UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

FERNANDA REGINA GODOY ROCHA

INFLAMASSOMOS NLRC4 E NLRP3 NA DOENÇA PERIODONTAL EXPERIMENTAL INDUZIDA POR *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

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Tese apresentada ao programa de Pós-Graduação em Odontologia, Área de concentração em Periodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista, para a obtenção do título de Doutor em Odontologia.

Orientador: Prof. Dr. Carlos Rossa Junior

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Tese para obtenção do grau de Doutor

Comissão julgadora

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Araraquara, 08 de abril de 2016

DADOS CURRICULARES

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(Colossenses 3:23)

”Em tudo dai graças...”

(I Tessalonissences 5:18)

Rocha FRG. Inflamassomos NLRC4 e NLRP3 na doença periodontal induzida por Aggregatibacter actinomycetemcomitans [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2016.

RESUMO

Inflamassomos são complexos multi protéicos capazes de amplificar o sinal inflamatório em condições de interações microbiota-hospedeiro e destruição tecidual, como a doença periodontal crônica. Devido à escassez de informações sobre o papel dos inflamassomos NLRC4 e NLRP3 na doença periodontal, utilizamos neste estudo um modelo de doença periodontal induzida por bactérias em camundongos WT, Ipaf-knockout (Ipaf-KO), Caspase 1-knockout (Casp1-KO) e NLRP3-knockout (NLRP3-KO) para descrever o efeito destes na inflamação e reabsorção óssea alveolar. *Aggregatibacter actinomycetemcomitans* (Aa) inativadas pelo calor foram injetadas nos tecidos gengivais palatais adjacentes aos primeiros molares dos camundongos normais e knockout, e os grupos controle receberam o mesmo volume do veículo de suspensão (PBS). A severidade da reabsorção óssea foi quantificada por análise de µCT. A inflamação foi avaliada por imunofluorescência, verificando-se presença e intensidade da coloração por marcadores de leucócitos (CD45) e neutrófilos (Ly6G). O número de osteoclastos foi determinado por TRAP e a expressão gênica de RANKL, MMP-13, TNF-a, IL-6 e IL-10 nos tecidos gengivais avaliada por RT-qPCR. A publicação 1 mostra uma reabsorção óssea inflamatória significativamente maior nos camundongos Ipaf-KO, sem diferenças, porém, no número de osteoclastos entre WT e Ipaf-KO. A expressão dos genes-alvo aumentou com a indução da doença periodontal, exceto de TNFa e IL-10, que foram altas nos animais Ipaf-KO mesmo em ausência da doença periodontal, podendo-se concluir que o inflamassomo NLRC4 teve um papel protetor na reabsorção óssea inflamatória neste modelo experimental. Na publicação 2 observamos que a severidade da reabsorção óssea não foi afetada pela ausência do inflamassomo NLRP3, mas foi significantemente reduzida nos camundongos Casp1-KO, sendo acompanhada nestes por um aumento significante no número de osteoclastos. Não houve, no entanto, diferenças no infiltrado inflamatório ou na expressão dos genes-alvo, concluindo-se que NLRP3 não teve papel significante na inflamação e reabsorção óssea, e caspase-1 contribuiu para a reabsorção óssea nas condições deste estudo.

Palavras-chave: Doença periodontal. Inflamação. Inflamassomos. Imunidade inata. Aggregatibacter actinomycetemcomitans.

Rocha FRG. NLRC4 and NLRP3 inflammasome in periodontal disease induced by Aggregatibacter actinomycetemcomitans [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2016.

ABSTRACT

Inflammasomes are multi-protein complexes that can amplify the inflammatory signal in situations involving host-microbial interactions and host tissue destruction, such as chronic periodontal disease. There is a relative scarcity of information on the role of NLRC4 and NLRP3 inflammasomes in periodontal disease. In this study, we used a model of bacteria-initiated periodontal disease in WT, Ipaf-knockout (Ipaf-KO), Caspase 1-knockout (Casp1-KO) and NLRP3-knockout (NLRP3-KO) mice to describe the effect of those inflammasomes on inflammation and alveolar bone resorption. Heat-killed *Aggregatibacter actinomycetemcomitans* (Aa) were injected in the gingival tissues on the palatal aspect adjacent to first molars of wild-type (WT), Ipaf-KO, Casp1-KO and NLRP3-KO mice, and control animals received the suspension vehicle (PBS). Severity of bone resorption was quantitated by µCT analysis. Inflammation was assessed by immunofluorescence, verifying the presence and intensity of a pan-leukocyte (CD45) and a neutrophil (Ly6G) markers. Osteoclast number was determined by TRAP and gene expression of RANKL, MMP-13, TNF-a, IL-6 and IL-10 in the gingival tissues was evaluated by RT-qPCR. In the first publication, µCT analysis showed a significantly greater inflammatory bone resorption in Ipaf-KO mice; however there was no difference between WT and Ipaf-KO on osteoclast numbers of inflammatory infiltrate. Expression of candidate genes was also similarly increased by the induction of experimental periodontal disease, except for the expression of TNF-alpha and IL-10, which was already significantly higher in the gingival tissues of Ipaf-KO mice in the absence of experimental periodontal disease. We conclude that NLRC4 inflammasome has a protective role on inflammatory bone resorption in this experimental model. In the second publication we observed that severity of bone resorption was not affected by the lack of NLRP3 inflammasome, but it was reduced in Casp1-KO mice. Interestingly, the attenuation of alveolar bone resorption in Casp1-KO mice was accompanied by increase on the number of osteoclasts, whereas there were no significant changes on the inflammatory infiltrate or expression of candidate genes. The conclusion was that NLPR3 inflammasome does not play a significant role in induced inflammation and bone resorption and caspase-1 has a pro-resorptive role in these conditions.

Keywords: Periodontal disease. Inflammation. Inflammasome. Innate immunity. Aggregatibacter actinomycetemcomitans.

SUMÁRIO

1 INTRODUÇÃO

1.1 Doenças periodontais como modelo de estudo das interações microbiota-hospedeiro

As diferentes formas de doença periodontal destrutiva representam a lesão lítica de tecido ósseo mais prevalente na espécie humana (Pacios[40], 2012) Atualmente consideradas como infecções mediadas e moduladas pela resposta do hospedeiro, as doenças periodontais tem como agente etiológico extrínseco fundamental microrganismos do biofilme dental. As doenças periodontais proporcionam um modelo único para o estudo das interações microbiota-hospedeiro. Mais de 500 diferentes espécies microbianas podem ser encontradas no biofilme dental (Paster[42], 2001) contudo apenas algumas são frequentemente associadas às doenças periodontais (Socransky[49,50], 1998, 1999). Assim, a cavidade bucal é continuamente colonizada por microrganismos não-patogênicos, de forma similar a outras superfícies mucosas, em especial a mucosa intestinal.

A discriminação entre espécies patogênicas e não-patogênicas requer mecanismos endógenos de regulação negativa da sinalização por receptores de reconhecimento padrão (PRRs) para evitar o desenvolvimento de uma resposta imune exagerada e com consequências deletérias ao próprio hospedeiro. No entanto, é justamente a resposta imune desregulada e crônica a responsável pela maior parte da destruição tecidual característica de diversas doenças

inflamatórias, incluindo as doenças periodontais. Existem evidências sugerindo que a desregulação da resposta imune localizada no periodonto representa um dos principais mecanismos biológicos para a possível influência negativa das infecções periodontais sobre órgãos e sistemas à distância, como os sistemas cardiovascular, respiratório e reprodutivo (Prizzo[45], 2010), sendo este fato, se confirmado, suficiente para aumentar a relevância desta condição local para a saúde geral dos indivíduos.

Histologicamente, as doenças periodontais se caracterizam por um infiltrado inflamatório crônico, com predominância de linfócitos e macrófagos, porém com presença também de neutrófilos (Page[41], 1976). A caracterização bioquímica das doenças periodontais é complexa devido a ausência de um consenso quanto aos critérios diagnósticos de atividade de doença. Assim, existem descrições na literatura de um desvio para um padrão de resposta Th1, Th2 e participação de células Th17 (Garlet[18], 2010; Graves[20], 2011). Ainda que células T sejam consideradas as principais reguladoras da resposta imune nas doenças periodontais e também a principal fonte de RANKL no microambiente periodontal, existem evidências de que os linfócitos B também tem papel relevante na destruição tecidual associada às doenças periodontais (Eastcott,[13] 1994; Teng[55] 1999; Baker[1] 1999; Kawai,[28] 2006). Mais recentemente, a polarização de macrófagos (M1/M2) presentes nos tecidos afetados por diferentes condições

infecciosas também tem sido relacionada com a destruição tecidual e desfecho clínico destas condições (Benoit[6], 2008). De qualquer forma, existem abundantes informações sobre a expressão de citocinas, quimiocinas e outros mediadores inflamatórios nas doenças periodontais, como membros da família TNF, IL-1, INF, Cicloxigenases, Prostaglandinas e, coletivamente, estas informações indicam uma importante e complexa ativação da resposta imune (Yu[65], 2011; Graves[20], 2011).

Tradicionalmente dividida em imunidade inata e adaptativa, a resposta imune tem a função básica de proteger o organismo, sendo responsável pelo reconhecimento inicial do desvio da normalidade e/ou da presença de microrganismos. Este reconhecimento é mediado por células e receptores da resposta imune inata, os quais podem ativar a resposta imune adaptativa. Além de reconhecer agentes externos como microrganismos, seus fragmentos e fatores de virulência, por exemplo, os sensores da imunidade inata também reconhecem padrões moleculares derivados do próprio hospedeiro, que são indicativos de um desvio da homeostasia e, portanto, entendidos como sinais de perigo resultantes do dano e destruição tecidual. Estes são denominados padrões moleculares associados ao dano (DAMPs, *damage-associated molecular patterns*) (Pedra[44], 2009). No entanto, uma vez estabelecida a resposta imune em quadros inflamatórios crônicos, os braços inato e adaptativo da resposta imune estão continuamente ativos, e apresentam regulação recíproca. Estes dados estabelecem a doença periodontal como um

problema relevante Também como um importante modelo de estudo de condições envolvendo interações microbiota-hospedeiro e inflamação crônica.

1.2 Inflamassomos: sensores microbianos da resposta imune inata

Uma questão fundamental em Imunologia diz respeito ao início da resposta do hospedeiro à presença de microrganismos. A identificação de receptores específicos capazes de reconhecer diferentes ligantes microbianos, os padrões moleculares associados à microrganismos (MAMPS), representa um dos maiores avanços científicos no entendimento da resposta imune inata, reconhecido com o prêmio Nobel de Medicina em 2011.

Receptores de membrana com homologia ao gene Toll da mosca de fruta atuam como mediadores no reconhecimento de ligantes microbianos presentes no meio externo e também interno de diversos tipos celulares (receptores semelhantes à Toll, TLRs), em especial nas células participantes da resposta imune inata (Garlet[18], 2010; Graves[20], 2011). Outros sensores moleculares da resposta imune inata vem sendo identificados, cujo papel não é redundante ao dos TLRs, como as proteínas com domínio de ligação a nucleotídeos e oligomerização (Nod) presentes no citosol e que apresentam como ligantes componentes derivados primariamente de microrganismos Gram-negativos (Nod1) ou derivados tanto de microrganismos Gram-positivos quanto Gram-negativos (Nod2). Estudo de nosso grupo de pesquisa em andamento

com apoio da FAPESP visa caracterizar o papel de Nod1 e Nod2 na reabsorção óssea associada à doença periodontal e os resultados obtidos até o momento indicam que Nod1 tem papel protetor, inibindo a reabsorção óssea associada à doença periodontal experimental. Estes sensores da imunidade inata são altamente conservados evolucionariamente, indicando sua relevância na resposta do hospedeiro.

Inflamassomos são uma família de complexos multiprotéicos citosólicos que participam no início e modulação da resposta imune iniciada por patógenos microbianos (MAMPs), além de sinais de dano tecidual (DAMPs) e grande número de espécies microbianas e fatores de virulência derivados de microorganismos (Yilmaz Lee[64], 2015). Estes complexos tem proteínas semelhantes à receptores Nod (NLR, *Nod-like receptors*) que incluem 22 proteínas citosólicas. Estas proteínas NLR representam o centro do complexo multiprotéico e dão o nome ao inflamassomo, como NLRP1, NLRP3 e NLRC4, por exemplo, que são os únicos inflamassomos com função fisiológica demonstrada até o momento (Jim[26] 2007). Além da proteína central NLR, os inflamassomos também incluem as proteínas caspase-1 e a proteína adaptadora denominada proteína semelhante à partícula associada à apoptose contendo um domínio CARD (ASC, *apoptosis-associated speck-like protein containing a CARD*). A relevância do inflamassomo para a resposta imune é demonstrada pela associação entre mutações nos genes codificando proteínas componentes do complexo protéico e condições inflamatórias

autoimunes, como vitiligo, síndrome de Muckle-Wells e síndrome periódica associada à criopirina (cryopyrin-associated periodic syndrome, CAPS) (Hoffman[23], 2001; Valk[60], 2010). Outras condições associadas à desregulação da resposta imune, como as neoplasias, também estão associadas a alterações na ativação dos inflamassomos, incluindo câncer de pulmão (Hu[24], 2010). Em camundongos, a ativação de NLRC4 tem efeito preventivo em câncer de cólon, possivelmente por meio de modulação da proliferação e apoptose de células epiteliais da mucosa intestinal (Miao[38], 2010). Os diferentes inflamassomos são montados e ativados por diversos sinais exógenos e endógenos. Enquanto NLRP3 pode ser ativado por MAMPs e DAMPs, NLRC4 é ativado preferencialmente por MAMPs, incluindo DNA dupla-fita e proteínas bacteriana específicas. A proteína efetora dos inflamassomos é a protease caspase- 1, que tem papel importante na morte celular (especificamente, por pirocitose) e também no processamento final de citocinas inflamatórias IL-1β, IL-18 e IL-33. Assim, o ganho de função com ativação constitutiva do inflamassomo e, consequentemente, de caspase-1 pode levar à exacerbação da resposta inflamatória, com aumento da secreção de IL-1β ativa e aumento da apoptose; enquanto que a perda ou redução da função do inflamassomo pode atenuar a resposta inflamatória e inibir a resposta imune, reduzindo a capacidade do hospedeiro detectar e reagir aos MAMPs. A importância dos inflamassomos na resposta imune é demonstrada por mecanismos de

evasão desenvolvidos por diferentes microrganismos, como *S. typhimurium* e NLRC4 (Gong[19], 2012).

Independentemente dos ativadores e ligantes específicos para NLRC4 e NLRP3, a doença periodontal, como uma condição inflamatória crônica, é caracterizada por altos níveis de espécies reativas de oxigênio (ROS) e também por degradação tecidual, com acúmulo, portanto, de DAMPs no microambiente periodontal. Tanto ROS como DAMPs (os quais também podem gerar ROS) podem ativar outros inflamassomos além de NLRC4 e NLRP3, incluindo AIM2, NLRP1, NLRP6 (Yilmaz Lee[64], 2015). A ativação dos inflamassomos pode, então, ser proveniente de interações diretas do reconhecimento dos ligantes bacterianos pela proteínas/sensores centrais dos inflamassomos, ou também pode pela detecção de alterações celulares induzidas por estímulos microbianos ou estresse (Franchi[21], 2012).

Coletivamente, estas informações indicam o papel relevante dos inflamassomos na resposta imune e sugerem que o melhor entendimento de suas funções biológicas e dos mecanismos biológicos envolvidos em sua ativação pode levar a novas estratégias de intervenção terapêutica direcionadas à modulação de vias reguladas pelos inflamassomos envolvidas na patogênese de diversas condições associadas à interações microbiota-hospedeiro e à inflamação crônica.

Especificamente no contexto da doença periodontal, há relativamente escassas informações disponíveis até o momento. Um

aumento na expressão gênica de NLRP3 e NLRP2, mas não de ASC-1, foi relatado em presença de doença periodontal em humanos, e estes níveis elevados foram relacionados a aumento de RNAm também das citocinas IL-1beta e IL-18, que são processadas pelos inflamassomos (Bostanci[7], 2009). Elevada expressão de NLRP3 e AIM2 em tecido gingival de pacientes com doença periodontal já foi também correlacionado com níveis de IL-1beta e IL-18 também por outros autores, sugerindo uma possível participação de diversos inflamassomos na doença periodontal (Park[43], 2014; Ramos-Junior[46] 2015). Não foi encontrado, no entanto, até o momento nenhum estudo in vivo utilizando a indução de doença periodontal em modelos geneticamente modificados, o que evidencia o aspecto de interesse e novidade em nosso trabalho.

1.3 O inflamassomo NLRC4 (Ipaf)

Os principais MAMPs capazes de iniciar a ativação do NLRC4, também conhecido como Ipaf, são flagelinas bacterianas e proteínas componentes do sistema secretor bacteriano tipo III e IV, utilizado pelos microorganismos para injetar fatores de virulência nas células do hospedeiro (Figura 1). Estas proteínas são comuns a vários microorganismos Gram-negativos causadores de infecções graves, como *Yersinia spp* (peste e desordens gastrointestinais), *Shigella spp* (disenteria), *Salmonella spp* (febre tifóide), *Eschericia coli* (infecções

alimentares), *Legionella pneumophila* (pneumonia) e *Pseudomonas spp* (diversas infecções, incluindo doenças periodontais) (Jin[15], 2007). A ativação de NLRC4 por patógenos representa o nível de sofisticação da resposta imune inata na detecção diferencial de patógenos e microrganismos comensais. Espécies comensais não patogênicas de *E. coli* presentes na flora intestinal devem ser distinguidas de *strains* da mesma espécie com fatores de virulência associados à doenças, como a *E. coli* enteropatogênica (EPEC). Recentemente, o sistema excretório tipo III do patógeno *Shigella flexneri* tem sido reconhecido como ativador indireto do inflamassomo NLRC4 (Suzuki[54], 2014). Forma-se um complexo de ativação do sistema complemento que pode induzir indiretamente a ativação dos inflamassomos, por alterações celulares associadas como a lise e alterações na concentração de cálcio do meio intracelular (Tirantafilou[57], 2015). A produção diferencial de citocinas inflamatórias como a IL-1β e IL-18 por células da resposta imune inata em resposta à espécies patogênicas e não à espécies comensais envolve a ativação de inflamassomos (Miao[39], 2010), uma vez que estes complexos protéicos funcionam como mediadores do processamento final destas citocinas e podem modular a resposta adaptativa ao promoverem a polarização da resposta de linfócitos T auxiliares para um perfil Th1 e também a ativação de células *natural killer* (Dinarello[12], 2010).

Há evidências de que o inflamassomo NLRC4 tem funções efetoras únicas em diferentes tipos celulares. A ativação de NLRC4 em macrófagos

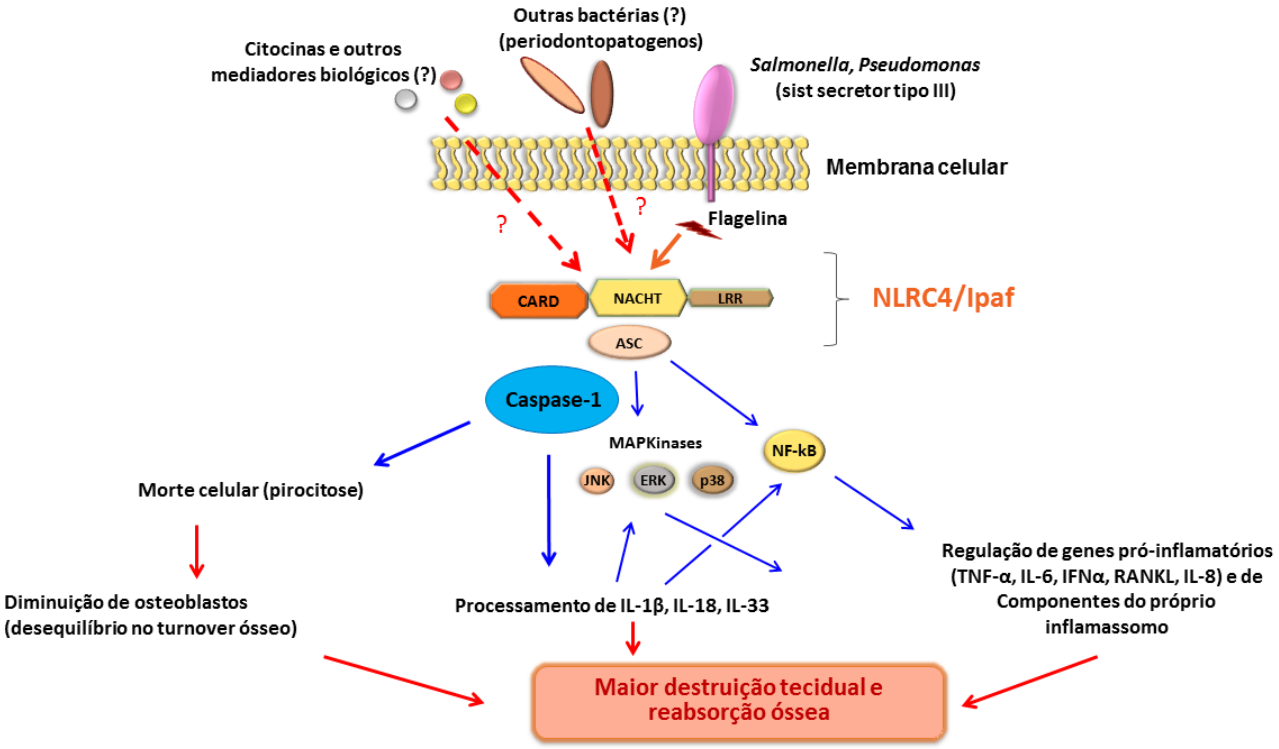
peritoneais em camundongos, por exemplo (mas não macrófagos derivados da medula óssea, resulta em aumento da produção de lipídeos pró-inflamatórios (Von Moltke[63], 2012). Há recentes relatos também de que a ativação de NLRC4 pode levar células epiteliais, mas não neutrófilos, a morte celular por piroptose (Vance[61], 2015).

Tal ativação de NLRC4, assim como a de outros inflamassomos, tem como resultado o recrutamento e dimerização de caspase-1, necessária para sua ativação. Assim, caspase-1, que é a proteína efetora de NLRC4, bem como dos outros inflamassomos, é responsável tanto pela morte celular por pirocitose como pelo processamento final e ativação das citocinas inflamatórias IL- 1β, IL-18 e IL-33 (De Santis[11], 2013).

IL-1β é a citocina inflamatória prototípica, com diversas ações biológicas importantes para a inflamação com a estimulação da proliferação de timócitos, da produção e liberação de colagenase e prostaglandinas e da diferenciação de osteoclastos, entre outras funções biológicas (Tripodi[58], 2011). IL-18 é outra citocina pró-inflamatória capaz de induzir a produção de interferon-gama por linfócitos T, polarizando a resposta para um padrão Th1 e também de ativar células *natural killer* (Schimitz[47], 2005) IL-33, por outro lado, estimula a produção de citocinas relacionadas ao padrão de resposta Th2 e inibe a produção de interferon-gama por células Th1 (Barksby[2], 2007) porém seu papel em condições inflamatórias crônicas é pouco conhecido (Figueiredo[14],

2008). Maiores níveis de IL-18 (Becerik[4], 2012) e IL-1β (Sutterwala[52], 2007) são associados à presença de doença periodontal. Não há informações específicas sobre o papel de IL-33 e caspase-1 na doença periodontal.

FIGURA 1 – Esquema representativo da ativação do inflamassomo NLRC4 por flagelinas bacterianas e proteínas componentes do sistema secretor bacteriano tipos III e IV, não sendo confirmadas ainda outras vias de ativação, como citocinas, outros mediadores biológicos, e periodontopatógenos, por exemplo.



Fonte: Elaboração Própria

1.4 *O inflamassomo NLRP3 (Nalp3)*

NLRP3 (ou Nalp3) é o inflamassomo mais extensamente estudado até o momento, e sua ativação em humanos envolve a associação da proteína central NLRP3, com duas proteínas contendo o domínio CARD (Cardinal e ASC), permitindo assim o recrutamento de duas unidades de Caspase-1 por interações CARD-CARD. Como não foi identificado um homólogo murino da proteína Cardinal, diferentemente dos humanos, acredita-se que a ativação de NLRP3 em roedores resulte no recrutamento de apenas uma molécula de caspase-1 (Sutterwala[53], 2006). Classicamente, a ativação do inflamassomo NLPR3 envolve interação microbiota-hospedeiro, via ativação de TLRs (Figura 2). A produção de IL-1β e IL-18 por macrófagos infectados com *Staphylococcus aureus* ou *Listeria monocytogenes* é dependente de NLRP3 (Martinson[35], 2006). Como trata-se de um complexo multi-protéico localizado no citoplasma, inicialmente considerou- se que os inflamassomos seriam particularmente relevantes na defesa contra microrganismos e parasitas intracelulares. No entanto, macrófagos deficientes em NLRP3 estimulados com agonistas de TLR não produziram IL-1β e IL-18 em virtude da não ativação de caspase-1, responsável pela clivagem e ativação destas citocinas. A relevância do NLRP3 foi confirmada in vivo, verificando que camundongos geneticamente deficientes foram mais resistentes ao choque endotoxêmico por injeção de LPS (Bostanci[7], 2009). Além dos microrganismos, o inflamassomo NLRP3 pode ser

ativado por sinais endógenos de perigo de origem não microbiana (DAMPs), como por exemplo o ácido úrico presente em condições como a gota e também liberado por células necróticas (Bauernfeind[3], 2009).

Especificamente em relação ao papel de NLRP3 em interações microbiota-hospedeiro nas doenças periodontais, estas já foram também relatadas, como quando foi investigada a expressão de diversos inflamassomos em células mononucleares em respota a estímulo com A. Actinomycetemcomitans. Foi encontrado um nível elevado de NLRP3, bem como IL-1β e IL-18 (Belibasakis[5], 2012). A. actinomycetemcomitans também induziu secreção de IL-1β e ativação de caspase-1 a partir da ativação do inflammassomo AIM2 em macrófagos THP-1 (Kim[29], 2016). Outro periodontopatógeno relacionado à ativação de NLRP3 e transcrição de IL-1β foi T. Denticola, que por uma proteína de sua superfície, Td92, a qual interagiu diretamente com integrina α5β1 para a ativação do inflamassomo NLRP3 e caspase-1, aumentando os níveis de IL-1β em macrófagos (Jun[27], 2012).

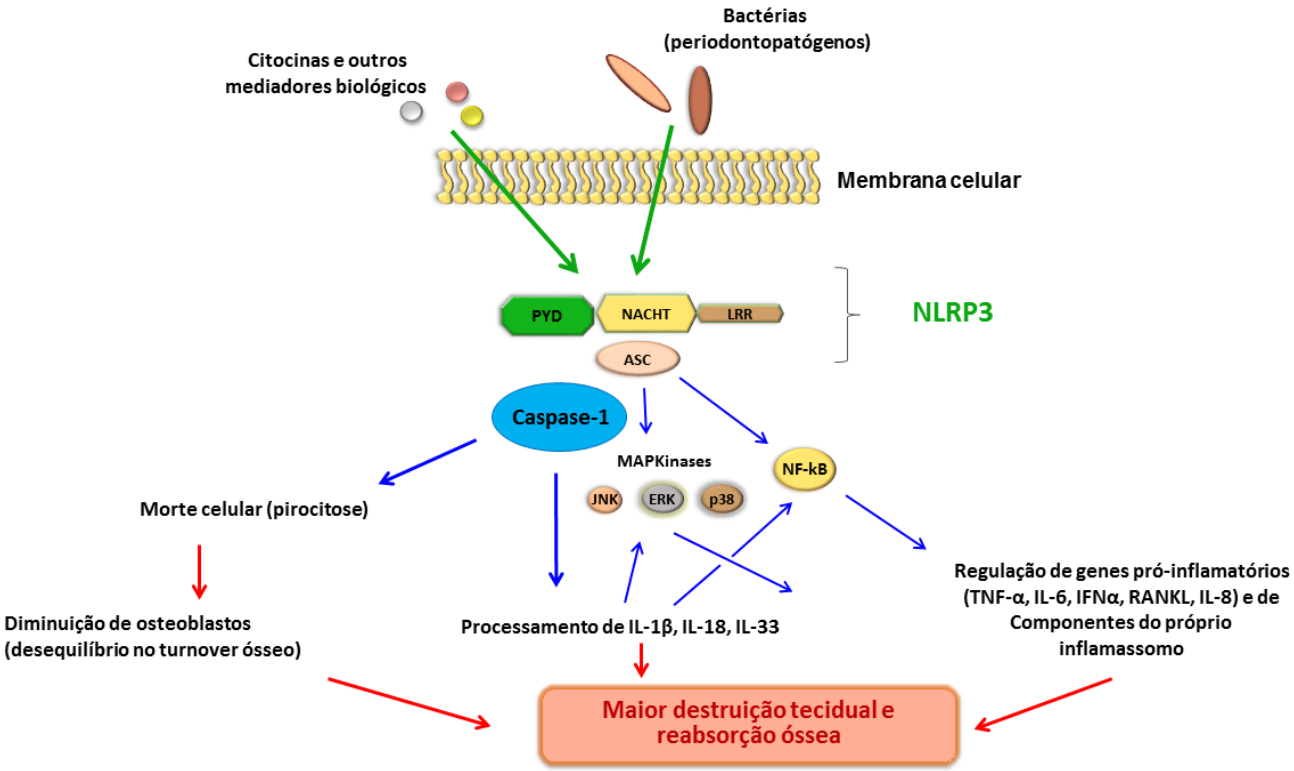
Estudos em humanos já relacionaram também o inflamassomo NLRP3 e doença periodontal. O estado de hiperglicemia mostrou ter exacerbado a inflamação pela ativação de NLRP3 em tecidos gengivais coletados pacientes com diabetes tipo 2 com periodontite. Maiores níveis de NLRP3 e IL-1β foram encontrados em pacientes com ambas as doenças, e a inibição de NLRP3 contribuiu para diminuição de caspase-1 e IL-1β (Huang [25], 2015). Outro estudo em humanos demonstrou que a

expressão gênica de NLRP3 em biópsias de tecidos gengivais inflamados foi significativamente aumentada em comparação à observada em tecidos normais, estando diretamente correlacionada à expressão gênica de IL-1β e IL-18 nestes tecidos. Estes autores verificaram ainda que o estímulo de macrófagos humanos in vitro com sobrenadantes de culturas de *Porphyromonas gingivalis*, bactéria Gram-negativa associada à doença periodontal, aumentou a expressão gênica de NLRP3, IL-1β e IL-18. Assim, a ativação de NLRP3 pode requerer o estímulo de TLRs como iniciador/indutor da expressão dos componentes do inflamassomo, sendo este estímulo inicial via TLRs também responsável pela indução do substrato (pro-IL-1β e pro-IL-18, por ex.) de ação do inflamassomo (Park[43], 2014). Mais recentemente, o aumento da expressão dos componentes do inflamassomo NLRP3 em tecidos gengivais inflamados foi confirmada em nível protéico e correlacionada positivamente com os níveis das citocinas inflamatórias IL-1β e TNF-α. In vitro, estes autores verificaram que macrófagos humanos estimulados com *P.gingivalis* vivos apresentaram aumento significativo da expressão protéica de NLRP3, a qual foi necessária para o aumento da ativação de caspase-1, mediando a ativação de IL-1β e também a morte celular por pirocitose (Ciraci[9], 2012). NLRP3 foi ativado também, além de via dependente de ATP (Mariathasan[33], 2006) após infecção com P. gingivalis, via sinalização de TLR2 e TLR4, levando a secreção de IL-1b e morte celular por piroptose (Park[43], 2014). Estes estudos sugerem novas abordagens da resposta

imune inata contra periodontopatógenos, sendo este um possível alvo para futuras estratégias no tratamento da doença periodontal.

Estes dados indicam que o inflamassomo NLRP3 tem importante papel na regulação da expressão de mediadores inflamatórios em situações envolvendo interações microbiota-hospedeiro e dano tecidual, sugerindo sua relevância em processos inflamatórios de natureza infecciosa como as doenças periodontais (Mariott[34], 2004; McCall[36], 2008).

FIGURA 2 – Esquema representativo da ativação já conhecida do inflamassomo NLRP3, por meio de interação microbiota-hospedeiro, via ativação de TLRs, levando à produção de IL-1β e IL-18. NLRP3 pode ser ativado por sinais endógenos de perigo de origem não microbiana (DAMPs).



Fonte: Elaboração Própria

1.5 Inflamassomos e imunidade adaptativa

Clássica e didaticamente, a resposta imune é dividida em dois braços: inato e adaptativo. Os limites teórico-didáticos entre imunidade inata e adaptativa são questionáveis, no entanto, na medida em que células da resposta imune adaptativa apresentam PRRs funcionais, e também considerando a estreita interação e modulação recíproca dos dois tipos de resposta imune, especialmente em condições crônicas como artrite reumatóide e as doenças periodontais. O papel da ativação dos TLRs na modulação da resposta imune é extensamente estudado; no entanto, a questão óbvia, mas pouco explorada até o momento, é o papel dos inflamassomos na regulação da imunidade adaptativa.

A produção de citocinas é essencial para o estabelecimento da resposta imune inata competente e também para a subsequente ativação da imunidade adaptativa. Como já mencionado, citocinas como IL-1, IL-18 e IL-33 são processadas pelos inflamassomos e tem importante papel na polarização da resposta de linfócitos T CD4+ nos perfis Th17, Th1, e Th2, respectivamente (Kool[30], 2008; Lasiglie[31], 2011). Uma sugestão do papel dos inflamassomos na imunidade adaptativa é derivada do modelo experimental de alergia respiratória em camundongos, que utiliza *alum* (mistura de alumínio e sais de magnésio), um ativador do inflamassomo NLRP3, como agente indutor da reação alérgica (Hoffman[16], 2006; Gris[22], 2010). Em modelo experimental de encefalite autoimune, camundongos geneticamente deficientes para

NLRP3 apresentaram redução na progressão da doença, que foi associada à menor produção de IL-18, IFN-γ e IL-17, indicando inibição da polarização da resposta Th1 e Th17 (Shaw[48], 2010). Outro estudo utilizando modelo de encefalite experimental demonstrou que a deficiência de caspase-1 (efetor comum dos inflamassomos) é relacionada à menor progressão da doença (Van de Veerdonk[62], 2011), sugerindo um possível papel para outros inflamassomos além do NLRP3. Outras evidências do papel dos inflamassomos na modulação da imunidade adaptativa, incluem a resposta a microorganismos. Animais deficientes em caspase-1 são mais susceptíveis à infecção disseminada por fungos, a qual foi associada à inibição da polarização de células CD4+ nos perfis Th1 e Th17 (Tomalka[56], 2011). O inflamassomo NLRC4 também foi implicado na resistência à infecção por fungos (Li[32], 2011). Assim, há ainda escassez de informações sobre o papel dos inflamassomos na modulação da imunidade adaptativa, em especial do inflamassomo NLRC4, porém as evidências disponíveis sugerem que a ativação destes complexos protéicos citoplasmáticos tem papel relevante na resposta do hospedeiro.

2 PROPOSIÇÃO GERAL

Considerando a relevância das doenças periodontais como uma condição de elevada prevalência associada à destruição de tecidos mineralizados e não mineralizados, a adequação de modelos de doença periodontal para estudo de condições associadas à interação microbiota-hospedeiro e à inflamação crônica, as evidências sugerindo um importante papel dos inflamassomos na modulação e amplificação da imunidade adaptativa e a relativa escassez de informações específicas sobre o papel dos inflamassomos NLRC4 e NLRP3 em situações associadas à inflamação crônica in vivo, o presente estudo tem como hipótese geral: os inflamassomos NLRC4 e NLRP3 tem um importante papel na inibição da resposta imune associada às doenças periodontais e marcante influência na reabsorção inflamatória do osso alveolar.

Propõe-se avaliar o papel dos inflamassomos NLRC4 e NLRP3 na resposta imune e reabsorção óssea na doença periodontal experimental induzida por Aa. Levando em conta os diferentes inflamassomos aqui avaliados, os papéis dos mesmos foram tratados em dois capítulos distintos, de forma a facilitar a análise e compreensão dos resultados inerentes à hipótese geral do estudo.

3 MATERIAL E MÉTODO

3.1 Animais

Foram utilizados 41 camundongos (*Mus musculus*) machos e adultos (5 a 8 semanas) da linhagem C57BL/6, sendo 12 animais normais (WT) e 12 geneticamente deficientes (KO) para Caspase1 (Casp1-KO), 12 NLRC4 (Ipaf-KO) e 5 NLRP3 (NLRP3-KO).

Os animais foram obtidos junto ao Biotério Central e Biotério de Animais Geneticamente Modificados dos Departamentos de Bioquímica e Imunologia e Biologia Celular, Molecular e Bioagentes Patogênicos da Faculdade de Medicina de Ribeirão Preto (FMRP) da Universidade de São Paulo (USP). A manutenção dos animais se deu em estante isoladora em condições livres de patógenos específicos (SPF), no biotério da Faculdade de Odontologia de Araraquara – UNESP, em acordo com o Conselho Nacional de Controle de Experimentação Animal (CONCEA) e a Comissão de Ética no Uso de Animais (CEUA) desta (protocolo número 10/2010; Anexo A) Os animais permaneceram acomodados em gaiolas de polipropileno, em número máximo de 5 animais por gaiola, e receberam alimentação granulada (Labina/Purina) e água *ad libitum,* sendo pesados semanalmente. Os sacrifícios foram realizados 28 dias após o início das injeções, por deslocamento cervical.

3.2 Modelo experimental e obtenção das amostras in vivo

Foi utilizado um modelo de indução de doença periodontal por meio de injeções de *Aggregatibacter actinomycetemcomitans* (Aa,

linhagem JP1) inativado pelo calor, possibilitando a comparação dos dados. Os animais foram brevemente anestesiados com isoflurano em injeção bilateral de 3 µL de uma suspensão de $1x10^9$ UFC/mL de Aa ou do mesmo volume do veículo (PBS, pH 7.2) nos tecidos gengivais palatinos entre primeiro e segundo molares superiores. Estas injeções foram realizadas 3x/semana, durante 4 semanas. Foi induzida a doença periodontal em 6 camundongos dos genótipos WT, Ipaf-KO e Casp1-KO e 3 animais do genótipo NLRP3-KO, com as injeções de Aa. O controle, sem doença, incluiu 6 animais dos genótipos WT, Ipaf-KO e Casp1-KO e 2 animais NLRP3-KO, que receberam injeções de mesmo volume de PBS.

O maxilar superior foi dividido, no momento do sacrifício, em duas hemi-maxilas. Um dos lados de todos os animais de cada genótipo (WT, Casp1-KO, Ipaf-KO e NLRP3-KO) em cada condição experimental (indução de doença periodontal ou controle) foi fixado em paraformadeído 4% a 4 ºC durante 24 horas, transferido para etanol 70%, e armazenado novamente a 4 ºC. Estas amostras foram utilizadas na avaliação da perda óssea por microtomografia computadorizada e posteriormente (após descalcificação em EDTA 0.5 M, pH 7,8 em temperatura ambiente, durante 4 a 6 semanas, com troca da solução 2x/semana, seguido de inclusão em blocos de parafina) nas avaliações histológicas e imunohistoquímicas. Nas demais hemi-maxilas dos animais de cada genótipo os tecidos periodontais não mineralizados da região

palatina dos primeiros e segundos molares superiores foram dissecados cuidadosamente e processados para a extração do RNA total para posterior utilização deste nos experimentos subsequentes.

3.3 Avaliação quantitativa - microtomografia computadorizada: uCT

As hemi-maxilas de cada genótipo e condição experimental (com e sem indução de doença periodontal) foram analisadas pela varredura de feixe de raios-X em um sistema de microtomografia computadorizada (Skyscan 1176, Aartselaar, Bélgica). Cada *scan* (varredura) foi reconstruído em uma matriz retangular e as imagens tridimensionais foram geradas para cada amostra. Estas imagens foram então rotacionadas em uma orientação padrão no plano sagital e foi estabelecido um limite de contraste para distinguir tecidos mineralizados de não-mineralizados, utilizando-se os softwares NRecon\DataViewer\CTan\CTvol (Skyscan, Aartselaar, Bélgica). A região de interesse (ROI) das peças de aproximadamente 4 x 16 mm^3 foi determinada medindo-se a área a partir da junção cemento-esmalte mesial do 1º molar até a junção cemento-esmalte mesial do 2º molar (dimensão ântero-posterior), com uma altura cérvico- apical de 2 mm a partir do limite coronário localizado no teto da furca do 1o. molar e com espessura (sentido vestíbulo-lingual) de 1,8 mm (60 cortes de 18 µm cada) a partir da tábua óssea vestibular. Com o auxílio dos programas CTan e CTvol, as seguintes análises foram realizadas: percentual de volume da ROI preenchida por tecido ósseo e densidade de superfície

óssea (BS/TV) do tecido presente na ROI. Estas análises foram realizadas na própria Faculdade de Odontologia de Araraquara – UNESP, segundo metodologia já utilizada em trabalhos anteriores de nosso grupo de pesquisa[65, 10, 59, 17].

3.4 Avaliação da Expressão Gênica: RT-qPCR

Para este experimento foi isolado o RNA total a partir de tecido gengival extraído da região palatal em que foram feitas as injecões de Aa, sendo analisadas 6 amostras de cada genótipo, para cada condição (com e sem doença). A extração do RNA foi feita com um sistema de colunas de afinidade para o isolamento e purificação do RNA total, incluindo tratamento com DNAse (RNAqueous-4-PCR, Ambion). Após quantificação do RNA total em espectrofotômetro UV de microvolumes (Nanovue, GE HealthSciences), 500 ng de RNA total foram utilizados na síntese de cDNA utilizando random hexamers primers e moloney leukemia vírus reverse transcriptase, segundo as instruções do fornecedor do kit (High Capacity cDNA synthesis kit, Applied Biosystems). A reação de PCR em tempo real foi feita utilizando conjuntos de primers e sondas pré desenhados (Gene expression assays, Applied Biosystems) para detecção dos genes-alvo (TNFα, lL-6, IL-10, RANKL, MMP-13) e do gene constitutivo (GAPDH), utilizando o sistema TaqMan (TaqMan Universal PCR Master Mix, Applied Biosystems) e o equipamento StepOne Plus (Applied Biosystems) com as condições de ciclagem otimizadas pelo fabricante (10 min a 95ºC para desnaturação inicial seguido de 40 ciclos de 15 segundos a 95ºC e 1 min a

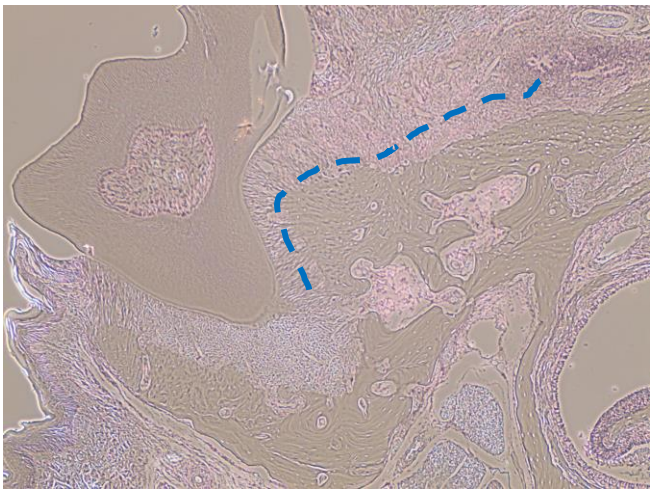
60ºC). Os valores *cycle threshold* (Ct) de cada poço foram calculados pelo software da termocicladora e analisados pelo método $\Delta(\Delta Ct)$.

3.5 H/E e TRAP

As mesmas peças (hemi-maxilas) submetidas à análise por microtomografia computadorizada foram posteriormente utilizadas, após a descalcificação e processamento histológico de rotina, para obtenção de cortes semi-seriados das peças incluídas em parafina. Estes cortes, com 4 µm de espessura, foram montados em lâminas silanizadas e corados com Hematoxilina & Eosina (H&E) para análise descritiva e estereométrica das condições histológicas.

Após o processamento histológico, 9 lâminas de cada animal foram utilizadas para detecção da enzima fostatase ácida resistente ao tartarato (TRAP), utilizando metodologia baseada em Naphtol[8, 37], em que os cortes foram incubados em tampão acetato 0.05 mol/L, pH5.0, contendo naphtol A-MX fosfato 0.27 mmol/L (Sigma-Aldrich, St. Louis, MO, EUA), 1% N,Ndimetilformamida, 1.6 mmol/L Fast Red LB e 50 mmol/L de tartarato de sódio a 37C durante 15 minutos. Após a incubação, foi feita a contra coloração com hematoxilina (Sigma, St, Louis, MO, USA) durante 1,5 minuto. A contagem dos osteoclastos foi feita na regiao de interesse descrita na figura 3, considerando-se como osteoclastos as células TRAP-positivas com dois ou mais núcleos.

FIGURA 3 – Representação da região de interesse considerada para contagem de osteoclastos marcados por TRAP, destacada na figura com traçado pontilhado em azul, correspondendo à região do palato em que foram feitas as injeções de Aa, entre o primeiro e segundo molares (Aumento 40x). As células foram contadas considerando-se 500 µm a partir da crista óssea em direção ao centro do palato, e no contorno da raiz palatina ate o ápice desta; ou então a partir do ponto mais alto do osso alveolar até a direção da artéria palatina, no caso de as raízes não estarem aparentes no corte.



Fonte: Elaboração própria

3.6 Imunofluorescência

Os cortes histológicos foram primeiro desparafinizados em duas trocas de xilol, de 15 e 5 minutos cada, e então desidratados em álcool 100% durante 2 minutos. Procedeu-se então a reidratação dos cortes em álcool 95% e 70%, em imersões de dois minutos cada, seguindo-se a imersão em água destilada durante um minuto. As lâminas foram então

submersas em Trilogy buffer (Cell Marque, Hot Springs, AR) por 25 minutos, dentro de banho-maria a 95ºC.

A seguir, os cortes foram mantidos por 5 minutos em água destilada para esfriar e então enxaguados em 1xTBS (Tris-Buffered Saline) e incubados em solução 10% de soro de cabra (Life Technologies) em 1xTBS com 0,1% Triton X-100 (Fisher Scientific) e 0,5% Tween 20 (Fisher Scientific). Os cortes foram então enxaguados 3 vezes de 5 minutos cada em 1xTBS e incubados com os anticorpos primários a 4ºC *overnight:* CD45, para marcação de células inflamatórias, concentração 1:200 (Purified Anti-mouse CD45, Biolegend) ou Ly6G, para marcação específica de neutrófilos, concentração 1:00 (Purified Anti-mouse Ly6G, Biolegend). Os cortes foram então lavados 3 vezes de 5 minutos cada em 1xTBS e incubados em anticorpo secundário Alexa Fluor 594 goat anti-rat (Biolegend) 1:650 e 1:200, respectivamente, durante 2 horas em temperatura ambiente. Foi utilizado DNA dye 4’,6-diamino-2phenylindole (DAPI) 1:50000 para coloração dos núcleos. As imagens foram obtidas em microscópio digital invertido EVOS FL (Life Technologies).

Os dados de intensidade de fluorescência média foram gerados utilizando o programa de análise de imagem ImageJ (https://imagej.nih.gov/ij/). As imagens foram separadas em canais individuais para marcação com DAPI e CD45 ou Ly6G, respectivemente. A intesidade média de fluorescência foi mensurada para a mesma região de interesse (ROI) em cada imagem, por meio da ferramenta de análise, e os

valores para infiltrado de leucócitos (CD45) e PMN (Ly6G) foram normalizados pelo número total de células presentes, de acordo com a marcação por DAPI. Os dados correspondem à média de três cortes diferentes por animal, para cada condição experimental.

3.7 Análise dos resultados

Os dados foram tabulados e a distribuição normal ou não-normal foi determinada para posterior aplicação de testes estatísticos adequados à distribuição dos dados e objetivo da análise utilizando Software GraphPad Prism 5 (La Jolla, CA, USA). O objetivo primário das análises foi identificar diferenças significantes nos desfechos de interesse segundo os diferentes genótipos dos animais (WT, NLRC4 KO, NLRP3 KO e Caspase-1 KO) e condições experimentais (com e sem indução de doença periodontal). Alguns aspectos foram avaliados de forma descritiva/qualitativa. O nível de significância adotado nas análises foi de 95% (p=0.05).

4 PUBLICAÇÃO 1

NLRC4 INFLAMMASOME HAS A PROTECTIVE ROLE ON INFLAMMATORY BONE RESORPTION IN A MURINE MODEL OF PERIODONTAL DISEASE *

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Abstract

Objectives: Inflammasomes are multi-protein complexes activated in situations involving host-microbial interactions, which can cause host tissue destruction, such as chronic periodontal disease. There is virtually no information on the possible influence of NLRC4 inflammasome on inflammation and bone resorption associated with destructive periodontal diseases. We used a model of bacteria-initiated periodontal disease in WT and Ipaf-knockout mice to assess the role of NLRC4 inflammasome on inflammation and alveolar bone resorption. **Materials and Methods:** Heat-killed *Aggregatibacter actinomycetemcomitans* (Aa) were injected in the gingival tissues on the palatal aspect adjacent to both first molars of wild-type (WT) and Ipaf-KO mice. Control animals were injected with the same volume of the vehicle (PBS). Severity of bone resorption was assessed by µCT. Inflammation was studied by immunofluorescence detection of a pan-leukocyte marker (CD45) and a neutrophil marker (Ly6G), osteoclast number was determined by biochemical staining for TRAP and gene expression of RANKL, MMP-13, TNF-a, IL-6 and IL-10 in the gingival tissues was evaluated by RT-qPCR.

Results: Ipaf-/KO mice demonstrated a significantly higher level of bone resorption; however there was no difference between WT and Ipaf-KO in the frequency of osteoclasts or inflammatory infiltrate. Expression of candidate genes were significantly increased by the induction of experimental periodontal disease, with exception of TNF-alpha and IL-10, which were already significantly higher in the gingival tissues of Ipaf-KO mice. **Conclusion:** NLRC4 inflammasome has a protective role on inflammatory bone resorption in this experimental model. **Clinical Relevance:** Lack of functional NLRC4 aggravates inflammatory bone resorption, suggesting a bone-sparing role for this inflammasome.

Keywords: Periodontal disease, Inflammation, NLRC4 Inflammasome, Innate Immunity, Aggregatibacter actinomycetemcomitans

Introduction

Periodontal disease is initiated and maintained by microorganisms of the dental biofilm, but is considered to be mediated and modulated by the immune response [1]. The mediators and cells of the immune system are deemed responsible for most of the tissue destruction in many inflammatory diseases, including periodontal diseases [2-4].

Inflammasomes are a family of cytosolic multiprotein complexes involved in the initiation and modulation of immune response triggered by microbial pathogens (MAMPs) and also by signals of tissue damage (DAMPs). Once assembled, these protein complexes include proteins similar to Nod receptors (NLR, Nod-like receptors), caspase-1 protein and an adapter protein associated with apoptosis containing CARD domain (ASC, apoptosis-associated speck-like protein containing a CARD) [5]. The relevance of the inflammasome to the immune response is demonstrated by the association between mutations in the genes encoding protein components of the inflammasome protein complex and autoimmune inflammatory conditions, or dysregulation of the immune response [6-8].

NLRC4 inflammasome (also called Ipaf or CARD12) is preferentially activated by MAMPs, including DNA and specific bacterial proteins. Its influence in experimental periodontal disease has not been studied, but may involve complex biological mechanisms related to the cascade of cytokines and intracellular signaling pathways activated in chronic

inflammation, which may result in reciprocal influences between NLRC4 activation and the immune response.

Caspase-1, the effector protein of all inflammasomes, plays an important role in cell death and final processing of inflammatory cytokines IL-1β, IL-18 and IL-33, whereby these cytokines can be induced independent of the activation of inflammasome. In this regard, there is evidence that other inflammatory cytokines, such as TNF-α, can induce the production and secretion of active IL-1β [9]. TNF-α, IL-1α and IL -1β can induce the activation of caspase-1 and secretion of IL-1β [10], even in the absence of microbial stimulation (MAMPs). This activation of caspase-1 mediated by TNF-α involves the activation of NF-kB, suggesting the possibility of indirect activation of inflammasomes [11], amplifying the inflammatory response, and bone mediators as RANKL and MMP-13 may lead to bone destruction.

There is evidence indicating that ASC, a core protein component of inflammasomes, can activate cytokines and chemokines that are not processed by the inflammasomes themselves. For instance, monocytic cell lines infected with the periodontal pathogen *Porphyromonas gingivalis*, ASC is required for the activation of several cytokines, including TNF-α, IL-6, IL-8 and IL-10. This activation was independent of IL-1R activation and also of caspase-1, but was correlated with activation of NF-kB [12]. Functionally, ASC can be an activator of NF-kB; however, this activity was not confirmed in macrophages [13, 14]. Other cytokines activated by ASC in humans and mice, include TNF- α, IL-6, IL-8 and CXCL2, which are all at least partially

controlled by the transcription factor AP-1, in turn, a downstream target of MAPKinases ERK, JNK and p38 [15]. These data indicate that the ASC may exert independent functions, by mediating the activation of NF-kB and MAPKinases and participate in the transcriptional activation of cytokines not processed by inflammasomes. In another point of convergence and cross-regulation, expression of the protein components of the inflammasome are transcriptionally controlled by signaling pathways and transcription factors activated by inflammatory cytokines [16].

Considering the different possible pathways for activation and its role in regulating final processing of inflammatory mediators, it is likely that activation of NLRC4 inflammasome has a role in inflammatory bone resorption associated with periodontal disease, despite the reduced presence of classic specific activators of this inflammasome (microorganisms with type III and IV secretion system) [17, 18]. IL-1β, which is directly processed by the inflammasomes, may modulate osteoclastogenesis; as it long been recognized as an indisputable and powerful stimulus to induce bone resorption, both in vitro [19] and in vivo [20]. Its effects can be either directly on osteoclasts [21] or indirectly by modulating the expression of RANKL (ligand for receptor activator of nuclear factor-kappa-B) in other cell types [22]. The activation of inflammasomes can amplifie the inflammatory activity by influencing several cells of the innate immune response, including macrophages and dendritic

cells as well as the adaptive response, including T and B lymphocytes [23, 24].

A better understanding of mechanisms involved in the activation of these immune cell populations may lead to new strategies for therapeutic intervention aimed at modulating pathways regulated by inflammasomes involved in the pathogenesis various conditions associated host-microbial interactions and chronic inflammation.

In this study we investigated whether the NLRC4 inflammasome has an important role in modulating the immune response and inflammatory bone resorption in an experimental periodontal disease model.

Materials and Methods

Periodontal disease model

A total of 24 C57BL/6 male adult mice (age between 6 and 8 weeks) were used, including 12 wild-type (WT) and 12 mice that were genetically deficient for NLRC4/Ipaf/CARD12 (Ipaf-KO), the central NLR protein in the NLRC4 inflammasome. All mice were obtained from the center for genetically modified and transgenic mice, School of Medicine at Ribeirao Preto-University of Sao Paulo (USP). Experimental periodontal disease was induced in 6 mice of each genotype (WT and Ipaf-KO) by direct injections of a 3 µL PBS suspension of heat-killed *Aggregatibacter actinomycetemcomitans (Aa, JP1 serotype)* at $1x10^9$ UFC/mL. Control mice (n=6 for each genotype) received injections of the same volume of the PBS

vehicle. These injections were performed using a Hamilton-type micro syringe (33 gauge needle) three times/week, for 4 weeks under mild general anesthesia with isofluorane (Baxter Healthcare, Deerfield, IL) directly on the gingiva at the palatal aspect of the upper molars, between the first and second molars. 28 days after the induction of disease, all animals were sacrificed. (?)

The maxillary bones were hemisected, and half of the specimens had the gingival tissue from the palatal aspect of the first molars carefully dissected for extraction of total RNA for RT-qPCR. The remaining specimens including the maxillary bone was used for microcomputed tomographic analysis of the alveolar bone resorption. The other half of the specimens, including the molars and surrounding tissues were submitted to routine histological processing for the histological analysis.

This study was carried out in accordance with the principles stated by the Brazilian College of Animal Experimentation and was approved by the Ethical Committee on Animal Experimentation (protocol number 10/2010) of the School of Dentistry at Araraquara,UNESP-SP, Brazil.

Microcomputed tomography analysis (µCT scanning)

The maxillae specimens from which the soft gingival tissue was dissected were fixed in 4% buffered formalin for 24h and transferred to 70% alcohol until scanning at 18 µm resolution using a µCT system (Skyscan, Aastselaar, Belgium). Tridimensional images were reconstructed and the resulting images were re-oriented spacially in a standardized manner (using

anatomical landmarks) using NRecon and DataViewer softwares (Skyscan, Aastselaar, Belgium). A standardized 5.4 mm^3 region of interest (ROI) was set with 1.5x4.0x0.9 mm (vertical or cervico-apical x horizontal or mesio-distal x lateral or buccal-palatal) was defined using the following references: cervical/coronal reference was the roof of the furcation area between mesial and distal roots of the upper first molar and mesially the distal aspect of the mesial root of the first molar. The thickness of the ROI was set to 50 slices (900 µm) counted towards the palatal /medial direction on the sagital plane beginning from the image that included the center of the upper first molar in its buccal-palatal width. On the analysis, a standardized threshold was set to distinguish between non-mineralized and mineralized tissues. Considering that variations on the size and mineralization of the tooth structures included in the ROI were irrelevant among the different animals, the analysis assessed the percentage of mineralized tissue (MT) within the total volume (TV) of the ROI, presented as the ratio MT/TV analysis. A decrease on this ratio is interpreted as indicative of bone resorption.

Osteoclast count

Sections (n=9 per animal/genotype) obtained from the paraffin-embedded tissues were used to determine the presence of osteoclasts by histochemical staining of tartarate-resistant acid phosphatase (TRAP), using a reagent kit according to the supplier's instructions (Acid phosphatase, leukocyte (TRAP) kit, Sigma-Aldrich Co.). Briefly, unstained sections were incubated in a solution of acetate buffer 0.05 mol/L, pH 5.0,

with Napthol AS-MX phosphate 0.27 mmol/L, 1% N,N dimethylformamide, 1.6 mmol/L Fast Red LB and 50 mmol/L of Sodium Tartarate at 37 C for 15 minutes. Sections were counterstained with hematoxylin (Sigma, St, Louis, MO, USA) for 90 seconds, and washed in water. Osteoclasts, defined as large, multinucleated and TRAP-positive cells in proximity with the alveolar bone, were counted by a single trained examiner, which was blind to the experimental groups. Osteoclasts were counted from the apical portion of the palatal root of the first molar until the depression in the palatal bone associated with the palatine artery/nerve,

Quantitative Reverse-Transcription Real-Time PCR

Total RNA was extracted from tissue samples using an affinity column system (RNAqueous-4PCR, Ambion Inc) according to the manufacturer's protocol. The quantity and purity of total RNA were determined by UV spectrophotometry and by the 260/280 nm ratio, respectively. 500 ng of total RNA was converted into cDNA with random hexamer primers and moloney leukemia virus reverse transcriptase in a reaction volume of 20 µL (High Capacity cDNA Synthesis kit, Applied Biosystems). The qPCR reactions were performed in a 20µL total volume reaction, including TaqMan qPCR mastermix (TaqMan Fast Advanced, Applied Biosystems), cDNA template, deionized water, and mouse-specific pre-designed and optimized sets of primers and probe (TaqMan gene expression assays). Cycling conditions were pre-optimized by the supplier of the sets of primers/probe and master mix, and 40 cycles were run on a StepOne Plus

qPCR thermocycler (Applied Biosystems). Determination of the relative levels of gene expression by the $\Delta(\Delta Ct)$ method was performed using the thermocycler's software using automated detection of the Ct. Expression levels of GAPDH in the same samples were used to normalize the results of the target genes. Results are presented as the normalized relative expression of the target gene from duplicate measurements of each sample (6 samples per group and genotype), and expressed as fold change over the levels of expression of the normalized target gene determined in cDNA samples from control (PBS-injected) gingival tissues in WT mice.

Immunofluorescence analysis of the inflammatory infiltrate

Formalin-fixed, paraffin-embedded tissue sections (9 per animal and experimental group) were deparaffinized in two changes of xylenes for 15 and 5 minutes, and then dehydrated in 100% ethanol for 2 minutes. The slides were then rehydrated through a graded ethanol series (95% and 70%) for 2 minutes each, followed by a wash in dH_20 for 1 minute, and then placed in Trilogy buffer (Cell Marque, Hot Springs, AR) at 96.5 C for 25 minutes. Slides were cooled in a dH_20 bath for 5 minutes, and rinsed in 1xTBS (Tris-Buffered Saline). Sections were permeabilized with 0.1% Triton X-100 (Fisher Scientific) and blocked in 1X PBS, 0.5% PBS-Tween 20 (Fisher Scientific) (PBST) containing 10% normal goat serum (Life Technologies) at room temperature for 30 minutes. Slides were washed with 1xTBS and incubated with primary antibodies diluted in 1xTBT for 24 hours at 4C temperature. Tissues were incubated with the following

antibodies: CD45 at 1:200 dilution (rat IgG, purified anti-mouse CD45, Biolegend), for 'general' leukocyte infiltration, and Ly6G at 1:100 dilution (rat IgG, purified anti-mouse Ly6G, Biolegend), for specific neutrophil (PMN). Tissues were washed three times for 5 minutes each in 1xTBS and incubated with Alexa Fluor 594 goat anti-rat secondary (ThermoFisher) 1:650, for 2 hours, at room temperature. The DNA dye 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain cell nuclei. Images were collected on an EVOS FL digital inverted microscope (ThermoFisher Scientific).

Mean fluorescence intensity data were generated using the image analysis program ImageJ (https://imagej.nih.gov/ij/). Raw images were separated into individual channels for DAPI and CD45 or Ly6G staining, respectively. The mean fluorescence intensity of the gray area was measured for the same region of interest (ROI) for each image using the Analyze tool in ImageJ. The values for general leukocyte infiltration (CD45), and specific PMN infiltration (Ly6G) were normalized to the total number of cells present as stained by DAPI. Three distinct sections per animal were averaged over three animals per experimental group.

Statistical Analysis

The statistical analysis was performed using GraphPad 5.0 (GraphPad Software Inc., San Diego, CA, USA). Central tendency and dispersion measures were calculated from different experiments. Pairwise comparisons between experimental conditions both within each genotype

group (PBS-injected x Aa-injected) and between the genotype groups (e.g., periodontal disease WT x periodontal disease Ipaf-kO) were performed using non-paired Student's t-test, considering each sample as an independent event. Significance level was 95% ($p < 0.05$) for all analysis.

Results

Increased inflammatory bone resorption in Ipaf-KO mice

Interestingly, the volume of mineral tissue in non-diseased, PBS-injected mice was greater in Ipaf-KO mice in comparison with WT animals (Figure 1), which suggests an influence of NLRC4 inflammasome on bone turnover even without overt inflammation. Injections of heat-killed Aa effectively induced inflammatory bone resorption, as the mineralized tissue volume was significantly reduced in both WT and Ipaf-KO mice. Comparison of the ratio MT/TV in Aa-injected mice indicates (Comentario da Shannon aqui: “so, is the change in MT/TV from baseline greater in the lpaf-KO? Since they start with less MT?”) that Ipaf-KO mice had a significantly greater inflammatory bone resorption ($p<0.05$). Together these data suggest a possible protective role for the NLRC4 inflammasome, once its absence caused an increase of inflammatory bone resorption associated with periodontal diseases.

Osteoclastogenesis in the periodontal disease model was not influenced by NLRC4 inflammasome

Since more severe inflammatory bone resorption is observed in Ipaf-KO mice, we wondered if this could be due to a direct effect on osteoclastogenesis in the inflammatory condition. Counting of osteoclasts in the region of interest (ROI) of maxillae tissue sections, indicated that induction of periodontal disease by Aa injections significantly increased osteoclast number ($p<0.05$) in both WT and Ipaf-KO mice. However, we did not find a significant difference on osteoclast numbers between WT and Ipaf-KO animals (Figure 2).

NLRC4 inflammasome and cytokine gene expression

Even though the biological role of inflammasomes in primarily on final post-translational processing of cytokines, considering the differences observed on inflammatory bone resorption and the possibility of a 'cascade-like effect' in which variations on the abundance of inflammasome-processed cytokines may affect the immune response in host-microbial interactions, we compared the expression of candidate inflammatory genes, based on their relevance for the pathogenesis of periodontal disease and also for the destruction of both mineralized and non-mineralized connective tissues. Induction of experimental periodontal disease resulted in significant increases of RANKL, MMP-13 and IL-6 gene expression in both WT and Ipaf-KO mice (Figure 3), but without significant differences between WT and Ipaf-KO mice. Interestingly, expression of both TNF-alpha and IL-10 was

significantly increased in tissues subjected to injections of Aa only in WT mice.

Ipaf-KO mice presented significantly higher levels of TNF-alpha and IL-10 gene expression in PBS-injected / non-disease control tissues in comparison with PBS-injected/non-disease control tissues of WT mice, and in contrast with the results from Wt mice, injections of Aa in Ipaf-KO mice did not further upregulated expression of TNF-alpha and IL10 (Figure 3).

NLRC4 does not affect the inflammatory infiltrate in the periodontal disease model

Since variations in the intensity of inflammatory infiltration or on the relative presence of different immune cells may be related with the differences in the severity of inflammatory bone resorption and also with the changes in inflammatory gene expression, we assessed the intensity of the inflammatory infiltrate and specifically the presence of acute-phase neutrophils (PMNs) by immunofluorescence using CD45 as a general marker for inflammatory cells/leukocytes and and Ly6G, as a specific marker for neutrophils.

Induction of experimental periodontal disease by injections of heat-killed Aa significantly increased the inflammatory infiltrate, as showed in the H/E representative images (Figure 4a). Increased number of CD45-positive cells in the gingival tissues in both Wt and Ipaf-KO mice, and no differences on

the intensity of the inflammatory infiltrate were observed between the different genotypes (Figure 4b).

Similarly, immunopositivity for Ly6G was also significantly increased in the gingival tissues of Aa-injected mice, and no differences were observed on the intensity of PMN infiltration between the genotypes (Figure 4b).

Discussion

Inflammasomes have been a keen topic of interest in chronic inflammatory conditions involving host-microbial interactions and autoimmune conditions, including infections caused by Lysteria, Legionella, Shigella, Mycobacterium; as well as in 'sterile' inflammatory conditions, such as diabetes, cancer, gout and rheumatoid arthritis [25].

Thus, there is plenty of novel information published recently that adds to the body of knowledge about the activation and functionality of inflammasomes in various conditions [26]. As an example, a number of other activating molecules for NLRC4 inflammasome have been added to those initially described microbial-derived molecules (needle/rod components of type III secreting system bacteria, and bacterial flagella), including host-derived danger-associated molecular patterns (DAMPs), as intracellular proteins, ATP and DNA, for example, and an increasing number of microbial species and microbial-derived virulence factors [25]. These new information further supports the rationale and fosters the relevance of our study, which is focused on the role of the one of most studied inflammasomes, NLRC4, in

periodontal disease, a bacterial-initiated and maintained chronic inflammatory condition.

It is now considered that activation of some inflammasomes, such as NLRP3, requires two signals: the first signal is the exogenous microbial-derived PAMP, which triggers the production of cytokine precursors (e.g., pro-IL1); and a second signal, represented by the interaction of DAMPs with their PRRs (e.g., RAGE). There is accumulating evidence indicating the relevance of this second signal of activation, particularly of extracellular ATP in the regulation of NLRP3 inflammasome through purinergic receptors (P2X), which are important mediators of apoptosis and initiators of inflammation [27].

In the case of NLRC4 this requires the recognition of its microbial ligands by NLR family of Apoptosis-Inducing Proteins (NAIPs) that function as cytosolic PRRs. Once bound to the bacterial ligand, these NAIPs (three have been described in mice, only one in humans) then recruit and co-assemble with the central protein leading to the assembly and activation of this inflammasome. However, less is known about alternative ligands/activators of NLRC4. The bacterial enzyme E3 ubiquitin ligase effector protein, which is introduced in the host eukaryotic cell via type III secreting system of *Shigella flexneri* has been recently recognized as an indirect activator of NLRC4 inflammasome [28]. The membrane attack complex formed at the final step of the activation of the complement system may also induce activation of the inflammasomes indirectly, by inducing

cellular changes associated with lysis and changes in intracellular Calcium concentrations [29].

Regardless of specific ligands/activators for NLRC4, as a chronic inflammatory condition, periodontal disease is characterized by high levels of reactive oxygen species (ROS) and also by increased tissue degradation, with accumulating DAMPs in its microenvironment. Both ROS and DAMPs (which may also generate ROS) can activate multiple inflammasomes besides NLRC4, including NLRP3, AIM2, NLRP1 and NLRP6 [25]. Thus, inflammasome activation may derive from direct recognition/interaction of bacterial ligands by the inflammasome central/sensor proteins or by the detection of cell changes induced by external microbial/stress stimuli [30].

Interestingly, we observed a higher volume of mineralized tissue in the non-diseased control Ipaf-KO animals, which suggests that Ipaf/NLRC4 inflammasome has a role in regulating bone resorption during the physiological bone turnover. However, in the presence of bacterial-induced inflammation, the lack of NLRC4 inflammasome was associated with a significantly greater bone resorption; which again suggests a protective role for NLRC4 on inflammation-induced bone resorption. It is important to bear in mind that the Ipaf-KO mice retained expression of protein components of other inflammasomes, and these results in vivo may be a function of the shift in the relative activation of these different inflammasomes in the absence of NLRC4. McCall et al 2008 did not observe NLRC4 expression in osteoblasts of mice, whereas these cells expressed NLRP3 [31], suggesting

that the increased alveolar bone volume observed in non-diseased Ipaf-KO mice may not be related with a direct influence of NLRC4 inflammasome on formative functions of osteoblasts, but could be related with indirect influences on the production of soluble biologically-active mediators by other cell types. In fact, there is evidence that the NLRC4 inflammasome has unique and distinct effector functions in different cell types. For example, activation of NLRC4 in 'resident' peritoneal macrophages from mice (but not in macrophages derived from bone marrow) results in increased production of pro-inflammatory lipids (eicosanoids) [32]. Similarly, activation of NLRC4 may lead to cell death by pyroptosis in epithelial cells, but not in neutrophils [33].

Interestingly, the significantly greater severity of inflammatory bone resorption in the experimental periodontal disease model was not accompanied by an increase in osteoclastogenesis/osteoclast numbers or by a significant increase on the inflammatory infiltrate. We speculate that this greater inflammatory bone resorption may be associated with increased activity of osteoclasts derived from NLRC4-deficient precursors and in vitro experiments are currently under way to assess this hypothesis. The increased basal expression of TNF-alpha and IL-10 in gingival tissues of Ipaf-KO mice is also suggestive of a functional role for NLRC4 inflammasome, since we did not observe significant differences on the intensity of the overall inflammatory infiltrate and also on the infiltration of gingival tissues by neutrophils. Interestingly, higher levels of TNF-alpha

protein were previously reported in Ipaf-KO mice. In fact, TNF-alpha can affect osteoclastogenesis through direct interference on induction of RANKL [34, 35].

In our study we have used a whole-genome knockout model, causing a 'global' deficiency of NLRC4 gene, and this precludes the interpretation of the specific role of NLRC4 in specific cell types in an in vivo model. Although we did not find any literature evidence of alternative or compensatory compensatory activation of distinct inflammasomes, we cannot rule out the possibility of a shift towards increased activation of other inflammasomes (e.g., NLRP3) in our experimental model. It is also important to consider the limitations of our model, in which we use heat killed Gram-negative bacterial species associated with periodontal disease in humans directly injected into the tissues, by-passing the need for colonization and interactions with the outer epithelial layer of the oral mucosa/gingiva. In spite of these limitations, this model effectively induces inflammation and the associated resorption of alveolar bone, mimicking the two major characteristics of periodontal disease in humans; and has the added advantage of allowing for a greater control of the intensity and frequency of the disease-inducing stimulus.Considering the absolute lack of information on the role of NLRC4 in the context of periodontal disease and the scarcity of information related with the influence of NLRC4 on bone turnover, this study provides novel information indicative that NLRC4 inflammasome has a protective role in inflammatory bone resorption.

Moreover, based on the lack of influence on osteoclastogenesis, on the severity of inflammatory infiltrate and on the increased basal expression of TNF-alpha and IL-10, we speculate that the NLRC4 inflammasome has a functional effect on osteoclasts and immune cells. The insights provided by this study will be further explored in in vitro experiments that are already under way to improve the understanding of NLRC4 inflammasome's role in RANKL-induced osteoclastogenesis and osteoclast activity.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Figure Legends:

Figure 1 – (a) Representative three-dimensional reconstructed images of upper hemi-maxillae of the experimental mice, according to the experimental groups. (b) Bone resorption was evaluated by the ratio of mineralized tissue ('MT') and the total volume ('TV') of the standardized ROI in 6 different animals from each group/genotype (*$p<0.05$; ANOVA followed by Tukey multiple comparison's test performed for each experimental condition). * indicates comparison to WT genotype, same experimental condition (non-disease/PBS-injected or disease/Aa-injected), and # indicates comparison between diseased (Aa-injected) with non-disease control (PBS-injected) in a given genotype

Figure 2 – (a) Representative images of biochemical staining of TRAP in the tissue sections from both non-disease control (PBS-injected) and diseased (Aa-injected) mice, according to the different genotypes (WT and Ipaf-KO). Red shade is indicative of TRAP-positive cells in the vicinity of the alveolar bone. 40X magnification. (b) Quantification of osteoclasts in a standardized region of interest, considering TRAP-positive cells in the vicinity of alveolar bone, presenting two or more visible nuclei. NUmber of ostaoclasts was averaged for each experimental condition (non-disease control / PBS-injected and diseased/Aa-injected) and genotype (WT or Ipaf-KO). (*$p<0.05$; ANOVA followed by Tukey multiple comparison's test performed for each experimental condition: 'PBS/control' or 'Aa/diseased').

# indicates comparison between diseased (Aa-injected) with non-disease control (PBS-injected) in a given genotype.

Figure 3 – Expression of candidate genes associated with the pathogenesis of periodontal disease and mineralized / non-mineralized tissue destruction in host-microbial inflammation. Total RNA was extracted from gingival tissues of non-diseased control (PBS-injected) and diseased (Aa-injected) from Wt and Ipaf-KO mice (n=6 for each experimental condition/genotype) and used to synthesize cDNA. Gene expression was assessed by qPCR using TaqMan chemistry and pre-designed / optmized sets of primers and probe and the results analyzed by the $\Delta(\Delta Ct)$ method, using the expression of GAPDH as the unchanged constitutive/housekeeping normalizing gene. Bars represent average fold change and standard deviation of normalized target gene expression in comparison to the non-diseased (PBS-injected) control group of the WT group. (*$p<0.05$; ANOVA and Tukey tests for pairwise comparisons). * indicates comparison to WT genotype, same experimental condition (non-disease control/PBS-injected or diseased/Aa-injected), and # indicates comparison to the non-disease (PBS-injected) control in the WT group.

Figure 4 – (a) Representative images of H/E-stained sections of each experimental group (non-disease control/PBS-injected or diseased/Aa-injected) according to the genotype (Wt or Ipaf-KO) at 20X magnification. (b) Representative images of immunofluorescence detection of the pan-leukocyte marker CD45 and the neutrophil marker Ly6G in the gingival

tissues of WT and Ipaf-KO mice, according to the experimental condition (non-disease control/PBS-injected or diseased/Aa-injected). (c) Results for the quantitation of mean fluorescence intensity (MFI) of CD45 or Ly6G according to the experimental condition and genotype. These values represent averages from MFI values from three animals per group and experimental condition, using semi-serial sections spanning 200 µm in the antero-posterior or mesio-distal direction. Nuclei were counterstained with Draq5, scale bar 100µm.

References

1. Pacios S, Kang J, Galicia J, Gluck K, Patel H, Ovaydi-Mandel A, Petrov S, Alawi F, Graves DT (2011) Diabetes aggravates periodontitis by limiting repair through enhanced inflammation. FASEB J Apr;26(4):1423-30. doi: 10.1096/fj.11-196279
2. Teng YT, Nguyen H, Hassanloo A, Ellen RP, Hozumi N, Gorczynski RM (1999) Periodontal immune responses of human lymphocytes in Actinobacillus actinomycetemcomitans-inoculated NOD/SCID mice engrafted with peripheral blood leukocytes of periodontitis patients. J Periodontal Res Jan;34(1):54-61
3. Eastcott JW, Yamashita K, Taubman MA, Harada Y, Smith DJ (1994) Adoptive transfer of cloned T helper cells ameliorates periodontal disease in nude rats. Oral Microbiol Immunol Oct;9(5):284-9
4. Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY, Goncalves RB, Valverde P, Dibart S, Li YP, Miranda LA, Ernst CW, Izumi Y, Taubman MA (2006) B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. Am J Pathol Sep;169(3):987-98
5. Pedra JH, Cassel SL, Sutterwala FS (2009) Sensing pathogens and danger signals by the inflammasome. Curr Opin Immunol Feb;21(1):10-6. doi: 10.1016/j.coi.2009.01.006
6. Jin Y, Mailloux CM, Gowan K, Riccardi SL, LaBerge G, Bennett DC, Fain PR, Spritz RA (2007) NALP1 in vitiligo-associated multiple autoimmune disease. N Engl J Med Mar 22;356(12):1216-25
7. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet Nov;29(3):301-5

8. Valk K, Vooder T, Kolde R, et al. Gene expression profiles of non-small cell lung cancer: survival prediction and new biomarkers. Oncology 79: 283-292
9. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA Jr, O'Connor JV (1986) Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med Jun 1;163(6):1433-50
10. Franchi L, Eigenbrod T, Núñez G (2009) Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. J Immunol Jul 15;183(2):792-6. doi: 10.4049/jimmunol.0900173
11. Barker BR, Taxman DJ, Ting JP (2011) Cross-regulation between the IL-1β/IL-18 processing inflammasome and other inflammatory cytokines. Curr Opin Immunol Oct;23(5):591-7. doi: 10.1016/j.coi.2011.07.005
12. Taxman DJ, Zhang J, Champagne C, Bergstralh DT, Iocca HA, Lich JD, Ting JP (2006) Cutting edge: ASC mediates the induction of multiple cytokines by Porphyromonas gingivalis via caspase-1-dependent and -independent pathways. J Immunol Oct 1;177(7):4252-6
13. Masumoto J, Dowds TA, Schaner P, Chen FF, Ogura Y, Li M, Zhu L, Katsuyama T, Sagara J, Taniguchi S, Gumucio DL, Núñez G, Inohara N (2003) ASC is an activating adaptor for NF-kappa B and caspase-8-dependent apoptosis. Biochem Biophys Res Commun Mar 28;303(1):69-73
14. Hasegawa M, Imamura R, Kinoshita T, Matsumoto N, Masumoto J, Inohara N, Suda T (2005) ASC-mediated NF-kappaB activation leading to interleukin-8 production requires caspase-8 and is inhibited by CLARP J Biol Chem Apr 15;280(15):15122-30
15. Hasegawa M, Imamura R, Motani K, Nishiuchi T, Matsumoto N, Kinoshita T, Suda T (2009) Mechanism and repertoire of ASC-

mediated gene expression. J Immunol Jun 15;182(12):7655-62. doi: 10.4049/jimmunol.0800448

16. Anderson JP, Mueller JL, Misaghi A, Anderson S, Sivagnanam M, Kolodner RD, Hoffman HM (2008) Initial description of the human NLRP3 promoter. Genes Immun Dec;9(8):721-6. doi: 10.1038/gene.2008.66
17. Deal C (2012) Bone loss in rheumatoid arthritis: systemic, periarticular, and focal. Curr Rheumatol Rep Jun;14(3):231-7. doi: 10.1007/s11926-012-0253-7
18. Zhao B, Grimes SN, Li S, Hu X, Ivashkiv LB (2012) TNF-induced osteoclastogenesis and inflammatory bone resorption are inhibited by transcription factor RBP-J. J Exp Med Feb 13;209(2):319-34. doi: 10.1084/jem.20111566
19. Lorenzo JA, Sousa SL, Alander C, Raisz LG, Dinarello CA (1987) Comparison of the bone-resorbing activity in the supernatants from phytohemagglutinin-stimulated human peripheral blood mononuclear cells with that of cytokines through the use of an antiserum to interleukin 1. Endocrinology Sep;121(3):1164-70
20. Sabatini M, Boyce B, Aufdemorte T, Bonewald L, Mundy GR (1988) Infusions of recombinant human interleukins 1 alpha and 1 beta cause hypercalcemia in normal mice. Proc Natl Acad Sci U S A Jul;85(14):5235-9
21. Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, Suda T (1999) Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. Exp Cell Res Feb 25;247(1):84-93
22. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S (1999) Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone Sep;25(3):255-9

23. Baker PJ, Evans RT, Roopenian DC (1994) Oral infection with Porphyromonas gingivalis and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. Arch Oral Biol Dec;39(12):1035-40
24. Teng YT, Nguyen H, Gao X, Kong YY, Gorczynski RM, Singh B, Ellen RP, Penninger JM (2000) Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. J Clin Invest Sep;106(6):R59-67
25. Yilmaz Ö, Lee KL (2015) The inflammasome and danger molecule signaling: at the crossroads of inflammation and pathogen persistence in the oral cavity. Periodontol 2000 Oct;69(1):83-95. doi: 10.1111/prd.12084
26. Vanaja SK, Rathinam VA, Fitzgerald KA (2015) Mechanisms of inflammasome activation: recent advances and novel insights. Trends Cell Biol May;25(5):308-15. doi: 10.1016/j.tcb.2014.12.009
27. Gombault A, Baron L, Couillin I (2013) ATP release and purinergic signaling in NLRP3 inflammasome activation. Front Immunol Jan 8;3:414. doi: 10.3389/fimmu.2012.00414
28. Suzuki S, Mimuro H, Kim M, Ogawa M, Ashida H, Toyotome T, Franchi L, Suzuki M, Sanada T, Suzuki T, Tsutsui H, Núñez G, Sasakawa C (2014) Shigella IpaH7.8 E3 ubiquitin ligase targets glomulin and activates inflammasomes to demolish macrophages. Proc Natl Acad Sci U S A Oct 7;111(40):E4254-63. doi: 10.1073/pnas.1324021111
29. Triantafilou M, Hughes TR, Morgan BP, Triantafilou K (2016) Complementing the inflammasome. Immunology Feb;147(2):152-64. doi: 10.1111/imm.12556
30. Greaney AJ, Leppla SH, Moayeri M (2015) Bacterial Exotoxins and the Inflammasome. Front Immunol Nov 10;6:570. doi: 10.3389/fimmu.2015.00570

31. McCall SH, Sahraei M, Young AB, Worley CS, Duncan JA, Ting JP, Marriott I (2008) Osteoblasts express NLRP3, a nucleotide-binding domain and leucine-rich repeat region containing receptor implicated in bacterially induced cell death. J Bone Miner Res Jan;23(1):30-40
32. von Moltke J, Trinidad NJ, Moayeri M, Kintzer AF, Wang SB, van Rooijen N, Brown CR, Krantz BA, Leppla SH, Gronert K, Vance RE (2012) Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature Oct 4;490(7418):107-11. doi: 10.1038/nature11351
33. Vance RE (2015) The NAIP/NLRC4 inflammasomes. Curr Opin Immunol Feb;32:84-9. doi: 10.1016/j.coi.2015.01.010. 33
34. DeSantis DA, Ko CW, Liu Y, Liu X, Hise AG, Nunez G, Croniger CM (2013)
Alcoholinduced liver injury is modulated by Nlrp3 and Nlrc4 inflamma somes in mice Mediators Inflamm doi: 10.1155/2013/751374
35. Graves DT, Oates T, Garlet GP (2011) Review of osteoimmunology and
the host response in endodontic and periodontal lesions. J Oral Microbiol Jan 17;3. doi: 10.3402/jom.v3i0.5304

Figure 1

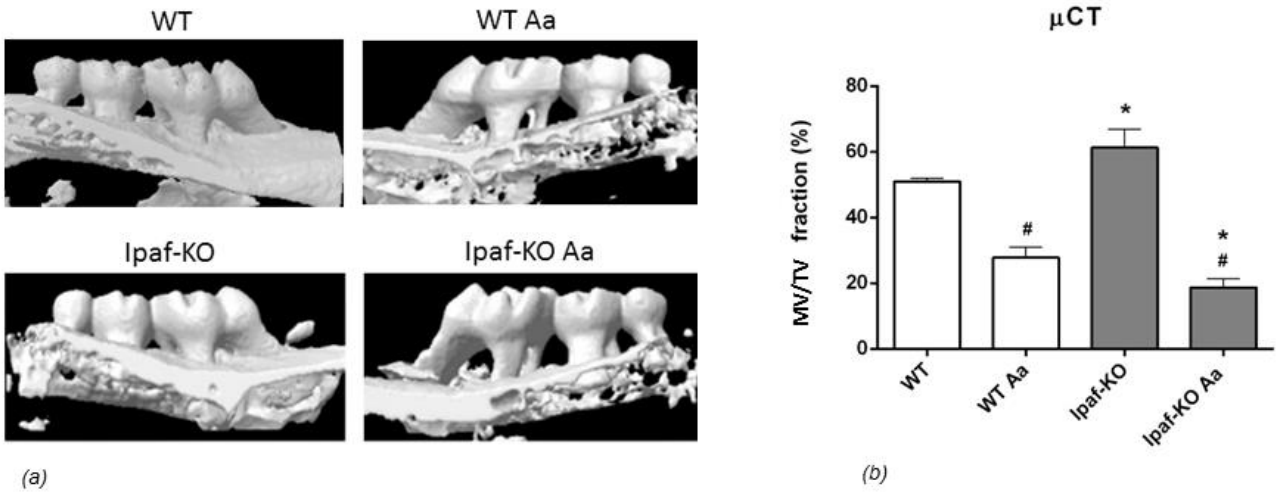


Figure 2

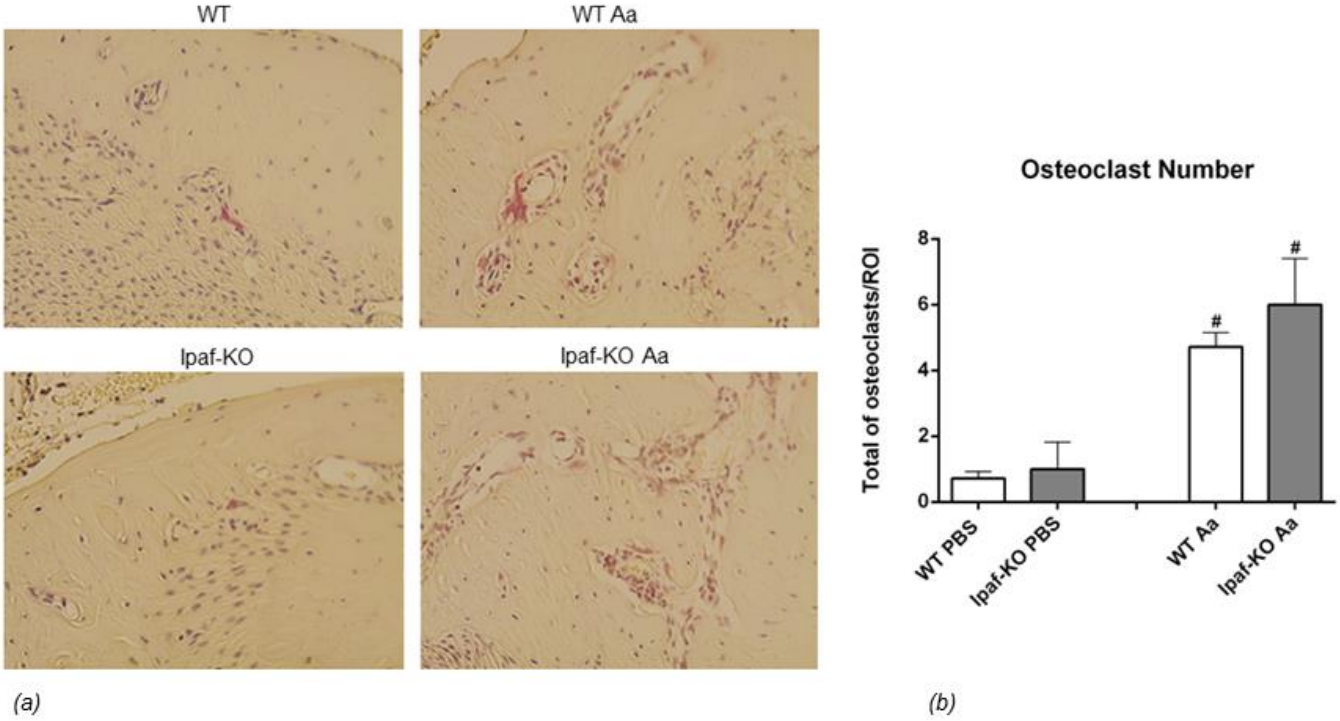


Figure 3

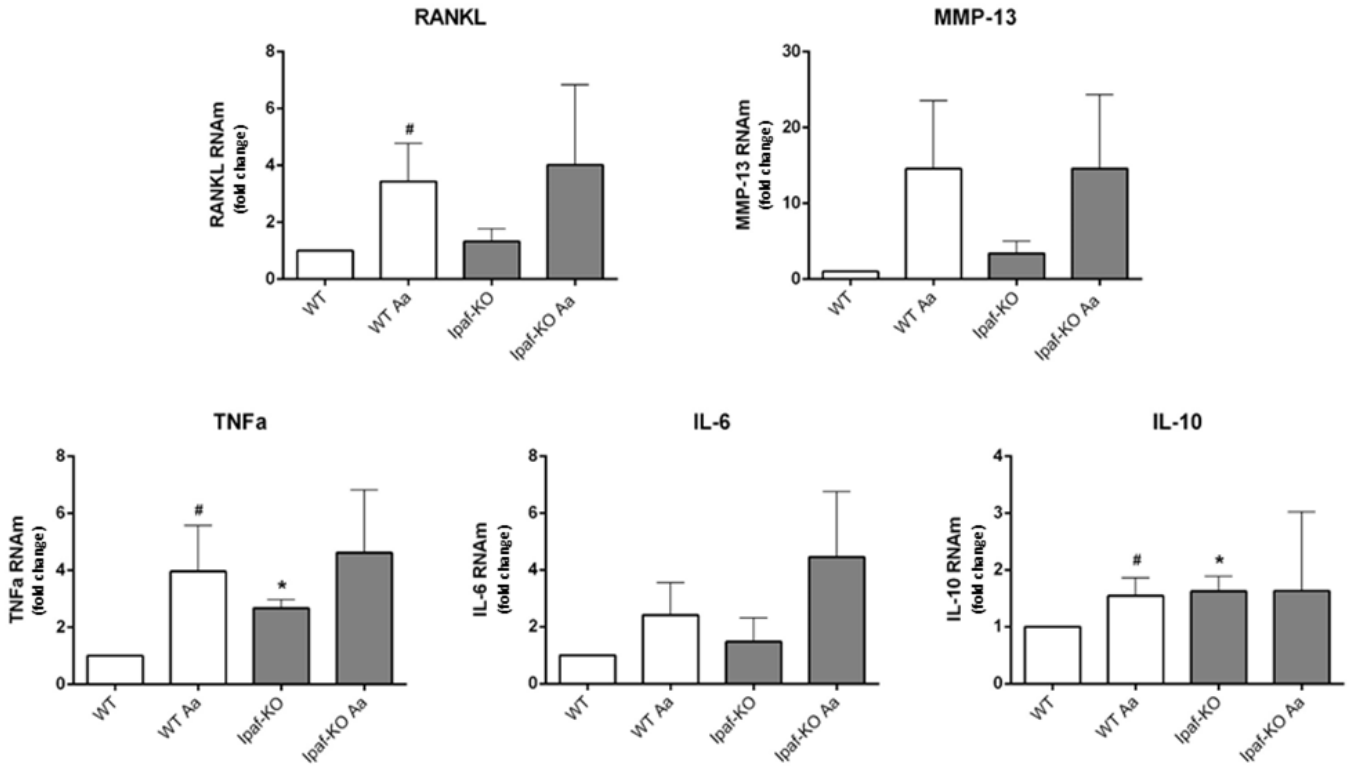
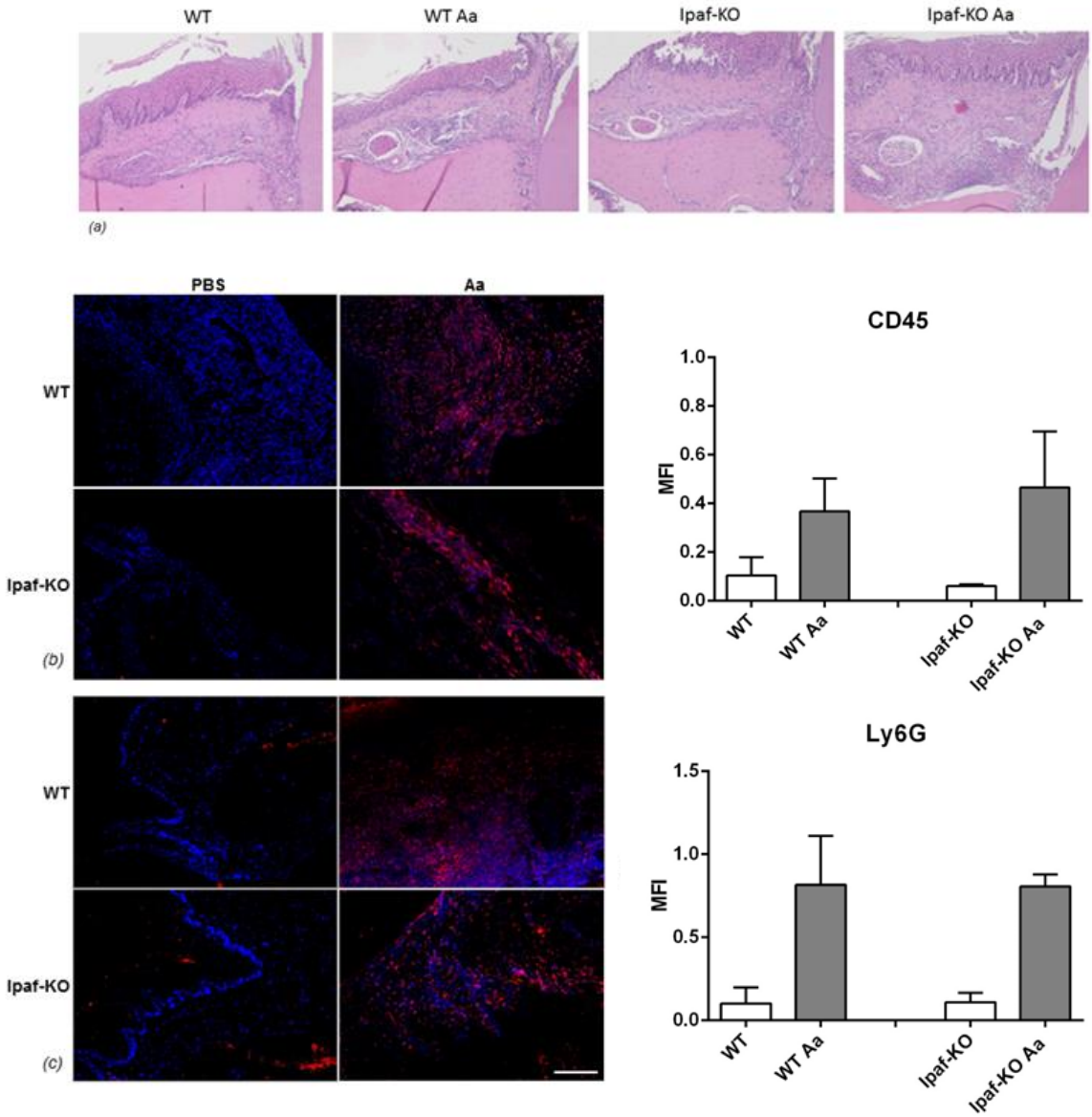

RANKL
RANKL RNAm (fold change)
MMP-13
MMP-13 RNAm (fold change)
TNFa
TNFa RNAm (fold change)
IL-6
IL-6 RNAm (fold change)
IL-10
IL-10 RNAm (fold change)
WT
WT Aa
Ipaf-KO
Ipaf-KO Aa

Figure 4


WT
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Ipaf-KO
Ipaf-KO Aa
(a)
PBS
Aa
WT
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(b)
WT
Ipaf-KO
(c)
CD45
MFI
1.0
0.8
0.6
0.4
0.2
0.0
WT
WT Aa
Ipaf-KO
Ipaf-KO Aa
Ly6G
MFI
1.5
1.0
0.5
0.0
WT
WT Aa
Ipaf-KO
Ipaf-KO Aa

5 PUBLICAÇÃO 2

LACK OF CASPASE-1, BUT NOT OF NLRP3 INFLAMMASOME, ATTENUATES INFLAMMATORY BONE RESORPTION IN EXPERIMENTAL PERIODONTAL DISEASE *

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Abstract

Background: NLRP3 inflammasome can be activated by a variety of stimuli, including microbial- (MAMPs) and host-derived (DAMPs). Periodontal disease is a chronic inflammatory condition initiated and maintained by a complex biofilm of bacteria, which presents increased levels of inflammasome-processed cytokines IL-1β and IL-18. We investigated the role of NLRP3 inflammasome and of Caspase 1 in inflammation and bone resorption associated with periodontal disease.

Methods: 3uL of $1x10^9$ UFC/mL of heat-killed *Aggregatibacter actinomycetemcomitans* (Aa) were injected in the gingival tissues on the palatal aspect of both first molars of Wild Type (WT), NLRP3-KO and Caspase1-KO mice 3x/week for 4 weeks. Control animals were injected with the same volume of the vehicle (PBS). Bone resorption was determined by μCT, osteoclast number was assessed by biochemical staining of TRAP. Inflammation was assessed in H/E-stained slides and also by immunofluorescence detection of leukocytes (CD45+ cells) and PMNs (Ly6G+ cells) in the gingival tissues. Expression of RANKL, MMP-13, TNF-a, IL-6 and IL-10 genes in the gingival tissues was studied by RT-qPCR.

Results: The severity of bone resorption was not affected by the lack of NLRP3 inflammasome, but was significantly reduced in Casp1-KO mice. Interestingly, the attenuation of alveolar bone resorption in Casp1-KO mice was accompanied by a discrete and significant increase on the number of

osteoclasts, whereas there were no significant changes on the inflammatory infiltrate or on expression of candidate genes.

Conclusion: We conclude that NLPR3 inflammasome does not play a significant role in inflammation and bone resorption and that caspase-1 has pro-resorptive role in experimental periodontal disease.

Keywords: Inflammation, Periodontal disease, Caspase-1, NLRP3 Inflammasome, Innate Immunity, Aggregatibacter actinomycetemcomitans

Introduction

Inflammasomes are cytosolic multiprotein complexes activated in response to microbial-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPS). These complexes include proteins structurally similar to Nod receptors (NLR, Nod-like receptors), an adapter protein associated with apoptosis containing CARD domain (ASC, apoptosis-associated speck-like protein containing a CARD) and caspase-1 as their main effector protein [1]. The relevance of inflammasomes to the immune response is demonstrated by the association between mutations in the genes encoding their protein components and autoimmune inflammatory conditions, or dysregulation of the immune response [2-3].

NLRP3 is one of the most studied and better characterized inflammasomes, and has been associated with various diseases and conditions characterized by chronic inflammation, including gout, cancer, type 2 diabetes and rheumatoid arthritis, besides periodontal diseases [4-6].

Periodontal disease is a chronic inflammatory condition, initiated and maintained by microbial insult and is the most prevalent lytic lesion of bone in humans [7-9]. Thus, this condition represents an excellent model to study the role of inflammasomes, due to the abundance of MAMPs and DAMPs and the elevated proportion of macrophages in the tissue microenvironment. However, the overwhelming diversity of the dental biofilm may dissuade some researchers from considering periodontal disease as a study model, particularly if the main purpose is to understand and describe activating ligands and the mechanism of inflammasome activation. Importantly, the study of inflammasomes in the context of periodontal disease provides unique opportunity to assess their functional role on microbial-triggered inflammatory bone resorption, the main hallmark of destructive periodontal diseases. Interestingly, there is a relative scarcity of information on the biological roles of inflammasomes derived from clinical or pre-clinical studies in periodontal disease. Increased expression of IL-1ß and IL-18 in gingival tissues and gingival crevicular fluid of patients with various forms of periodontal disease [10-12] correlates positively with increased expression of NLRP3 mRNA in this microenvironment [13], suggesting the participation of this inflammasomes in periodontal disease pathogenesis.

Stimulation of primary gingival epithelial cells with *Porphyromonas gingivalis* (*P.g.*), resulted in downregulation of NLRP3 and increase of pro-interleukin-1beta expression, suggesting an inhibition of inflammasome function, which

is consistent with other immunosuppressive properties associated with this particular bacterial species.

The effector protein of all inflammasomes is the protease caspase-1, which cleaves the proform of inflammatory cytokines IL-1β, IL-18 and IL-33 to their mature and secreted forms. Caspase-1 can also induce cell death by pyroptosis [14]. Osteoblasts express inflammasome components, including the core protein of NLRP3 inflamasome, NALP3 [15]. Activation of caspase-1 as the effector protein of inflammasomes leads to cell death by pyroptosis of osteoprogenitor cells [16], which would affect bone turnover. Cytokines processed by the effector caspase-1, particularly IL-1β, may modulate the osteoclastogenesis by direct effects on osteoclasts [17] or by indirectly modulating the expression of RANKL (ligand for receptor activator of nuclear factor-kappa-B) in other cell types [18].

A better understanding of biological functions and mechanisms involved in inflammasome activation may lead to new strategies for therapeutic intervention aimed at modulating pathways involved in the pathogenesis of various conditions associated host-microbial interactions and chronic inflammation. There are no studies assessing the role of NLRP3 inflammasome or/and Caspase-1 in in vivo models of periodontal disease. Thus, considering the relevance of periodontal diseases as a highly prevalent condition associated with the destruction of mineralized and non-mineralized tissues, we assessed the role NLRP3 inflammasome and of caspase-1 as the common downstream effector of most inflammasomes o

inflammation and alveolar bone resorption in a murine model of bacterial-induced experimental periodontal disease.

Materials and Methods

Periodontal disease model

A total of 36 C57BL/6 male adult mice (age between 6 and 8 weeks) were used, including 12 wild-type (WT) mice, 5 mice genetically deficient (knockout) for NALP3 (NRLP3-KO), the central NLR protein in the NLRP3 inflammasome, and 12 mice genetically deficient for Caspase-1 (Casp1-KO). All mice were obtained from the Center for genetically modified and transgenic mice, School of Medicine at Ribeirao Preto-University of Sao Paulo (USP). Experimental periodontal disease was induced in 18 mice, including 6 animals of each genotype (WT, NLRP3-KO and Casp1-KO) by direct bilateral injections of a 3 µL PBS suspension heat-killed *Aggregatibacter actinomycetemcomitans (Aa, JP1 serotype)* at $1x10^{9}$ UFC/mL. 18 non-disease control mice (n=6 for each genotype) received bilateral injections of the same volume of the PBS vehicle. These injections were performed under mild general anesthesia with isofluorane (Baxter Healthcare, Deerfield, IL) using a Hamilton-type micro syringe (33 gauge needle) three times/week for 4 weeks, directly on the gingiva at the palatal aspect of the upper molars, between the first and second molars. 28 days after the beginning of injections, all animals were sacrificed by cervical dislocation.

The maxillary bones were hemisected, and one of the hemimaxillae had the gingival tissue from the palatal aspect of the first molars carefully dissected for extraction of total RNA for RT-qPCR. The remaining sample including the maxillary bone and teeth was used for microcomputed tomographic analysis of alveolar bone resorption. The other half of the blocks, including the molars and intact surrounding soft tissues were submitted to routine histological processing for the histological analysis.

This study was carried out in accordance with the principles stated by the Brazilian College of Animal Experimentation and was approved by the Ethical Committee on Animal Experimentation (protocol number 10/2010) of the School of Dentistry at Araraquara,UNESP-SP, Brazil.

Microcomputed tomography analysis (µCT scanning)

The hemimaxillae from which the soft gingival tissue was dissected were fixed in 4% buffered formalin for 24h and transferred to 70% alcohol until scanning using 56 kV, 300 mA and a 0.5 mm aluminum attenuation filter, with the resolution of the slices set to 18 µm using a uCT system (Skyscan, Aastselaar, Belgium). Tridimensional images were reconstructed and the resulting images were re-oriented spacially in a standardized manner using anatomical landmarks with the NRecon and DataViewer softwares (Skyscan, Aastselaar, Belgium). A standardized 5.4 mm^3 region of interest (ROI) was set with 1.5x4.0x0.9 mm (vertical or cervico-apical x horizontal or mesio-distal x lateral or buccal-palatal) was defined using the following references: cervical/coronal reference was the roof of the furcation area

between mesial and distal roots of the upper first molar; mesially we used the distal aspect of the mesial root of the first molar. The thickness of the ROI was set to 50 slices (900 µm) counted towards the palatal /medial direction on the sagital plane beginning from the image that included the center of the upper first molar in its buccal-palatal width. For the analysis, a standardized threshold was set to distinguish between non-mineralized and mineralized tissues. Considering that variations on the size and mineralization of the tooth structures included in the ROI were irrelevant among the different animals, the analysis assessed the percentage of mineralized tissue (MT) within the total volume (TV) of the ROI, presented as the ratio MT/TV analysis. A decrease on this ratio is interpreted as indicative of bone resorption.

Histological analysis

Hemimaxillae samples that included the surrounding soft tissues were fixed in 4% buffered formalin for 48h, decalcified in EDTA (10%, 0.5M) for 45 days at room temperature, and embedded in paraffin. Semi-serial sections of 4µm thickness were obtained in the buccal-lingual (frontal plane) direction and stained with hematoxylin and eosin (H/E).

Immunohistochemical staining of TRAP

Nine sections from each paraffin-embedded hemimaxillae spanning 1000 µm on the antero-posterior direction (sagittal plane, n=6 animals/experimental condition and genotype) were used for the biochemical detection of tartarate resistant acid phosphatase (TRAP), using

a substrate-based kit (Acid Phosphatase, Leukocyte (TRAP) kit, Sigma-Aldrich Co.) Briefly, the sections were sequentially incubated in acetate buffer (0.05 mol/L, pH 5.0), Napthol AS-MX phosphate (0.27 mmol/L), 1% N,Ndimethylformamide, Fast Red stain (1.6 mmol/L) and Sodium tartarate (50 mmol/L) at 37 C for 15 minutes. Counterstaining was done with hematoxylin (Sigma-Aldrich Co.) for 90 seconds, followed by a 2 min wash in running water. Osteoclasts were identified as TRAP-positive large cells, containing two or more nuclei located in the vicinity of the alveolar bone. A single trained examiner who was blind to the coding identifying the experimental groups and genotypes counted the osteoclasts located from the apical portion of the palatal root of the first molar towards the center of the palate, adjacent to the depression on the palatal bone associated with the major palatine artery and nerve.

Immunofluorescence analysis of the inflammatory infiltrate

Distinct formalin-fixed, paraffin-embedded tissue sections of 4 µm thickness (9 sections per animal and experimental group) were deparaffinized in two changes of xylenes for 15 and 5 minutes, and then dehydrated in 100% ethanol for 2 minutes. The slides were then rehydrated through a graded ethanol series (95% and 70%) for 2 minutes each, followed by a wash in dH_20 for 1 minute, and then placed in Trilogy buffer (Cell Marque, Hot Springs, AR) at 96.5 C for 25 minutes. Slides were cooled in a dH_20 bath for 5 minutes, and rinsed in 1xTBS (Tris-Buffered Saline). Sections were permeabilized with 0.1% Triton X-100 (ThermoFisher Scientific) and

blocked in 1X PBS, 0.5% PBS-Tween 20 (Fisher Scientific) (PBST) containing 10% normal goat serum (Life Technologies) at room temperature for 30 minutes. Slides were washed with 1xTBS and incubated with primary antibodies diluted in 1xTBT for 24 hours at 4C. Tissues were incubated with the following antibodies: CD45 at 1:200 dilution (rat IgG, purified anti-mouse CD45, Biolegend), for 'general' leukocyte infiltration, and Ly6G at 1:100 dilution (rat IgG, purified anti-mouse Ly6G, Biolegend), for specific neutrophil (PMN) staining. Tissues were washed three times for 5 minutes each in 1xTBS and incubated with goat anti-rat secondary antibody conjugated with AlexaFluor 594 (ThermoFisher Scientific) at 1:650 and 1:200 dilutions, respectively, for 2 hours, at room temperature. The DNA dye 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain cell nuclei. Images were obtained on an EVOS FL digital inverted microscope (ThermoFisher Scientific).

Mean fluorescence intensity data were generated using the image analysis program ImageJ (https://imagej.nih.gov/ij/). Raw images were separated into individual channels for DAPI and CD45 or Ly6G staining, respectively. The mean fluorescence intensity of the gray area was measured for the same region of interest (ROI) for each image using the Analyze tool in ImageJ. The values for general leukocyte infiltration (CD45), and specific PMN infiltration (Ly6G) were normalized to the total number of cells present as stained by DAPI. Three distinct sections per animal were averaged over three animals per experimental group.

Quantitative Reverse-Transcription Real-Time PCR

Total RNA was extracted from tissue samples using an affinity column system (RNAqueous-4PCR, Ambion Inc) according to the manufacturer's protocol. The quantity and purity of total RNA were determined by UV spectrophotometry and by the 260/280 nm ratio, respectively. 500 ng of total RNA was converted into cDNA with random hexamer primers and moloney leukemia virus reverse transcriptase in a reaction volume of 20 µL (High Capacity cDNA Synthesis kit, Applied Biosystems). The qPCR reactions were performed in a 20µL total volume reaction, including TaqMan qPCR mastermix (TaqMan Fast Advanced, Applied Biosystems), cDNA template, deionized water, and mouse-specific pre-designed and optimized sets of primers and probe (TaqMan gene expression assays). Cycling conditions were pre-optimized by the supplier of the sets of primers/probe and master mix, and 40 cycles were run on a StepOne Plus qPCR thermocycler (Applied Biosystems). Determination of the relative levels of gene expression by the $\Delta(\Delta Ct)$ method was performed using the thermocycler's software using automated detection of the Ct. Expression levels of GAPDH in the same samples were used to normalize the results of the target genes. Results are presented as the normalized relative expression of the target gene from duplicate measurements of each sample (6 samples per group and genotype), and expressed as fold change over the levels of expression of the normalized target gene determined in cDNA samples from control (PBS-injected) gingival tissues in WT mice.

Statistical Analysis

The statistical analysis was performed using GraphPad 5.0 (GraphPad Software Inc., San Diego, CA, USA). Central tendency and dispersion measures were calculated from different experiments. Pairwise comparisons between experimental conditions (PBS-injected x Aa-injected) were performed using non-paired Student's t-test, whereas comparison among the different genotypes (WT, NLRP3-KO and Casp1-KO) in each experimental condition were perfomed using ANOVA followed by Tykey post-hoc test for pairwise comparisons. Significance level was 95% ($p < 0.05$) for all analysis.

Results

Inflammatory bone resorption is attenuated in Casp1-KO mice

Interestingly, µCT analysis showed a greater volume of mineralized tissue (MT) in non-disease control (PBS-injected) NLRP3-KO and Casp1-KO animals. Injection of heat-killed Aa effectively induced alveolar bone resorption in all genotypes, but the severity of resorption was significantly attenuated in Casp1-KO mice (Figure 1). These results suggest that NLRP3 and Caspase-1 may have a role in physiological bone turnover and that Caspase-1, but not NLRP3, has a role in promoting inflammatory bone resorption,

Osteoclast number in increased in Casp-1 KO mice

Surprisingly, the number of osteoclasts was increased similarly with the induction of experimental periodontal disease in all genotypes. Strikingly, Casp1-KO mice, which showed some protection for inflammatory bone loss by µCT, even had a significant increase on the numbers of osteoclasts in the ROI. These results indicate that the osteoclast differentiation in this inflammatory microenvironment is not affected by the lack of NLRP3 or Caspase-1 (Figure 2).

Characterization of the local inflammatory infiltrate

Experimental periodontal disease caused increase of the total inflammatory infiltrate, as showed in the H/E representative images (Figure 4a). Were also observed a significant increase on leukocyte (CD45+) infiltration including infiltration of PMNs (Ly6G+) in all genotypes. There was no difference on the overall inflammatory infiltrate (CD45+) in periodontal disease tissues among genotypes; however there was a significant decrease on the PMN (Ly6G+) infiltrate in Caspase-1 KO mice. Moreover, Casp1-KO mice showed the least relative increase on PMN infiltration with the induction of experimental periodontal disease (Figure 3).

Caspase-1 does not affect gene expression of IL-6, IL-10, TNF-alpha RANKL and MMP-13 in the experimental periodontal disease model

Since WT and NLRP3 mice showed similar severity of alveolar bone resorption and also no differences in the inflammatory infiltrate in the experimental model used, we compared the expression of selected candidate genes related with inflammation and bone resorption in the

gingival tissues of Caspase-1 KO and WT mice. Gene expression of IL-6, IL-10, TNF-alpha, RANKL and MMP-13 were all markedly increased in the Aa-injected gingival tissues in both WT and Casp1-KO mice; however there were no differences between Casp-1 KO and WT mice in the expression of all these candidate genes (Figure 4).

Discussion

To our knowledge, this is the first study to assess the role of NLRP3 inflammasome and Caspase-1 in an in vivo model of bacterial-induced periodontal disease. The results indicated that the NLRP3 inflammasome does not have a relevant role in the inflammation and inflammatory bone resorption in this model. Interestingly, gene deletion of Caspase-1 significantly attenuated inflammatory bone resorption, but strikingly this effect was not accompanied by a change in the osteoclast numbers, on the inflammatory infiltrate or on the expression of genes associated with inflammation and mineralized and non-mineralized soft tissue degradation.

In this study we used heat-killed Gram-negative bacteria associated with periodontal disease in humans to avoid issues with possible fluctuation on bacterial cell viability and also issues with adherence/colonization in the oral environment. The goal of our model was to induce an inflammatory response and the associated inflammatory alveolar bone resorption, the two major hallmarks of periodontal disease. This model provides the two signals that are required for NLRP3 inflammasome activation: exogenous microbial-

derived PAMPs, which triggers the production of cytokine precursors (e.g., pro-IL1); and a second signal, represented by the interaction of DAMPs with their PRRs (e.g., RAGE). The two-signal model of activation of NLRP3 was already demonstrated in the context of periodontal disease by stimulating primary gingival epithelial cells with *Porphyromonas gingivalis*, which resulted in downregulation of NLRP3 expression and increase of pro-interleukin-1beta expression. Increased secretion of interleukin-1beta was only detected upon stimulation of with extracellular ATP as the danger signal/second signal [19].

Interestingly, *P. gingivalis* have been reported to decrease expression of NLRP3 and IL-1β also in gingival fibroblasts [20], whereas in monocytes it increases expression of NLRP3, IL-1β and IL-18 [21]. A number of other studies have reported activation of NLRP3 by periodontal disease-associated bacteria, including *Fusobacterium nucleatum* [22] and *Treponema denticola* [23]. *Aggregatibacter actinomycetemcomitans* (Aa, used in this study) has been shown distinct stimulatory effects on NLRP3 and IL-1beta expression in vitro, including human monocytes [24] and human PBMCs [25]. Some studies report that leukotoxin secreted by Aa is a crucial virulence factor mediating the induction of IL-1beta and IL-18 by this microorganism [26]; however, another in vitro study using human monocytes infected with mutant strains of Aa (knockout for leukotoxin and cytolethal dystending toxin genes) also showed increased expression of IL-1β, IL-18 and NLRP3, suggesting that other molecules derived from Aa may

activate inflammasomes [25]. This supports the possibility of inflammasome activation in our model using heat-killed Aa.

Regardless of specific ligands/activators for NLRP3, as a chronic inflammatory condition, periodontal disease is characterized by high levels of reactive oxygen species (ROS) and also by tissue degradation, with accumulating DAMPs in its microenvironment. Both ROS and DAMPs (which may also generate ROS) can activate multiple inflammasomes besides NLRP3, including AIM2, NLRP1, NLRC4 and NLRP6 [27]. Thus, inflammasome activation may derive from direct recognition/interaction of bacterial and host-derived ligands by the inflammasome central/sensor proteins coupled with the detection of cell changes induced by external microbial/stress stimuli [28]. Specifically regarding periodontal disease, there is scarce information available. Increased gene expression of NLRP3 and NLRP2, but not of ASC-1, was reported in the presence of periodontal disease in humans, and these increased levels of inflammasome core genes were correlated with increased mRNA of IL-1β and IL-18, cytokines processed by the inflammasomes [13]. Increased expression of NLRP3 and AIM2 in the gingival tissues of patients with periodontal disease is correlated with the levels of IL-1β and IL-18 suggesting that various inflammasomes may participate in the microbial-induced inflammation in periodontal diseases [29]. In our experimental model, it is important to consider that in NLRP3-KO mice only this particular inflammasome was disrupted, and this may cause a preponderance of biological effects

associated with the activation of the other still functional inflammasomes (NLRC4, AIM2). Nevertheless, the lack of functional NLRP3 did not affect the inflammatory infiltrate and alveolar bone resorption in this model. In contrast, genetically modified mice with a global gain of function mutation of NLRP3 [30] demonstrated that activation of NLRP3 increased production of proinflammatory mediators, which was associated with altered bone turnover and reduced bone mass; however when NLRP3 gain of function mutation was limited to osteoclasts there was no increase in proinflammatory cytokines accompanying the decrease of bone mass [31].

Since caspase-1 is the main effector of all inflammasomes, Casp1-KO mice may be considered as representative of the loss of function of all inflammasomes, which in our model was associated with a significant attenuation of inflammatory bone resorption. Strikingly, inflammatory infiltrate, osteoclastogenesis and expression of candidate inflammatory genes were not affected by the lack of caspase-1 in our experimental model. We speculate that lack of caspase-1 may cause a functional impairment and/or a phenotypical change through both direct and indirect cascade of events in inflammatory cells and osteoclasts; a possibility that we will explore in future experiments.

The suggestion is that inflammasome activation may affect bone turnover indirectly (i.e., via modulation of expression of proinflammatory mediators) or directly (i.e., via an osteoclast-specific effect). Osteoclast specific effect on bone turnover may not be necessarily associated with change in

osteoclast number (i.e., osteoclast differentiation), but rather with increased osteoclast activity. Selective blockage of NLRP3 in vitro has been shown to reduce osteoclastogenesis [32]; which we have not observed in our in vivo model with the concurrent participation of multiple cell types (including non-inflammatory stromal cells, particularly osteoblasts), diverse cytokines/biological mediators and also of other inflammasomes.

In summary, this is the first study to assess the role of NLRP3 inflammasome and of Caspase-1, the effector protein of all inflammasomes, in an in vivo model of bacterial induced periodontal disease. NLRP3 did not play a significant role in the inflammation and bone resorption in this model, whereas lack of caspase-1 attenuated inflammatory bone resorption. Insights from this study will drive further research into the role of inflammasomes in microbial-initiated and maintained chronic inflammatory conditions associated with the destruction of both soft and mineralized connective tissues.

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Figure Legends

Figure 1 – (a) Representative tridimensional reconstructed images of microcomputed tomography scans of the hemimaxillae from WT, NLRP3-KO and Casp1-KO mice, according to the experimental groups (non-disease control/PBS-injected or diseased/Aa-injected). (b) Alveolar bone resorption was evaluated as the change in the ratio between mineralized tissue (MT) to the total volume (TV) of the ROI in 6 different animals from each group (*$p<0.05$; ANOVA followed by Tukey multiple comparison's test performed for each experimental condition). *indicates comparison with WT genotype in the same experimental condition. #indicates comparison with the non-disease (PBS-injected) control in the same genotype. *!*indicates the comparison with other genotype within the same experimental condition.

Figure 2 – (a) Representative images of biochemical staining of TRAP in the perioodontal tissues, both in non-disease control (PBS-injected) and diseased (Aa-injected) mice, according to the different genotypes. Red shade staining of TRAP positive cells, near to the alveolar bone. 40x magnification. (b) Quantification of osteoclasts considered as TRAP-positive cells located in the vicinity of alveolar bone and presenting two or more visible nuclei. Number of osteoclasts was averaged for each experimental condition (non-disease control / PBS-injected and diseased/Aa-injected)

and genotype (WT, NLRP3-KO and Casp1-KO). The quantification was performed in 9 different and non-sequential sections spanning 1000 µm on the antero-posterior direction (sagittal plane) from 6 different animals in each experimental condition (non-disease control/PBS-injected and diseased/Aa-injected) and genotype (WT, NLRP3-KO and Casp1-KO). (ANOVA followed by Tukey multiple comparison's test. *indicates $p<0.05$ for the comparison with the WT genotype in the same experimental condition (non-diseased/PBS-injected or diseased/Aa-injected), and # indicates $p<0.05$ for the comparison with the non-diseased control (PBS-injected) in the same genotype.

Figure 3 – (a) Representative images of H/E-stained sections of each experimental group (non-disease control/PBS-injected or diseased/Aa-injected) according to the genotype (WT, NLRP3-KO or Casp1-KO) at 20X magnification. (b) Representative images of immunofluorescence detection of the pan-leukocyte marker CD45 and the neutrophil marker Ly6G in the gingival tissues of WT, NLRP3 and Casp1-KO mice, according to the experimental condition (non-disease control/PBS-injected or diseased/Aa-injected). (c) Results for the quantitation of mean fluorescence intensity (MFI) of CD45 or Ly6G according to the experimental condition and genotype. These values represent averages from MFI values from three animals per group and experimental condition, using semi-serial sections

spanning 200 µm in the antero-posterior or mesio-distal direction. Nuclei were counterstained with DAPI, scale bar 100µm.

Figure 4– Expression (mRNA) of selected candidate genes associated with destruction of mineralized and non-mineralized soft tissues in the periodontal disease model. Since there was no difference in the severity of bone loss and inflammatory infiltrate between WT and NLRP3-KO mice, we focused on the possibility of differential regulation of these genes between WT and Casp1-KO mice. Significantly lower expression of IL-6, TNF and RANKL in non-diseased tissues of Casp1-KO mice. Induction of experimental periodontal disease increased expression of Expression of all candidate genes, and no difference was observed between WT and Casp-1 KO mice. Bars represent average fold change and standard deviation of normalized target gene expression in comparison to the non-diseased (PBS-injected) control group of the WT group. (*$p<0.05$; ANOVA and Tukey tests for pairwise comparisons). * indicates comparison to WT genotype, same experimental condition (non-disease control/PBS-injected or diseased/Aa-injected), and # indicates comparison to the non-disease (PBS-injected) control in the WT group.

References

1. Pedra JH, Cassel SL, Sutterwala FS. Sensing pathogens and danger signals by the inflammasome. Curr Opin Immunol 2009;v.21(1):10-6

2. Jin Y, Mailloux CM, Gowan K, Riccardi SL, LaBerge G, Bennett DC, Fain PR, Spritz RA. NALP1 in vitiligo-associated multiple autoimmune disease. N Engl J Med 2007;v.22;356(12):1216-25
3. Valk K, Vooder T, Kolde R, et al. Gene expression profiles of non-small cell lung cancer: survival prediction and new biomarkers. Oncology 79: 283-292
4. Schroder & Tschopp. The Inflammasomes. Cell 2010; v.140(6): 821-32
5. Mathews RJ, Robinson JI, Battellino M, et al. Evidence of NLRP3-inflammasome activation in rheumatoid arthritis (RA); genetic variants within the NLRP3-inflammasome complex in relation to susceptibility to RA and response to anti-TNF treatment. Ann Rheum Dis 2014; v.73 (6): 1202, 2014
6. Larsen CM, Faulenbach M, Vaag A, Ehses JA, Donath MY, Mandrup-Poulsen T. Sustained effects of interleukin-1 receptor antagonist treatment in type 2 diabetes. Diabetes Care 2009; v.32 (9): 1663-8
7. Teng YT, Nguyen H, Hassanloo A, Ellen RP, Hozumi N, Gorczynski RM. Periodontal immune responses of human lymphocytes in Actinobacillus actinomycetemcomitans-inoculated NOD/SCID mice engrafted with peripheral blood leukocytes of periodontitis patients. J Periodontal Res 1999; v.34(1):54-61
8. Eastcott JW, Yamashita K, Taubman MA, Harada Y, Smith DJ. Adoptive transfer of cloned T helper cells ameliorates periodontal disease in nude rats. Oral Microbiol Immunol 1994;Oct;9(5):284-9
9. Pacios S, Kang J, Galicia J, Gluck K, Patel H, Ovaydi-Mandel A, Petrov S, Alawi F, Graves DT. Diabetes aggravates periodontitis by limiting repair through enhanced inflammation. FASEB J 2011; v.26(4):1423-30
10. Orozco A, Gemmell E, Bickel M, Seymour GJ. Interleukin-1beta, interleukin-12 and interleukin-18 levels in gingival fluid and serum of

patients with gingivitis and periodontitis. Oral Microbiol Immunol. 2006 Aug;21(4):256-60

11. Johnson RB, Serio FG. Interleukin-18 concentrations and the pathogenesis of periodontal disease. J Periodontol 2005; v.76(5): 785-90
12. Engebretson SP, Grbic JT, Singer R, Lamster IB. GCF IL-1beta profiles in periodontal disease. J Clin Periodontol. 2002 Jan;29(1):48-53
13. Bostanci N, Emingil G, Saygan B, Turkoglu O, Atilla G, Curtis MA, Belibasakis GN. Expression and regulation of the NALP3 inflammasome complex in periodontal diseases Clin Exp Immunol 2009; Sep;157(3):415-22et al., Clin Exp Immunol; v.157: 415
14. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature. 2009 Mar 26;458(7237):509-13
15. McCall SH, Sahraei M, Young AB, Worley CS, Duncan JA, Ting JP, Marriott I. Osteoblasts express NLRP3, a nucleotide-binding domain and leucine-rich repeat region containing receptor implicated in bacterially induced cell death. J Bone Miner Res 2008; Jan;23(1):30-40
16. Almeida MQ, Tsang KM, Cheadle C, Watkins T, Grivel JC, Nesterova M, Goldbach-Mansky R, Stratakis CA. Protein kinase A regulates caspase-1 via Ets-1 in bone stromal cell-derived lesions: a link between cyclic AMP and pro-inflammatory pathways in osteoblast progenitors. Hum Mol Genet. 2011 Jan 1;20(1):165-75
17. Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, Suda T. Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. Exp Cell Res 1999; Feb 25;247(1):84-93

18. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S () Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 1999; Sep;25(3):255-9
19. Yilmaz Ö, Lee KL. The inflammasome and danger molecule signaling: at the crossroads of inflammation and pathogen persistence in the oral cavity. Periodontol 2000 2015; Oct;69(1):83-95. doi: 10.1111/prd.12084
20. Belibasakis GN, Guggenheim B, Bostanci N. Down-regulation of NLRP3 inflammasome in gingival fibroblasts by subgingival biofilms: involvement of Porphyromonas gingivalis. Innate Immun 2013; Feb;19(1):3-9
21. Hamedi M, Belibasakis GN, Cruchley AT, Rangarajan M, Curtis MA, Bostanci N. Porphyromonas gingivalis culture supernatants differentially regulate interleukin-1beta and interleukin-18 in human monocytic cells. Cytokine. 2009 Feb;45(2):99-104
22. Taxman DJ, Zhang J, Champagne C, Bergstralh DT, Iocca HA, Lich JD, Ting JP. Cutting edge: ASC mediates the induction of multiple cytokines by Porphyromonas gingivalis via caspase-1-dependent and -independent pathways. J Immunol 2006; Oct 1;177(7):4252-6
23. Jun HK, Lee SH, Lee HR, Choi BK. Integrin α5β1 activates the NLRP3 inflammasome by direct interaction with a bacterial surface protein. Immunity 2012; May 25;36(5):755-68
24. Kelk P, Abd H, Claesson R, Sandström G, Sjöstedt A, Johansson A. Cellular and molecular response of human macrophages exposed to Aggregatibacter actinomycetemcomitans leukotoxin. Cell Death Dis 2011; Mar 10;2:e126
25. Belisabakis GN, Xiong J, Miller VM, Li Y, Jayachandran M. Microvesicles at the crossroads between infection and

cardiovascular diseases. J Cardiovasc Pharmacol 2012; Feb;59(2):124-32

26. Kelk P, Claesson R, Chen C, Sjöstedt A, Johansson A. IL-1beta secretion induced by Aggregatibacter (Actinobacillus) actinomycetemcomitans is mainly caused by the leukotoxin. Int J Med Microbiol 2008; Jul;298(5-6):529-41
27. Yilmaz Ö, Lee KL. The inflammasome and danger molecule signaling: at the crossroads of inflammation and pathogen persistence in the oral cavity. Periodontol 2000 2015; Oct;69(1):83-95. doi: 10.1111/prd.12084
28. Greaney AJ, Leppla SH, Moayeri M. Bacterial Exotoxins and the Inflammasome. Front Immunol 2015; Nov 10:6:570.
29. Park E, Na HS, Song YR, Shin SY, Kim YM, Chung J. Activation of NLRP3 and AIM2 inflammasomes by Porphyromonas gingivalis infection. Infect Immun 2914; v.82(1): 112-23, 2014
30. Bonar SL, Brydges SD, Mueller JL, McGeough MD, et al. Constitutively activated NLRP3 inflammasome causes inflammation and abnormal skeletal development in mice. PLos One 2012;v7:e35979
31. Qu C, Bonar SL, Hickmand-Brecks CL et al. NLRP3 mediates osteolysis through inflammation-dependent and -independent mechanisms. FASEB J 2015; v.29(4): 1269-79
32. Greenhill CJ, Jones GW, Nowell MA, Newton Z, et al., Interleukin-10 regulates the inflammasome-driven augmentation of inflammatory arthritis and joint destruction Arthritis Res Ther 2014; v.16(4): 419

Figure 1

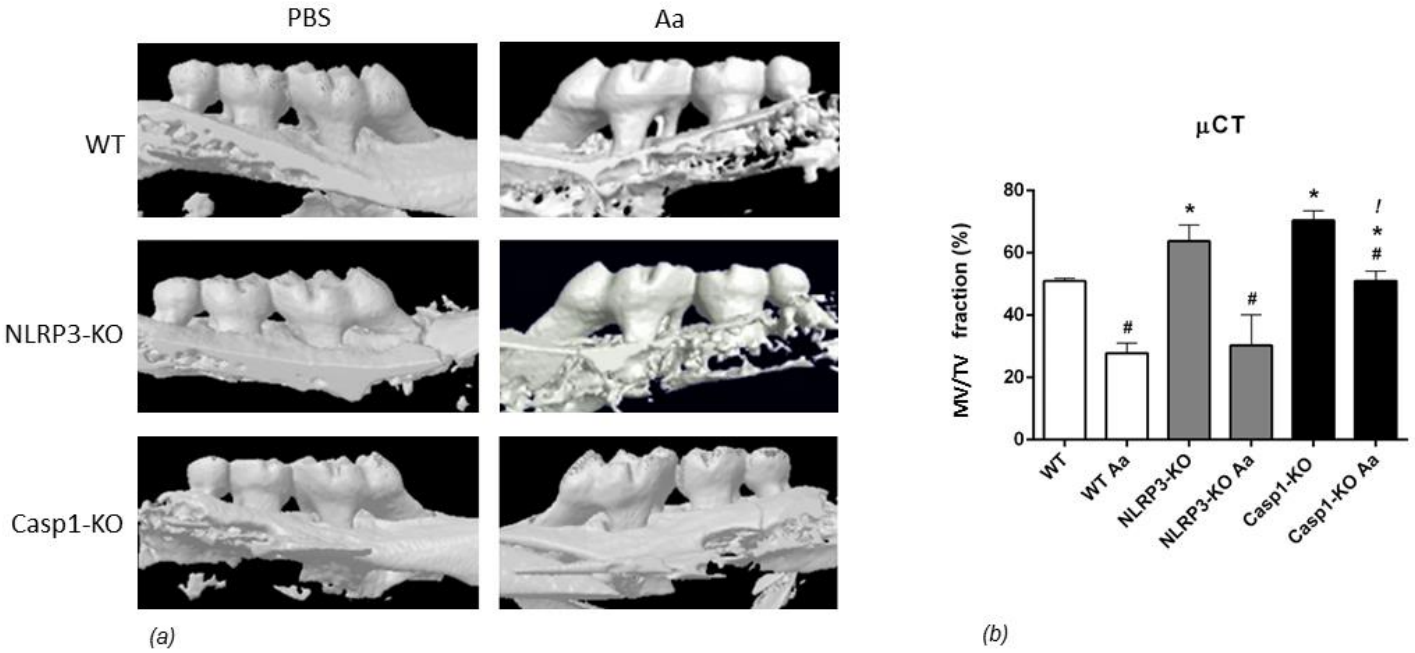


Figure 2

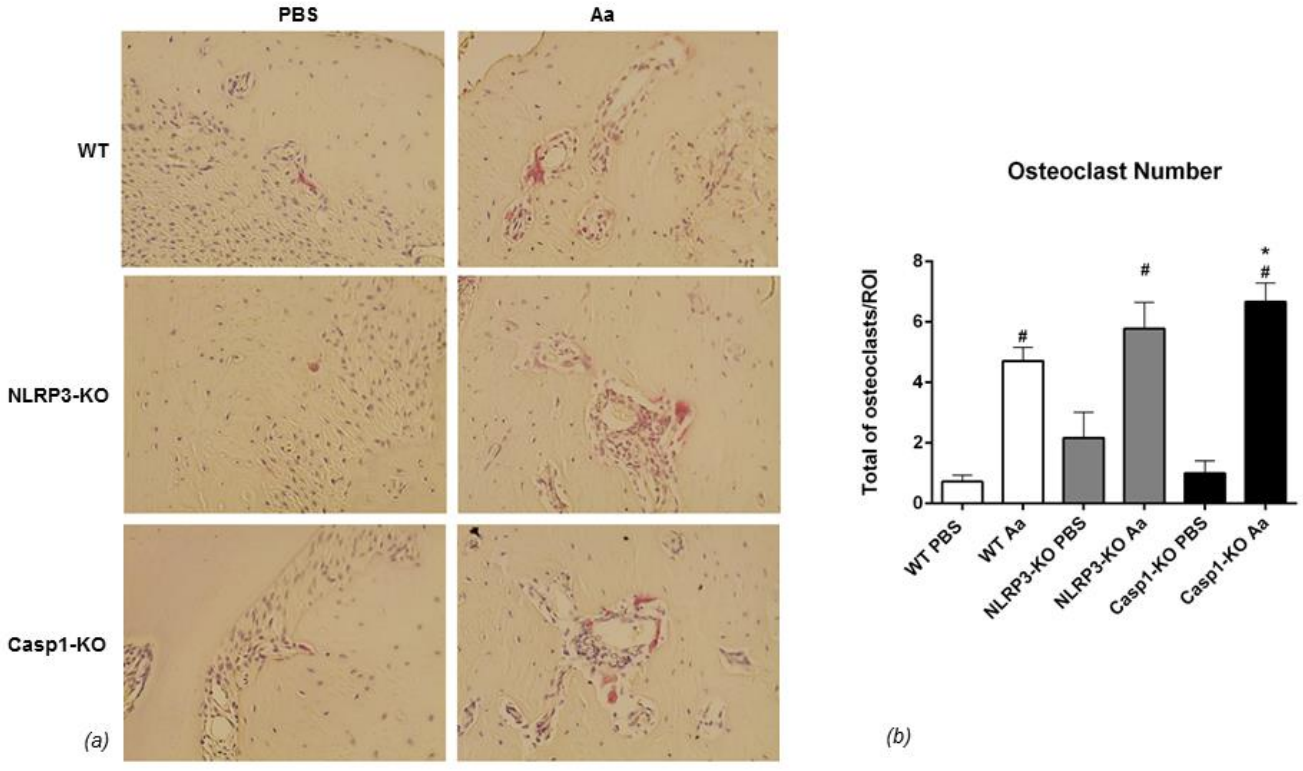
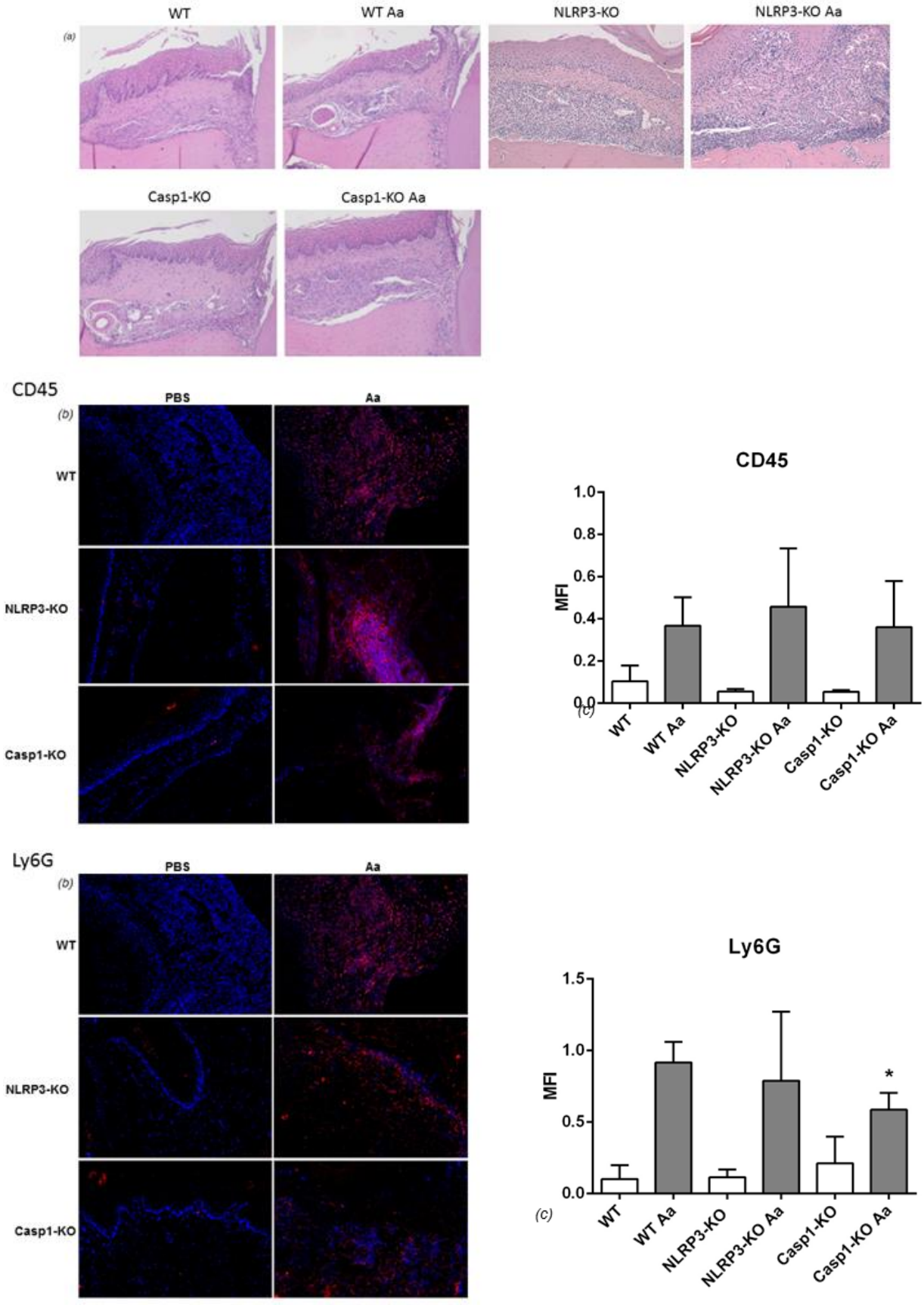


Figure 3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NLRP3-KO
NLRP3-KO Aa
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Casp1-KO Aa
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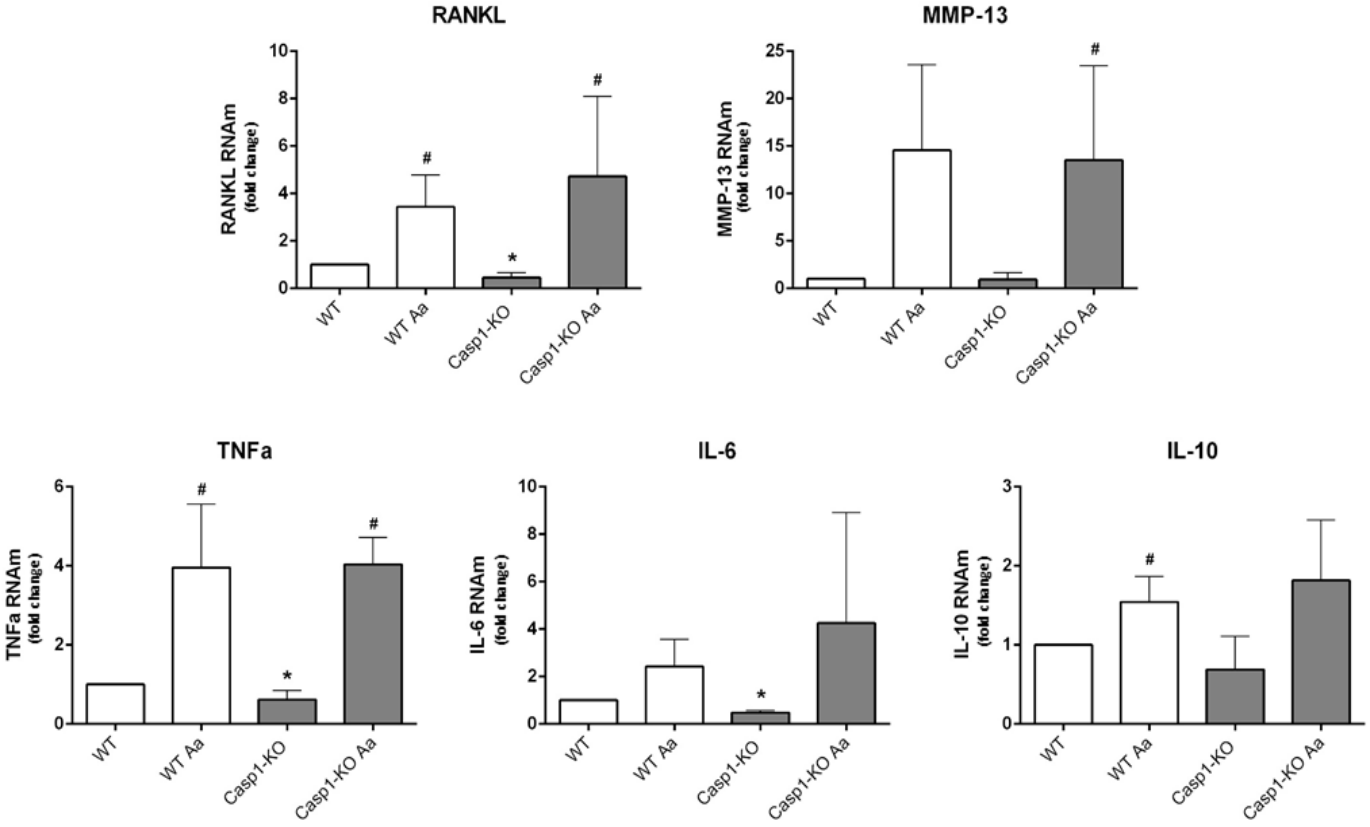
Figure 4


RANKL
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MMP-13
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TNFa
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IL-6
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IL-10
IL-10 RNAm (fold change)
WT
WT Aa
Casp1-KO
Casp1-KO Aa

6 CONCLUSÃO

Considerando a falta de informações sobre o papel de NLRC4 no contexto da doença periodontal e também sua influência no turnover ósseo, este estudo traz novas informações, indicando que o inflamassomo NLRC4 desempenha um papel protetor na reabsorção óssea inflamatória. Há evidências, por exemplo, de que NLRC4 teria um papel único e distinto em diferentes tipos celulares (McCall[32], 2008 e Vance[33], 2015). Com base na falta de influência da osteoclastogênese na severidade do infiltrado inflamatório e no aumento da expressão basal de TNF-alfa e IL-10, podemos especular possível papel do inflamassomo NLRC4 no efeito funcional em osteoclastos e células imunes.

Este também é o primeiro estudo que avalia o papel do inflamassomo NLRP3 e Caspase-1, proteína efetora de todos os inflamassomos, em um modelo de doença periodontal induzida por bactérias in vitro. NLRP3 não teve um papel significante na inflamação e reabsorção óssea, enquanto que a falta de caspase-1 atenuou a reabsorção óssea inflamatória.

Utilizamos neste estudo animais com deleção global dos genes NLRC4, NLRP3 e Caspase-1. Embora não sejam encontrados na literatura evidências de vias alternativas conpensatórias de diferentes inflamassomos e o possível alteração de sua ativação em nosso modelo experimental, é importante considerar as limitações deste modelo, no qual não há necessidade de colonização e interação bacteriana com células epiteliais da mucosa oral e gengiva. Apesar das limitações, este modelo induz

efetivamente a inflamação e reabsorção óssea alveolar, simulando assim as maiores características da doença periodontal em humanos, e tendo a vantagem de possibilitar grande controle da intensidade e frequência do estímulo indutor da doença.

Os resultados deste estudo ainda necessitam da finalização de experimentos in vitro que já tem sido feitos para aumentar o entendimento do papel de NLRC4 e NLRP3 na osteoclastogênese induzida por RANKL e atividade osteoclástica. Juntos, estes achados levarão a futuras investigações sobre o papel dos inflamassomos em condições inflamatórias crônicas associadas com a destruição tecidual.

REFERÊNCIAS*

1. Baker PJ, Dixon M, Evans RT, Dufour L, Johnson E, Roopenian DC. CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. Infect Immun. 1999; 67(6): 2804-9.

2. Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol. 2007; 149(2): 217-25.

3. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol. 2009; 183(2): 787-91.

4. Becerik S, Ozturk VO, Atmaca H, Atilla G, Emingil G. Gingival crevicular fluid and plasma acute- phase cytokine levels in different periodontal diseases. J Periodontol. 2012; 83(10): 1304-13.

5. Belisabakis GN, Xiong J, Miller VM, Li Y, Jayachandran M. Microvesicles at the crossroads between infection and cardiovascular diseases. J Cardiovasc Pharmacol. 2012; 59(2): 124-32.

6. Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. J Immunol. 2008; 181(6): 3733-9.

7. Bostanci N, Emingil G, Saygan B, Turkoglu O, Atilla G, Curtis MA, et al. Expression and regulation of the NALP3 inflammasome complex in periodontal diseases. Clin Exp Immunol. 2009; 157(3): 415-22.

8. Burstone, MS. Histochemical demonstration of acid phosphatases with naphtol AS-phosphates. J Natl Cancer Inst. 1958; 21(3):523-39.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/#biblioteca/manual.

9. Ciraci C, Janczy JR, Sutterwala FS, Cassel SL. Control of innate and adaptive immunity by the inflammasome. Microbes Infect. 2012; 14(14): 1263-70.

10. de Aquino SG, Guimaraes MR, Stach-Machado DR, da Silva JA, Spolidorio LC, Rossa C, Jr. Differential regulation of MMP-13 expression in two models of experimentally induced periodontal disease in rats. Arch Oral Biol. 2009; 54(7): 609-17.

11. DeSantis DA, Ko CW, Liu X, Hise AG, Nunez G, Croniger CM. Alcohol-induced liver injury is modulated by Nlrp3 and Nlrc4 inflammasomes in mice. Mediators Inflamm. 2013; 2013: 751374.

12. Dinarello CA. IL-1: discoveries, controversies and future directions. Eur J Immunol. 2010; 40(3): 599-606.

13. Eastcott JW, Yamashita K, Taubman MA, Harada Y, Smith DJ. Adoptive transfer of cloned T helper cells ameliorates periodontal disease in nude rats. Oral Microbiol Immunol. 1994; 9(5): 284-9.

14. Figueredo CM, Rescala B, Teles RP, Teles RP, Fischer RG, Haffajee AD, et al. Increased interleukin-18 in gingival crevicular fluid from periodontitis patients. Oral Microbiol Immunol. 2008; 23(2): 173-6.

15. Franchi L, Kamada N, Nakamura Y, Burberry A, Kuffa P, Suzuki S, et al. NLRC4-driven production of IL-1beta discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. Nat Immunol. 2012; 13(5): 449-56.

16. Franchi L, Nunez G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol. 2008; 38(8): 2085-9.

17. Garcia de Aquino S, Manzolli Leite FR, Stach-Machado DR, Francisco da Silva JA, Spolidorio LC, Rossa C, Jr. Signaling pathways associated with the expression of inflammatory mediators activated during the course of two models of experimental periodontitis. Life Sci. 2009; 84(21-22): 745-54.

18. Garlet GP. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. J Dent Res. 2010; 89(12): 1349-63.

19. Gong YN, Shao F. Sensing bacterial infections by NAIP receptors in NLRC4 inflammasome activation. Protein Cell. 2012; 3(2): 98-105.

20. Graves DT, Oates T, Garlet GP. Review of osteoimmunology and the host response in endodontic and periodontal lesions. J Oral Microbiol. 2011 Jan 17; 3; doi:10.3402/jom.v3i0.5304.

21. Greaney AJ, Leppla SH, Moayeri M. Bacterial Exotoxins and the Inflammasome. Front Immunol. 2015; 6: 570.

22. Gris D, Ye Z, Iocca HA, Wen H, Craven RR, Gris P, et al. NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. J Immunol. 2010;185(2): 974-81.

23. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet. 2001; 29(3): 301-5.

24. Hu B, Elinav E, Huber S, Booth CJ, Strowig T, Jin C, et al. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4. Proc Natl Acad Sci U S A. 2010; 107(50): 2135-40.

25. Huang X, Yang X, Ni J, Xie B, Liu Y, Xuan D, Zhang J. Hyperglucose contributes to periodontitis: involvement ofthe NLRP3 path way by engaging the innate immunityof oral gingival epithelium. J Periodontol. 2015; 86(2):327-35.

26. Jin Y, Mailloux CM, Gowan K, Riccardi SL, LaBerge G, Bennett DC, et al. NALP1 in vitiligo-associated multiple autoimmune disease. NEngl J Med. 2007; 356(12): 1216-25.

27. Jun HK, Lee SH, Lee HR, Choi BK. Integrin α5β1 activates the NLRP3 inflammasome by direct interaction with a bacterial surface protein. Immunity 2012; 36(5): 755-68.

28. Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY, et al. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. Am J Pathol. 2006; 169(3): 987-98.

29. Kim S, Park MH, Song YR, Na HS, Chung J. Aggregatibacter actinomycetemcomitans induced Aim2 inflammasome activation is suppressed by xylitol indifferentiated Thp-1 macrophages. J Periodontol. 2016 Fev 15: 1-16 [Epub ahead of print].

30. Kool M, Petrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M, et al. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol. 2008; 181(6): 3755-9.

31. Lasiglie D, Traggiai E, Federici S, Alessio M, Buoncompagni A, Assogli A, et al. Role of IL-1 beta in the development of human T(H)17 cells: lesson from NLPR3 mutated patients. PLoS One. 2011; 6(5):200-14.

32. Li Q, Yu H, Zinna R, Martin K, Herbert B, Liu A, et al. Silencing mitogen-activated protein kinase-activated protein kinase-2 arrests inflammatory bone loss. J Pharmacol Exp Ther. 2011; 336(3): 633-42.

33. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-GirmaM, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 2006; 440(7081): 228-32.

34. Marriott I, Gray DL, Tranguch SL, Fowler VG Jr, Stryjewski M, Scott Levin L, et al. Osteoblasts express the inflammatory cytokine interleukin-6 in a murine model of Staphylococcus aureus osteomyelitis and infected human bone tissue. Am J Pathol. 2004; 164(4): 1399-406.

35. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 2006; 440(7081): 237-41.

36. McCall SH, Sahraei M, Young AB, Worley CS, Duncan JA, Ting JP, Marriot I. Osteoblasts express NLRP3, a nucleotide-binding domain and leucine-rich repeat region containing receptor implicated in bacterially induced cell death. J Bone Miner Res. 2008; 23(1): 30-40.

37. McHugh KP, Shen Z, Crotti TN, Flannery MR, O'Sullivan RP, Purdue PE, Goldring SR. The role of cell-substrate interaction in regulating osteoclast activation: potential implications in targeting bone loss in rheumatoid arthritis. Ann Rheum. 2010; 1 (1): 83-5.

38. Miao EA, Mao DP, Yudkovsky N, Bonneau R, Lorang CG, Warren SE. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proc Natl Acad Sci U S A. 2010; 107(7): 3076-80.

39. Miao EA, Warren SE. Innate immune detection of bacterial virulence factors via the NLRC4 inflammasome. J Clin Immunol. 2010; 30(4): 502-6.

40. Pacios S, Kang J, Galicia J, Gluck K, Patel H, Ovaydi-Mandel A, et al. Diabetes aggravates periodontitis by limiting repair through enhanced inflammation. FASEB J. 2012; 26(4): 1423-30.

41. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab Invest. 1976; 34(3): 235-49.

42. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. J Bacteriol. 2001; 183(12): 3770-83.

43. Park E, Na HS, Song YR, Shin SY, Kim YM, Chung J. Activation of NLRP3 and AIM2 Inflammasomes by Porphyromonas gingivalis Infection. Infect Immun. 2014; 82(1): 112-23.

44. Pedra JH, Cassel SL, Sutterwala FS. Sensing pathogens and danger signals by the inflammasome. Curr Opin Immunol. 2009; 21(1): 10-6.

45. Pizzo G, Guiglia R, Lo Russo L, Campisi G. Dentistry and internal medicine: from the focal infection theory to the periodontal medicine concept. Eur J Intern Med. 2010; 21(6): 496-502.

46. Ramos-Junior ES, Morandini AC, Almeida-da-Silva CL, Franco EJ, Potempa J, Nguyen KA, et al. A Dual Role for P2X7 Receptor during Porphyromonas gingivalis Infection. J Dent Res. 2015; 94(9): 1233-42.

47. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005; 23(5): 479-90.

48. Shaw PJ, Lukens JR, Burns S, Chi H, McGargill MA, Kanneganti TD. Cutting edge: critical role for PYCARD/ASC in the development of experimental autoimmune encephalomyelitis. J Immunol. 2010; 184(9): 4610-4.

49. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25(2): 134-44.

50. Socransky SS, Haffajee AD, Ximenez-Fyvie LA, Feres M, Mager D. Ecological considerations in the treatment of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis periodontal infections. Periodontol 2000. 1999; 20: 341-62.

51. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012 (7381); 481: 278-86.

52. Sutterwala FS, Ogura Y, Szczepanik M, et al. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity. 2006; 24(3): 317-27.

53. Sutterwala FS, Ogura Y, Flavell RA. The inflammasome in pathogen recognition and inflammation. J Leukoc Biol. 2007; 82(2): 259-64.

54. Suzuki S, Mimuro H, Kim M, et al. Shigella IpaH7.8 E3 ubiquitin ligase targets glomulin and activates inflammasomes to demolish macrophages. Proc Natl Acad Sci U S A. 2014; 111(40): 4254-63.

55. Teng YT, Nguyen H, Hassanloo A, Ellen RP, Hozumi N, Gorczynski RM. Periodontal immune responses of human lymphocytes in Actinobacillus actinomycetemcomitans-inoculated NOD/SCID mice engrafted with peripheral blood leukocytes of periodontitis patients. J Periodontal Res. 1999; 34(1): 54-61.

56. Tomalka J, Ganesan S, Azodi E, Patel K, Majmudar P, Hall BA, et al. A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen Candida albicans. PLoS Pathog. 2011; 7(12): 1071-9.

57. Triantafilou M, Hughes TR, Morgan BP, Triantafilou K. Complementing the inflammasome. Immunology. 2016; 147(2): 152-64.

58. Tripodi D, Maccauro G, Anogeianaki A, Castellani ML, Pandolfi F, Felaco P, et al. Impact of IL-18 on inflammation. J Biol Regul Homeost Agents. 2011; 25(1): 7-11.

59. Trombetta-Esilva J, Yu H, Arias DN, Rossa C, Jr., Kirkwood KL, Bradshaw AD. LPS induces greater bone and PDL loss in SPARC-null mice. J Dent Res. 2011; 90 (4): 477-82.

60. Valk K, Vooder T, Kolde R, Reintam MA, Petzold C, Vilo J, et al. Gene expression profiles of non-small cell lung cancer: survival prediction and new biomarkers. Oncology. 2010; 79(3-4): 283-92.

61. Vance RE. The NAIP/NLRC4 inflammasomes. Curr Opin Immunol. 2015; 32:84-9.

62. van de Veerdonk FL, Joosten LA, Shaw PJ, Smeekens SP, Malireddi RK, van der Meer JW, et al. The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. Eur J Immunol. 2011; 41(8): 2260-8.

63. von Moltke J, Trinidad NJ, Moayeri M, Kintzer AF, Wang SB, van Rooijen N, et al. Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature. 2012; 490(7418): 107-11.

64. Yilmaz Ö, Lee KL. The inflammasome and danger molecule signaling: at the crossroads of inflammation and pathogen persistence in the oral cavity. Periodontol 2000. 2015; 69(1): 83-95.

65. Yu H, Li Q, Herbert B, Zinna R, Martin K, Junior CR, et al. Anti-inflammatory effect of MAPK phosphatase-1 local gene transfer in inflammatory bone loss. Gene Ther. 2011; 18(4): 344-53.

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Câmpus de Araraquara

FACULDADE DE ODONTOLOGIA

Proc. CEEA nº 10/2010

Araraquara, 05 de julho de 2010

Senhores Pesquisadores:

O Comitê de Ética em Experimentação Animal-CEEA desta Faculdade reunido em 05/07/2010, após a avaliação do projeto de sua responsabilidade intitulado "Papel das proteínas NOD na modulação da resposta imune nas doenças periodontais" (Proc. CEEA nº 10/2010) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para JULHO/2013.

Atenciosamente.

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Araraquara, 08 de abril de 2016

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