10x
(d)
MPO levels
Optical density. mg tissue-1
0.20
0.15
0.10
0.05
0.00
**
#
#
Control
CrO
Control
CrO
WT
P2X7-/-
(e)
IL1\ levels
pg mg tissue-1
800
600
400
200
0
**
#
#
Control
CrO
Control
CrO
WT
P2X7-/-
(b)
Ear swelling (mg)
10
8
6
4
2
0
NS
#
#
Control
CrO
Control
CrO
WT
P2X7-/-
(c)
PMN (cells mm2)
20
15
10
5
0
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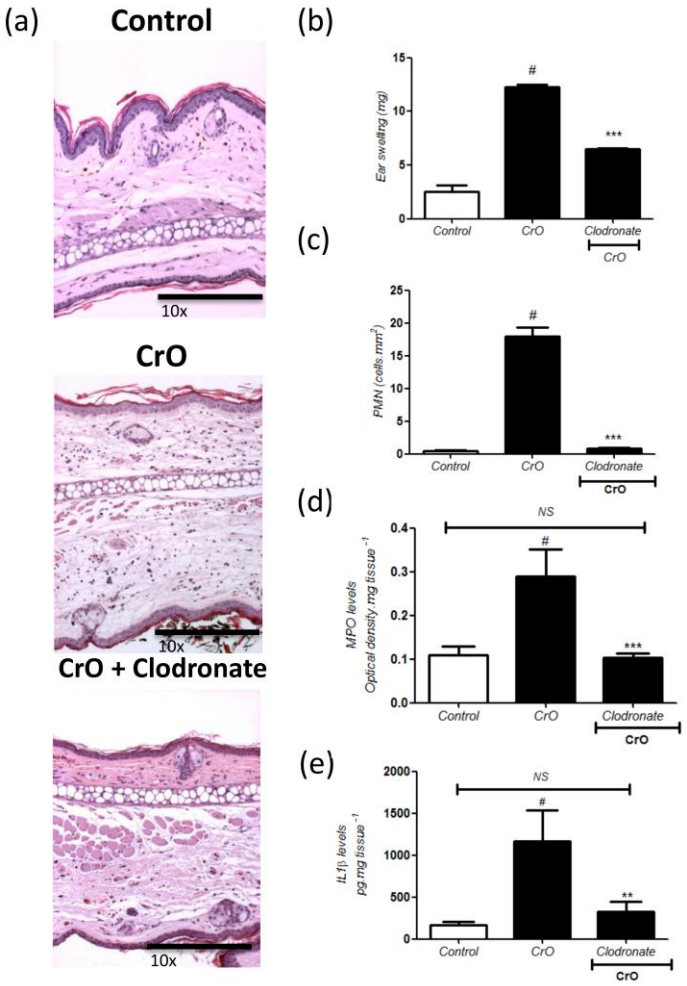
#
#
Control
CrO
Control
CrO
WT
P2X7-/-

Figure 3


(a)
Control
10x
CrO
10x
CrO + Clodronate
10x
(b)
Ear swelling (mg)
#
***
Control
CrO
Clodronate
CrO
(c)
PMN (cells.mm²)
#
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Control
CrO
Clodronate
CrO
(d)
NS
#
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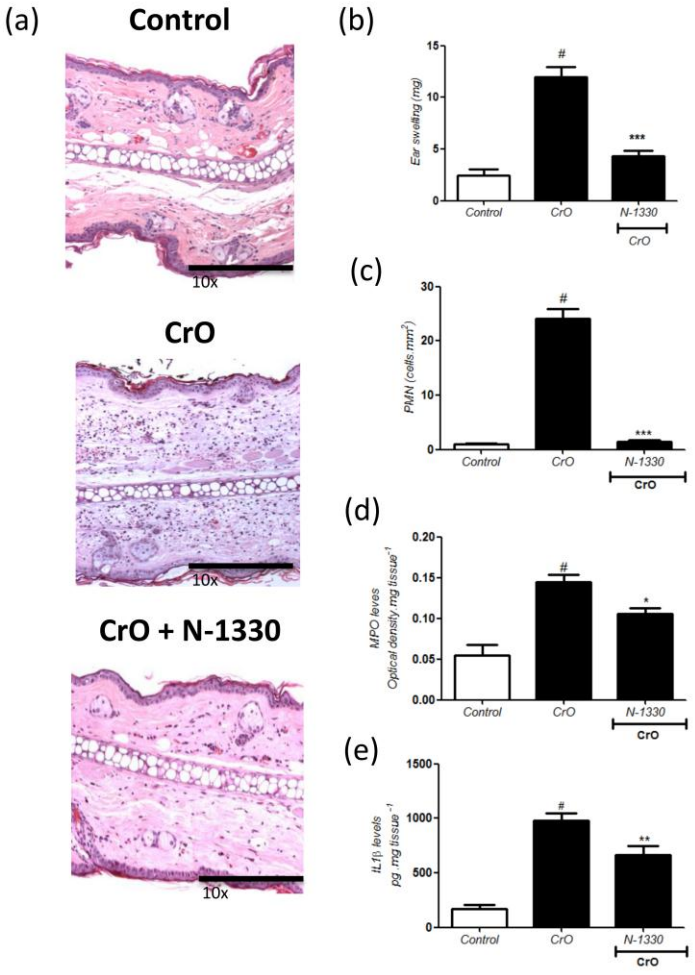
MPO levels
Optical density mg tissue⁻¹
Control
CrO
Clodronate
CrO
(e)
NS
#
**
IL1β levels
pg mg tissue⁻¹
Control
CrO
Clodronate
CrO

Figure 4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Control
10x
CrO
10x
CrO + N-1330
10x
(b)
Ear swelling (mg)
15
10
5
0
#
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Control
CrO
N-1330
CrO
(c)
PMN (cells.mm²)
30
20
10
0
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Control
CrO
N-1330
CrO
(d)
MPO leves
Optical density mg tissue⁻¹
0.20
0.15
0.10
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Control
CrO
N-1330
CrO
(e)
IL1β levels
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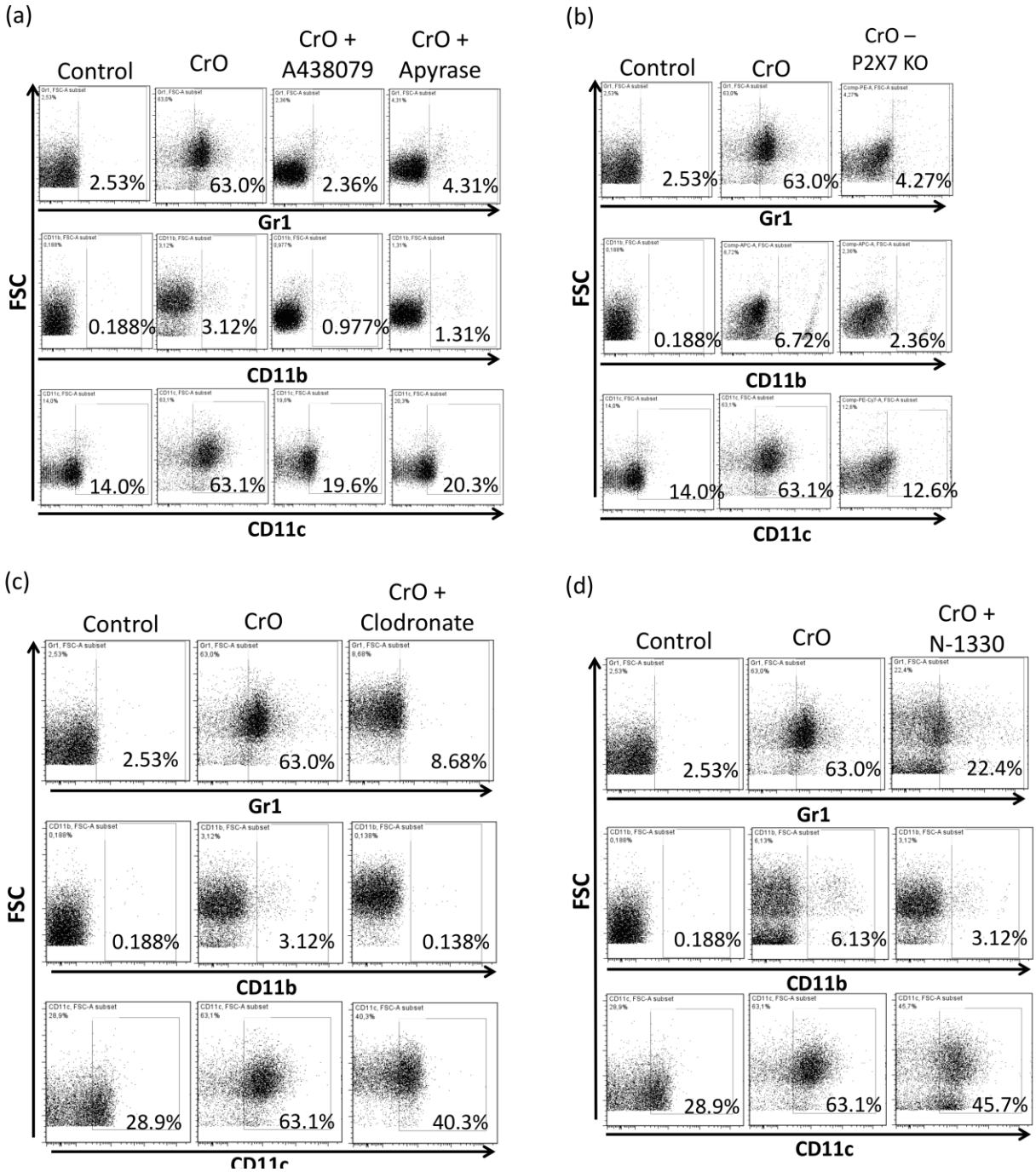
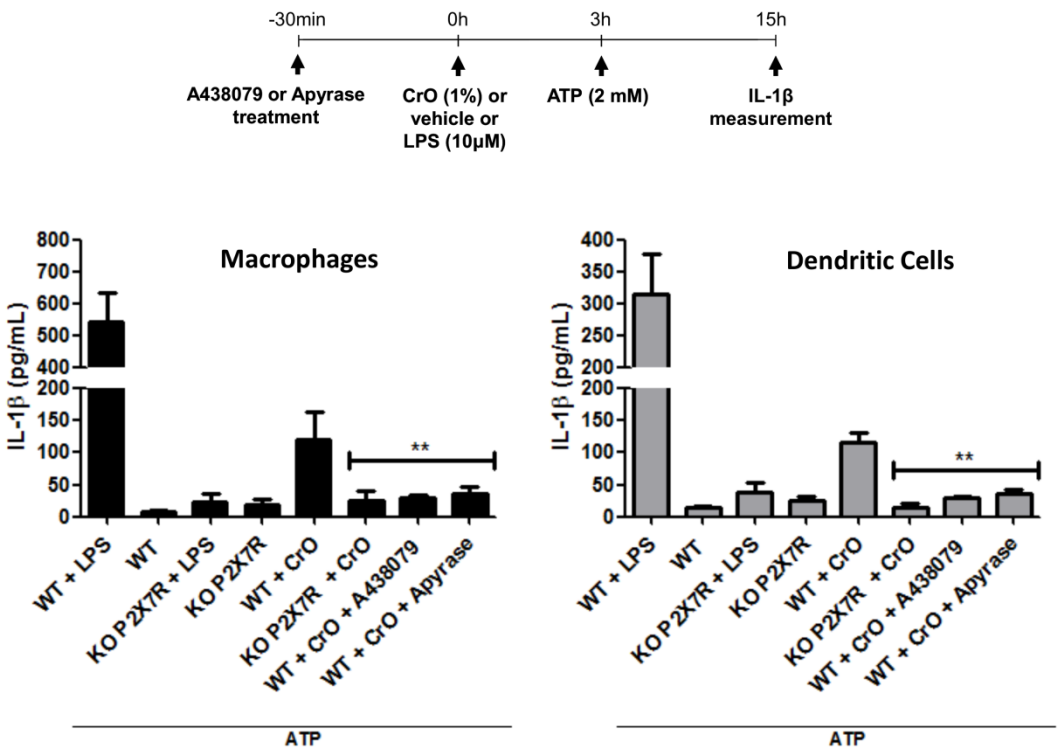
Control
CrO
N-1330
CrO

Supplementary Figure 1



Supplementary Figure 2



CAPÍTULO II

ARTIGO CIENTÍFICO

Decrease of adenine nucleotides hydrolysis in mice blood serum with irritant contact dermatitis: possible P2X7 receptor involvement

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Artigo a ser submetido ao periódico: Purinergic Signaling

2013

DECREASE OF ADENINE NUCLEOTIDES HYDROLYSIS IN MICE BLOOD SERUM WITH IRRITANT CONTACT DERMATITIS: POSSIBLE P2X7R INVOLVEMENT

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ABSTRACT

Extracellular adenosine 5'-triphosphate (ATP) has significant effects on a variety of physiopathological conditions and it is the main physiological agonist of P2X7 purinergic receptor (P2X7R). Recent work from our group demonstrated that the P2X7R is required to neutrophil recruitment in a mice model of irritant contact dermatitis (ICD) induced by croton oil (CrO). The present study investigated the effects of CrO upon ATP, ADP and AMP hydrolysis in the blood serum of mice, as well as citotoxicity in keratinocytes cell line. The topical application of CrO induced a decrease on soluble ATP/ADPase activities, and the treatment with the selective P2X7R antagonist A438079 reversed these effects. Furthermore, we showed that CrO decreased the cellular viability and caused necrosis in keratinocytes. The necrosis induced by CrO was prevented by the pre-treatment with the selective P2X7R antagonist A438079. The results presented herein suggest that CrO exerts an inhibitory effect on the activity of NTPDase-1 (CD39) in mice blood serum, reinforcing the idea that ICD has a pathogenic mechanism dependent of CD39. Furthermore, we showed evidences that ATP released from keratinocytes may act as an inducer of the inflammatory response observed in ICD via P2X7R activation.

Key words: P2X7R, keratinocytes, ATP, mice blood serum, nucleotide hydrolysis, contact dermatitis.

1 INTRODUCTION

The purine nucleotide adenosine triphosphate (ATP) is the universal energy molecule of intracellular biological reactions (Burnstock, 2006a; Eltzschig et al., 2012). Following Geoffrey Burnstock's pioneering studies, ATP appears as is an important extracellular messenger (Burnstock, 1972). In addition, the breakdown products of ATP, adenosine 5'-diphosphate (ADP), adenosine monophosphate (AMP) and adenosine, have been recognized as signaling molecules that have major effects on a variety of biological processes (Deaglio and Robson, 2011; Rittiner et al., 2012).

In healthy tissues, ATP is almost exclusively localized intracellularly (Di Virgilio et al., 2009), whereas in pathological conditions as inflammation, the tissue injury leads to the release of large amounts of ATP to the extracellular medium (Eltzschig et al., 2012). ATP can be hydrolyzed by ectonucleotidases that are situated on the surface of cells or by soluble forms in the interstitial medium, or within body fluids (Zimmermann et al., 2012). ATP, ADP and AMP are hydrolysed by the ecto-nucleoside triphosphatase diphosphohydrolase enzyme family (NTPDases), nucleotide phosphatase inhibitor/phosphodiesterase family (NPPs), alkaline phosphatases and ecto-5'-nucleotidase (Robson et al., 2006). These nucleotidases control the availability of nucleotides for purinergic receptors, and consequently, control the duration and extent of receptors activation (Souza et al., 2011).

Currently, experimental observations indicate that extracellular ATP acting via purinergic receptors plays a relevant role on skin inflammation (Burnstock, 2006a; Mizumoto et al., 2003; Pastore et al., 2007). The P2X7 receptor (P2X7R) is the most peculiar subtype of P2X purinergic receptors, since the activation of P2X7R by extracellular ATP can induce different actions such as: cell death, maturation,

membrane trafficking and release of pro-inflammatory cytokines such as IL-1β and IL-18 during inflammatory process (Ferrari et al., 2006). Recently, our group reported that P2X7R activation induce IL-1β release and tissue neutrophil recruitment in a mouse model of irritant contact dermatitis (ICD) (da Silva et al., 2013a). ICD is an inflammation reaction of the skin characterized by edema and erythema, initiated from damage of epidermal cells generally caused by exposure to chemical irritants (Han et al., 2007; Nosbaum et al., 2009).

The epidermal keratinocytes represent the major cellular constituents of the skin and these are the first cells to respond to noxious agents, giving them an essential role in the initiation and development of skin inflammation (Nosbaum et al., 2009). Previous works demonstrated that keratinocytes release ATP in response to chemical irritants (Mizumoto et al., 2003), and the inflammatory response induced by the irritants may be dependent of NTPDase1/CD39 (Mizumoto et al., 2002a), a member of NTPDases family that hydrolyzed ATP and ADP almost equally (Heine et al., 1999; Zimmermann, 2001). Furthermore, laboratory or clinical studies using pharmacological compounds that increase extracellular ATP and ADP hydrolysis, showed therapeutic effects in patients with inflammatory diseases (Eltzschig et al., 2012).

In this context, the present study investigated the effects of the chemical irritant croton oil (CrO) upon nucleotides hydrolysis in an ICD mouse model, as well as evaluated keratinocytes citotoxicity *in vitro*. We showed that CrO reduced ATP and ADP hydrolysis in mice blood serum and caused necrosis in a keratinocyte cell line. The results were correlated to the activation of P2X7R. Taken together, the data presented herein support the idea that the ATP acting via P2X7R is an important inflammatory mediator in ICD.

2 MATERIALS AND METHODS

2.1 Drugs and chemicals

A438079 was obtained from Tocris (Ellisville, MO, USA); adenosine 5'-triphosphate (ATP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A); Croton oil, nucleotides, Trizma Base, EGTA sodium salt, ouabain, oligomycin, sodium azide, orthovanadate, NEM (N-ethylmaleimide), lanthanum chloride and levamisole were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All others reagents were of analytical grade.

2.2 Animals

Male Swiss mice (5 per group, 25–30 g) were used in this study. The animals were maintained in controlled temperature (22 ± 1^0C) and humidity (60–70%), under a 12-hour light–dark cycle (lights on 7:00 AM). Food and water were available *ad libitum*. Animals were acclimatized to the laboratory for at least 1 hour before testing and were used only once throughout the experiments. All the tests were performed between 7:00 AM and 7:00 PM. The experimental procedures reported in this manuscript followed the ‘‘Principles of Laboratory Animal Care’’ from National Institutes of Health (NIH) (Zimmermann, 1983), and were approved by the Institutional Animal Ethics Committee (protocol number: 10/00206).

2.2.1 Irritant contact dermatitis model

Mice received topical application of 1% croton oil (CrO), according to the method described by (Mizumoto et al., 2002b). CrO was dissolved in acetone and used as an inducer of ICD. 20 µl of CrO (1%) was applied to the dorsal surface of ears. The extension of inflammatory response induced by CrO was evaluated by measuring the edema extension. In separate experiments, blood samples were collected and immediately centrifuged at 3,000 *g* for 10 min at room temperature. The serum samples obtained were stored at –20^{o}C for up to 10 days and used for the measurement of nucleotide hydrolysis and IL-1β levels. For measurement of nucleotides hydrolysis and IL-1β levels on the serum samples, the animals were treated with A438079 (80 µM/kg i.p.), Apyrase (0,2 U/ear s.c. into the ear) and with the control drug, dexamethasone (0,5 mg/kg s.c.) before and after CrO application.

2.2.2 Measurement of edema extension

The thickness of the mice ears was measured before of the CrO application using a digital paquimeter (Mitutoyo®). Mensurement of ear thickness was also carried out 1, 2, 3, 4, 5 and 6 hours after CrO application. Animals were treated with the selective P2X7R antagonist A438079, 30 min before (for prophylactic treatment) or 2 hours after (for therapeutic treatment) the CrO application. After measuring ear thickness, six millimeters diameter sections of the right and left ears were removed using a circular punch and weighed on an analytic balance. The extent of the edema (edema weight) was expressed as the difference between the weight (in mg) of the section removed from the

right ear (which received the irritant agent) and the weight (in mg) of the section removed from the left ear (which received vehicle used to dilute the irritant agent).

2.2.3 Mensurament of nucleotides hydrolysis

Nucleotides hydrolysis was determined using the method previously described by Morrone et al. (2009). The reaction mixture containing ATP or ADP as substrate (3 mM), 112.5 mM Tris-HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37^{o}C for 40 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid. The samples were chilled on ice and the amount of liberated inorganic phosphate was measured by the malachite green method. All samples were centrifuged at 5,000 *g* for 5 minutes and the supernatant was used for the colorimetric assay. AMP hydrolysis was determined under the same conditions for ATP, except that the substrate was AMP and at pH 7.5. For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. The addition of the enzyme preparation after the addition of TCA was used as a control to correct for nonenzymatic hydrolysis of the substrates. Enzyme activities were expressed as μmol of Pi liberated/min/ per liter (U/L). Protein was measured by the Coomassie Blue method using bovine serum albumin as standard (Morrone et al., 2009).

2.2.4 Determination of IL-1β levels

The serum samples were collected as previously described in this paper. IL-1β levels were measured using an enzyme-linked immunosorbent assay kit according to supplier recommendations (DuoSet Kit; R&D Systems, Minneapolis, MN, USA). The results were expressed as pg/ml serum.

2.3 Cell culture

HaCaT cell lines (immortalized human keratinocytes) were gently donated from Prof. Dr. Décio dos Santos Pinto Jr. (Oral Pathology Laboratory, FO-USP, Brazil). Cells were cultured in Dubelco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2.

2.3.1 Cell viability

Keratinocytes were seeded at 6 x 10^3 cells per well in 96-well plates and grown for 24 h. Initially, the cells were treated with ATP (0.1, 0.5, 3 and 5 mM) or croton oil (2 µg/ml; in acetone 2%). To evaluate the receptor activity, the cells were pre-treated with the selective P2X7R antagonist A438079 (10 µM) and after 20 min with ATP (5 mM) or croton oil (2 µg/ml; in acetone 2%) for 24 h. At the end of this time, the medium was removed, the cells were washed with calcium magnesium-free medium (CMF) and 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) (5 mg/ml in PBS in 90% DMEM/10% FBS) was added to the cells and

incubated for 3 h. The formazan crystals were dissolved with 100 µl of dimethyl sulfoxide (DMSO). The absorbance was quantified in 96-well plates (Spectra Max M2e, Molecular Devices) at 595 nm. Cell viability was calculated considering the control group as 100%.

2.3.2 Annexin V/PI flow cytometry staining technique

Keratinocytes were seeded at 20 x 10^6 cells per well in 24-well plates. The cells were treated with croton oil (2 µg/ml) for 24 hours. After that, the dead cells were quantified by annexin V-FITC and propidium iodide (PI) double staining, using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's instructions. The data were acquired by BD FACSCanto II flow cytometer and the results were analyzed using FlowJo Software (Tree Star).

2.4 Statistical analysis

Results are expressed as the Mean ± Standard Error Mean (SEM). The statistical analysis was performed by one or two way analysis of variance (ANOVA), followed by Tukey's post-hot test, depending on the experimental protocol. *P* values smaller than 0,05 were considered as significant. All tests were performed using the GraphPad® 5 Software (USA).

3 RESULTS

CrO decreases ATPase and ADPase activities in mice blood serum and this effect is reversed by P2X7R antagonist

It has been reported that a considerable number of compounds alter or inhibit extracellular nucleotide hydrolysis by NTPDases (Robson et al., 2006). Here, we examined the effect of CrO on nucleotides hydrolysis in mice blood serum. The topical application of CrO induced a decrease in soluble ATPase (Fig. 1a) and ADPase (Fig. 1b) activities and the treatment with apyrase (0,2 U/ear) significantly reversed these effects (Fig. 1a; 1b). Interestingly, the treatment with the selective P2X7R antagonist, A438079 (80 μM/kg), also reversed the increase of ATPase (Fig. 1a) and ADPase (Fig. 1b) activities evoked by CrO, indicating that P2X7R activation is involved in the inflammatory response evoked by CrO. AMPase activity was not altered when the animals were treated with either apyrase or A438079 (Fig. 1c). Otherwise, the treatment with the control drug, dexamethasone reversed the AMPase activity, corroborating with results previously described (Bavaresco et al., 2007; Detanico et al., 2011).

CrO increases serum IL-1β levels

The involvement of P2X7R on the local release of IL-1β in CrO-induced ICD has been recently reported by our group (Da Silva et al., 2013b). In this study, we showed that CrO significantly increases serum IL-1β levels, indicating that topical application of CrO cause systemic effects. The elevation of serum IL-1β levels evoked

by CrO was partially diminished by the systemic administration of the P2X7R antagonist A438079. This finding together with the observation that CrO decreased ATPase activity leads us to consider that the topical application of CrO increases the extracellular levels of ATP in the blood, resulting in P2X7R activation and IL-1β release (Fig. 1d).

CrO-induced edema is time-dependent

To further investigate the role of P2X7 receptor in ICD development, we also tested the effect of the systemic treatment with the selective P2X7R antagonist A438079 (80 μM/kg; i.p.) before and after of the CrO application. We observed that the topical application of CrO evoked a time dependent edema and the treatment with A438079 starts to decrease this edema only after the second hour (Fig. 2a). In an interesting way, we verified that the prophylactic treatment with the P2X7R antagonist (30 min before the croton oil application) did not reduce ear swelling, while therapeutic application of A438079 (2 hours after croton oil application) significantly reduced the ear edema in 42% ± 5 (Fig. 2b), suggesting that CrO-induced ATP release, and that P2X7R activation may be occurring in a time dependent way.

CrO-induced cell death in HaCaT lineage by necrosis via P2X7R

Keratinocytes has been described as initiators of ICD (Nosbaum et al., 2009). Considering the finding that P2X7R is involved in the inflammatory response evoked by CrO, we decided to investigate the citotoxity evoked by CrO using a human keratinocyte cell line (HaCaT). Firstly, we utilized MTT assay to investigate the effect

of ATP, the main physiological agonist of P2X7R, upon the viability of HaCaT. Cell viability was not altered when keratinocytes were treated with ATP at lower concentrations (0.3; 0.5 or 3.0 mM). Otherwise, the treatment with a high ATP concentration (5 mM) significantly diminished the cell viability of HaCaT (58% ± 9) (Fig. 3a). Next, we investigated whether ATP-induced cytotoxicity in HaCaT cell line might be related to the activation of P2X7R. As shown in the Fig. 3a, the selective P2X7R antagonist A438079 prevented the cytotoxicity caused by ATP 5 mM (from 58,25% to 85,28%), indicating that this receptor is probably implicated in the cytotoxic effects displayed by ATP. We verified that CrO (2 µg/ml) also significantly decreased HaCaT cell viability (53% ± 4) and this effect was reversed by A438079 (10 µM) (from 52,6% to 99%), indicating that the activation of P2X7R is probably implicated in the cytotoxic effects displayed by CrO (Fig. 3b).

In addition, we also used Annexin V/PI flow cytometric staining to demonstrate that the cell death induced by CrO exposition occurred mainly by necrosis. As shown in the Fig. 3c., the treatment with CrO (2 µg/ml) induced a clear increase in propidium iodide (PI) population, and the treatment with the P2X7R selective antagonist significantly reduced the PI population. This set of results, suggest that ATP is probably released after exposure to CrO irritant chemical agent causing the cells death and besides it could be ATP source to the bloodstream.

4 DISCUSSION

ATP binds to P2X7R and may also serve as substrate for ectonucleotidases. The most well-characterized ectonucleotidase is CD39 or NTPDase-1, which converts ATP (or ADP) in AMP (Longhi et al., 2013). Mizumoto et al. (2002) demonstrated a nucleotide-mediated pathogenic mechanism and a CD-39-dependent protective mechanism of ICD. In this study, we hypnotized that chemical irritants exert toxic effects by elevating the extracellular ATP levels and decreasing ATP hydrolysis by ectonucleotidases. To further investigate this hypothesis, we measured the nucleotides hydrolysis in the serum of mice six hours after application of CrO. Figure 1 shows that CrO decreases ATP and ADP hydrolysis. In contrast, CrO did not alter the AMPase activity. We believe that these results are consistent with the finding that CrO significantly decreases ATPase activity leading to a high concentrations of extracellular ATP levels, inhibiting ecto-5'-nucleotidase (AMPase) activity, as reported by Zimmermann (2001). We also tested the effect of the selective P2X7R antagonist A438079 on nucleotides hydrolysis and the IL-1β release on the blood serum of mice that received topical CrO application CrO. The blockage of P2X7R by A438079 reversed the effects evoked by CrO upon the nucleotide hydrolysis (Fig. 1a) and significantly decreased serum IL-1β levels (Fig. 1d). These data brings up the P2X7R antagonists as possible pharmacological strategies to decrease the pro-inflammatory effect evoked by ATP. This observation is confirmed by the finding that post treatment with A438079 (after 2 hours of the CrO application) decreased the edema (Fig. 2a-b), whereas the prophylactic therapy with A438079 (30 min before the CrO application) failed to decrease the edema (Fig. 3b), suggesting that P2X7R antagonists could be suitable alternatives for the treatment, but not to prevention, of ICD.

The role of ATP as a chemothatic signal for phagocytes and the effects of ectoenzymes on inflammatory cells are well known. Ectoenzymes can function physically as adhesion receptors and can regulate the recruitment of cells through their catalytic activities (McDonald et al., 2010; Salmi and Jalkanen, 2005). Recently, our group reported that the activation of P2X7R on phagocytes is required to neutrophils recruitment to the tissue in CrO-induced ICD (da Silva et al., 2013a). Here, we showed some points of evidence that CrO may exerts its toxic effects by decreasing the nucleotides hydrolysis, leading to an increase of extracellular ATP levels and P2X7R activation. Furthermore, our findings suggest that the soluble ectonucleotidases are important controllers of acute inflammatory response evoked by chemicals irritants.

Previous studies have shown that ATP released from keratinocytes in response to chemical irritants can act as an initiator of skin inflammation (Boeynaems and Communi, 2006). In this way, we also investigated the effect of ATP and CrO on keratinocytes viability *in vitro*. According to Welss et al. (2004), there is a correlation between irritant potential and cell viability, and the measurement of viability (e.g. MTT) may be suitable to evaluate the irritant potential to chemicals. Therefore, we firstly used MTT assay to show that the chemical irritant CrO causes decrease of cellular viability of keratinocytes and subsequently we used flow citometry to demonstrate that the cell death evoked by CrO in keratinocytes occurs per necrosis. Furthermore, the necrosis induced by CrO was prevented by the pre-treatment with the selective P2X7R antagonist A438079, suggesting that this receptor is implicated on the cell death effects displayed by CrO (Fig. 3c). ATP is the only known physiological activator of P2X7R. Since the cytoplasmic ATP concentration is in the millimolar range, acute cell death can cause massive ATP release into the extracellular milieu. P2X7R are unique among the P2X receptor family as they are activated by high ATP concentrations (>100 µM), this

fact led researchers to consider the possibility that this receptor functions as a danger sensor (Carroll et al., 2009; Ferrari et al., 2006). To further investigate the activation of P2X7R by ATP in keratinocytes, we treated the cells with different ATP concentrations and verified that only the most high concentration (5 mM) induced cell death, and this effect was partially reversed by the pre-treatment with A438079 (Fig. 1a). This result, together with the finding that P2X7R is involved with the citotoxicity evoked by CrO, led us to suggest that activation of P2X7R by ATP in keratinocytes could be the initiator of the inflammatory response evoked by CrO. This set of results indicates that these cells can release ATP upon exposure to CrO, as previously reported for other irritants (Burrell et al., 2005; Mizumoto et al., 2002b; Welss et al., 2004b). Therefore, we recognized that keratinocytes can participate of the inflammatory response induced by CrO via activation of P2X7R.

Purinergic signaling regulates a wide range of inflammatory diseases. In this report, we showed that CrO chemical irritant decreases blood serum ATP/ADP hydrolysis suggesting an effect on the activity of NTPDase-1, reinforcing the idea that ICD has a pathogenic mechanism dependent of CD39. Furthermore, we showed evidences that CrO evokes ATP release from keratinocytes, and this nucleotide acts as an inducer of inflammatory response observed in ICD. Considering that the selective P2X7R antagonist A438079 was able to revert the effects triggered by CrO, it is tempting to suggest that the control of ATP extracellular levels and the P2X7R blockage could be an interesting pharmacological strategy to treat ICD.

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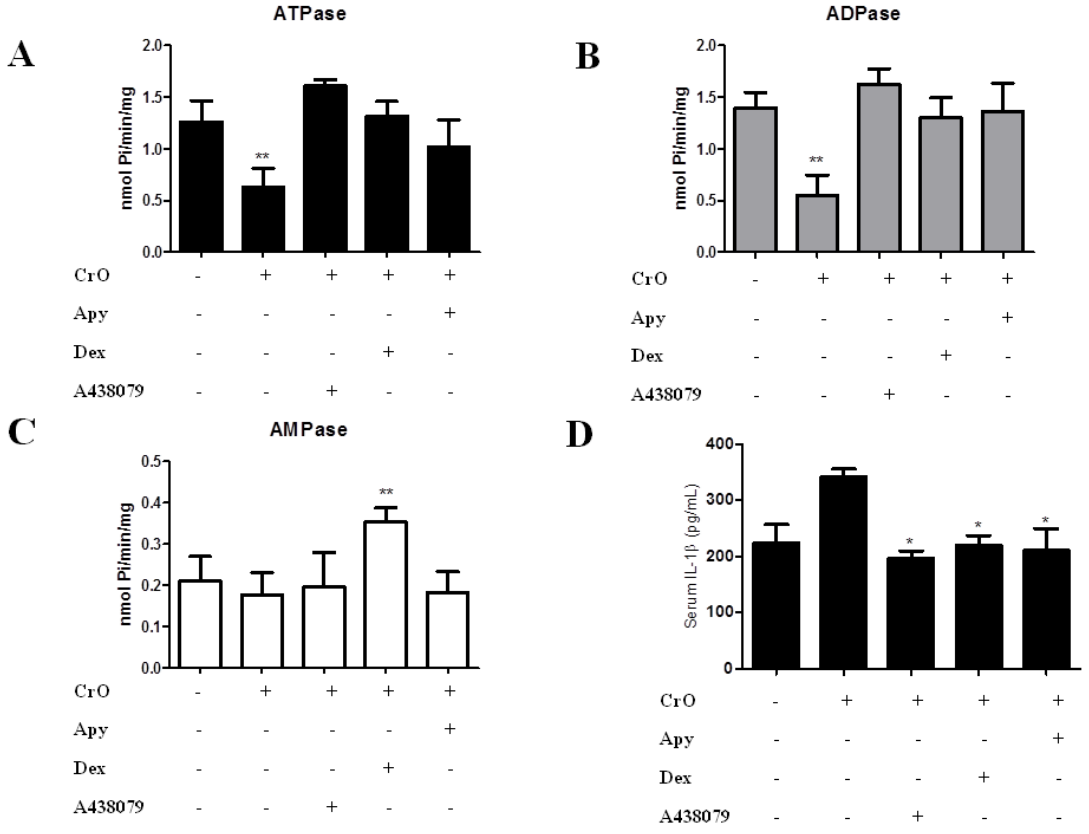
FIGURE LEGENDS

FIGURE 1. In vivo effect of irritant contact dermatitis induced by CrO on nucleotides hydrolysis and IL-1β release in mice blood serum. (A) Black bars, ATPase; (B) Grey bars, ADPase; (C) White bars, AMPase. (D) Serum IL-1β levels (pg/ml). Vehicle or treatments: A438079 (80 µmol/kg, i.p.), Apyrase (0,2 U/ear) and Dexamethasone (0,5 mg/kg) were administrated 30 min before and 2 hours after CrO application. The data represent three experiments ± SEM with different serum preparations. Number of animals per group 6-8. (*) P<0,5; (**) P<0.01; indicates significant differences from control group, one-way ANOVA with Tukey's post-hoc test.

FIGURE 2. In vivo effect of A438079 on croton oil-induced ear swelling. *Swiss* mice were treated with 2% croton oil (CrO) in acetone or acetone alone (vehicle). (A) Vehicle or A438079 (80 µM/kg) were administered i.p. 2 hours after CrO application. Ear thickness was measured at the time of CrO application and at the indicated time intervals (1-6 h later). Number of animal per group 6-8. (*) P<0,001 indicate significant differences from CrO group; ($^{\#}$) indicate significant differences from CrO + A438079 or CrO group, two-way ANOVA with Tukey's post-hoc test. (B) Vehicle or A348079 (80 µMol/kg) were administered i.p. 30 min before or 2 hours after CrO application. The extent of the edema was expressed as the difference between the weight (in mg) of ear that received the application of CrO and the weight (in mg) of the ear that received the vehicle used to dilute the CrO. Number of animal per group 6-8. (***) P<0,001 indicate significant differences from CrO group, one-way ANOVA with Tukey's post-hoc test.

FIGURE 3. Effect of croton oil in keratinocyte cell line HaCaT. (A) Effect of the treatment with CrO (2 μg/ml) on cell viability of HaCaT. The cells had been primed with CrO in the presence or absence of A438079 (10 μM). Data represent the mean ± SEM of three experiments. ***P<0,001 denote the significance in comparison to control, one-way ANOVA with Tukey's post-hoc test. The experiments were carried out at least three times in triplicate. (B) Effect of treatment with ATP 0,3 μM; 0,5 μM; 3 mM; 5 mM on cell viability of HaCaT. The cells had been primed with ATP in the presence or absence of A438079 (10 μM). Data represent the mean ± SEM of three experiments. ***P<0,001 denote the significance in comparison to control, one-way ANOVA with Tukey's post-hoc test. The experiments were carried out at least three times in triplicate. (C) Dot plot with percentage of Annexin V/PI positive HaCaT cells 24 h after the treatments. Each sample has 50,000 cells. Data shown is representative of at least two independent experiments.

FIGURE 1


A
ATPase
nmol Pi/min/mg
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CrO - + + + +
Apy - - - - +
Dex - - - + -
A438079 - - + - -
B
ADPase
nmol Pi/min/mg
2.0
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**
CrO - + + + +
Apy - - - - +
Dex - - - + -
A438079 - - + - -
C
AMPase
nmol Pi/min/mg
0.5
0.4
0.3
0.2
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CrO - + + + +
Apy - - - - +
Dex - - - + -
A438079 - - + - -
D
Serum IL-1β (pg/mL)
400
300
200
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0
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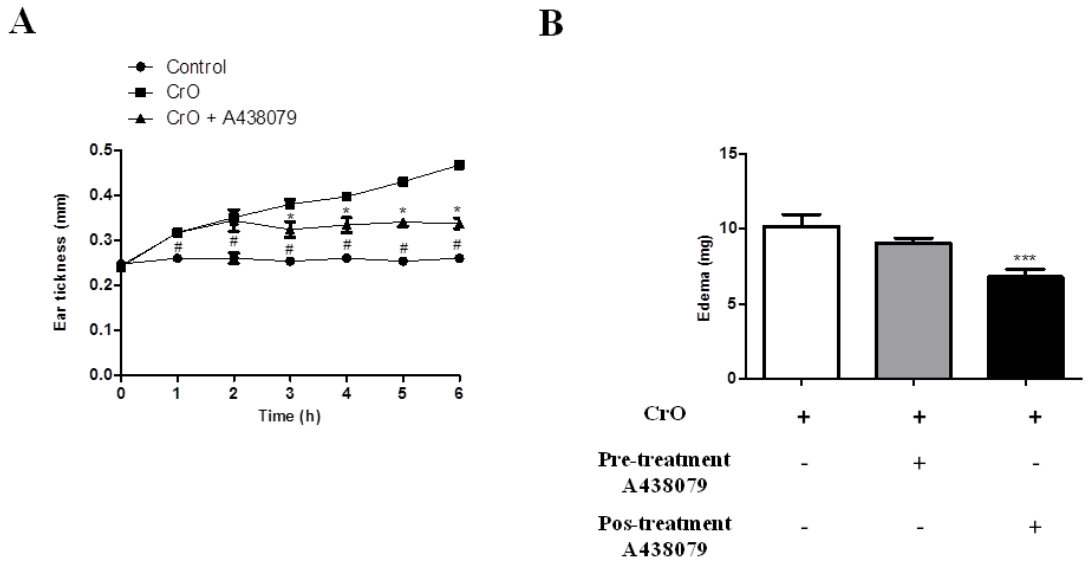
CrO - + + + +
Apy - - - - +
Dex - - - + -
A438079 - - + - -

FIGURE 2


A
Control
CrO
CrO + A438079
Ear tickness (mm)
0.0
0.1
0.2
0.3
0.4
0.5
0
1
2
3
4
5
6
Time (h)
B
Edema (mg)
0
5
10
15
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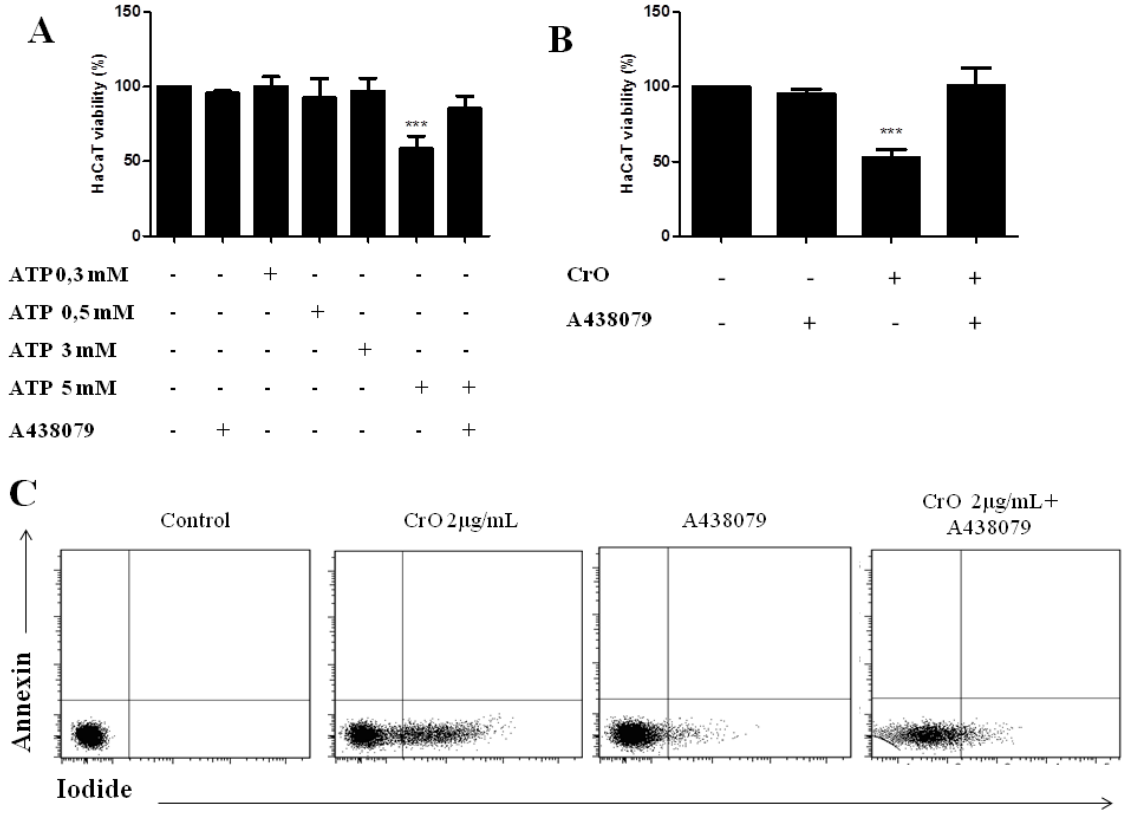
CrO + + +
Pre-treatment A438079 - + -
Pos-treatment A438079 - - +

FIGURE 3


A
HaCaT viability (%)
150
100
50
0
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ATP 0,3 mM - - + - - - -
ATP 0,5 mM - - - + - - -
ATP 3 mM - - - - + - -
ATP 5 mM - - - - - + +
A438079 - + - - - - +
B
HaCaT viability (%)
150
100
50
0
***
CrO - - + +
A438079 - + - +
C
Control
CrO 2µg/mL
A438079
CrO 2µg/mL+ A438079
Annexin
Iodide

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4 DISCUSSÃO

A dermatite de contato é uma doença que ocorre em resposta à exposição a substâncias químicas que desencadeiam respostas imunes. Foram identificados dois tipos de dermatites de contato: dermatite de contato irritante (ICD) e dermatite de contato alérgica (ACD). Apesar de ambas possuírem sinais clínicos e histológicos semelhantes, elas diferem de acordo com seus mecanismos imunopatológicos. A ICD é uma dermatose inflamatória inespecífica causada pelo contato com substâncias irritantes tóxicas à pele que desencadeiam a inflamação por ativação direta de células do sistema imune inato. Por outro lado, a ACD é relacionada à ativação da imunidade adquirida sendo considerada uma reação de hipersensibilidade do tipo IV cuja inflamação é desencadeada por ativação de células T antígeno específicas (Nosbaum et al., 2009; Rozieres et al., 2009; Zhang and Tinkle, 2000).

A ICD é o tipo de dermatite de contato mais comum, correspondendo a cerca de 80% dos casos de dermatite (Slodownik et al., 2008). É uma patologia multifatorial cujos mecanismos são pouco conhecidos (Levin and Maibach, 2002). Um exemplo bastante comum é a dermatite ocupacional (OCD, *occupational contact dermatitis*) apresentada por pessoas que trabalham em indústrias e/ou laboratórios e estão constantemente expostas a uma ampla variedade de compostos químicos. A OCD pode ser facilmente evitada através do uso de cremes hidratantes oclusivos e/ou equipamentos de proteção individual, como luvas protetoras (Smith et al., 2002). Entretanto, a maioria dos casos de ICD não podem ser prevenidos, pois atualmente estamos constantemente expostos a uma ampla variedade de substâncias químicas presentes em plantas, alimentos, cosméticos, perfumes e produtos de higiene pessoal, tornando-se praticamente impossível prever quando um processo irritativo pode surgir.

Irritantes químicos podem causar danos diretos às células de epiderme, principalmente queratinócitos, desencadeando uma resposta inflamatória estéril responsável pelos sinais clínicos observados em casos de dermatite de contato irritante, como edema e eritema. O mecanismo exato pelo qual os irritantes exercem sua ação tóxica não é completamente entendido e as alternativas de tratamento da doença se restringem ao uso de corticóides tópicos, uma prática que tem sido questionada na literatura (Yamaura et al., 2012). Neste contexto, tornam-se necessários estudos que esclareçam os mecanismos patogênicos da ICD e identifiquem novos alvos para ação de fármacos.

Considerando que cada vez mais estudos apontam o receptor P2X7 como um possível alvo farmacológico para o tratamento de doenças inflamatórias e que os mecanismos da ICD ainda são pouco esclarecidos, no primeiro capítulo desta tese nosso objetivo foi investigar os mecanismos imunológicos dependentes da ativação de P2X7 em ICD. Para atingir este objetivo, nós induzimos ICD em camundongos através da aplicação tópica do irritante químico óleo de cróton e utilizamos diversas abordagens farmacológicas para comprovação das hipóteses que foram sendo levantadas ao longo do desenvolvimento deste capítulo. Considerando que a utilização de camundongos geneticamente modificados são importantes estratégias para avaliar o papel de receptores em diferentes processos, nós também usamos camundongos *knockout* para o receptor P2X7 em alguns experimentos.

O primeiro achado obtido durante o desenvolvimento deste trabalho foi que tanto a deleção gênica do receptor P2X7, quanto o tratamento farmacológico com um antagonista seletivo deste receptor (A438079) diminuíram o recrutamento de neutrófilos induzido pelo irritante químico CrO. Este achado nos levou a pensar que a ativação de P2X7R por ATP estava envolvida no recrutamento de neutrófilos, por isso nós usamos

o tratamento farmacológico com apirase, uma ecto-enzima que degrada ATP, para confirmar esta hipótese. De fato, o tratamento com apirase provocou um efeito similar ao observado com o antagonista do P2X7R, sugerindo que o ATP extracelular via ativação do P2X7R poderia ser um dos responsáveis pelo recrutamento de neutrófilos observado neste modelo. A deleção gênica do P2X7R, o tratamento com A438079 ou com apirase também diminuíram a liberação local de IL-1β induzida pelo óleo de cróton. Todos estes achados iniciais estão de acordo com dados apresentados em estudos anteriores onde foi demonstrado o envolvimento do receptor P2X7 com a migração de leucócitos e com a secreção de IL-1β (Martins et al., 2012; Moncao-Ribeiro et al., 2011).

Para melhor investigar os mecanismos imunológicos dependentes de P2X7 em ICD e aprofundar nosso estudo, nós levantamos a hipótese de que o recrutamento de neutrófilos provocado pela aplicação tópica de CrO poderia ser dependente da liberação de IL-1β via ativação de P2X7 em outras células do sistema imune inato. Então, nós utilizamos clodronato encapsulado em lipossomas para depletar APCs. A administração local e sistêmica de clodronato 24 horas antes da aplicação de CrO promoveu uma diminuição significativa da população de macrófagos e células dendríticas (DCs) no tecido. Esta diminuição de APCs foi acompanhada da redução do recrutamento de neutrófilos e da redução da liberação local de IL-1β, sugerindo uma correlação entre o recrutamento de neutrófilos mediado por CrO e a liberação de IL-1β por DCs e macrófagos. Então, nós decidimos investigar se aplicação tópica de CrO provoca a liberação local de IL-1β via ativação do receptor P2X7 em DCs e macrófagos utilizando um estudo *in vitro*. Nós diferenciamos e isolamos DCs e macrófagos extraídos da medula óssea de camungondos e mostramos que o pré-tratamento destas células com A438079 ou apirase reduziu significativamente a liberação de IL-1β provocada pela

estimulação com CrO e ATP, confirmando a nossa hipótese e apontando o envolvimento de DCs e macrófagos na iniciação do processo inflamatório desencadeado por irritantes químicos.

Para finalizar este primeiro capítulo, nós também utilizamos um inibidor irreversível de caspase-1 (N-1330) para demonstrar que a liberação de IL-1β observada no nosso modelo de ICD é parcialmente dependente da ativação de caspase-1. O tratamento com o N-1330 também provocou uma diminuição do recrutamento de neutrófilos para o tecido, mostrando mais uma evidência de que o recrutamento de neutrófilos observado neste modelo é dependente da liberação de IL-1β. Sabendo que o ATP via ativação do receptor P2X7 pode funcionar como um sinal que ativa o inflamossomo NLRP3 promovendo ativação de caspase-1 com consequente maturação e liberação de IL-1β, nós também sugerimos que o recrutamento de neutrófilos induzido por óleo de cróton neste modelo pode ser dependente da ativação de NLRP3 via P2X7R.

Portanto, no primeiro capítulo deste estudo, evidenciou-se que a ativação do receptor P2X7 pelo ATP extracelular é necessária para o recrutamento de neutrófilos para o tecido e para os mecanismos imunopatológicos observados no modelo de dermatite de contato irritante induzido por CrO. Entretanto, o mecanismo pelo qual o CrO poderia iniciar a resposta imune observada no nosso modelo não ficou esclarecido. Portanto, no segundo capítulo desta tese nós decidimos avaliar os mecanismos pelos quais o CrO poderia iniciar uma resposta inflamatória e promover o recrutamento de leucócitos ao local da lesão. Em 2002, um estudo com animais mostrou que a deleção gênica de CD39 (NTPDase-1; ecto-apirase) desencadeia uma resposta inflamatória exacerbada a irritantes químicos (Mizumoto et al., 2002a) e em 2007, um estudo *in vitro* mostrou que o ATP extracelular possui efeitos estimulatórios sobre a expressão e

liberação de IL-6 via receptores purinérgicos em queratinócitos (Inoue et al., 2007). Estes e outros estudos apontam os queratinócitos como células iniciadoras do processo inflamatório observado na ICD. Portanto, nós levantamos a hipótese de que os irritantes químicos exercem suas atividades tóxicas promovendo a morte celular de queratinócitos com consequente aumento dos níveis de ATP extracelular. Para investigar esta hipótese, nós primeiramente avaliamos o efeito do CrO sobre a viabilidade de uma linhagem de queratinócitos humanos (HaCaT) utilizando o ensaio do MTT. Os dados apresentados mostraram que a exposição ao CrO causa uma diminuição da viabilidade de queratinócitos. Posteriormente, nós usamos citometria de fluxo para demonstrar que a morte celular induzida por CrO ocorre primariamente por necrose, fornecendo evidências de que ocorre a liberação de ATP para o meio extracelular quando queratinócitos são expostos a este irritante químico.

É sabido que o aumento da concentração de ATP no meio extracelular pode levar à ativação do receptor purinérgico P2X7 causando a abertura reversível de um poro na membrana plasmática da célula que pode posteriormente levar à morte celular (Di Virgilio et al., 1998). Portanto, nós também investigamos o envolvimento deste receptor nos efeitos de morte celular desencadeados pelo óleo de cróton. Para isto, nós utilizamos o A438079, um antagonista que demonstrou ser altamente seletivo para P2X7R (Carroll et al., 2009). Nossos dados mostraram que a morte celular provocada por CrO é revertida quando os queratinócitos são pré-tratados com A438079, sugerindo que a ativação de P2X7R está envolvida nos efeitos de morte celular provocados por CrO. Sabendo que o aumento da hidrólise de ATP via ectonucleotidades promove o fechamento do poro e o reestabelecimento da homeostase celular, nós investigamos também o efeito de CrO sobre a atividade de ectonucleotidades *in vivo*, para isto nós utilizamos o modelo de dermatite de contato irritante induzida por óleo de cróton em

camundongos e mostramos que a aplicação tópica de CrO promove uma diminuição da hidrólise de ATP e ADP no soro, indicando um efeito inibitório sobre a NTPDase-1 e confirmando a hipótese de Mizumoto et al. (2002). Foi observado também que o tratamento com o antagonista A438079 reverte este provável efeito inibitório sobre NTPDase-1, apontando o bloqueio farmacológico do receptor P2X7 como uma estratégia para o tratamento da ICD. Digno de nota, o tratamento com A438079 duas horas após a aplicação do CrO reduziu o edema, um dos principais sinais clínicos observados na ICD. Os achados mostrados no segundo capítulo desta tese sugerem que irritantes químicos podem exercer seus efeitos tóxicos ao promoverem a morte celular de queratinócitos com consequente liberação de ATP para o meio extracelular, ativação do receptor P2X7 e diminuição da atividade de ectonucleotidases.

5 CONCLUSÃO

Os dados apresentados nesta tese apontam para hipótese de que o óleo de cróton exerce seus efeitos tóxicos primariamente promovendo a morte celular de queratinócitos e diminuindo a atividade de ectonucleotidades, o que sugere um aumento dos níveis extracelulares de ATP. O ATP extracelular via ativação de P2X7R em DCs e macrófagos provoca a liberação de IL-1β que é parcialmente responsável pelo recrutamento de neutrófilos observado na dermatite de contato irritante induzida por óleo de cróton. Além disso, nós sugerimos que o receptor P2X7 pode ser um alvo farmacológico importante para o desenvolvimento de novas terapias para o tratamento da ICD. A figura abaixo (Figura 5) representa de forma esquemática os resultados obtidos nos dois capítulos apresentados nesta tese.

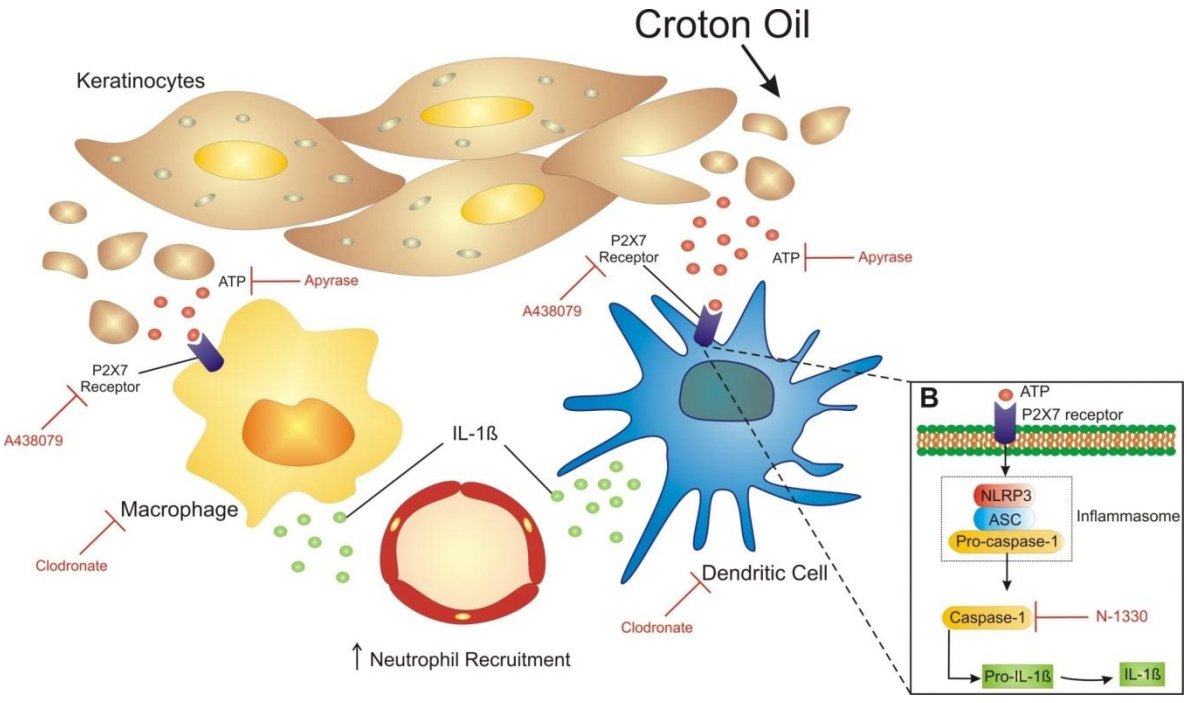

Figura 5. Representação esquemática do envolvimento do ATP e do receptor P2X7 na dermatite de contato irritante induzida por óleo de croton.

6 PERSPECTIVAS FUTURAS

Entre as perspectivas de experimentos a serem realizados estão:

- Dosagem de nucleotídeos no soro dos camundongos por HPLc.

- Dosagem de ATP no sobrenadante da cultura de queratinócitos, a fim de avaliar a liberação deste nucleotídeo.

- Análise da expressão de ectonucleotidases por RT-PCR nas células e no tecido.

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ANEXO A - Documento de aprovação da Comissão de Ética para Uso de Animais

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 034/11 – CEUA

Porto Alegre, 10 de março de 2011.

Senhora Pesquisadora:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 10/00206, intitulado: **"Papel dos receptores purinérgicos P2X7 em processos inflamatórios cutâneos em modelos *in vitro e in vivo*".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA/PUCRS

Ilma. Sra.
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ANEXO B – Artigo publicado no periódico Experimental Dermatology

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Original Article

P2X7 receptor is required for neutrophil accumulation in a mouse model of irritant contact dermatitis

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Abstract: Irritant contact dermatitis (ICD) is an inflammatory reaction caused by chemical toxicity on the skin. The P2X7 receptor (P2X7R) is a key mediator of cytokine release, which recruits immune cells to sites of inflammation. We investigated the role of P2X7R in croton oil (CrO)-induced ICD using *in vitro* and *in vivo* approaches. ICD was induced *in vivo* by CrO application on the mouse ear and *in vitro* by incubation of murine macrophages and dendritic cells (DCs) with CrO and ATP. Infiltrating cells were identified by flow cytometry, histology and myeloperoxidase (MPO) determination. Effects of the ATP scavenger apyrase were assessed to investigate further the role of P2X7R in ICD. Animals were also treated with N-1330, a caspase-1 inhibitor, or with clodronate, which induces macrophage apoptosis. CrO application induced severe inflammatory $Gr1^+$ cell infiltration and increased MPO levels in the mouse ear. Selective P2X7R antagonism with A438079 or genetic P2X7R deletion reduced the neutrophil infiltration. Clodronate administration significantly reduced $Gr1^+$ cell infiltration and local IL-1β levels. *In vitro* experiments confirmed that A438079 or apyrase treatment prevented the increase in IL-1β that was evoked by macrophage and DC incubation with CrO and ATP. These data support a key role for P2X7 in ICD-mediated inflammation via modulation of inflammatory cells. It is tempting to suggest that P2X7R inhibition might be an alternative ICD treatment.

Key words: irritant contact dermatitis – neutrophils – P2X7R

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Introduction

The skin is the primary defense between the body and the environment [1]. Contact dermatitis is a common inflammatory skin disease that involves activation of the innate and adaptive immune systems[1]. Contact dermatitis comprises both irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) [2]. ICD is defined as a locally arising reaction that appears after chemical irritant exposure. The chemical agents are directly responsible for cutaneous inflammation because of their inherent toxic properties, which cause tissue injury [3]. This inflammatory response activates innate immune system cells, such as macrophages, DCs and neutrophils [3–5]. Conversely, ACD is a delayed-type hypersensitivity response, which is triggered by specific T-cell activation and proliferation [6]. Croton oil (CrO) is a chemical irritant that causes topical inflammation when applied to mouse skin [7,8]. CrO application induces marked oedema and cell migration with massive neutrophil migration, which are features of an ICD model [5,9].

The P2X7 receptor (P2X7R) is an ATP-gated cation channel that is expressed on inflammatory cells [10]. This receptor reportedly controls diverse pro-inflammatory cellular signalling based on its ability to initiate post-translational cytokine processing, such as that of IL-1β [11,12]. P2X7R is activated by ATP [13,14] and is an important stimulator of the NLRP3 inflammasome [12]. Interference of leucocyte migration is an effective approach to treat skin inflammation [15]. NLRP3 is a signalling pathway that drives proteolytic activation of caspase-1 and IL-1β release [16], which recruits leucocytes to sites of infection and/or injury leading to host tissue damage [17]. Different cell types produce IL-1β, and it is not known which of these cell types mediate the inflammatory response during the ICD effector phase.

We used a CrO-induced ICD model to investigate the nature of the inflammatory cells and the role of P2X7R in this process. We verified that P2X7R activation is required for neutrophil accumulation and cytokine production in this experimental paradigm. Therefore, we suggest that P2X7R inhibition might represent a potential therapeutic alternative for ICD treatment.

Material and methods

Drugs

A438079 was obtained from Tocris (Ellisville, MO, USA). CrO, apyrase, dexamethasone and clodronate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-1330 (Ac-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Bachem Americas, Inc. (Torrance, CA, USA).

Animals

Male Swiss mice, or C57BL/6 WT and C57BL/6 $P2X7^{-/-}$ (6–8 weeks, 25–30 g) were used in this study. Swiss and C57BL/6 mice were obtained from the Federal University of Pelotas (UFPEL; Pelotas, RS, Brazil), and $P2X7^{-/-}$ mice were donated by Dr. Robson Coutinho-Silva, Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil). The $P2X7^{-/-}$ mice were generated

using the method developed by Dr. James Mobley (PGRD; Pfizer Inc., Groton, CT, USA). The $P2X7^{-/-}$ receptor-deficient mice used in this study were inbred to C5BL/6.

The animals were maintained at controlled temperature (22 ± 1°C) and humidity (60–70%) with a 12-h light–dark cycle (lights on 7:00 AM). Food and water were available *ad libitum*. Animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. All of the tests were performed between 7:00 AM and 7:00 PM. The experimental procedures reported in this manuscript followed the 'Principles of Laboratory Animal Care' from the National Institutes of Health [18] and were approved by the Institutional Animal Ethics Committee (protocol number: 10/00206).

Irritant contact dermatitis model

Swiss mice received a topical application of 1% CrO on the right ear and vehicle (acetone) on the left ear according to the method described by Mizumoto et al. [8]. Briefly, 6 h after CrO application, the animals were euthanized, and the ears were collected for analysis.

In a separate experiment, P2X7 receptor involvement was assessed using animals that had genetic deletion of this receptor. C57BL/6 mice were used as controls for this series of experiments. ICD was induced as described previously. All of the experiments were performed with a minimum of five animals per group and were repeated at least three times.

Ear oedema measurement

The animals were euthanized 6 h after CrO application. A 6-mm-diameter disc from the right and the left ears was removed with a circular metal punch and was weighed on an analytical balance. CrO-induced swelling was assessed as the weight difference (mg) between the right (inflamed) and the left (vehicle-treated) ears.

Pharmacological treatments

To characterize the role of P2X7R in the CrO-induced inflammatory response, *Swiss* mice were treated with the P2X7R antagonist A438079 (80 μmol/kg, i.p.) or the ATP scavenger apyrase (0.2 U/ear, s.c.) 2 h after CrO application. A separate group was treated with the positive control dexamethasone (0.5 mg/kg, s.c.). To evaluate whether IL-1β was involved in the processes, *Swiss* mice were also treated with the caspase-1 inhibitor N-1330 (6.25 μmol/kg) according to Mathiak et al. [19].

Depletion of phagocytic cells with clodronate

Liposomally encapsulated clodronate (dichloromethylene diphosphonate) is a macrophage and DC apoptosis inducer [20]. To verify the role of these cells in the inflammatory process, clodronate (dichloromethylene diphosphonic acid; Sigma–Aldrich) or sterile PBS-containing liposomes were instilled subcutaneously (locally – in the ears) and intravenously (25 mg/kg) 24 h before CrO application.

Myeloperoxidase activity

Neutrophil recruitment to the ears was quantified by tissue myeloperoxidase (MPO) activity according to the method described by Pereira et al. [21] with minor modifications. The ear tissue was homogenized in 5% (w/v) ethylenediamine tetra acetic acid (EDTA)/NaCl buffer (pH 4.7) and centrifuged at 6500 rpm for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethylammonium bromide buffer (pH 5.4), and the samples were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were recentrifuged under the same conditions mentioned previously (4400 *g*, 15 min, 4°C), and 25 μl supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM Na_3PO_4 and 0.3 mM hydrogen peroxide. Absorbance was measured at 650 nm, and the results are expressed as optical density per milligram of tissue.

Determination of IL-1β levels in ear tissue

Ear tissues were homogenized in phosphate-buffered saline (PBS) containing 0.4 M NaCl, 0.1 M phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 0.05% Tween-20, 0.5% bovine serum albumin and 2 μg/ml aprotinin A. Samples were centrifuged at 5000 rpm for 10 min at 4°C, and the supernatants were used for the assay. IL-1β levels were measured using an enzyme-linked immunosorbent assay kit according to supplier recommendations (DuoSet Kit; R&D Systems, Minneapolis, MN, USA). The results were expressed as pg/mg tissue.

Flow cytometry

Skin cells were obtained from mouse ears according to McLachlan et al. [22]. Fc receptors were blocked by resuspending cells in culture supernatant containing 24G2 antibody (ATCC) plus 1% mouse serum and 1% rat serum (FcBlock). Samples were later stained for surface markers as described below. Macrophages were stained with anti-CD11b (APC – clone M1/70), neutrophils were stained with anti-Gr1 (PE – clone RB6-8C5) and dendritic cells were stained with anti-CD11c (PE-Cy7 – clone HL3). All of the antibodies were purchased from BD Biosciences (San Jose, CA, USA). Cells were analysed using FACSCantoII (Becton Dickinson, Franklin Lakes, NJ, USA) and BD FACSDiva software, and FACS data were analysed with Flowjo software (version 7.6.5; Tree Star, Inc, Ashland, OR, USA).

Histological analysis

The collected ears were fixed in buffered formalin solution (10%) for 24 h, and the samples were subsequently paraffin embedded. Slices that were 5 μm thick were obtained and stained with haematoxylin and eosin. A pathologist who was blinded to the treatment reviewed each specimen for leucocyte infiltration.

Murine macrophage and dendritic cell culture

In accordance with Inaba et al. [23], C57BL/6 murine DCs were grown from bone marrow with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 (both BD Biosciences). On culture day 6, the cells were separated into adherent (macrophages) and non-adherent (dendritic cells). Cells were incubated with 25 μM A438079 and Apyrase (2 U) for 30 min followed by stimulation with 1% CrO or nothing (0 h). After 3 h, all of the cells were incubated with 2 mM ATP for 12 h, and IL-1β expression was analysed in the supernatant with an ELISA kit (DuoSet Kit; R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Results are expressed as the mean ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. P-values < 0.05 were considered to be significant. All of the tests were performed using GraphPad® 5 Software (version 5.0; Graphpad Software Inc., San Diego, CA, USA).

Results

P2X7R mediates neutrophil recruitment in ICD

To investigate whether neutrophil recruitment in our ICD model was dependent on P2X7R, we verified the histological sections. The analysis demonstrated that CrO-induced neutrophil infiltration in the ear tissue compared with the control, whereas apyrase, A438079

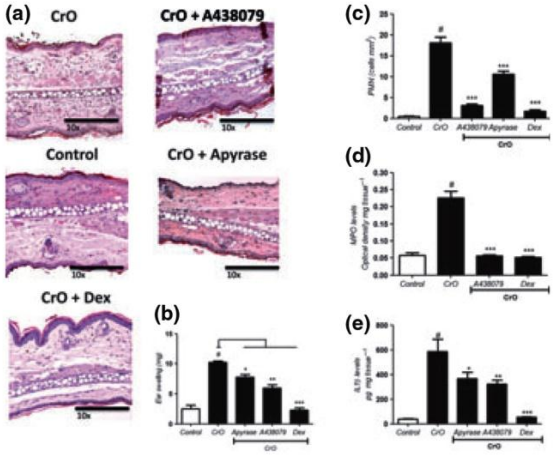

Figure 1. A438079 and apyrase treatment decreased the croton oil (CrO) chemical irritant-mediated inflammatory response. Irritant contact dermatitis (ICD) was elicited as described in Materials and methods. (a) Ear sections were stained with haematoxylin and eosin (10×) 4 h after topical 1% CrO application in the presence or absence of apyrase (0.2 U/ear, s.c.), A438079 (80 μmol/kg, i.p.) and dexamethasone (0.5 mg/kg, s.c.). (b) Swiss mice were treated with 1% CrO, acetone (vehicle), apyrase, A438079 and dexamethasone. Oedema (ear weight) was measured 6 h after CrO application. (c) Histological polymorphonuclear (PMN) cell quantification. (d) Myeloperoxidase (MPO) activity and (e) IL1β secretion in the ears of the Swiss mice that received 1% CrO topically in acetone or acetone alone (control) in the presence or absence of apyrase (0.2 U/ear, s.c.), A438079 (80 μmol/kg, i.p.) and dexamethasone (0.5 mg/kg, s.c.). Data represent the mean ± SEM of five animals. Significantly different from the control $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ significantly different from CrO treatment. $\#P < 0.05$ significantly different from vehicle treatment. All of the parameters were measured 6 h after the application of CrO.

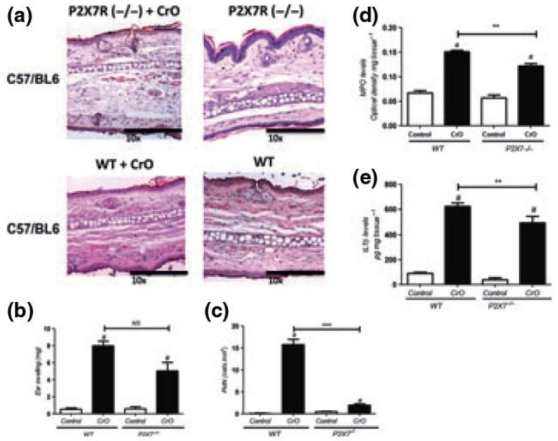

Figure 2. P2X7R genetic deletion decreased the croton oil (CrO) chemical irritant-evoked inflammatory response. Irritant contact dermatitis (ICD) was elicited as described in the Materials and methods section. (a) Ear sections from WT and P2X7$^{-/-}$ mice were stained with haematoxylin and eosin (10×) 4 h after topical 1% CrO application. (b) P2X7$^{-/-}$ and WT mice were treated with 1% CrO and acetone (vehicle) on the ear. Oedema (ear weight) was measured 6 h after CrO application. (c) Polymorphonuclear (PMN) cell quantification from histology. (d) Myeloperoxidase (MPO) activity (e) IL1β secretion in P2X7$^{-/-}$ and C57BL/6 WT mouse ears that received topical 1% CrO application. Data represent the mean ± SEM of five animals. $**P < 0.01$ and $***P < 0.001$ significantly different from WT and P2X7$^{-/-}$ and $\#P < 0.001$ significantly different compared with CrO.

and dexamethasone reduced the neutrophil infiltration, which was also accompanied by decreased ear oedema (Fig. 1a–c). In addition, we measured MPO levels in the *Swiss* mouse ears in the presence or absence of P2X7R-specific antagonist, apyrase or dexamethasone after CrO application. CrO administration increased MPO levels in the ears (Fig. 1d), which was significantly reduced by P2X7R antagonist A438079 (75 ± 6%) and dexamethasone (72 ± 5%) treatment. IL-1β is a primary cause of inflammation, and growing evidence suggests that P2X7R is a key mediator of IL-1β release [11]. CrO application markedly increased IL-1β levels in the ears of the Swiss mice (Fig. 1e). The increased IL-1β levels were reduced 37 ± 9%, 45 ± 5% and 81 ± 4% by apyrase, A438079 or dexamethasone treatment, respectively, after CrO administration (Fig. 1d).

These data were further confirmed by flow cytometry. Figure S1a demonstrates that CrO application results in increased Gr1$^+$ cell recruitment into the ear, which was diminished after apyrase and A438079 treatments. Additionally, we observed an increase in the percentage of DCs (CD11c$^+$ cells) and macrophages (CD11b$^+$ cells) that were present after CrO administration, which were also reduced after apyrase and A438079 treatments.

To confirm the role of P2X7R in ICD *in vivo*, we assessed CrO-mediated neutrophil infiltration in C57BL/6 WT and P2X7$^{-/-}$ mice. Histological analysis demonstrated that P2X7$^{-/-}$ mice exhibited reduced neutrophil influx and had a partial reduction in ear oedema after CrO application (Fig. 2a–c). These results were further confirmed by flow cytometry (Fig. S1b). In addition, as shown in Fig. 2d, CrO application was associated with neutrophil accumulation in C57BL/6 WT mice, as indicated by a significant increase in MPO activity in the skin. CrO-induced MPO activity was significantly decreased by 35% in P2X7$^{-/-}$ mice compared with WT mice, suggesting the involvement of P2X7R in CrO-mediated neutrophil recruitment. Similar to the first experiments with the Swiss mice, IL-1β levels were elevated after CrO exposure in C57BL/6 WT mice (Fig. 2d). In contrast, CrO-mediated IL-1β induction in P2X7$^{-/-}$ mice was significantly reduced (28 ± 6%) compared with WT (Fig. 2e). Taken together, these data indicate that the P2X7 receptor is required for CrO-mediated neutrophil recruitment and suggest that IL-1β release may be associated with this process.

Neutrophil recruitment is significantly decreased after clodronate liposome administration

It has been demonstrated that other innate immune system cells are necessary for neutrophil recruitment [9,23,24]. To address this issue, we used liposome-encapsulated clodronate to deplete APCs. Flow cytometry (Fig. S1c) demonstrated that clodronate treatment primarily reduced CD11b- and CD11c-expressing cells. Furthermore, CrO did not increase MPO levels (Fig. 3c), IL-1β levels (Fig. 3d) or the Gr1$^+$ cell population (Fig. S1c) in mice that were pretreated with clodronate, thereby suggesting a correlation between neutrophil recruitment and CrO-mediated IL-1β release by DCs and macrophages. Additionally, histology corroborated with reduced neutrophil infiltration after clodronate treatment (Fig. 3a–c). Moreover, clodronate pretreatment significantly decreased CrO-mediated ear oedema (Fig. 3b).

We hypothesized that CrO treatment triggered ATP release, thus activating DCs and macrophages following P2X7 receptor stimulation and IL-1β secretion. To evaluate this hypothesis, we performed an *in vitro* experiment with macrophages and DCs that had been differentiated from mouse bone marrow. The cells were pretreated with A438079 or apyrase or were left untreated. The cells were subsequently stimulated with CrO or vehicle for 3 h followed by incubation with 2 mM ATP. After 12 h, IL-1β release

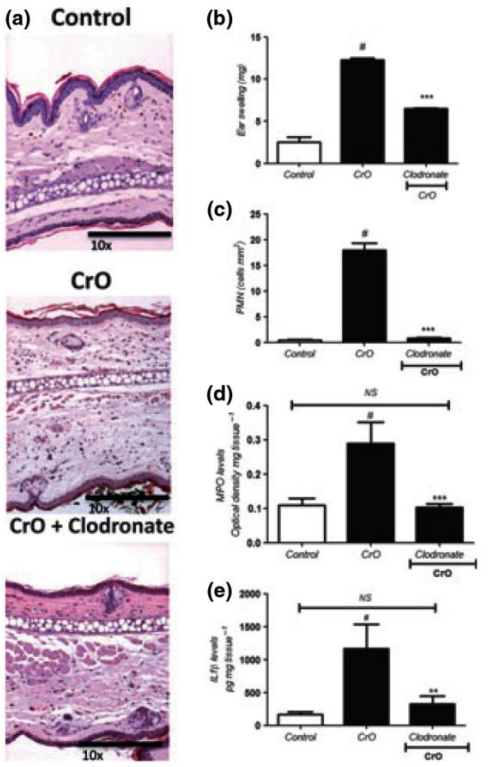

Figure 3. Depletion of macrophages and dendritic cells (DCs) decreased croton oil (CrO)-mediated inflammation. Irritant contact dermatitis (ICD) was elicited as described in the Materials and methods section. Clodronate was administered 24 h before ICD induction, and ears were collected 4 h later. (a) Ear sections were stained with haematoxylin and eosin (10×) 4 h after topical 1% CrO application in the presence or absence of Clodronate (25 mg/kg). (b) Swiss mice were treated with 1% CrO, acetone (vehicle) and clodronate. Oedema was measured 6 h after CrO application. (c) Polymorphonuclear (PMN) cell quantification from histology. (d) Clodronate application (25 mg/kg) on myeloperoxidase (MPO) activity and (e) IL1β secretion. Data represent the mean ± SEM of three experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ denote significance compared with control values; $^{\#\#}P < 0.001$; $^{\#}P < 0.01$ denote significance compared with control values.

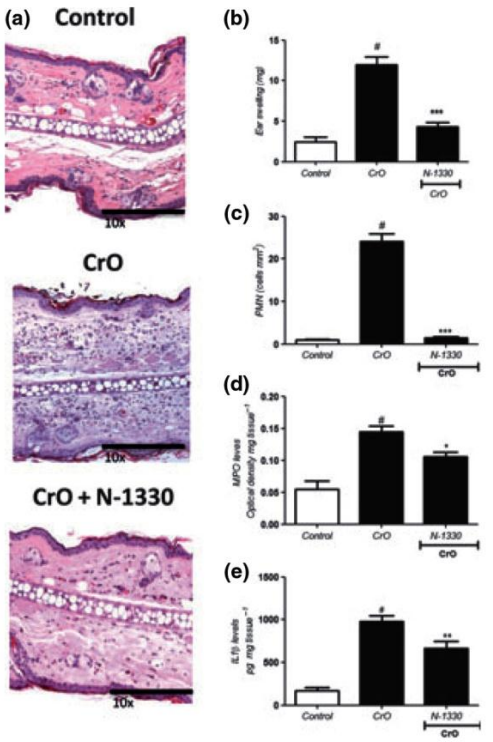

Figure 4. Caspase-1 inhibition decreased croton oil (CrO)-mediated neutrophil accumulation. Irritant contact dermatitis (ICD) was elicited as described in the Materials and methods section. N-1330 (6.25 μmol/kg), an irreversible inhibitor of caspase-1, was administered 30 min prior to ICD induction, and ears were collected 4 h later. (a) Ear sections were stained with haematoxylin and eosin (10×) 4 h after topical 1% CrO application in the presence or absence of N-1330 (6.25 μmol/kg). (b) Swiss mice were treated with 1% CrO, acetone (vehicle) and N-1330. Oedema (ear weight) was measured 6 h after CrO application. (c) Polymorphonuclear (PMN) cells were quantified from histology. (c) Myeloperoxidase (MPO) activity and (d) IL1β secretion. $^{*}P < 0.05$; $^{**}P < 001$ denotes the significance levels compared with control values; $^{\#}P < 0.001$ denotes the significance levels compared with vehicle values.

was measured by ELISA. Figure S2 demonstrates that CrO or ATP incubation evoked IL-1β release from macrophages and DCs, which was significantly reduced by pretreatment with A438079 or apyrase, suggesting that extracellular ATP-mediated P2X7R activation is important for IL-1β secretion by macrophages and DCs. Taken together, the results described previously indicate that MPs and DCs play a key role in CrO-induced ICD and suggest that P2X7R activation in MPs and DCs may be involved.

Reduced IL1-β secretion impairs neutrophil accumulation

P2X7R is a key player in IL-1β processing and release [11]. The IL-1β release following ATP-mediated P2X7 receptor stimulation occurs via the NLRP3 inflammasome [12]. NLRP3 activates caspase-1, an enzyme that cleaves inactive pro-IL1-β to active IL1-β [24]. To gain further insights in our experimental paradigm, we treated Swiss mice with the irreversible caspase-1 inhibitor N-1330 (Ac-Tyr-Val-Ala-Asp-chloromethylketone). Interestingly, N-1330 treatment significantly decreased neutrophil accumulation as assessed by histology (Fig. 4a,c). In addition, ear oedema was decreased in N-1330-treated animals compared with the control group. Furthermore, MPO levels (27 ± 4%) and IL-1β production (36 ± 11%) were also reduced by N-1330 treatment (Fig. 4d,e). Flow cytometry data (Fig. S1d) were also consistent with these results. In addition, $CD11b^+$ and $CD11c^+$ cells were also reduced. Taken together, these results reinforce the possible association between IL-1β-mediated neutrophil recruitment and ear oedema.

Discussion

Little is known about the involvement of purinergic signalling in skin inflammation. Recently, Weber et al. [25] reported a functional link in ACD between ATP, P2X7R and the NLRP3 inflammasome to regulate IL-1β release. In this study, we investigated the role of P2X7R in CrO-mediated neutrophil recruitment in ICD. We demonstrated that P2X7R is implicated in neutrophil recruitment and ear oedema. As was reported by Weber et al., we verified that $P2X7^{-/-}$ mice displayed a partial reduction in CrO-induced ear oedema (Fig. 2b). Interestingly, ear oedema was markedly reduced in mice that had been treated with the selective P2X7 receptor antagonist A438079. It is important to mention that genetically modified animals (including P2X7 KO mice) might exhibit compensatory changes in signal transduction; however, knockout strategies are still useful tools to confirm the functional data that is obtained using pharmacological approaches [26].

In this study, we demonstrated that CrO application increased neutrophil accumulation, which was confirmed by MPO levels,

$Gr1^+$ staining and histology of mouse ears. Remarkably, in $P2X7^{-/-}$ mice (Fig. 2), treatment with the selective P2X7R antagonist A438079 or administration of the ATP-degrading enzyme apyrase markedly reduced this effect (Fig. 1). Previous data demonstrated the involvement of P2X7R in tissue injury-mediated leucocyte infiltration [27–29]. Therefore, the results obtained herein reinforced the relationship between P2X7R activation and neutrophil accumulation in our ICD model.

Next, we reported that clodronate liposome administration subcutaneously and intravenously eliminated $CD11b^+$ cells (macrophages) and partially eliminated $CD11c^+$ cells (DCs; Fig. S1c). Elimination of these cells occurred concurrently with diminished neutrophil infiltration, similar to the effect observed in P2X7R knockout animals (Fig. 1a,c and d). These findings demonstrate a key role of DCs and macrophages in initiating the ICD inflammatory process. Of note, IL-1β levels were comparable with the control after clodronate liposome treatment, indicating that IL-1β is produced mainly via immune cells, as was described by Chen et al. [30].

P2X7R is implicated in IL-1β processing and release, which supports leucocyte migration to inflamed tissue [11]. In this study, we demonstrate that CrO increased IL-1β levels in the ear of the treated mice, and this effect was significantly reduced by A438079 or apyrase treatment (Fig. 1b). In addition, CrO-induced IL-1β accumulation was significantly decreased in $P2X7^{-/-}$ mice compared with C57BL/6 WT mice (Fig. 2d), suggesting IL-1β involvement following P2X7R activation in the CrO-ICD model. Extracellular ATP activates the caspase-1/Nlrp3 inflammasome complex via P2X7 receptor signalling, which generates inflammatory cytokines such as IL-1β [12, 13, 17, 31]. In this study, we demonstrated that simultaneous treatment with CrO and ATP induced the release of significant amounts of IL-1β via P2X7R in macrophages and DCs (Fig. 3e).

Croton oil induced keratinocyte necrosis (data not shown), suggesting that ATP is released during CrO exposure. We further demonstrated that caspase-1 inhibition reduced IL-1β formation (Fig. 4d) and consequently reduced neutrophil accumulation (Fig. 4a–c). Mc Donald et al. [7] demonstrated that ATP initiates the inflammatory response that causes neutrophil recruitment. Thus, we suggest that P2X7R-mediated IL-1β secretion is related to tissue neutrophil recruitment in the ICD model, and we also suggest participation of other pathways in addition to P2X7R and IL-1β-mediated neutrophil recruitment [15,32].

In summary, our compelling evidence indicates the importance of P2X7R and APCs (macrophages and DCs) in promoting neutrophil accumulation and ear oedema in ICD, which may be correlated in part, to enhanced IL-1β levels. Therefore, it is tempting to suggest that P2X7R inhibition represents a potential therapeutic alternative for ICD treatment.

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Author Contributions

G. Silva, N. Sperotto, T. Borges, C. Takya, and Rafael Zanin performed research and interpreted the data. C. Bonorino and R. Coutinho-Silva contributed essential reagents or tools. T. Cristina, R. Coutinho-Silva, M. M. Campos, R. Zanin and F.B. Morrone assisted with the data analysis. G. Silva wrote the draft of the manuscript. M.M Campos, R. Zanin, F.B. Morrone were responsible for study design, writing and manuscript correction.

Ethical approval

The experimental procedures reported in this manuscript followed the 'Principles of Laboratory Animal Care' from the National Institutes of Health (NIH) and were approved by the Institutional Animal Ethics Committee (protocol number: 10/00206).

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Dot plot with percentages of $Gr1^+$, $CD11b^+$ and $CD11c^+$ cells in ear tissue from Swiss mice that received topical 1% croton oil (CrO) application in presence or absence of A438079 (80 μmol/kg, i.p.) or apyrase (0.2 U/ear, s.c.). Figures represent at least three independent experiments with pooled ears from three mice per experiment. Flow cytometry analyses were performed 4 h after croton oil application. (b) Dot plots with the percentage of $Gr1^+$, $CD11b^+$ and $CD11c^+$ cells in ear tissue from C57/B6 and $P2X7^{-/-}$ mice that received topical 1% CrO application. (c) Dot plots with the percentage of $Gr1^+$, $CD11b^+$ and $CD11c^+$ cells in ear tissues. Figures are representative of at least three independent experiments with pooled ears from three mice per experiment. Flow cytometry analyses were performed 4 h after croton oil application. (d) Dot plots with the percentage of $Gr1^+$, $CD11b^+$ and $CD11c^+$ cells in ear tissues from Swiss mice that received topical 1% CrO application in the presence or absence of N-1330 (6.25 μmol/kg).

Figure S2. IL1-β was measured from murine dendritic cell (DC) and macrophage culture supernatants that had been primed with Croton oil (1%) CrO 3 h after ATP treatment (2 mM) in the presence or absence of A438079 (25 μM) or apyrase (2 U/ml).