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JOÃO ANTÔNIO CHAVES DE SOUZA

Papel das proteínas Nod na modulação da resposta imune nas doenças periodontais

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Tese apresentada ao Programa de Pós-Graduação em Odontologia – Área de Implantodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista, para obtenção do título de Doutor em Odontologia.

Orientador: ***Prof. Dr Carlos Rossa Junior***

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JOÃO ANTÔNIO CHAVES DE SOUZA

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RESUMO

Souza JAC. Papel das proteínas Nod na modulação da resposta imune nas doenças periodontais [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

RESUMO

As interações microrganismo-hospedeiro se iniciam pela detecção de padrões moleculares associados a microrganismos (MAMPs) por receptores semelhantes à Toll (TLR) e por proteínas com domínio de ligação à nucleotídeos e oligomerização (Nod) na resposta imune inata. No entanto, como a cavidade bucal saudável é continuamente colonizada por microrganismos não patogênicos que também apresentam MAMPs, deve haver um mecanismo endógeno de regulação negativa da resposta do hospedeiro para evitar uma resposta exagerada e desnecessária com consequências negativas ao hospedeiro. Os mecanismos associados à distinção de microrganismos comensais e patogênicos na mucosa bucal são ainda pouco compreendidos. As proteínas Nod foram inicialmente descritas como 'TLRs intracelulares' capazes de reconhecer MAMPs no citosol; no entanto, estudos in vitro indicam que Nod têm papel relevante na regulação da expressão de RANKL e OPG induzidas por antígenos microbianos, bem como na modulação da atividade de vias de sinalização intracelular associadas à expressão de citocinas diretamente relacionadas à regulação do turnover do tecido ósseo. Devido à escassez de informações sobre o papel das proteínas Nod na modulação das interações microrganismo-hospedeiro na mucosa oral e com base nestas informações, nossa hipótese é que as proteínas Nod tem um papel relevante na modulação da reação inflamatória e suas consequências, incluindo a reabsorção do osso alveolar. Para testar esta hipótese, os objetivos específicos propostos foram: avaliar em camundongos *knockout* para Nod1, Nod2 ou Rip2, através de microtomografia computadorizada e avaliações histológicas descritivas, estereométricas e imunohistoquímicas (TRAP), o papel das proteínas Nod na inflamação e reabsorção óssea associadas à doença periodontal experimental induzida por bactérias inativadas por calor. A influência das proteínas Nod nas redes de citocinas e na sinalização intracelular associadas com a doença periodontal foi determinada in vitro em culturas primárias de macrófagos através de ensaios baseados nas plataformas ELISA e PCR em tempo real. Nossos resultados mostraram que, enquanto que nos camundongos Nod1 KO a reabsorção óssea alveolar e o número de osteoclastos aumentou

significantemente, a deleção de Nod2 causou efeito contrário, diminuindo a reabsorção óssea. Os resultados in vitro foram correspondentes com os dados in vivo no caso de Nod2 (qPCR array, ELISA citocinas e vias de sinalização) e distintos no caso Nod1 (ELISA citocinas e vias de sinalização). Estes resultados demonstram que as proteínas Nod tem papel relevante na modulação das interações bactéria-hospedeiro associadas à doença periodontal induzida por bactérias inativadas por calor. Enquanto Nod1 parece exercer papel protetor na inflamação e reabsorção óssea, Nod2 atua como amplificador da resposta do hospedeiro.

Palavras-chave: Proteínas adaptadoras de sinalização NOD; doenças periodontais; reabsorção óssea.

ABSTRACT

Souza JAC. Role of Nod proteins in the modulation of the immune response in periodontal diseases [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013

Recognition of pathogenic bacteria by the host is initially mediated by the innate immune response through detection of microbe-associated molecular pattern (MAMPs) by Toll-like receptors (TLR) and Nucleotide-oligomerization domain (Nod) proteins. Since the oral cavity, as well as other mucosal surfaces, is continuously colonized with non-pathogenic bacteria that also present MAMPs, there has to be an endogenous negative regulatory mechanism in place to prevent an overt host response with deleterious consequences. Specifically in the oral mucosa, it is not clear how the immune system is able to quickly distinguish between commensal and pathogenic bacteria and tailor the host response. Nod proteins were initially described as 'intracellular TLRs' that recognize MAMPs associated with bacteria invading the cytosol; however these proteins have been shown to modulate the activation of various signaling pathways involved in the expression of inflammatory genes, including p38 MAPK and NF-κB in concert with TLR stimulation. There is paucity of information on the in vivo role of Nod proteins in the modulation of host-microbe interactions in the oral mucosa. Based on this information, our hypothesis is that Nod proteins play an important role in the modulation of the inflammatory reaction associated with periodontal diseases and its consequences, including alveolar bone resorption. To test this hypothesis, we propose the following specific aims: Assess the role of Nod proteins in the inflammation and bone resorption in experimentally-induced periodontal disease Describe the influence of Nod proteins on the cytokine and signaling networks associated with periodontal disease

Keywords: NOD signaling adaptor proteins; periodontal diseases; bone resorption.

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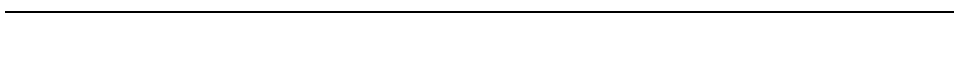
1 INTRODUÇÃO



1 INTRODUÇÃO

As interações microrganismo-hospedeiro modulam a resposta imune associada à destruição tecidual nas doenças periodontais

As doenças periodontais caracterizam-se por processos que culminam na degradação de tecido conjuntivo e ósseo, e são resultado da reação imune/inflamatória em resposta ao desafio microbiano originado pelos microrganismos do biofilme dental. Sendo assim, a progressão da doença é decorrente de um desequilíbrio no processo dinâmico saúde-doença entre a microbiota periodontopatogênica e a resposta do hospedeiro frente à agressão[28]. Estudos demonstrando maior susceptibilidade e severidade de doenças periodontais em indivíduos com alterações da resposta imune associadas à diversas condições sistêmicas proporcionam evidência epidemiológica para a relevância da resposta imune[10, 35]. Segundo o atual paradigma da etiopatogenia das doenças periodontais, a presença de microrganismos periodontopatogênicos é necessária para o início do processo patológico; no entanto a extensão e severidade da destruição tecidual são amplamente dependentes da natureza das interações microrganismo-hospedeiro. Estas interações são dinâmicas, uma vez que tanto a composição microbiana do biofilme dental quanto à competência da resposta do hospedeiro podem variar, num mesmo indivíduo, com o tempo.

A detecção e reconhecimento de microrganismos e seus antígenos (os MAMPs) pelo hospedeiro é mediada pela interação destes com receptores específicos, denominados receptores de reconhecimento de padrões (pattern recognition receptors, PRR)[3, 4]. PRRs são expressos por diversos tipos celulares, incluindo células residentes ou 'não imunes' (osteoblastos, fibroblastos) e células imunes (macrófagos, células dendríticas, leucócitos) e incluem a família dos receptores tipo Toll (Toll-like receptors, TLRs) e as proteínas com domínio de ligação à nucleotídeos e oligomerização (nucleotide-binding oligomerization domain proteins, Nod).

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[44]; contudo apenas algumas são frequentemente associadas às doenças periodontais[49, 50]. Assim, a cavidade bucal é continuamente colonizada por microrganismos não patogênicos, de forma similar à outras superfícies

mucosas, em especial à mucosa intestinal. A discriminação entre espécies patogênicas e não patogênicas requer mecanismos endógenos de regulação negativa da sinalização por PRRs para evitar o desenvolvimento de uma resposta imune exagerada com consequências deletérias ao próprio hospedeiro. A doença de Crohn é um exemplo das consequências de defeitos genéticos nos mecanismos endógenos de regulação da sinalização por PRRs na mucosa intestinal[32, 58].

Além disso, a presença de uma microbiota não patogênica pode ser benéfica ao hospedeiro. Embora na cavidade bucal estes benefícios não sejam bem definidos, no trato gastrointestinal a microbiota indígena auxilia na digestão dos nutrientes; previne infecções por patógenos por meio de competição direta pelos nichos de colonização ou pela sensibilização cruzada da resposta imune; estimula a vascularização e desenvolvimento das microvilosidades intestinais[13, 18]. As células epiteliais de revestimento do epitélio do sulco constituem a interface inicial de interação microbiota-hospedeiro na resposta imune inata. Tanto microrganismos associados à doença periodontal, como *Porphyromonas gingivalis* e *Aggregatibacter actinomycetemcomitans*, quanto microrganismos comensais, como *Streptococcus gordonii*, são encontrados no interior de células do epitélio do sulco gengival[9, 46], sugerindo que a natureza das interações entre estas diferentes espécies microbianas e o hospedeiro determinam uma coexistência pacífica ou o desenvolvimento de uma resposta imune para a eliminação do microrganismo.

A sinalização via PRRs é a interface mais importante nas interações microbiota-hospedeiro e as proteínas Nod podem modular a resposta imune inata e adaptativa

Desde a descrição inicial dos TLRs em meados dos anos 90[33], o estudo da resposta imune inata teve grande avanço e o papel destes receptores na modulação da resposta do hospedeiro tem sido intensamente estudado. TLRs são proteínas transmembrana expressas na superfície de diversos tipos celulares, com passagem única pela membrana celular. Estes receptores apresentam uma região extracelular amino-terminal rica em leucina responsável pelo reconhecimento dos ligantes, e uma região carboxi-terminal localizada no citoplasma que é similar à porção citoplasmática do receptor da interleucina-1 (interleukin-1 receptor, IL-1R)[2]. A família dos TLRs conta, atualmente, com 13 membros, cada um capaz de reconhecer diferentes MAMPs que são

expressos em componentes microbianos, incluindo LPS, lipopolipeptídeos, peptidoglicanos (PGN), ácido lipotéico, flagelina e motivos CpG no DNA. A sinalização destes receptores é complexa, envolvendo diversas proteínas adaptadoras (para revisão ver Kirkwood, K.L.)[29]. Existe grande complexidade nas proteínas adaptadoras envolvidas na sinalização intracelular de TLRs. As diferenças nas respostas celulares frente a um mesmo estímulo externo podem ser resultantes da ativação de vias de sinalização alternativas em tipos celulares distintos, incluindo a ativação de diferentes proteínas adaptadoras e a participação de possíveis modificadores da transdução de sinais, como as proteínas Nod.

As proteínas Nod representam a família dos receptores semelhantes às proteínas com domínio de ligação à nucleotídeos e oligomerização (nucleotide-binding oligomerization domain-like receptors, NLR), e são proteínas citoplasmáticas que também apresentam região rica em leucina e foram inicialmente descritas como 'TLRs intracelulares' com a função de reconhecer MAMPs de bactérias invadindo o citosol[23, 41]. Ainda que a função destas proteínas não seja completamente conhecida, esta descrição inicial da função das proteínas Nod é baseada em sua localização citoplasmática (ainda que TLR3, TLR7 e TLR8, envolvidos no reconhecimento de MAMPs de origem viral, sejam expressos no citoplasma, a localização dos demais TLRs na membrana celular não permitiria sua atuação no reconhecimento de patógenos bacterianos intracelulares) e também em sua similaridade estrutural com as proteínas R de plantas. Como plantas não apresentam resposta imune adaptativa, as proteínas R têm a função de reconhecer antígenos microbianos e patógenos intracelulares no citosol e iniciar a resposta do hospedeiro.

A família de NLRs conta, atualmente com 22 proteínas em humanos agrupadas em 5 subfamílias que possuem três domínios distintos: um domínio de interação proteína-proteína (CARD, ou caspase-activating and recruitment domain) na extremidade N-terminal, um domínio capaz de interação/ligação com nucleotídeos (NBS ou nucleotide-binding site) na porção central e na porção C-terminal um domínio com presença de repetições múltiplas de motivos ricos em leucina (LRR ou leucine-rich repeats domain)[7, 37] Nod1 (CARD4) e Nod2 (CARD15) são os membros mais bem estudados na família dos NLRs; enquanto Nod1 é amplamente expressa por diversos tipos celulares, a expressão de Nod2 é mais restrita, incluindo monócitos/macrófagos, células dendríticas e células intestinais de Paneth[22]; no entanto seus padrões de expressão podem variar segundo o tipo celular. Monócitos, por exemplo, expressam

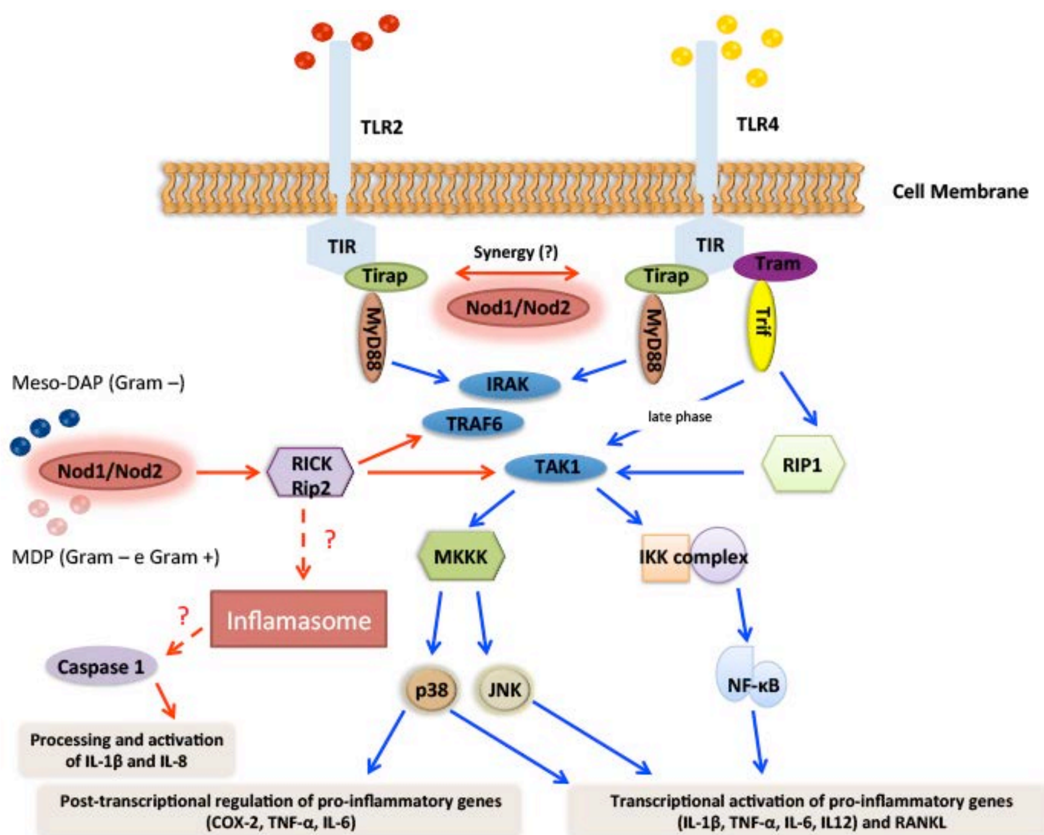
Nod2 de forma constitutiva[15]; enquanto em osteoblastos o RNAm de Nod2 só é detectado após estímulo com LPS, IL-1β ou TNF-α[60]. Estas proteínas reconhecem motivos estruturais distintos em peptideoglicanos da parede celular bacteriana e quando ativadas sofrem alterações de conformação tridimensional e auto-oligomerização: Nod1 é ativada por ácido γ-D-glutamil-meso-diaminopimélico (meso-DAP) presente em todas as bactérias Gram-negativas e apenas em determinadas bactérias Gram-positivas (incluindo as dos gêneros Listeria e Bacillus)[8]; enquanto Nod2 é ativada por muramil-dipeptídeo (MDP), presente em todas as bactérias Gram-positivas e Gram-negativas[14, 27]. Assim, tanto Nod1 quanto Nod2 podem participar na modulação da resposta imune no periodonto, tanto na saúde (em que predominam bactérias Gram-positivas) quanto na doença (com maior prevalência de bactérias Gram-negativas). A relevância destas proteínas para a resposta imune é evidenciada pela associação da doença de Crohn (CD, uma condição hiperinflamatória) com a existência de mutações no gene NOD2[21].

Além do papel como reconhecedoras de antígenos derivados da parede celular microbiana, Nod1 e Nod2 atuam como modificadores da transdução de sinais iniciada por antígenos microbianos, modulando a resposta celular quanto à expressão de citocinas inflamatórias ou indução de apoptose[11]. A caracterização inicial de Nod1 e Nod2 demonstrou que ambas podem ativar NF-kB de forma independente da ativação de TLRs[25]. Esta ativação ocorre via recrutamento, através do domínio CARD, e subsequente ubiquitinização de Rip2 (receptor interacting protein 2, também conhecida como RICK ou CARDIAK), a qual recruta TAK1 que é uma kinase necessária à ativação tanto do fator de transcrição nuclear kappa-B (nuclear factor kappa-B, NF-kB)[24, 39] quanto das proteínas-kinase ativadas por mitógenos (mitogen-activated protein kinases, MAPK) (Figura 1)[1, 16, 43]. Apesar do processo de ativação ser semelhante para Nod1 e Nod2, a ativação de Nod2 requer dois eventos de hidrólise de ATP, mediados por resíduos de aminoácido; ao contrário de um evento inicial único de hidrólise necessário para ativação Nod1[63]. Esta informação, combinada com a existência de diferentes ligantes sugerem que Nod1 e Nod2 podem ter papéis funcionais diferentes.

Tanto NF-kB quanto MAPK são vias de sinalização fundamentais na expressão de genes inflamatórios, demonstrando a relevância de Nod1 e Nod2 na modulação da resposta imune em diversas condições infecciosas, incluindo as doenças periodontais. A relevância destas proteínas na resposta imune à patógenos intracelulares foi demonstrada recentemente pela marcante inibição da produção de TNF-α, IL-12,

interferon gama e óxido nítrico sintase por macrófagos deficientes em Nod1 ou Nod2 e infectados com Trypanosoma cruzi[48].

Figura 1 – Ativação das proteínas Nod1 e Nod2 e as possíveis ações independentes e sinérgicas à sinalização via TLRs. Após ativação por seus ligantes ou de forma concomitante à ativação de TLRs as proteínas Nod recrutam a serina/treonina kinase Rip2/RICK por meio de interações via domínios CARD que, por sua vez, pode ativar diversos substratos (TRAF6, TAK1) envolvidos na ativação subsequente de vias de sinalização intracelular fundamentais para a expressão de mediadores inflamatórios (p38, JNK MAPKinases e NF-kB). A ativação Nod2 pode também resultar na formação de um complexo multiprotéico denominado inflamasome, possivelmente por meio de interação entre Nod2 e NLRP1, no qual ocorre a ativação de caspase-1 (NOD-like receptor protein 1). A ativação de caspase-1 leva ao processamento pós-traducional e ativação de IL-1 e IL-8[5, 36].



Embora NLRs possam atuar de forma independente da ativação de TLRs, exercendo efeitos não redundantes, existem evidências de que a ativação destas proteínas pode resultar em efeitos sinérgicos à ativação de TLRs, por exemplo, em células estimuladas apenas com peptídeoglicanos bacterianos (agonistas de Nod) ou concomitantemente com agonistas de TLR e Nod[38, 57]. Evidências indicam, ainda, que Nod2 está envolvida na modulação da resposta imune adaptativa por meio do estímulo à produção de anticorpos tipo IgG1 para antígenos de receptores de células T[31]; enquanto

a ausência de Nod2 resulta no aumento da produção de IL-12 e interferon-gamma (IFN-g) após estímulo com peptídeoglicano (PG, ligante de Nod1 e Nod2). In vivo, este estímulo com PG resultou em colite antígeno-específica do tipo Th1 apenas nos animais deficientes em Nod2; enquanto animais deficientes em Nod2 e TLR2 não desenvolveram a colite[59], sugerindo que a sinalização via Nod2 tenha efeito regulador negativo no desenvolvimento de uma reação imune do tipo Th1. A ativação de Nod1 por seu ligante específico também induz a resposta adaptativa predominantemente do tipo Th2; contudo quando ativada simultaneamente à sinalização via TLRs, Nod1 é crucial para o desenvolvimento de respostas adaptativas dos tipos Th1, Th2 e até mesmo Th17[12]. De fato, animais Nod1-/- foram mais susceptíveis à infecção por T. cruzi, com maiores níveis de parasitas presentes no tecido-alvo apesar de apresentarem pequenas alterações na produção de citocinas detectadas no soro[48]. Embora o mecanismo segundo o qual Nod1 proporcione resistência à infecção por T cruzi não seja conhecido, estes dados demonstram sua relevância para a resposta imune. Estas informações sugerem que as proteínas Nod tem um importante papel na ativação da resposta imune inata por células residentes (incluindo células epiteliais, fibroblastos, cardiomiócitos e células do estroma) e subsequente ativação da resposta adaptativa por estas células, por meio da modulação de sinais que precedem aqueles originados das células apresentadoras de antígeno ‘profissionais’.

O papel das proteínas Nod na modulação da inflamação e destruição tecidual nas doenças periodontais é pouco conhecido

Desde a demonstração da etiologia microbiana da gengivite em meados da década de 60, sabe-se que o desenvolvimento da inflamação gengival acompanha alterações quantitativas e qualitativas na microbiota do biofilme dental. Estas alterações incluem a redução de espécies Gram-positivas e aumento nas espécies Gram-negativas, com ativação de diferentes TLRs. Neste sentido, existe evidência sugerindo que microrganismos Gram-positivos ativam TLR2 com indução de IL-8, enquanto bactérias Gram-negativas ativam TLR4 e TLR2, induzindo a expressão de TNF-a[53]. De fato, relatos recentes indicam que a maior parte das bactérias Gram-negativas associadas à doença periodontal, incluindo *Porphyromonas gingivalis, Tannerella forsythensis, Prevotella intermedia, Prevotella nigrescences, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* e também a bactéria Gram-negativa não

associada à doença periodontal *Veillonela parvula,* são todas capazes de ativar TLR2; enquanto as duas últimas espécies (Aa e Vf) podem também ativar TLR4[6] . Ainda que todos estes microrganismos associados à doença ativem a sinalização via TLR2, esta via pode também ser ativada, in vitro, por microrganismos comensais, não associados à doença, e presentes no biofilme dental composto primariamente por bactérias Gram-positivas[61], uma vez que estes microrganismos apresentam ácido lipoteicóico (ligante de TLR2). O fato de TLR2 ser ativado tanto por microrganismos patogênicos quanto comensais é interessante por sugerir que a existência de mecanismos de modulação das vias de sinalização intracelular, os quais podem estar relacionados à diferenças na utilização de proteínas adaptadoras; ou à ativação concomitante de outros PRRs (incluindo TLRs e proteínas Nod) por múltiplos MAMPs derivados das diferentes espécies microbianas presentes no biofilme associado à doença ou à saúde periodontal. Esta hipótese é suportada por modelo avaliando a detecção de formas virulentas e avirulentas de *Legionella pneumophila*[47], em que formas virulentas estimulam uma resposta imune mais intensa envolvendo ativação independente de NLRs e sinergismo com TLRs. Assim, as diferenças na quantidade e qualidade dos MAMPs presentes resultaria na ativação de múltiplos PRRs, o que levaria à variações nas vias de sinalização ativadas e, consequentemente, à modulação da resposta imune e destruição dos tecidos periodontais.

Células epiteliais da cavidade bucal expressam elevados níveis de Nod1 e Nod2[52], e apesar de relatos recentes demonstrando o papel sinérgico das proteínas Nod com a sinalização via TLRs na expressão de citocinas inflamatórias[54, 56, 57], as informações sobre a influência das proteínas Nod na modulação da resposta imune associada às doenças periodontais in vivo são ainda escassas. Diversos estudos in vitro indicam que Nod1 e Nod2 têm papel relevante na regulação da expressão de RANKL e OPG induzidas por antígenos microbianos, bem como na modulação da atividade de vias de sinalização intracelular associadas à expressão de citocinas diretamente relacionadas à regulação do turnover do tecido ósseo[47, 60] porém sua importância in vivo, especialmente nas doenças periodontais, é amplamente desconhecida.

2 PROPOSIÇÃO

2 PROPOSIÇÃO

Nossa hipótese é que as proteínas Nod modulam a resposta imune associada a progressão da doença periodontal. O entendimento sobre os mecanismos pelos quais as proteínas Nod modulam as interações microbiota-hospedeiro nas doenças periodontais podem levar ao desenvolvimento de estratégias terapêuticas alternativas focadas na modulação da resposta imune.

Os objetivos deste trabalho foram:

1) – Determinar in vivo o papel das proteínas Nod na inflamação e reabsorção óssea associadas à doença periodontal experimental através de microtomografia computadorizada e avaliações histológicas descritivas, estereométricas e imunohistoquímicas (TRAP). (Apêndice)

2) – Descrever in vitro em culturas primárias de macrófagos a influência das proteínas Nod nas redes de citocinas e na sinalização intracelular associadas com a doença periodontal através de ensaios baseados nas plataformas ELISA e PCR em tempo real. (Apêndice)

3 CAPÍTULO 1

3 CAPÍTULO 1

Role of Nod2 and Rip2 in host-microbe interactions with Gram-negative bacteria. Insights from the periodontal disease model.

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Abstract

Nod2 is a member of the Nod-like receptors (NLRs) family of proteins that participate in the activation of the innate immune response to invading bacteria or to bacterial antigens present in the cytoplasm. The specific ligand that activates Nod2 is a bacterial peptidogycan structure (muramyl-dipeptide, MDP) that is common to both Gram-positive and -negative bacteria, which are present in both the intestinal and oral microbiota. There are currently no known links between periodontal disease and mutations of Nod2; however Nod2 ligands aggravate microbial-induced periodontal disease in vivo. This is the first evidence of the role of Nod2 and Rip2 on periodontal disease using genetically modified mice. We used mice with whole-genome deletion of Nod2 gene (or of the downstream kinase Rip2) in a microbial-induced periodontal disease model to assess the role of Nod2 in tissue destruction associated with inflammation induced by host-microbial interactions. In vitro experiments using primary bone marrow-derived macrophages from these mice provide insight into the role of Nod2 and Rip2 on inflammatory gene expression. uCT indicated that deletion of Nod2 significantly attenuated bone resorption in this model, but without a significant effect on inflammation observed histologically. In vitro, stimulation of macrophages with heat-killed Gram-negative bacteria induced the same biological processes in WT, Nod2- and Rip2-deficient cells; however there was a distinct decrease on the expression of pro-inflammatory mediators, assessed by qPCR arrays and multi-ligand ELISA, in Nod2 and to a lesser extent, in Rip2-deficient cells. The data indicate that deletion of Nod2 attenuates inflammation induced by Gram-negative bacteria.

Introduction

Nucleotide-binding oligomerization domain(NOD)-like receptors (NLRs) are proteins initially described as cytosolic sensors of bacterial infection or intracellular pathogen-recognition receptors (PRRs). There are 22 proteins included in the NLR family, which are further grouped into five subfamilies according to variations in their domain structures. The NLRC subfamily comprises 5 different proteins that are highly conserved in vertebrates: Nod1 (NLRC1), Nod2 (NLRC2) and the inflammasome-activating NLRs NLRC3, NLRC4 and NLRC5. Nod1 and Nod2 are the two most studied members and they recognize different structures in bacterial peptidoglycan: meso-diaminopimelic acid-containing peptidoglycan (meso-DAP) present in most Gram-negative and some Gram-positive bacteria is the ligand for Nod1; whereas Nod2 is activated by muramyl-dipeptide (MDP), which is a structure found in all bacterial peptidoglycan (1).

Upon recognition of their specific ligands, Nod proteins undergo a similar activation process involving self-oligomerization and interaction with a common downstream kinase called Rip2/RICK/CARDIAK that will primarily drive NF-kB and MAPKinase activation. Although simplistically the activation process is similar for Nod1 and Nod2, Nod2 activation requires two ATP hydrolysis events mediated by acidic aminoacid residues; as opposed to a single initial hydrolysis event in Nod1 activation (2). This information, combined with the existence of distinct ligands, suggests that Nod1 and Nod2 may have different functional roles.

Abundant evidence of the important role of Nod2 to host response is primarily derived from studies on host-microbial interactions in the gut mucosa (3) and conditions usually associated with infection by invasive bacteria, such as enteroinvasive *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri* and *Listeria monocytogenes*. Activation of Nod proteins was initially only associated with the presence of their peptidoglycan ligands in the intracellular environment due to bacterial invasion or by direct introduction by non-invasive bacteria presenting a type III or type IV secreting apparatus, such as *Helicobacter pylori* (4, 5). However, novel functions of Nod1 and Nod2 have been described more recently, including the activation of B lymphocytes (6), modulation of dendritic cell activation and antigen presentation (7), regulation of adaptive immunity by participating in Th17 (8) and Th1 polarization (9), particularly by

cross-talk with TLR signaling. Importantly, activated Nod proteins may function as signaling scaffolds and interact with as yet unknown functional partners and have a significant impact on the innate and adaptive host response. Thus it is possible that some NLRs in addition to their PRR role also act as signaling regulators amplifying the activation of different pathways and modulating other immune and non-immune processes (4, 10).

Periodontal disease is the most prevalent bone resorption-associated condition in humans (11). As a bacterial initiated and maintained pathology, the nature of host-microbial interactions largely dictates the extent and severity of tissue destruction. The role of Nod1/Nod2 activation in periodontal diseases in vivo is largely unknown. In fact, this study is the first information on the role of Nod2 in experimental periodontal disease using genetically modified mice. Our data demonstrates an important role for Nod2 as a positive regulator of inflammatory gene expression and bone resorption in vivo; which indicates that Nod2 may be a valid therapeutic target in immunomodulatory therapy. Other in vivo studies report that direct injections of peptidoglycan from Gram-positive bacteria (Nod2 ligand) in the gingival tissues of mice induced significant bone loss, whereas injections of peptidoglycan from Gram-negative bacteria (Nod1 ligand, weak Nod2 activator) did not. Moreover, the association of bacterial LPS and peptidoglycan from either Gram-positive or Gram-negative bacteria synergistically enhanced bone resorption induced by LPS in this model (12). In this study, we show that whole-genome deletion of Nod2 significantly attenuates the inflammatory response and bone loss, demonstrating a relevant modulatory role for Nod2 in host-microbial interactions in the periodontal disease model induced by heat-killed periodontopathogenic bacteria. Using a focused array assay approach on a qPCR platform, we also provide insight into the role of Nod2 on the regulation of host-microbial related inflammatory gene expression, indicating that Nod2 functions as an amplifier of the host response.

Material and methods

Animal use

We used wild-type (WT) and genetically-modified (whole genome deletion of Nod2 and Rip2: Nod2 KO and Rip2 KO, respectively) mice backcrossed to C57/BL6 background for eight generations (13, 14). All animals used in these studies were

between 8 and 10 weeks of age. Sacrifices were always performed by CO_2 inhalation and the Institutional Committee on the Use of Experimental Animals approved the study protocol (ANEXO). Primary bone marrow-derived macrophages (BMDM) were obtained from bone marrow flushed from tibias and femurs, differentiated and expanded in the presence of M-CSF, as previously described (15). Host-microbial interactions in vivo were studied using an experimental model of periodontal disease. In this model, heat-killed bacteria associated with periodontal disease were resuspended in PBS at 10^9 CFU/mL and 3 uL of this suspension were directly injected into the gingival tissues surrounding the teeth of the mice, three times/week for 4 weeks. Sacrifice was performed by CO_2 inhalation 2 days after the last injection. These in vivo studies used a total of 42 mice distributed equally among three different genotypes: WT, Nod2 KO and Rip2 KO. Of the 14 mice in each genotype, 6 were vehicle controls and received bilateral injections of PBS vehicle in the palatal aspect of upper first molars; whereas 8 mice received injections of $3x10^6$ CFU of heat-killed Gram-negative *Aggregatibacter actinomycetemcomitans* (Aa, JP1 serotype).

Assessment of alveolar bone loss and inflammation

Immediately after sacrifice, tissue blocks including the upper molars and surrounding tissues were carefully dissected from the animals, rinsed in PBS and fixed in 4% paraformaldehyde for 18 h at 4C. These samples were then rinsed in distilled water, transferred to 70% ethanol and maintained at 4C. uCT scanning of these samples was done on a Skyscan (Skyscan, Aartselaar, Belgium) at a resolution of 18 um and tridimensional images reconstructed, spacially re-oriented in a standardized orientation and analyzed using the equipment's software (NRecon/DataViewer/CTan/CTvol, Skyscan, Aartselaar, Belgium). A standardized region of interest (ROI) of 2.5 mm^3 was positioned on the tridimensional images using anatomical landmarks as reference points, and the fraction of the volume of the ROI occupied by mineralized tissue (BV%) was be determined using a standard threshold for detection of mineralized tissues. Considering that the variation in the volume of similar tooth roots in different animals is negligible, a decrease in the BV% in the ROI indicates bone loss.

After scanning, the same tissue blocks used on the uCT analysis were decalcified in 0.5 M EDTA (pH 8.0) and submitted to routine processing for paraffin embedding. 5 um thick, semi-serial sections were obtained on the mesio-distal plane and stained with

hematoxylin-eosin for descriptive assessment of inflammation an experienced examiner blinded to the experimental groups according to a severity score system ('0'-no significant inflammation; '1'-mild inflammation; '2'-moderate inflammation; '3'-severe inflammation) (16). A minimum of 6 equally-spaced semi-serial sections spanning 500 μm of the bucco-lingual diameter of the specimens. Sections from 3 to 4 different animals of each genotype were assessed. Scorings were performed three times with a minimum interval of 2 weeks between the assessments and the most prevalent score was used.

Immunohistochemical detection of TRAP was performed using a goat polyclonal antibody (sc-30833, Santa Cruz Biotechnology) and an biotin-streptavidin-DAB visualization system (LSAB2+, Dako USA). A minimum of 6 equally-spaced semi-serial sections of each experimental conditions (PBS or Aa injections) from 3 different animals of each genotype were stained. TRAP+ cells containing 2 or more nuclei present in the vicinity of the bone surface were considered osteoclasts. The number of osteoclasts in a linear extension of 400 um from the palatal aspect of the first molar by a trained examiner blind to the experimental groups.

In vitro studies

BMDM ($1x10^6$ cells/well in 12-well plates) were grown overnight in RPMI1640 supplemented with penicillin/streptomycin and 10% heat-inactivated FBS. After a 6-hour de-induction in medium containing 0.2% heat-inactivated FBS these cells were stimulated with $1x10^6$ UFC/mL of heat-killed Aa (1:1 ratio bacteria:cells) for 6 (RT-qPCR arrays) and 24 (multi-ligand ELISAs) hours. Negative controls were treated with the same volume of PBS vehicle used to resuspend the bacteria. A total of 6 samples (unstimulated and Aa-stimulated for 3 genotypes) were obtained from each experiment. Three independent experiments were performed, each one using cells obtained from 2 to 4 mice of each genotype.

RT-qPCR and qPCR arrays

Total RNA was harvested 6h after stimulation with the bacteria using an affinity column system (RNeasy micro, Qiagen), and 6 pools of RNA were prepared by combining 300 ng of total RNA obtained from each sample (unstimulated or Aa-stimulated BMDM from WT, Nod2 KO and Rip2 KO mice) in each independent

experiment. cDNA was synthesized from each of these six 900 ng pools of total RNA using the reagents and procedure indicated by the supplier of the PCR-based arrays (RT^2 First Strand cDNA kit, SABiosciences/Qiagen). Expression of 84 genes related with innate immunity in each sample was investigated using qPCR-based arrays (RT^2 $Profiler_{TM}$ PCR Array Mouse Toll-Like Receptor Signaling Pathway, SABiosciences/Qiagen) performed according to the instructions of the supplier on a StepOne Plus qPCR thermocycler (Applied Biosystems) using the indicated cycling conditions (10 min/95C, followed by 40 cycles of 15s/95C and e 60s/ 60C). Analysis was carried out using the ΔΔCt method initially using the thermocycler's software; and subsequently with an online tool provided by the supplier of the array. Normalization was performed using the expression of GAPDH, Beta-actin and beta-glucuronidase. These genes were automatically selected by the online analysis tool based on the panel of 6 housekeeping genes included in the array. The purpose of the analysis was to assess the relative regulation of the 84 target genes in comparison to the gene expression determined in unstimulated macrophages from WT mice.

Multi-ligand ELISAs

The conditioned culture media collected 24 h after stimulation of BMDM with heat-killed *A. actinomycetemcomitans* or with the same volume of PBS diluent was aliquoted and stored at -80C until use. Each aliquot was thawed on ice only once and immediately before its use in multi-ligand ELISAs that allow the detection of 6 cytokines and 6 chemokines associated with host-microbial interactions and chemotaxis of immune cells (SA Biosciences/Qiagen). The concentration of total protein in the conditioned media was initially determined by a Bradford assay and then the same quantities of total protein from each experimental condition was used in the ELISAs, normalizing the results and allowing for a comparison of the relative quantities of the different cytokines in each sample. Activation of intracellular signaling pathways associated with inflammatory gene expression was also determined using multi-ligand ELISAs (Cell Signaling). For these experiments, stimulation of BMDM with Aa or the same volume of PBS was performed for 10, 30 and 60 min. Cell lysates from three independent experiments (using cells from 4 - 6 mice) were harvested and pooled according to the experimental conditions (PBS or Aa stimulation) and genotype by combining 10 µg of total protein from each experiment. Data was analyzed as relative changes to

unstimulated control macrophages with the same genetic background (WT, Nod2 KO and Rip2 KO) in each period. The data in these experiments was also normalized by using the same quantity of total protein and also by the expression of total p65, as recommended by the supplier of the assays.

Data analysis

The statistical analysis aimed at comparing the results between different genotypes (WT, Nod2 KO, Rip2 KO) in instances where at least three data points from independent experiments were available. These comparisons were done using non-paired t-tests with Welch's correction for unequal variances, assuming complete independence between the results of the three genotypes. For these analyses, statistical significance was set at 95% (*$p<0.05$). The qPCR focused array data obtained with pooled samples was analyzed using an online bioinformatics tool, DAVID (Database for Annotation, Visualization and Integrated Discovery - http://david.abcc.ncifcrf.gov) (17, 18), in an exploratory manner consistent with an hypothesis-generating study (19). The purpose of these analyses was to assess how the gene functional clusters that were upregulated in macrophages stimulated with heat-killed bacteria were affected by deletions of Nod2 or Rip2 genes.

Results

Lack of Nod2 significantly reduces alveolar bone resorption without a corresponding decrease on the inflammation assessed histologically

In the LPS experimental periodontal disease model, deletion of Nod2 significantly attenuated alveolar bone resorption; which is supported by a significant decrease on osteoclast numbers (Figure 1); however strikingly this effect was not accompanied by a decrease on inflammation observed histologically (Figure 2). Lack of downstream kinase Rip2 was also associated with a reduction on the severity of alveolar bone resorption, but this decrease did not reach statistical significance. Similarly to Nod2 KO animals, there was no decrease on inflammation in the soft tissues adjacent to the bone. Since Nod2 is related primarily with innate immunity and host-microbial interactions, these results suggest that Nod2 may affect biological processes associated with osteoclastogenesis and/or osteoclast activity.

Figure 1- Bone resorption and osteoclastogenesis are significantly reduced in mice lacking Nod2. Alveolar bone resorption induced by injections of heat-killed *Aggregatibacter actinomycetemcomitans* is significantly attenuated in Nod2KO mice. (A) The images are representative of tridimensional reconstructions of mineralized tissues in the hemi-maxillae segments. The graph represents the average and standard deviation of the relative reduction in mineralized tissue content (BV fraction) in the standardized region of interest (ROI) assessed in comparison to the BV fraction of the ROI in WT control samples (set to 100%). Samples from at least 3 different animals were analyzed for each group. (B) Immunohistochemical staining for TRAP to identify osteoclasts in the region of disease induction. Representative low and high-magnification images of sections from animals of each genotype and experimental group. The graph depicts the results of osteoclast numbers according to the genetic background and presence or absence of disease induction. Asterisk (*) indicates significant difference between the indicated pair of bars (Student t-test with Welch's correction for unequal variances).

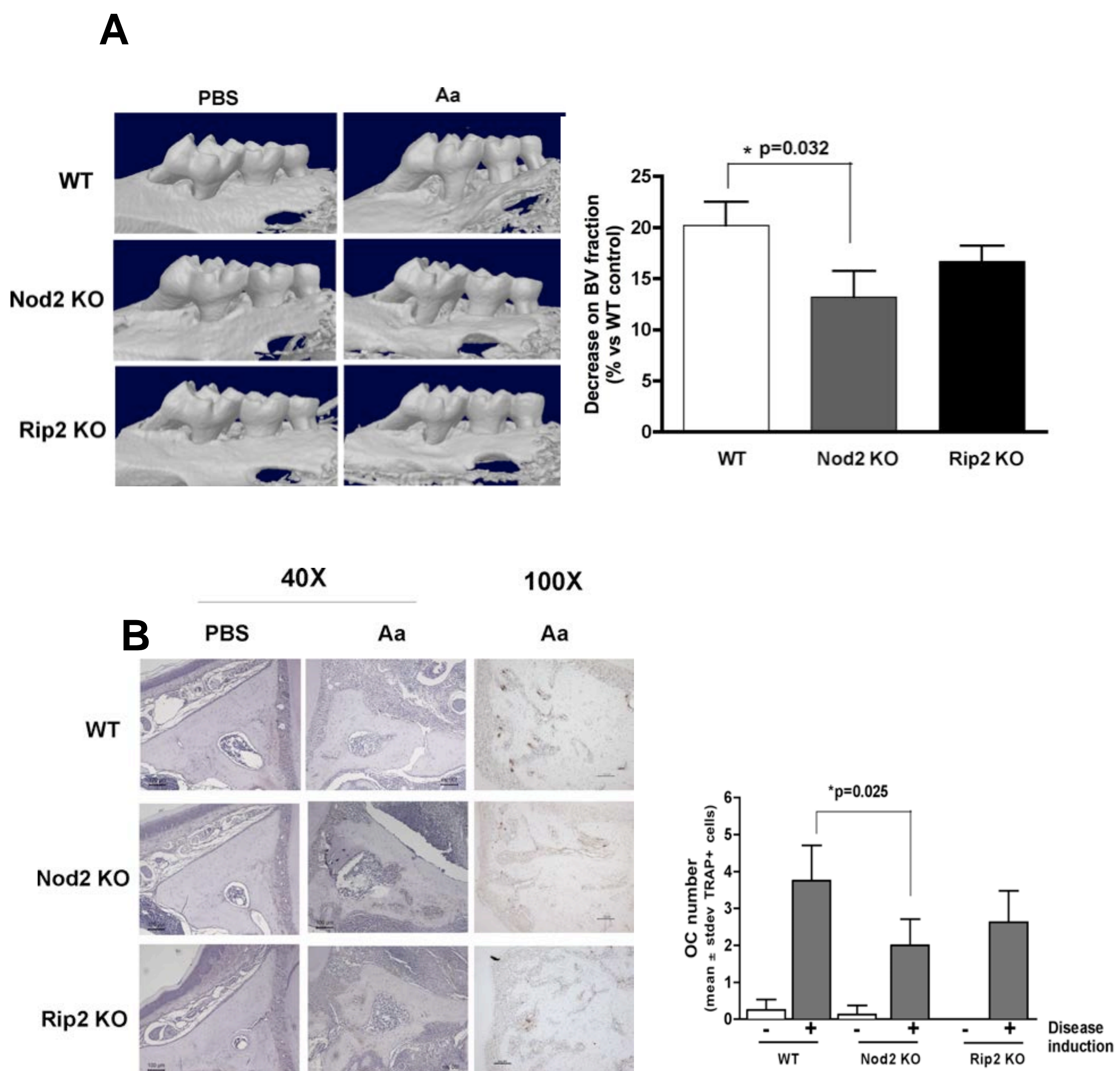
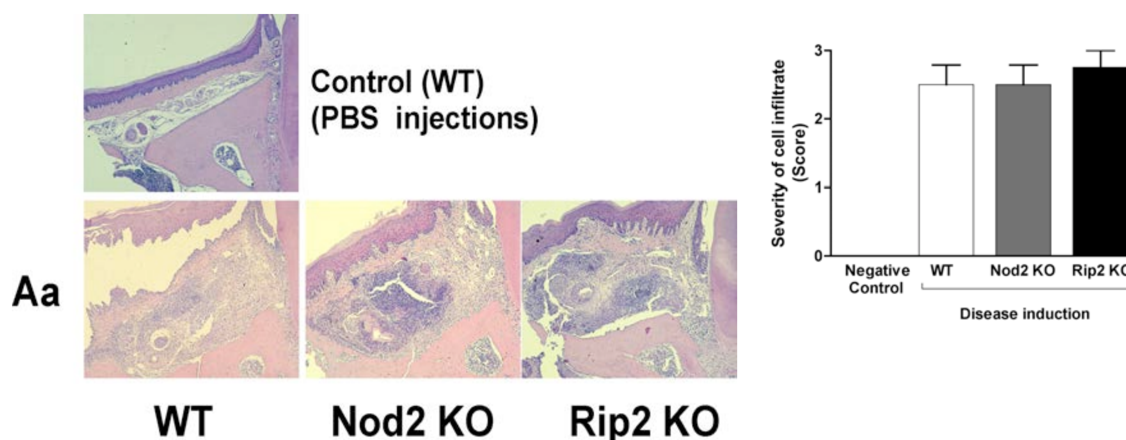


Figure 2- Cell infiltrate is not reduced in Nod2KO and Rip2 KO mice. Representative images of 5 um H/E-stained sections from upper first molars, according to the genotype of the animal. Marked increase of inflammation in the sections from Aa-injected Nod2 and Rip2 KO mice. No difference was observed in PBS-injected sections, regardless of genotype (representative image of a WT mouse is shown). The graph presents the analysis of cellular infiltrate using the severity score. At least 4 semi-serial sections obtained from samples from at least 3 different animals were analyzed for each group and these representative images were obtained at 100X magnification.



Lack of Nod2 and Rip2 modulates inflammatory gene expression profile in macrophages stimulated by heat-killed Aa

To further investigate the overall effect of Nod2 and Rip2 deletions on macrophage response to periodonpathogenic bacteria presenting both Nod1 and Nod2 ligands, we used a focused qPCR array, investigating the relative modulation of the expression of 84 genes associated with host-microbial interactions (Figure 3). There is a discernible decrease on the expression of several proinflammatory genes in Nod2KO and Rip2KO macrophages lacking Nod2 have shown a relative increase on the expression of anti-inflammatory IL-10 upon bacterial stimulation. These results were validated by RT-qPCR for IL-6 and TNF-α expression, performed using cDNA prepared from the RNA collected from the three independent experiments that was used to prepare the pooled sample used on the arrays. In these confirmatory experiment, decrease on proinflammatory gene expression reached statistical significance only for Nod2KO macrophages; even though there was a discrete decrease, particularly for the expression of TNF-α in Rip2KO macrophages.

Nod2 and Rip2 are required for maximum response of macrophages to periodontopathic bacteria: decreases of pro-inflammatory cytokine and chemokine production and of inflammation-associated signaling.

The changes on gene expression at mRNA level were also assessed at protein level using multi-ligand ELISAs, which indicated a marked decrease on the expression of various pro-inflammatory mediators and chemokines. and statistically significant decrease on the induction of proinflammatory TNF-α and IL-6 in macrophages lacking Nod2 or Rip2. Notably, IL-6 protein levels were significantly decreased in Rip2-deficient macrophages, whereas the mRNA levels were not, suggesting a possible post-transcriptional regulatory mechanism regulating production of IL-6 in these cells. The striking effect on the induction of proinflammatory genes in response to bacterial stimulation indicate a profound effect on the responsivity macrophages and point to the relevance of these genes in host-microbial interactions. Both Nod2 and Rip2-deficient macrophages showed impaired production of CCL22, which is strongly chemotatic for dendritic cells and chronically activated T cells, suggesting that lack of Nod2 and Rip2 may affect adaptive immunity by indirect and direct mechanisms. Interestingly, expression of CCL11, a chemotatic factor for eosinophils and primarily associated with allergic reaction and inflammation was impaired only in Nod2-deficient macrophages after bacterial stimulation.

Figure 3- Heat map of the expression of 84 genes associated with innate immunity in bone marrow-derived macrophages from WT, Nod2 KO or Rip2 KO mice stimulated with heat-killed *Aggregatibacter actinomycetemcomitans* for 24 h. Total RNA was harvested 6h after stimulation with the bacteria and the cDNA used in qPCR-based arrays. This data is based on the analysis of cDNA prepared from pooled RNA samples harvested from 3 independent experiments (cells obtained from a minimum of 3 animals in each independent experiment) and the experimental groups were automatically arranged by the online data analysis tool based on the pattern of gene regulation

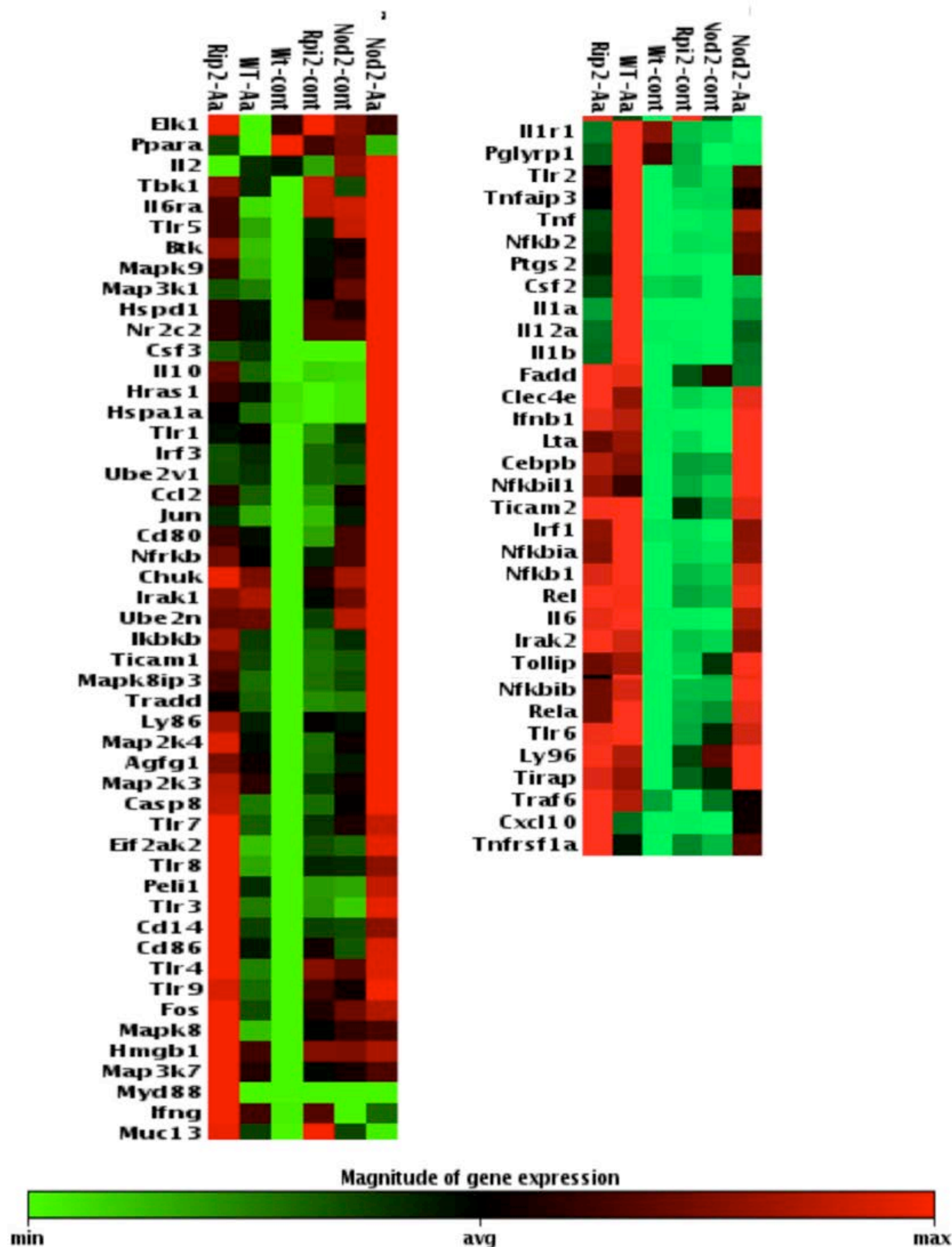
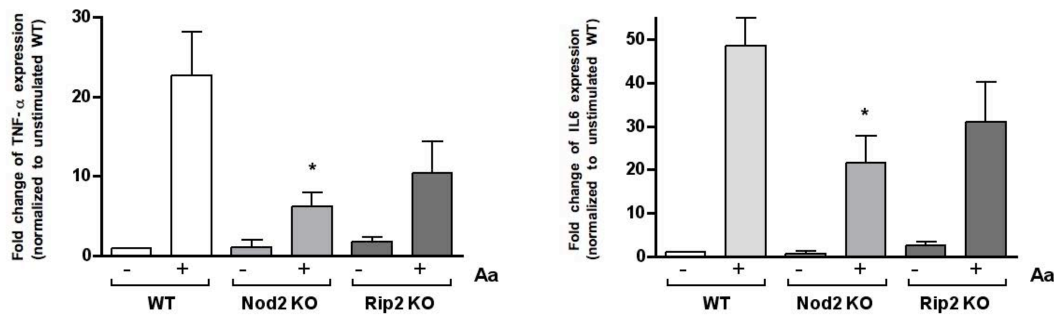


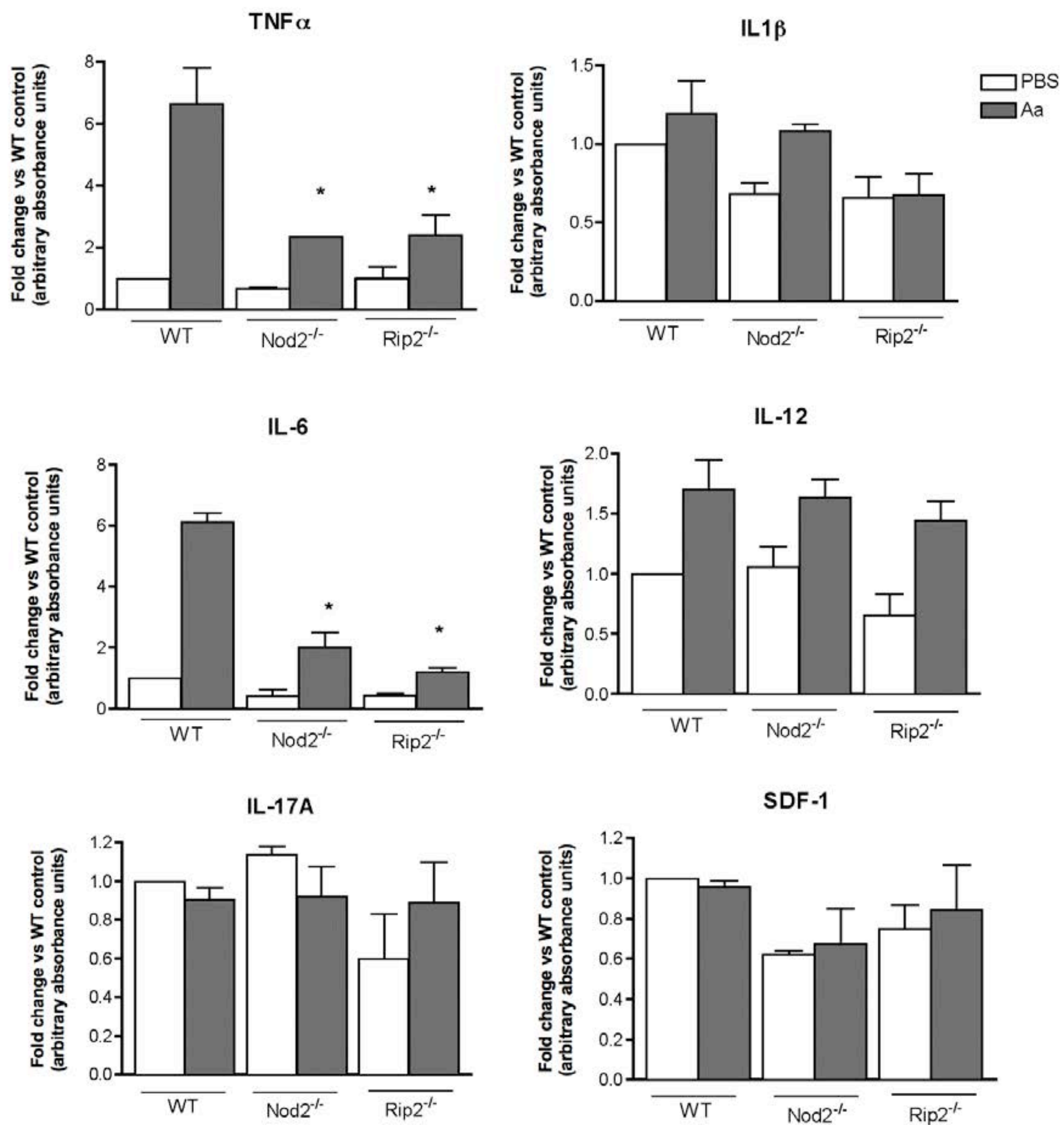
Figure 4- Validation of qPCR array data by RT-qPCR array using cDNA prepared from RNA obtained from bone marrow-derived macrophages differentiated from WT, Nod2 KO or Rip2 KO mice in three independent experiments. These were the same RNA samples combined to prepare the pool used in the array analysis. Target gene expression was normalized to beta-actin expression and fold regulation was calculated by comparison with the normalized gene expression in WT unstimulated control macrophages. Asterisk (*) indicates $p<0.05$ by unpaired Student t-tests with Welch's correction for unequal variances.



Modulation of the candidate pro-inflammatory genes and chemokines at the protein level in Nod2 and Rip2-deficient cells reached statistical significance only for TNFα, IL6, CCL22 and CCL11 (figure 5). This may be related with the attenuation of bone resorption observed in vivo, as IL-6 and TNFα are directly related with inflammatory-associated bone resorption. On the other hand, the lack of significant modulation of IL-1β, IL-12 and IL-17A in Nod2 and Rip2-deficient cells may be related with the absence of a noticeable change on the inflammation assessed histologically in vivo. These modulatory effects of Nod2 and Rip2 on inflammatory and chemokine gene expression appear to be modulated by the marked attenuation of NF-κB and p38 MAPK signaling (figure 6), two common downstream targets of PRR signaling in macrophages. The cross-talk between TLR and Jak-STAT3 signaling was demonstrated in macrophages after longer periods of stimulation (18 to 24 h), which supports and indirect mechanism mediated by autocrine activation of STAT3 by TLR-induced early response genes (e.g., IL-6) , thus our results of modulation of Aa-induced STAT3 activation in early periods (10, 30 min) of stimulation is unexpected and deserves further investigation (figure 6).

Figure 5- Deletion of Nod2 or of the downstream kinase Rip2 inhibits the production of cytokines (A) and chemokines (B) by macrophages stimulated for 24 h with heat-killed Aa. These results were obtained with multi-ligand ELISA assays using cell culture supernatants from three independent experiments. Asterisk (*) indicates $p<0.05$ in comparison with the same experimental condition in cells from WT mice by unpaired Student t-tests with Welch's correction for unequal variances.

A



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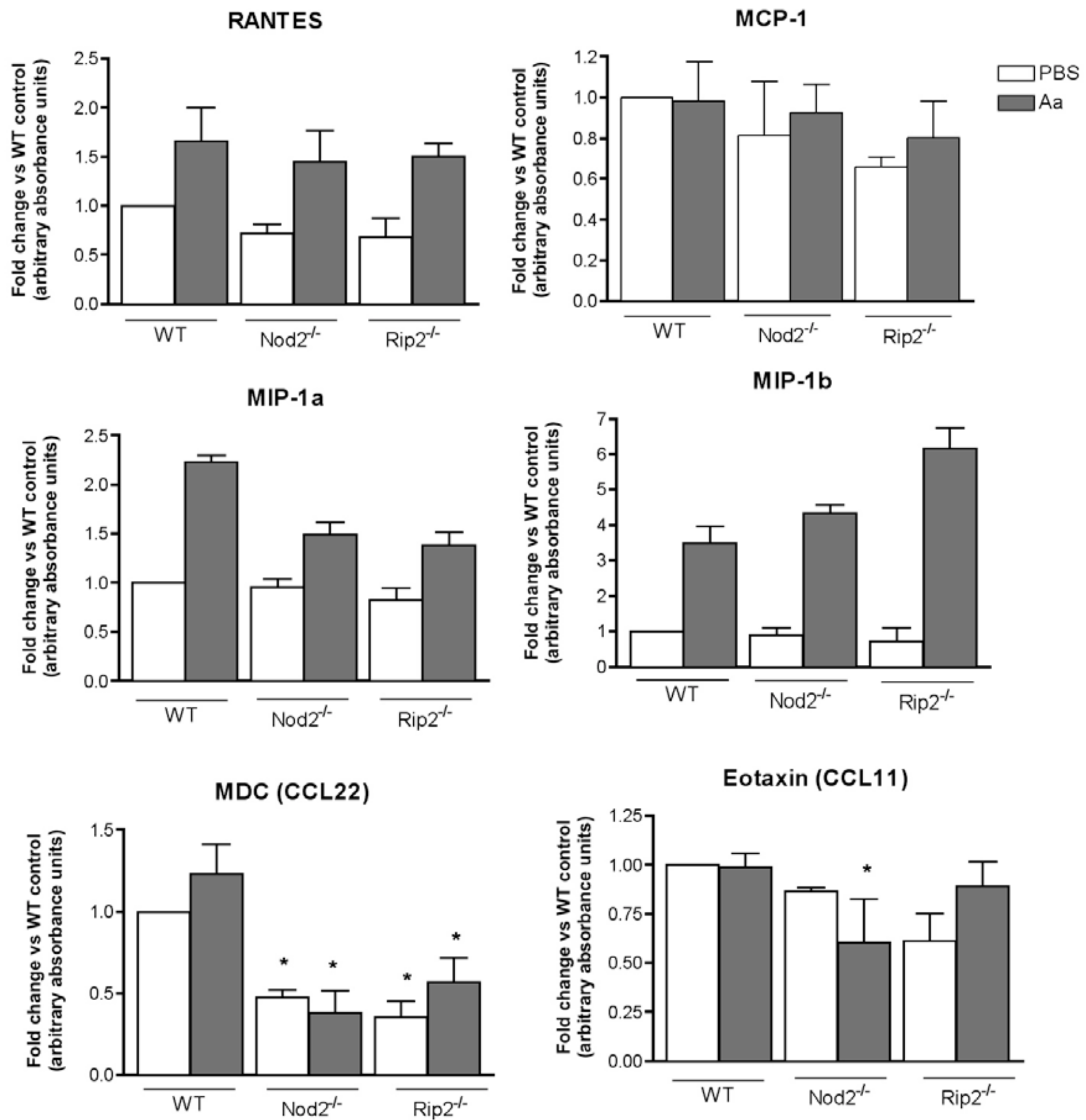
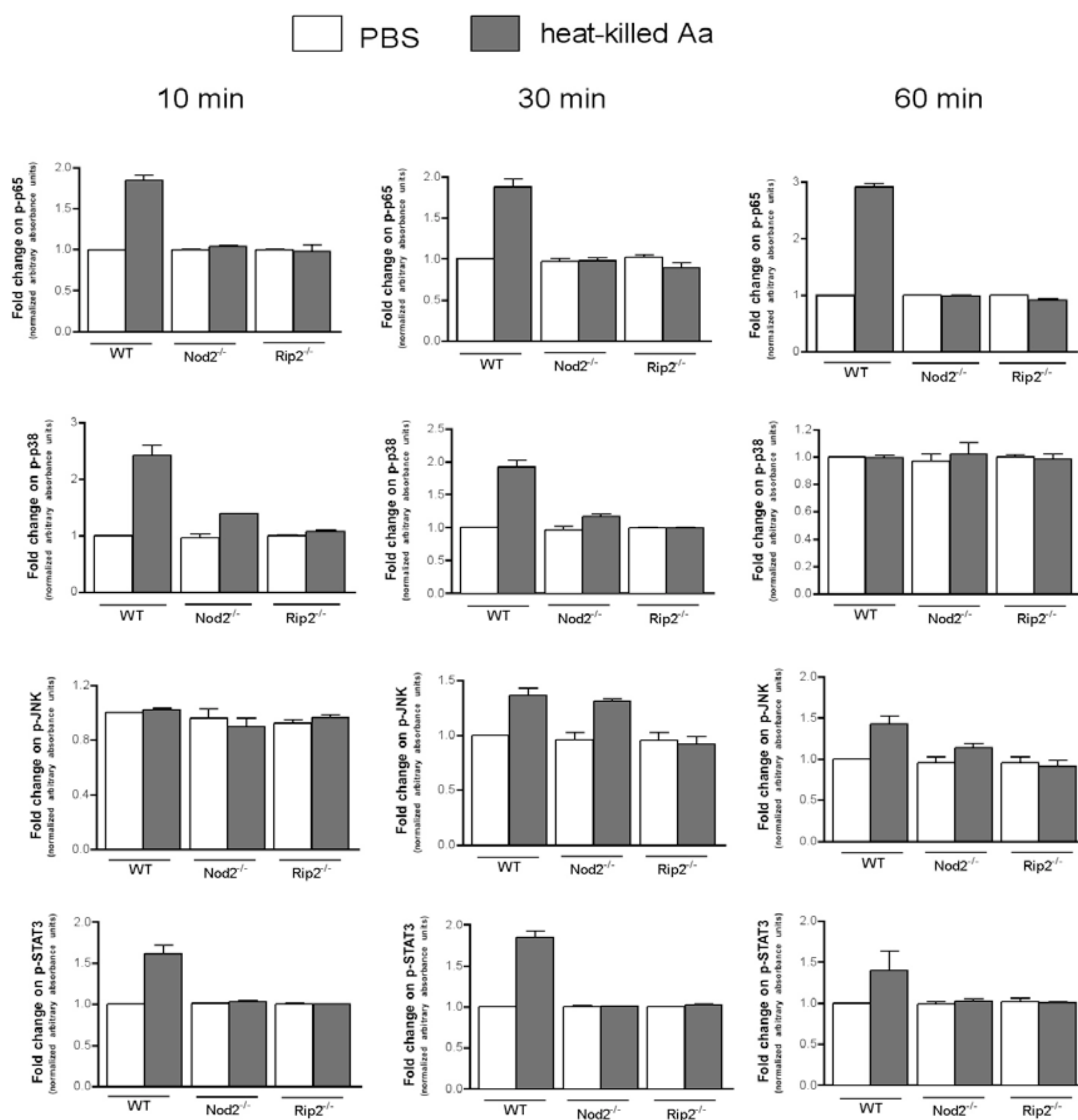


Figure 6- Nod2 and Rip2 deficiency attenuates activation of signaling pathways associated inflammatory gene expression by heat-killed Aa in macrophages. Macrophages differentiated from bone marrow of WT, Nod2KO or Rip2KO mice were stimulated with heat-killed Aa for 10, 30 and 60 min. Total cell lysates were harvested and 30 ug used in ELISA multilagnd assays to detect phosphorylated forms of p65, p38, JNK and STAT3. These results were further normalized by the expression of total p65. Data was analyzed as relative change (fold change) to WT control in each experimental period (10, 30 and 60 min). Bars indicate averages and standard deviations of duplicate measurements. Data obtained using a pool of cell lysates from three independent experiments using cells derived from 6 - 8 different animals of each genotype.



Functional clusters of genes modulated by heat-killed Aa in macrophages are similar, but in Nod2-deficient macrophages the expression of Th1-associated cytokines and TLR receptor genes are more markedly attenuated than in Rip2-deficient macrophages

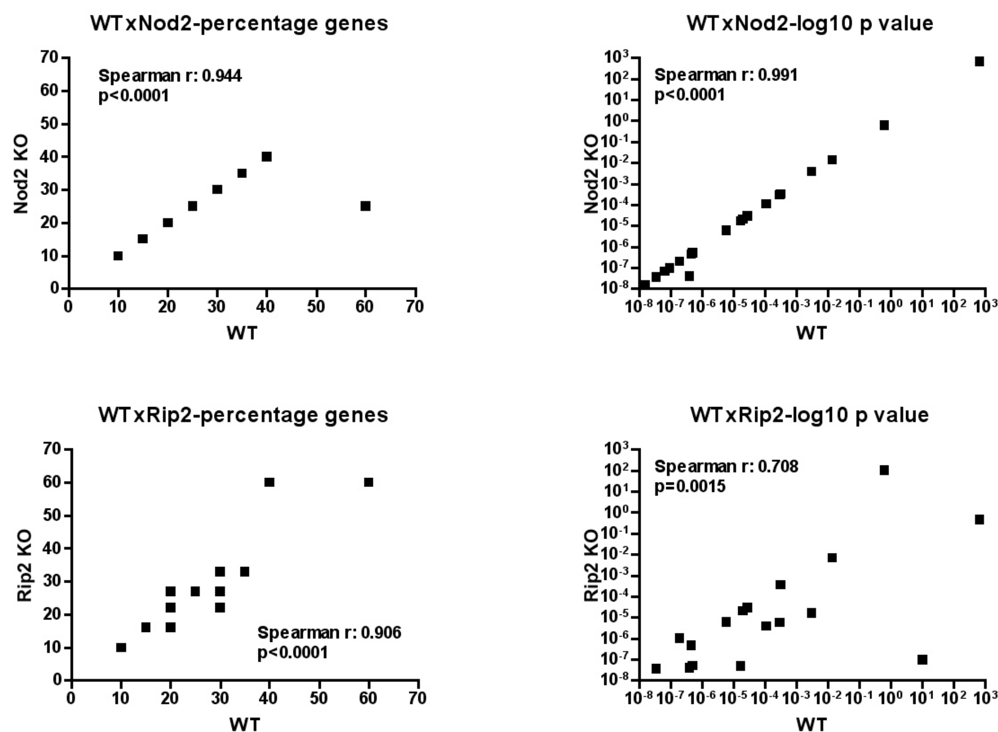
To obtain further insight into the role of Nod2 and Rip2 on macrophage response to Gram-negative bacteria, we initially assessed the overall effect of deletion of these genes by extracting terms defining biological function of the list of genes upregulated in stimulated macrophages using an online bioinformatics application (DAVID). To be considered 'upregulated', we used a minimum of four-fold increase over the expression level in unstimulated controls (or 2 fold increase on a $\log_2$ scale), which is more stringent than the guidelines for studies using treated primary cells in a microarray approach for hypothesis-generating studies (19). This stringent criterium was used due to the use of pooled samples in the qPCR array analysis to reduce Type I error, at the cost of accepting some false-negatives. In defining the functional gene clusters, DAVID parameters were also set to the highest stringency setting in order to generate less functional clusters including more tightly associated genes in each cluster. The 'score' represents the overall enrichment score for the gene cluster, with scores higher than 2 considered to be of biological significance (17, 18). To reduce the possibility of spurious associations, we have considered only the gene clusters associated with an enrichment score greater than 3. Table 1 indicates that the biological functions defined by clusters formed from the list of genes upregulated in macrophages after bacterial stimulation is exactly the same in WT and Nod2-deficient macrophages, whereas in Rip2-deficient macrophages the main functional annotations and their enrichment scores were different.

Table 1 - Comparison of Functional annotation terms and Enrichment scores for the top 5 gene clusters identified in the lists of genes up-regulated in macrophages by heat-killed Aa.

	WT / Nod2 -/-		**Rip2 -/-**	
Gene cluster	*Functional annotation (top 3)*	*score*	*Functional annotation (top 3)*	*score*
1	cytokine, cytokine activity, immune response	10.13	cytokine, cytokine activity, extracellular space	8.07
2	macrophage, four-helical cytokine, JAK-STAT signaling pathway	6.79	disulfide bond, signal	4.69
3	four-helical cytokine, JAK-STAT signaling pathway, disulfide bond	6.40	defense response, regulation of cytokine production, inflammatory response	4.15
4	macrophage, lymphokine, hematopoietic cell lineage	4.80	regulation of cytokine biosynthetic process, response to virus, immune response	4.09
5	macrophage, hematopoietic cell lineage, myeloid leukocyte differentiation	3.83	lymphokine, cytokines and inflammatory response, defense response to bacterium	3.16

Assessment of the pathways affected using the Kyoto Encyclopedia of Genes and Genomes (KEGG) also indicated a great similarity both on the percentage of genes and on the log10 of p values indicating the statistical significance included in the top 20 KEGG terms defining the functions and utilities of genes that were up-regulated by heat-killed Aa in macrophages obtained from WT, Nod2 KO and Rip2 KO mice (Figure 5).

Figure 7 - Correlations of percentage of genes included in each functional annotation term and of the enrichment statistical values obtained from DAVID Chart Reports. The correlation plots to measure the annotation agreement between genes up-regulated by heat-killed Aa stimulation in WT, Nod2 -/- and Rip2 -/- macrophages. The gene hit percentages and enrichment p values of top enriched terms between the lists of up-regulated genes show very strong correlation overall. There is a lower correlation between the data generated from WT and Rip2 -/- macrophages, suggesting that lack of Rip2 may have a more pronounced effect on the pathways modulated by heat-killed Aa than the lack of Nod2. Overall, this data indicates that heat-killed Aa induces common mechanisms/pathways in macrophages.



Since both the function of genes and the pathways modulated by heat-killed Aa in macrophages was similar for all genotypes assessed, we investigated: 1) the similarity of the genes included in the gene clusters and under the same KEGG pathway denomination term; and 2) if the potency of induction gene expression in terms of the fold-change increase in comparison to unstimulated WT macrophages was also similar. Table 2 presents the data on the fold change up-regulation of genes according to the genotype of the mice.

Table 2 - Fold up-regulation on gene expression in macrophages from WT, Nod2 KO and Rip2 KO mice after a 6h-stimulation with heat-killed Aa in comparison to WT untreated control

Gene name	WT	Nod2 KO	Rip2 KO
Ccl2	4.05		6.39
Cd14			4.00
Clec4e	4.73		5.54
Csf2	6.64	7.78	
Csf3	16.64	28.12	12.41
Cxcl10	13.91	15.36	39.12
Ifnb1	11.87	9.50	12.14
Ifng	5.69		7.89
Il10	12.36	26.95	24.99
Il12a	15.14		4.34
Il1a	83.56	12.67	16.38
Il1b	45.77	9.48	13.71
Il6	16.56	8.95	14.20
Myd88			1243.42
Nfkb2	4.94		
Nfkbia	7.93	4.07	5.32
Nfkbib	4.31		
Ptgs2	17.86	7.64	7.37
Tlr2	7.50		
Tnf	29.52	15.61	10.79
Tnfaip3	8.23		4.05
Tlr3			4.21
Tlr9			4.25

Overall, lack of Nod2 and Rip2 attenuated the expression of inflammatory cytokines, including IL-12, IL-1α and β, TNF-α, Ptgs2 (murine analog of Cox-2) and increased expression of anti-inflammatory IL-10. Interestingly, gene regulation in Rip2-deficient macrophages presented some punctual differences with Nod2-deficient macrophages that suggest an activation of a compensatory mechanism of response to microbial stimuli via TLR signaling, as suggested by upregulation of Cd14, Tlr3, Tlr9 and particularly by the strikingly potent up-regulation of MyD88 expression. IL6 and IFN-gamma expression were also more markedly inhibited in Nod2-deficient macrophages.

Interestingly, the expression of IL-1r1 (-7.23 fold decrease) and Pglyrp1 (-25.47 fold decrease) genes was inhibited after heat-killed Aa stimulation only in Nod2-deficient macrophages. Pglyrp1 is a pattern-recognizing receptor that plays a role in innate immunity, recognizing murein peptidoglycans of bacteria. This receptor has a bactericidal activity in Gram-positive and a bacteriostatic effect in Gram-negative

microorganisms. IL1r1 is a common receptor for IL-1-α, -β and -RA and its inhibition in Nod2-deficient macrophages may result in a more pronounced attenuation of inflammation.

Discussion

In this study, using in vivo and in vitro approaches we demonstrated that Nod2 has a positive modulatory role in the response to Gram-negative bacteria. For the in vitro studies, we have used macrophages derived from the bone marrow of long bones of these mice and comprehensively assessed the role of Nod2 and Rip2 on macrophage response to Gram-negative bacteria using qPCR-based focused arrays. Lack of Nod2 significantly attenuated alveolar bone resorption, whereas this effect was less marked and did not reach statistical significance in Rip2 KO mice. Inhibition of bone resorption was accompanied by a significant decrease on the number of osteoclasts in Nod2 KO mice and a less marked (non-significant) reduction in Rip2 KO mice. Nod2 ligand MDP has been shown to enhance osteoclastogenesis induced by LPS and inflammatory cytokines in co-cultures of osteoblasts and hematopoietic cells by modulating Nod2-mediated RANKL mRNA expression in osteoblasts (Yang et al., J Immunol.; v.175: 1956, 2005). This could be an explanation for the decrease on alveolar bone resorption without a marked effect on the inflammation assessed histologically. It is also possible that the modulation of selected genes, as indicated by the in vitro studies, such as TNF-α, IL-6, IL-10, IFN-δ, IL-12, IL1r1 and Pglyrp in Nod2-deficient macrophages (and to a lesser extent in Rip2-deficient macrophages) that may have occurred in the absence of obvious changes in the histological aspect of the cell infiltration. Thus, it is possible that lack of Nod2 and Rip2 affected osteoclastogenesis either directly (by affecting osteoclast precursor cells) or indirectly, by modulating expression of cytokines that are important for osteoclast differentiation. These possibilities will be investigated in subsequent studies. Importantly, the attenuation of bone resorption in our loss-of-function model using Nod2 KO mice is supported by a recent study in which Nod2 agonists were introduced in vivo (in a gain-of-function model) and caused an increase in bone resorption (12). Interestingly, a recent publication did not find any role for Nod2 on bone resorption in another model of experimental periodontal disease. Using the ligature-induced model in mice, Jiao et al. (20) show reduced alveolar bone loss in Rip2 KO mice, but not in Nod2 KO mice. The authors report a decrease on neutrophil

infiltration in Rip2 KO mice only, associating these effects to the increased prevalence of an endogenous Gram-negative bacterium (NI1060) with great genetic similarity to *A. actinomycetemcomitans*. The obvious difference in the experimental model (ligature for 10 days, versus sustained stimulation with heat-killed bacteria over 3 weeks) is demonstrated by the markedly greater severity of bone loss in the ligature model, which may be representative of a more acute inflammatory response than in our exogenous, heat-killed bacteria injection model. Moreover, we have not assessed the potential role of the endogenous microbiota, but we assume that since we injected the microorganisms their role on our results, if any, was minimal.

Rip2 is a kinase that is a common dowstream target of both Nod1 and Nod2. The differences between the outcomes assessed in the absence of Nod2 and Rip2 both in vivo and in vitro may be related with an associated impairment of Nod1 function in Rip2-deficient cell and animals. In general, attenuation of bone resorption and macrophage response was less pronounced in the absence of Rip2, which may be due to a compensatory activation of inflammation/immune response pathways due to the more severe inhibition of innate immune response. On the other hand, inhibition of Rip2 kinase decreases production of PGE_2 in human monocytes by inhibiting Cox-2 gene expression (21), which agrees with our results for Ptgs2 gene expression (the murine homolog of Cox-2) in murine macrophages, and is consistent with an inhibition of bone resorption (22). However, the magnitude of Ptgs2 inhibition we observed was nearly identical in Nod2 and Rip2-deficient cells, suggesting that this decrease is not the main responsible for the differences in attenuation of bone resorption between Nod2 KO and Rip2 KO mice in vivo.

It is important to consider that the data include the assessment of gene expression at the mRNA level, which does not necessarily reflect the protein level of the cytokines and enzymatic products (such as prostaglandins). Moreover, we used a qPCR focused array approach, which assesses a limited number of genes in comparison to a microarray approach; however this approach has the advantage of focusing on genes directly related with host-microbial interactions. The fact that we used a pooled sample from three independent experiments (each including cells from 3 - 4 mice) is a limitation; however we validated the array data by RT-qPCR using cDNA prepared from the same RNA samples used to prepare the pool. We consider that by pooling equal quantities of total RNA from three independent experiments (each experiment including cells obtained from multiple mice) we included the inherent experimental variability into the

data and used stringent criteria (consistent with an hypothesis-generating study) to select genes considered as up- or down-regulated, moreover the qPCR array data was validated by RT-qPCR using cDNA prepared from the RNA samples isolated from the individual experiments. IL-6 and TNFα cytokine production by Aa-stimulated macrophages was significantly inhibited in both Nod2- and Rip2-deficient cells, whereas IL-1β and SDF-1 production was discretely inhibited in Rip2- and Nod2-defficient cells, respectively. Modulation of gene expression at the mRNA and protein level is likely to be dependent on the marked inhibition of the activation of NF-kB and p38 MAPK. Surprisingly, our data indicate that Aa-induced activation of STAT3 was completely blocked in Nod2 and Rip2-deficient cells, which is unexpected as JAK-STAT is not a direct target of TLR/PRR signaling and suggests a role for Nod2/Rip2 in cross-talk leading to activation of this signaling pathway.

In any case correspondence between in vitro and in vivo data has to be considered with caution; in the in vitro studies we used only macrophages, as professional antigen-presenting cells and a major cell type in inflammation and innate immunity; but the participation of multiple cell types in vivo contributing to the cytokine network may result in distinct regulation of cytokines. Nod2 and Rip2 may also have important roles in other cell types, both from the innate and adaptive immunity and we have ongoing studies assessing the relevance of these genes in the regulation of inflammatory gene expression by lymphocytes. Interestingly, Nod2 and Rip2 significantly inhibits the expression of CCL22, a ligand for CCR4 with specific chemotactic activity for monocytes, dendritic cells and activated T cells of Th2 phenotype (23), suggesting that Nod2 and Rip2 may influence the adaptive immune response, possibly altering the Th-type response. CCL11 is classically associated with allergic response as it is chemotactic for eosinophils but not for neutrophils or mononuclear cells (24). TLR agonists were shown to induce its expression in in vivo models of sepsis (25); and we observed a significant attenuation of CCL11 production only in Aa-stimulated Nod2-deficient, but not in Rip2-deficient, macrophages. In fact, heat-killed Gram-negative bacteria inhibited expression of CCL11 by human airway smooth muscle cells, which may be a mechanism for the effects of bacterial immunotherapy for allergic lung conditions (26). However, these authors suggested that the inhibition of CCL11 was mediated by bacterial DNA and not by TLR4 or Nod receptors, which is contradictory to our data. NF-kB and GATA-3 are the major common transcription factors with binding sites in the promoters of both CCL22 and CCL11, indicating that NF-kB could

be an important pathway affected by the deletion of Nod2 gene in macrophages. The possible consequences of the modulation of chemokine expression on the adaptive immunity would only be perceived in vivo; and future studies will address the role of Nod2 in the nature of the adaptive immune response.

Nod2 was initially described as a cytosolic receptor, functioning as an intracellular PRR sensing bacterial peptidoglycan in innate immune cells. The basic assumption is that the ligands have to gain access to the cytosol to activate Nod2, which may happen by active fagocytosis or by invasion of the host cells by the bacteria. In this study we used heat-killed Gram-negative bacteria, which included many MAMPs that can activate multiple PRRs, including Nod1 and Nod2. In our model, activation of Nod2 and Rip2 may occur by three possibilities: 1) fagocytosis of heat-killed bacteria by the macrophages with internalization of the ligands; 2) indirect activation by cross-talk with TLR-mediated activation of intracellular signaling pathways; 3) indirect activation by autocrine or paracrine effects of cytokines produced by the activation of membrane-bound TLR by the bacterial antigens. We have not addressed which of these possibilities would be involved in Nod2 and Rip2 activation in macrophages, since our experimental model aimed to describe the role of Nod2 and Rip2 in macrophage response to Gram-negative bacteria, which may activate multiple TLRs and also Nod1 and Nod2. This type of microorganism is a common cause of various chronic infections, including periodontal disease. The data obtained indicated an important role for both Nod2 and Rip2 on gene expression in macrophages and for Nod2 in bone resorption in vivo and further exploration of the biological mechanisms may provide insight for therapeutic perspectives based on the modulation of Nod2 activation in conditions characterized by host-microbial interactions.

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4 CAPÍTULO 2

4 CAPÍTULO 2

Role of Nod1 in the modulation of host-microbial interactions in the periodontal disease model

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Abstract

Periodontal diseases are chronic inflammatory conditions characterized by destruction of non-mineralized and mineralized connective tissues. They are initiated and maintained by a shift of the microbial composition of the bacteria of the biofilm adjacent to teeth towards increased prevalence of Gram-negative microorganisms. Nod1 is a member of the Nod-like receptors (NLRs) family of proteins that participate in the activation of the innate immune response to invading bacteria or to bacterial antigens present in the cytoplasm. The specific ligand that activates Nod1 is a bacterial peptidogycan structure derived primarily from Gram-negative bacteria. We used mice with whole-genome deletion of Nod1 gene in a microbial-induced periodontal disease model using direct injections of heat-killed Gram-negative or Gram-negative/Gram-positive bacteria on the gingival tissues for 3 weeks to assess the role of Nod1 in tissue destruction associated with inflammation induced by host-microbial interactions. In vitro experiments using primary bone marrow-derived macrophages from WT and Nod1KO mice provide insight into the role of Nod1 on the macrophage response to Gram-negative and Gram-negative/Gram-positive bacteria. uCT analysis indicated that deletion of Nod1 significantly aggravated bone resorption induced by Gram-negative bacteria, which was significantly attenuated by the association with Gram-positive bacteria. This effect was accompanied by changes in the osteoclast numbers. In vitro, stimulation of macrophages with heat-killed Gram-negative bacteria induced the same biological processes in WT and Nod1-deficient cells; however there was a distinct increase on the expression of pro-inflammatory mediators, assessed by qPCR arrays, which was not observed at the protein level, as assessed by multi-ligand ELISAs. The data indicate that deletion of Nod1 aggravates bone loss by modulating osteoclast differentiation in vivo. Regulation of cytokine expression in concert with TLR activation is complex and likely involves post-translational mechanisms. Both in vivo and in vitro outcomes were attenuated by concomitant stimulation with Nod2-activating Gram-positive bacteria.

Introduction

Periodontal diseases are chronic inflammatory conditions initiated and maintained by a highly complex microbiota organized in a biofilm present adjacent to the teeth that represent the most prevalent lytic disease of bone humans (1). Besides the obvious relevance for the oral health as a major cause of tooth loss, accumulating evidence indicates that this long-persistent inflammation caused by host-microbial interactions in the surroundings of teeth may be influencing other systemic conditions of great morbidity, including diabetes, cardiovascular diseases/atherosclerosis and rheumatoid arthritis. Moreover, knowledge derived from the study of experimental periodontal disease models can provide relevant information on host-microbial interactions and the regulation of immune response.

The identification and study of pathogen-recognition receptors (PRRs) in the past 15 years has led to a great advance in the understanding of the mechanisms by which the host response recognize microbial-derived molecular patterns (MAMPs), including antigens and virulence factors. Importantly, this knowledge has been continuously increased with the identification of mechanisms and novel functions for the PRRs, including the sensing of host-derived damage-associated molecular patterns (DAMPs). Nod1 (*Card4*) and Nod2 (*Card15*) were the first described and remain the most studied PRRs from the Nucleotide-binding domain and Leucine-rich repeat-containing Receptor (NLR) family that includes 22 genes in humans and over 30 genes in mice (2, 3). Nod1 belongs to the NLR*C* family of NLRs, characterized by a Caspase-activating and recruitment domain (CARD) at the N-terminal. Initially described as a cytosolic sensor of innate immune cells that recognize peptidoglycan, more specifically D-glutamyl-meso-diaminopimelic acid (DAP) present in most Gram-negative bacteria and a few ram-positive bacteria including *Bacillus* and *Listeria* (4); however recent evidence suggests that Nod1 activation may be triggered by other non-bacterial and host-derived ligands and affect the autophagy and adaptive immune response (5, 6); as well as possibly playing a role in conditions such as asthma and inflammatory bowel diseases (7).

There is scarse information on the role of Nod1 in modulating host-microbe interactions in periodontal disease, but activation of Nod1 and Nod2 by direct injections of *E.coli*- or *S. aureus*-derived peptidoglycan synergistically enhanced LPS-induced alveolar bone loss in mice in vivo and also RANKL-induced osteoclastogenesis in vitro (8); whereas

recently Nod1 KO mice were shown to be less susceptible to ligature-induced periodontal disease. In this study we assessed the effect of whole-genome deletion of Nod1 on inflammation and bone resorption induced by direct injections of heat-killed Gram-negative bacteria associated with periodontal disease in humans alone or in association with heat-killed Gram-positive bacteria that are usually considered commensal, non-disease associated bacteria. We further explore the role of Nod1 in modulating macrophage responses to these microorganisms.

Material and methods

Animal use

We used wild-type (WT) C57/BL6 mice and Nod1 knockout mice backcrossed to C57/BL6 background for eight generations (9, 10). All animals used in these studies were between 8 and 10 weeks of age. Sacrifices were always performed by CO_2 inhalation and the Institutional Committee on the Use of Experimental Animals approved the study protocol. Primary bone marrow-derived macrophages (BMDM) were obtained from bone marrow flushed from tibias and femurs, differentiated and expanded in the presence of M-CSF, as previously described (11). Host-microbial interactions in vivo were studied using a microbial-induced model of experimental periodontal disease. In this model, 3μL of a suspension of PBS containing 10^9 CFU/mL of heat-killed Gram-negative *Aggregatibacter actinomycetemcomitans* (Aa, JP1 serotype) bacteria associated with periodontal disease combined or not with 10^9 CFU/mL of Gram-positive *Lactobacillus fermentans* (Lf) was directly injected into the palatal gingival tissues surrounding the upper molar teeth of the mice, three times/week for 3 weeks. These in vivo studies used 14 WT and 14 Nod1KO mice. Sacrifice was performed 2 days after the last injection. Six mice of each genotype were vehicle controls and received bilateral injections of sterile PBS in the palatal aspect of upper first molars; whereas 8 mice received injections heat-killed bacteria.

Assessment of alveolar bone loss and inflammation

Immediately after sacrifice, tissue blocks including the upper molars and surrounding tissues were carefully dissected from the animals, rinsed in PBS and fixed in 4% paraformaldehyde for 18 h at 4C. These samples were then rinsed in distilled water, transferred to 70% ethanol and maintained at 4C. uCT scanning of these samples was done on a Skyscan (Skyscan, Aartselaar, Belgium) at a resolution of 18 um and

tridimensional images reconstructed, spacially re-oriented in a standardized orientation and analyzed using the equipment's software (NRecon/DataViewer/CTan/CTvol, Skyscan, Aartselaar, Belgium). A standardized region of interest (ROI) of 2.5 mm^3 was positioned on the tridimensional images using anatomical landmarks as reference points, and the fraction of the volume of the ROI occupied by mineralized tissue (BV%) was be determined using a standard threshold for detection of mineralized tissues. Considering that the variation in the volume of similar tooth roots in different animals is negligible, a decrease in the BV% in the ROI indicates bone loss.

After scanning, the same tissue blocks used on the uCT analysis were decalcified in 0.5 M EDTA (pH 8.0) and submitted to routine processing for paraffin embedding. 5 um thick, semi-serial sections were obtained on the mesio-distal plane and stained with hematoxylin-eosin for descriptive assessment of inflammation an experienced examiner blinded to the experimental groups according to a severity score system ('0'-no significant inflammation; '1'-mild inflammation; '2'-moderate inflammation; '3'-severe inflammation). A minimum of 6 equally-spaced semi-serial sections spanning 500 µm of the bucco-lingual diameter of the specimens. Sections from 3 to 4 different animals of each genotype were assessed. Scorings were performed three times with a minimum interval of 2 weeks between the assessments and the most prevalent score was used. Immunohistochemical detection of TRAP was performed using a goat polyclonal antibody (sc-30833, Santa Cruz Biotechnology) and an biotin-streptavidin-DAB visualization system (LSAB2+, Dako USA). A minimum of 6 equally-spaced semi-serial sections of each experimental conditions (PBS, Aa or Aa+Lf injections) from 3 different animals of each genotype were stained. TRAP+ cells containing 2 or more nuclei present in the vicinity of the bone surface were considered osteoclasts. The number of osteoclasts in a linear extension of 400 um from the palatal aspect of the first molar by a trained examiner blind to the experimental groups.

In vitro studies

BMDM ($1x10^6$ cells/well in 12-well plates) were grown overnight in RPMI1640 supplemented with penicillin/streptomycin and 10% heat-inactivated FBS. After a 6-hour de-induction in medium containing 0.2% heat-inactivated FBS these cells were stimulated with $1x10^6$ UFC/mL of heat-killed Aa (1:1 ratio bacteria:cells) for 6 (RT-qPCR arrays) and 24 (multi-ligand ELISAs) hours. Negative controls were treated with the same volume of PBS vehicle used to resuspend the bacteria. A total of 6 samples

(unstimulated, Aa- and Aa+Lf-stimulated for WT and Nod1 -/- cells) were obtained from each experiment. Three independent experiments were performed, each one using cells obtained from 2 to 4 mice of each genotype.

RT-qPCR and qPCR arrays

Total RNA was harvested harvested 6 hours after stimulation with the bacteria or vehicle using an affinity column system (RNeasy micro, Qiagen), and 6 pools of RNA were prepared by combining 300 ng of total RNA obtained from each sample (unstimulated or Aa-stimulated BMDM from WT, Nod2 KO and Rip2 KO mice) in each independent experiment. cDNA was synthesized from each of these six 900 ng pools of total RNA using the reagents and procedure indicated by the supplier of the PCR-based arrays (RT^2 First Strand cDNA kit, SABiosciences/Qiagen). Expression of 84 genes related with innate immunity in each sample was investigated using qPCR-based arrays (RT^2 $Profiler_{TM}$ PCR Array Mouse Toll-Like Receptor Signaling Pathway, SABiosciences/Qiagen) performed according to the instructions of the supplier on a StepOne Plus qPCR thermocycler (Applied Biosystems) using the indicated cycling conditions (10 min/95C, followed by 40 cycles of 15s/95C and e 60s/ 60C). Analysis was carried out using the ΔΔCt method initially using the thermocycler's software; and subsequently with an online tool provided by the supplier of the array. Normalization was performed using the expression of GAPDH, Beta-actin and beta-glucuronidase. These genes were automatically selected by the online analysis tool based on the panel of 6 housekeeping genes included in the array. The purpose of the analysis was to assess the relative regulation of the 84 target genes in comparison to the gene expression determined in unstimulated macrophages from WT mice.

Multi-ligand ELISAs

The conditioned culture media collected 24 h after stimulation of BMDM with heat-killed *A. actinomycetemcomitans* associated or not with heat-killed *L. fermentans,* or with the same volume of PBS diluent was aliquoted and stored at -80C until use. Each aliquot was thawed on ice only once and immediately before its use in multi-ligand ELISAs that allow the detection of 6 cytokines and 6 chemokines associated with host-microbial interactions and chemotaxis of immune cells (SA Biosciences/Qiagen). The concentration of total protein in the conditioned media was initially determined by a

Bradford assay and then the same quantities of total protein from each experimental condition was used in the ELISAs, normalizing the results and allowing for a comparison of the relative quantities of the different cytokines in each sample. Activation of intracellular signaling pathways associated with inflammatory gene expression was also determined using multi-ligand ELISAs (Cell Signaling). For these experiments, stimulation of BMDM with Aa or the same volume of PBS was performed for 10, 30 and 60 min. Cell lysates from three independent experiments (using cells from 4 - 6 mice) were harvested and pooled according to the experimental conditions (PBS, Aa or Aa+Lf stimulation) and genotype by combining 10 μg of total protein from each experiment. Data was analyzed as relative changes to unstimulated control macrophages with the same genetic background (WT and Nod1 KO) in each period. The data in these experiments was also normalized by using the same quantity of total protein and also by the expression of total p65, as recommended by the supplier of the assays.

Data analysis

The statistical analysis aimed at comparing the results obtained with each experimental conditions (control/unstimulated, Aa-only stimulation and Aa+Lf stimulation) between different genotypes (WT and Nod1 KO) in instances where at least three data points from independent experiments were available. These comparisons were done using non-paired t-tests with Welch's correction for unequal variances, assuming complete independence between the results of the two genotypes. For these analyses, statistical significance was set at 95% (* $p<0.05$). The qPCR focused array data obtained with pooled samples was analyzed using an online bioinformatics tool, DAVID (Database for Annotation, Visualization and Integrated Discovery - http://david.abcc.ncifcrf.gov) (12, 13), in an exploratory manner consistent with an hypothesis-generating study (14). The purpose of these analyses was to assess how the gene functional clusters that were upregulated in macrophages stimulated with heat-killed bacteria were affected by deletions of Nod1 gene.

Results

Increased inflammation and bone resorption upon stimulation with Gram-negative bacteria in Nod1 KO mice. Concomitant stimulation with Gram-positive bacteria attenuates inflammation and bone resorption

In the Gram-negative bacteria-induced experimental periodontal disease model, deletion of Nod1 significantly accentuated alveolar bone resorption; which is supported by a significant increase of osteoclast numbers (Figure 1) and an aggravation of inflammation observed histologically (Figure 2). However, the simultaneous stimulation with Gram-positive bacteria in Nod1 KO mice significantly attenuated bone resorption, inflammation and also reduced osteoclast numbers. Considering that Nod2 is present in Nod1 KO mice, and also that its ligand, muramyl-dipeptide (MDP), is present in most bacteria, particularly Gram-positive, this attenuation effect may be the result of Nod2 activation.

Figure 1- Alveolar bone resorption induced by injections of heat-killed *Aggregatibacter actinomycetemcomitans* is significantly increased in Nod1 KO mice. The concomitant injection of Gram-positive bacteria in the gingival tissues attenuates bone loss (A) and reduces osteoclast numbers (B) The images are representative of tridimensional reconstructions of mineralized tissues in the hemi-maxillae segments. The graph represents the average and standard deviation of the relative reduction in mineralized tissue content (BV fraction) in the standardized region of interest (ROI) assessed in comparison to the BV fraction of the ROI in WT control samples (set to 100%). Asterisk (*) indicates significant difference between the indicated pairs of bars by unpaired Student t-tests with Welch's correction for unequal variances. Samples from at least 6 different animals were analyzed for each group. (B) Immunohistochemical staining for TRAP to identify osteoclasts in the region of disease induction. Representative low and high-magnification images of sections from animals of each genotype and experimental group. The graph depicts the results of osteoclast numbers according to the genetic background and presence or absence of disease induction. Plus sign (+) indicates significant difference (unpaired Student T-test with Welch's correction for unequal variances) in comparison with osteoclast number in sections from control (PBS-injected) WT mice. Asterisk (*) indicates significant difference between the indicated pairs of bars (unpaired Student t-test with Welch's correction for unequal variances).

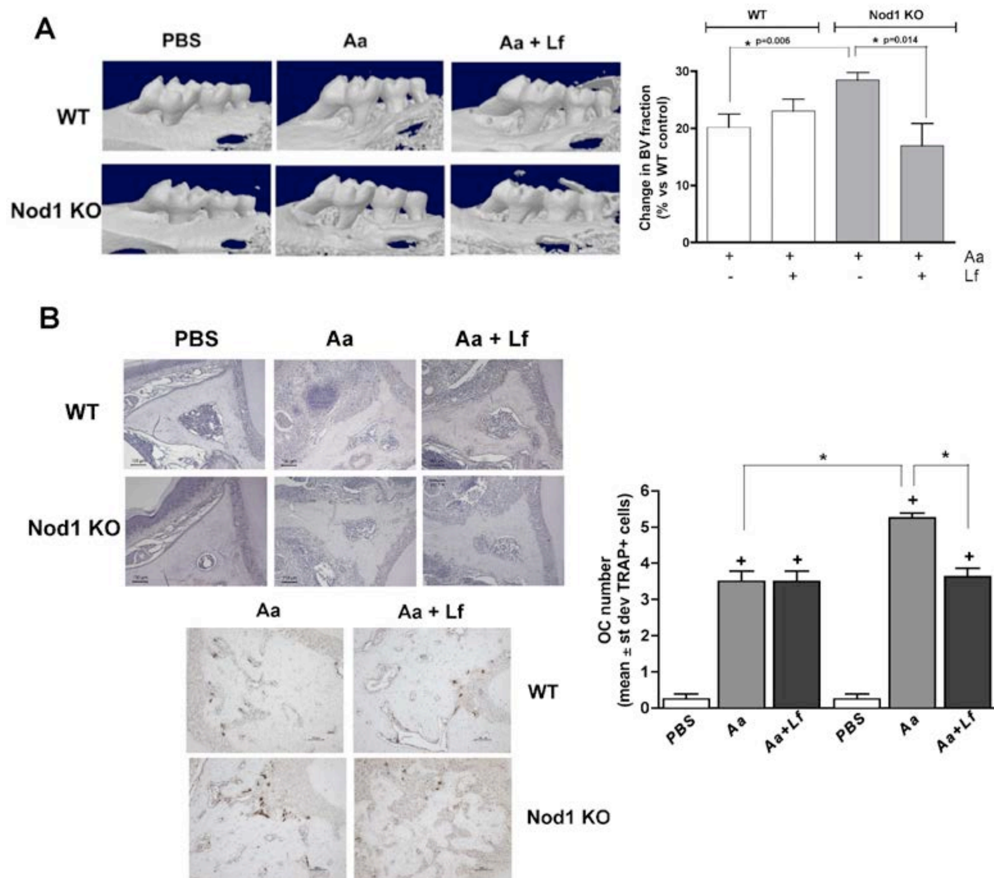


Figure 2- Representative images of 5 um H/E-stained sections in the bucco-lingual (frontal) plane depicting the palatal aspect of upper first molars, according to the genotype of the animal and experimental condition (PBS, Aa- or Aa+Lf injections). (A). Bar graph presents the assessment of inflammation (cellular infiltrate) severity by the scoring system according to the genotype and experimental condition. Asterisk (*) indicates significant difference between the indicated pair of bars by unpaired Student t-test with Welch's correction for unequal variances (B). Samples from at least 6 different animals were analyzed for each group and these representative images were obtained at 100X magnification.

A

Control (WT)
(PBS injections)

Aa

Aa
+
Lf

WT

Nod1 KO

B

* p=0.015

Severity of cell infiltrate (Score)

0
1
2
3

Control
Aa
Aa+Lf
Aa
Aa+Lf

WT

Nod1 KO

Deletion of Nod1 reduces TNF-α and CCL22 production and inflammation-associated signaling in bacteria-stimulated macrophages.

Based on the bone resorption data in vivo, we expected that deletion of Nod1 would markedly enhance the expression of inflammatory markers. We used multi-ligand ELISAs to assess the effect of Nod1 on the production of inflammation-associated chemokines, cytokines and the activation of signaling pathways. Surprisingly, there was a decrease on the expression of both proinflammatory TNF-α (Figure 3A) and activated T lymphocyte-attracting chemokine CCL22 (Macrophage-Derived Chemokine, MDC) (Figure 3B). Moreover, deletion of Nod1 markedly attenuated the activation of inflammation-associated signaling pathways in bacterial-activated macrophages, including STAT3, p38 MAPK and NF-kB (Figure 4).

Figure 3- Deletion of Nod1 attenuates the production of cytokines (A) and chemokines (B) by macrophages stimulated for 24 h with heat-killed Gram-negative Aa alone or associated with Gram-positive Lf. Production of TNFα and of the chemokine Eotaxin (CCL11) were significantly reduced in Nod1-deficient macrophages stimulated with Aa+Lf, whereas MDC (CCL22) was significantly inhibited only after stimulation with Aa. These results were obtained with multi-ligand ELISA assays using cell culture supernatants from three independent experiments. * indicates $p<0.05$ and ! indicates $p<0.10$ by unpaired t-tests with Welch's correction for unequal variances.

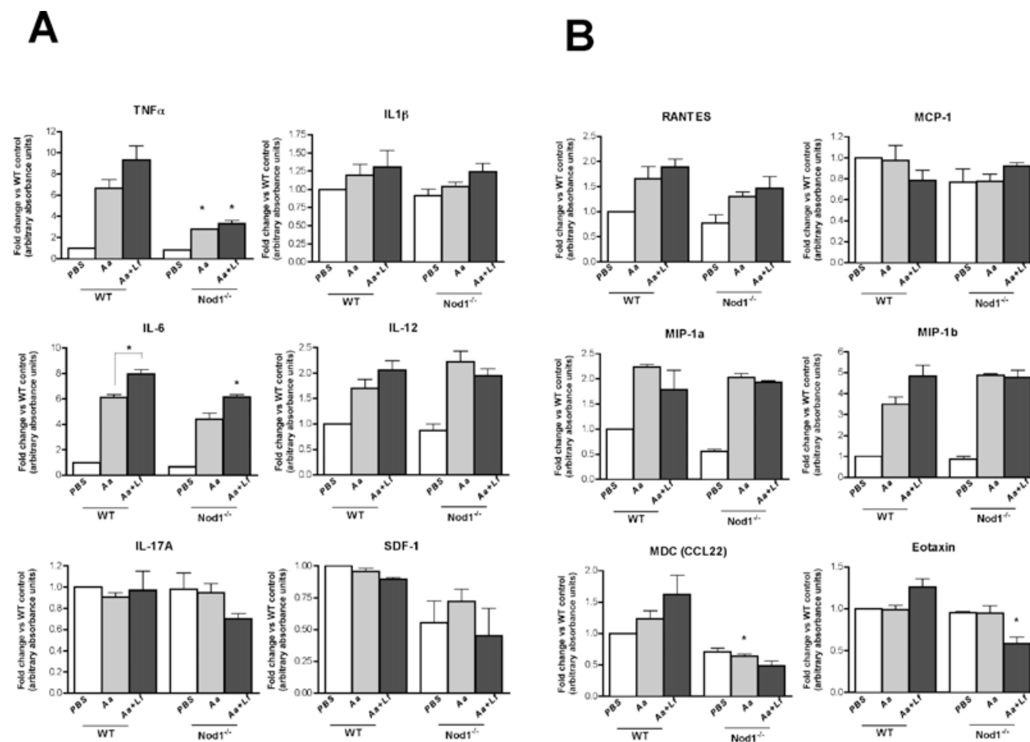
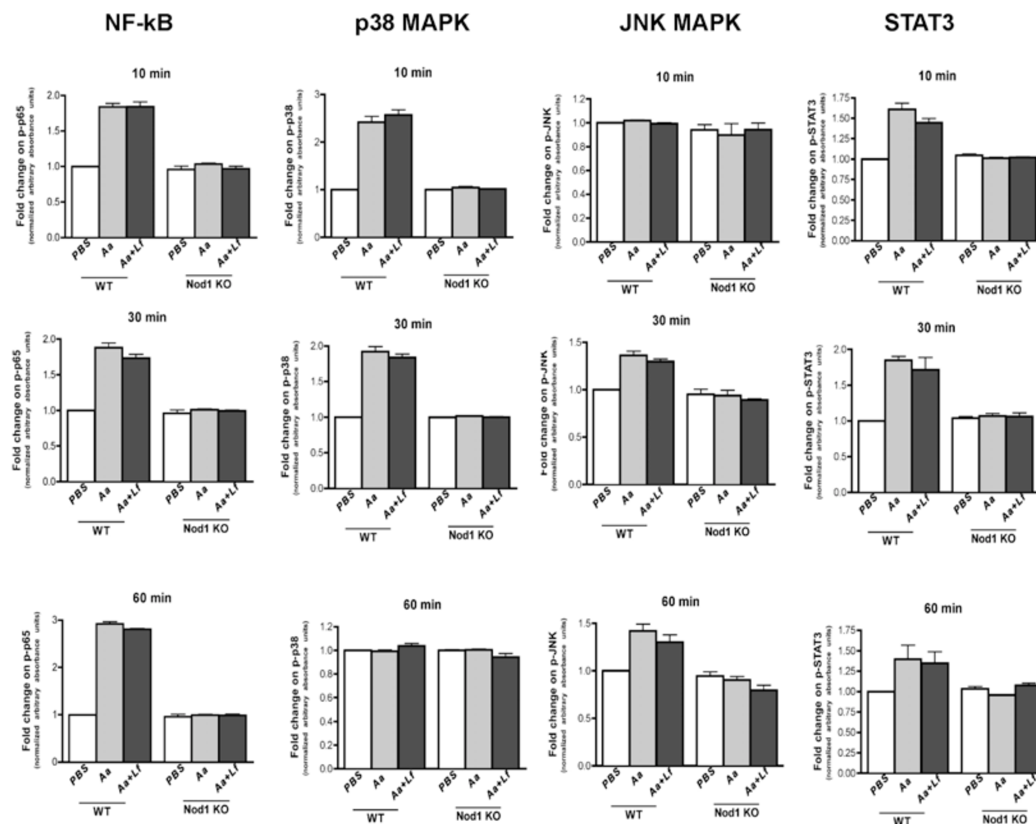


Figure 4- Nod1 is required for the microbial-induced activation of various inflammation-associated signaling pathways in macrophages. There was a nearly complete inhibition of activation of NF-kB (p-p65), p38 and JNK MAPK, and surprisingly of STAT3 in Nod1-deficient macrophages stimulated with Aa or with Aa+Lf. Data was analyzed as relative change (fold change) to WT control in each experimental period (10, 30 and 60 min). Bars indicate averages and standard deviations of duplicate measurements. Data obtained using a pool of cell lysates from three independent experiments using cells derived from 6 - 8 different animals of each genotype.



Lack of Nod1 increased mRNA expression of proinflammatory genes in bacteria-stimulated macrophages. Concomitant stimulation with Gram-positive bacteria increased expression of IL10 mRNA and attenuated expression of macrophage activation-related genes

To further investigate the overall effect of Nod1 deletion on macrophage response to periodontopathogenic and non-disease associated Gram-positive bacteria presenting both Nod1 and Nod2 ligands, we used a focused qPCR array, investigating the relative modulation of the expression of 84 genes associated with host-microbial interactions. Interestingly, unstimulated Nod1 -/- cells presented increase expression of TLR4, 5, 7, 8

and 9 (but not of TLR1, 2, 3, and 6) in comparison with unstimulated WT macrophages. Besides PRRs, expression of Ly86 and Ly96, which are important for macrophages activation by TLR4/LPS, were also upregulated in unstimulated Nod1 -/- macrophages (Figure 5). It is possible that this represents a compensatory mechanism to augment the responsiveness of macrophages as innate immune cells.

Upon stimulation with Gram-negative Aa, in comparison with WT macrophages Nod1-deficient cells showed a clear increase on the expression of several proinflammatory genes, including macrophage activation-related genes (CD80, CD14, Csf2) NF-kB signaling-related genes (Nfkb1, Nfkb2, Nfkbia, Nfkbib, Rel) and inflammatory cytokines (IL1b, Tnf, IL12a) (Figure 5). Interestingly, concomitant stimulation of Nod1-deficient macrophages with Gram-positive and Gram-negative bacteria attenuated the expression of some pro-inflammatory genes (IL12a, IL1a), whereas the expression of other inflammatory genes remained upregulated (IL1b, TNF) or was even further upregulated in comparison to Gram-negative simulation alone (Ptgs2, IL6). Notably, concomitant stimulation of Nod1 -/- macrophages with Gram-negative and Gram-positive did increase the expression of anti-inflammatory cytokine IL10 and attenuated the expression of some macrophage activation-related genes (CD80, Csf2) in comparison to Nod1 -/- macrophages stimulated with heat-killed Aa only (Figure 5, Table 2); but the expression of NF-kB signaling-related genes was not modulated in comparison with Gram-negative stimulation alone. These results were validated by RT-qPCR for IL6 and TNF-α expression, which was performed using cDNA prepared from the RNA collected from the three independent experiments that was used to prepare the pooled sample used on the arrays. In this confirmatory experiment, TNF-α and IL6 expression were significantly increased after Aa and Aa+Lf stimulation; however in spite of a noticeable increase of TNF expression in Nod1-deficient macrophages in comparison to WT cells, this change was not statistically significant.

Figure 5- Heat map of the expression of 84 genes associated with innate immunity in bone marrow-derived macrophages from WT and Nod1 KO mice stimulated with heat-killed *Aggregatibacter actinomycetemcomitans* (Aa) alone or associated with Gram-positive *Lactobacillus fermentans* (Lf) for 6h. Total RNA was harvested and the cDNA used in qPCR-based arrays. This data is based on the analysis of cDNA prepared from pooled RNA samples harvested from 3 independent experiments (cells obtained from a minimum of 3 animals in each independent experiment).

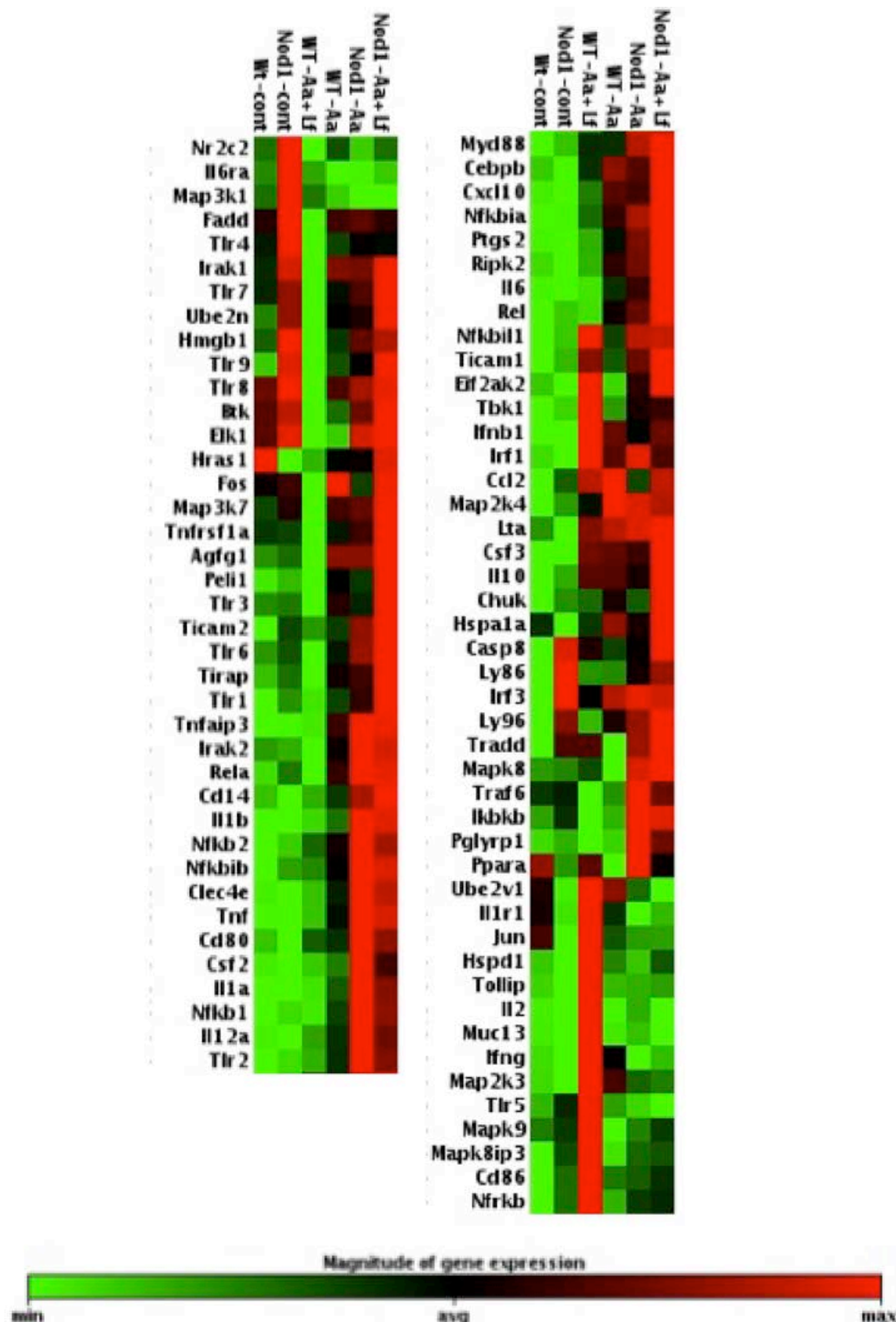
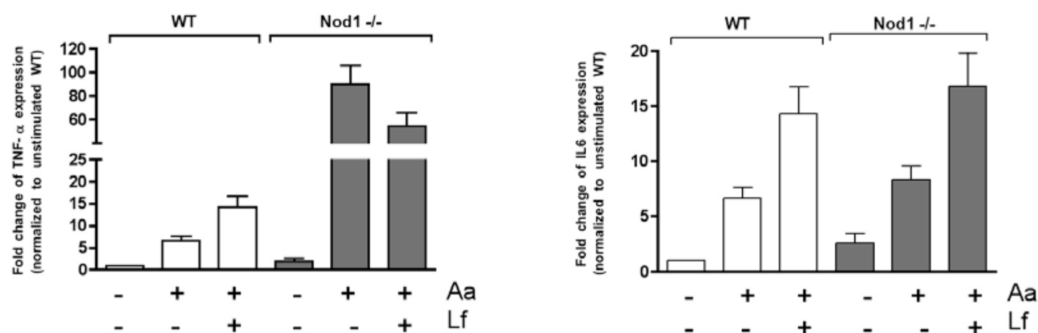


Figure 6- Validation of qPCR array data by RT-qPCR array using cDNA prepared from RNA obtained from bone marrow-derived macrophages from WT and Nod1 KO mice in three independent experiments (a total of 4-6 mice of each genotype were used). These were the same RNA samples combined to prepare the pool used in the array analysis. Target gene expression was normalized to beta-actin expression and fold regulation was calculated by comparison with the normalized gene expression in WT unstimulated control macrophages.



Functional clusters of genes modulated by heat-killed bacteria in macrophages are similar, but in Nod2-deficient macrophages the expression of Th1-associated cytokines and TLR receptor genes are more markedly attenuated than in Rip2-deficient macrophages

To obtain a better understanding of the functional role of Nod1 on the macrophage response to Gram-negative bacteria associated or not with Gram-positive bacteria, we initially assessed the overall effect of Nod1 deletion by extracting terms defining biological function of the list of genes upregulated in stimulated macrophages using an online bioinformatics application (DAVID). To be considered 'upregulated', we used a minimum of four-fold increase over the expression level in unstimulated controls (or 2 fold increase on a log_2 scale), which is more stringent than the guidelines for studies using treated primary cells in a microarray approach for hypothesis-generating studies (14). This stringent criterium was used to reduce Type I error due to the use of pooled samples in the qPCR array analysis, at the cost of accepting some false-negatives. In defining the functional gene clusters, DAVID parameters were also set to the highest stringency setting in order to generate less functional clusters including more tightly associated genes in each cluster. The 'score' represents the overall enrichment score for the gene cluster, with scores higher than 2 considered of biological significance (12,

13). To reduce the possibility of spurious associations, we have considered only the gene clusters associated with an enrichment score greater than 3. Table 1 indicates that overall the biological functions defined by clusters formed from the list of genes upregulated in macrophages after stimulation with heat-killed Aa is very similar in WT and Nod1-deficient macrophages, with a greater enrichment score in the first cluster of genes functionally-defined as inflammation-associated genes.

Table 1 - Comparison of Functional Annotation Terms and Enrichment Scores for the top 5 gene clusters identified in the lists of genes up-regulated in macrophages by heat-killed Aa. associated or not with Lf.

	Aa stim / WT		**Aa stim / Nod1 -/-**	
Gene cluster	*Functional annotation*	*score*	*Functional annotation*	*score*
1	defense response, inflammatory response, positive regulation of multicellular organism process, response to wounding	10.78	immune response, defense response, inflammatory response, response to wounding	17.54
2	immune response, cytokine, cytokine activity, macrophage, lymphokine	8.52	cytokine, cytokine activity, macrophage, lymphokine, JAK-STAT signaling pathway	8.53
3	hematopoietic cell lineage, JAK-STAT signaling activity, growth factor activity	6.40	response to bacterium, regulation of interleukin-6 production, response to molecule of bacterial origin, positive regulation of NF-kB transcription factor activity	6.16
4	response to bacterium, regulation of IL-6 production, response to molecule of bacterial origin, positive regulation of cytokine production	5.92	positive regulation of protein kinase cascade, positive regulation of cytokine production, positive regulation of signal transduction	5.98
5	immunoregulation, acute-phase response, acute inflammatory response, homeostatic process	5.23	macrophage, immunoregulation, negative regulation of transport, acute inflammatory response	5.18

	Aa+Lf stim / WT		Aa+Lf stim / Nod1 -/-	
Gene cluster	*Functional annotation (top 5)*	*score*	*Functional annotation (top 5)*	*score*
1	immune response, cytokine, cytokine activity, JAK-STAT signaling pathway, cytokine network	11.66	regulation of IkB/NF-kB cascade, response to bacterium, regulation of IL-6 production, regulation of cellular response to stress	8.97
2	defense response, inflammatory response, response to wounding	10.09	regulation of transcription factor activity, innate immune response-activating signal transduction, defense response to Gram-positive bacterium	6.93
3	macrophage, immune system development, leukocyte differentiation	4.57	regulation of TNF production, response to lipoteichoic acid	6.34
4	lymphokine, regulation of cytokine production, response to bacterium, immunoregulation	4.49	cytokine, cytokine activity, macrophage, lymphokine, IL-10 anti-inflammatory signaling pathway	6.32
5	signal, disulfide bond, glycoprotein	4.24	immune system development, leukocyte differentiation,	5.47

On the other hand, the comparison of functional clusters of genes upregulated by stimulation with the association of heat-killed Aa and Lf in macrophages from WT and Nod1 KO mice (Table 1) shows some differences in the functional defining terms of the gene clusters, particularly the inclusion genes related with NF-kB cascade in Nod1-/- macrophages (versus JAK-STAT in WT macrophages), and the presence of gene clusters functionally-defined by IL-10 anti-inflammatory signaling, TNF production and response to Gram-positive bacterium/lipoteichoic acid in the Nod1-/- macrophages only. Collectively, this information may be interpreted as an indication that upon stimulation with Gram-negative bacteria, Nod1 has a role in the modulation of the intensity of the macrophage response, whereas some qualitative changes occur when both Gram-negative and -positive bacteria are present, with a shift from JAK-STAT pathway to NF-kB pathway and the activation of IL-10 anti-inflammatory pathway.

Assessment of the pathways affected using the Kyoto Encyclopedia of Genes and Genomes (KEGG) also indicated a great similarity on both the percentage of genes and on the log10 of the p values indicating the statistical significance for the association in the top 20 KEGG terms defining the functions and utilities of genes that were up-

regulated in macrophages by heat-killed Aa in macrophages obtained from WT and Nod1 KO mice (Figure 7).

Figure 7 - Correlations of percentage of genes included in each functional annotation term and of the enrichment statistical values (log 10 p value) obtained from DAVID Chart Reports. The correlation plots measure the annotation agreement between genes up-regulated by heat-killed Aa or Aa+Lf stimulation in WT and Nod1-deficient macrophages. The gene hit percentages and enrichment p values of top enriched terms between the lists of up-regulated genes show very strong correlation upon Aa stimulation. The comparison of WT and Nod1-deficient macrophages upon Aa+Lf stimulation shows a weak correlation between the percentage of genes upregulated and no correlation between the the p values, indicating a qualitative difference in the response of Nod1-deficient macrophages upon Gram-negative and Gram-positive stimulation.

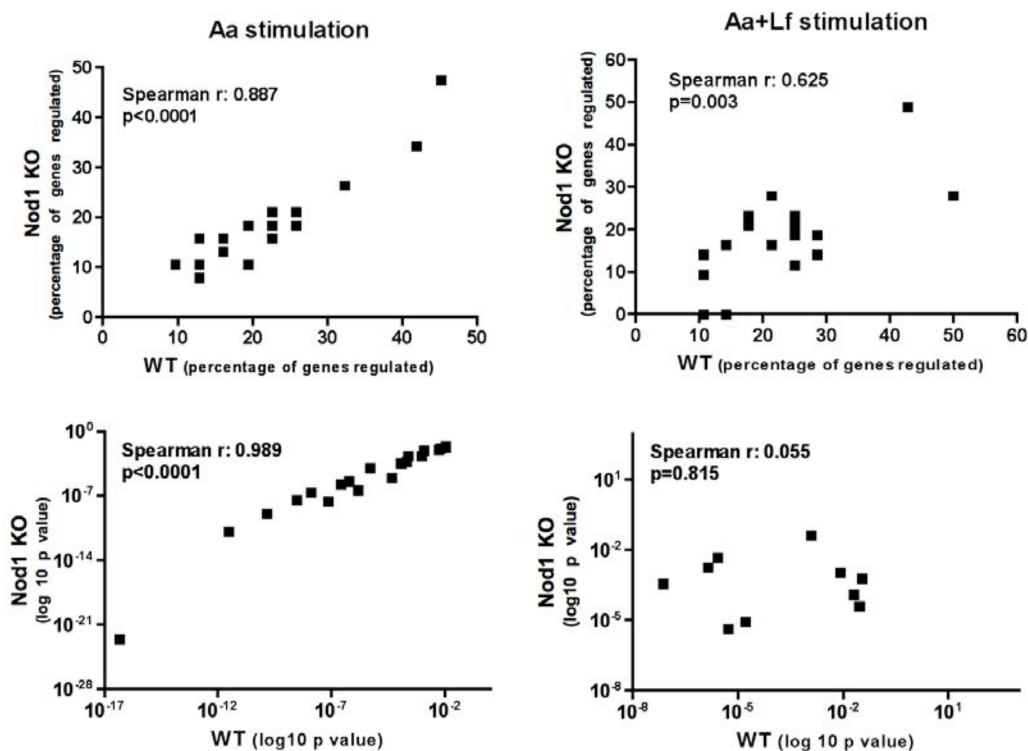


Table 2 presents the data on the fold change up-regulation of genes according to the genotype of the mice and the stimulation to provide further insight into the role of Nod1 in regulating macrophage response to Aa and Aa+Lf from and intensity/quantitative and qualitative standpoint.

Table 2 - Genes presenting at least 4-fold up-regulation of their expression in macrophages from WT and Nod1 KO mice after a 6h-stimulation with heat-killed Aa associated or not with heat-killed Lf in comparison to WT untreated control.

Gene name	**Aa simulation**		**Aa+Lf stimulation**	
	WT	**Nod1 -/-**	**WT**	**Nod1 -/-**
Ccl2	4.40		4.00	4.03
Cd14				4.45
Cd86			5.01	
Clec4e	5.08	10.68		9.52
Csf2	6.71	26.89		17.08
Csf3	16.08	14.54	16.86	22.76
Cxcl10	15.60	14.62	5.92	22.85
Ifnb1	12.92	9.22	17.67	12.96
Ifng	6.18		11.92	
Il10	13.36	11.04	13.42	19.36
Il12a	15.28	37.58	6.67	27.24
Il1a	90.94	281.95	12.79	217.05
Il1b	45.80	166.39	12.34	159.89
Il2			19.11	
Il6	16.56	24.77		40.05
Irf1		5.29	5.45	
Lta		4.29		4.52
Muc13			11.19	
Nfkb1		6.59		5.40
Nfkb2	5.39	9.16		7.79
Nfkbia	7.90	10.83	4.31	12.57
Nfkbib	4.27	7.45		7.12
Ptgs2	19.23	30.43	6.28	40.60
Rel		4.83		6.44
Ripk2				4.97
Tlr1		4.28		6.45
Tlr2	8.12	18.21		14.10
Tlr9				4.36
Tnf	29.23	61.02	7.55	57.45
Tnfaip3	8.32	12.89		12.67

Lack of Nod1 markedly increased the expression of some pro-inflammatory cytokines upon heat-killed Aa stimulation in comparison with WT macrophages, notably IL-1α and β, IL-12, TNF-α and Ptgs2 (murine analog of Cox-2). On the other hand, there was a marked inhibition of Ppara expression (-8.81 fold) in Aa-stimulated WT macrophages that was not observed in the comparison between WT control and Aa-stimulated Nod1-/- macrophages. When both Aa and Lf were associated, expression of Elk1, Fadd, Irak2, Tlr7 and Tlr8 were all inhibited more than 2 fold in comparison to control

(unstimulated) WT macrophages; whereas none of the 84 genes assessed in the qPCR array were markedly inhibited in Nod1-/- macrophages stimulated with Aa+Lf. Interestingly, stimulation with the association Aa+Lf reduced the expression of IL-1α and β, IL-6, TNF-α and Ptgs2 in comparison to stimulation with Aa alone in WT macrophages. This suggests that activation of Nod2 by the association with the Gram-positive Lf has attenuated the macrophage response. In Nod1-deficient macrophages, in spite of this attenuation of IL-1α and β gene expression with the association of Aa and Lf; expression of IL-6, IL-10, TNF-α and Ptgs2 were up-regulated in comparison to Nod1-deficient macrophages stimulated with Aa only.

Discussion

We showed that genomic deletion of Nod1 in vivo accentuates inflammatory-driven bone resorption associated with the exposure to heat-killed Gram-negative bacteria in an experimental periodontal disease model, representative of host-microbial interactions. This effect was supported by significantly increased numbers of osteoclasts in Nod1KO mice, indicating an influence of Nod1 on osteoclastogenesis. Interestingly, the association of Gram-positive, Nod2-activating microorganism in the model attenuated the bone resorption, cellular infiltrate and also reduced the number of osteoclasts in the microenvironment. This influence may be either directly affecting the osteoclast precursor cells or indirectly modulating the external signals regulating the osteoclast differentiation process and will be investigated in subsequent studies. A possible indication of the latter possibility is the upregulation of IL10 gene expression in Nod1 -/- macrophages stimulated with Aa+Lf in comparison with Nod1-/- cells stimulated with Aa only, which was not observed in WT cells (Table 2). Gram-positive bacteria of the Lactobacillus genus were shown to induce IL10 when used as vaccine adjuvants in murine asthma model (15). Similarly, there is in vivo and in vitro evidence that administration of probiotic Gram-positive bacteria (including Lactobacillus species) attenuate colitis in murine experimental models by decreasing proinflammatory cytokine expression (16, 17). However, this induction of IL-10 by Gram-positive bacteria is dependent on the bacterial species and strain, as *L. crispatus* (strain M206119) aggravate experimental dextran-induced colitis in mice by increasing expression of proinflammatory genes and decreasing IL-10 (18).

Interestingly, our in vitro data using bone marrow-derived macrophages shows a marked increase on the expression of Csf2/G-CSF in Nod-1 deficient cells upon microbial stimulation, and there is evidence for increased osteoclast differentiation in mice upon in vivo administration of G-CSF alone (19). Moreover, MDP (which can activate both Nod1 and Nod2) has been shown to enhance osteoclastogenesis induced by LPS and inflammatory cytokines in co-cultures of osteoblasts and hematopoietic cells by modulating Nod2-mediated RANKL mRNA expression in osteoblasts (20). It is important to bear in mind that Nod1-/- animals and the macrophages derived from these animals present functional Nod2.

In fact, Nod1KO mice still present all other PRRs, including TLRs and NLRs, and thus the effect on inflammatory bone resorption may have shifted the MAMP- and DAMP signaling to other pathways, modulating the cytokine network and thus the phenotypical characteristics of immune cells and the inflammatory microenvironment. Alternatively, Nod1 may have a direct inhibitory role in osteoclastogenesis and its deletion allowed other signaling pathways to exert more influence on this process. In fact, simultaneous stimulation of osteoclast precursor cells with RANKL and Nod1 agonist inhibited osteoclast differentiation in vitro (8).

We used heat-killed microorganisms to avoid colonization issues and also bias introduced by bacterial-secreted products, moreover there is evidence indicating that osteoclast differentiation induced in mice by injecting live or heat-killed bacteria in mice is similar (21) and also that both live and heat-killed bacteria can activate Nod signaling (22). Nod1 was originally considered a cytosolic PRR, therefore especially suited for the detection of invading microorganisms; however it has been shown that viability or even the presence of bacteria is not necessary for the stimulation of Nod1 (23) and even with live bacteria the intracellular localization of bacteria is also not a requisite for the activation of Nod1 (24). These findings support the functional relevance of Nod1 in non-phagocytosing cells; whereas it is also possible that in our model the specific Nod1 and Nod2 ligands gained access to the cytoplasm of macrophages and neutrophils by phagocytosis or pinocytosis (25).

Since the microbial-induced periodontal disease model we used is characterized by sustained stimulation over a period of 4 weeks, there are multiple cell types involved in the microenvironment, including stromal cells (osteoblasts, fibroblasts, epithelial cells) and neutrophils, macrophages, dendritic cells, T and B cells. The effect observed in vivo is the net outcome of Nod1 genomic deletion, which affects all these cell types, which

also present other TLRs, particularly TLR2 and 4 that are activated by major MAMPs of the microorganisms used, respectively lipoteichoic acid (from Gram-positive Lf) and LPS (from Gram-negative Aa). Even in the specific cell type assessed in vitro, gene expression of various TLRs, particularly of TLR4 and its co-activators Ly86 and Ly96, was increased in unstimulated macrophages. Moreover, over the course of the 4-week experimental period, host-derived ligands from damaged tissue and cells may also activate PRRs as danger signals and add another layer of complexity to the interpretation of the observations. Specifically, Nod signaling has been shown to be induced by TLR ligands, IFN-γ and TNF-α (4, 26) that accumulated over time. Even though these facts preclude a direct mechanistic interpretation of the results, it provides an in vivo perspective of the pathological relevance of Nod1 in inflammatory-driven bone resorption, involving a sustained exposure to Gram-positive and Gram-negative bacteria. This was an initial study that will be further expanded to assess the relative relevance of Nod1 gene in stromal/resident cells and leukocytes and bone-marrow derived cells for the host response in this host-microbial interaction model.

Interestingly, a recent publication presents completely opposite results using another model of experimental periodontal disease. Using the ligature-induced model in mice, Jiao et al. (27) show reduced alveolar bone loss in Nod1KO mice. The authors report a decrease on the expression of inflammatory cytokine mRNA and neutrophil infiltration, associating these effects to the increased prevalence of an endogenous Gram-negative bacterium (NI1060) with great genetic similarity to *A. actinomycetemcomitans*. The obvious difference in the experimental model (ligature for 10 days, versus sustained stimulation with heat-killed bacteria over 4 weeks) may account for the diametrically opposite findings. Moreover, we have not assessed the potential role of the endogenous microbiota, but we assume that since we injected the microorganisms their role on our results, if any, was minimal.

To obtain insight on the role of Nod1 in the modulation of cell response to Gram-negative and Gram-positive bacteria, we chose to use bone marrow-derived macrophages, as these are professional antigen-processing and presenting innate immune cells, and are also the prototypical Nod-expressing cells and osteoclast precursors. Within the limitations of an in vitro assessment, the response to the Gram-positive and Gram-negative microorganisms was attenuated in Nod1-deficient macrophages in comparison to macrophages from WT mice. This was unexpected based on the in vivo data, but it is consistent with the removal of a PRR involved in

macrophage activation by MAMPs from Gram-negative microorganisms. In fact, in Nod1-deficient macrophages we observed a marked attenuation of MAPK and NF-kB signaling pathways (the major targets of Nod signaling), alongside a decrease on STAT3 activation, which was shown previously in Nod2-deficient cells (28, 29). It is tempting to speculate that since the activation of NF-kB and p38 MAPK was attenuated in Nod1-deficient cells, other signaling pathways and mechanisms were preferentially activated, particularly those leading to the activation of AP-1 transcription factor, since JNK MAPK activation was not affected in Nod1 -/- macrophages. For example, activation of NFAT-c1, the necessary transcription factor for osteoclast differentiation, may be mediated by NF-kB/AP-1/c-Fos and calcium signaling (30).

In sharp contrast to the modulation of cytokine secretion assessed by ELISA, the comprehensive assessment of the regulation of gene expression by RT-qPCR focused arrays indicated that microbial stimulation induced a marked increase on the gene expression of some inflammatory cytokines in Nod1-deficient macrophages in comparison to WT macrophages, most notably IL-1α, IL-1β, TNF-α and IL-12a, whereas IFN-γ was upregulated only in WT macrophages. This is most striking, considering the marked attenuation on the activation of inflammatory signaling pathways observed in these same cells and under the same stimulations. In fact, the marked attenuation of p38 MAPK and NF-kB signaling occurred in spite of the expression of other PRRs that could be engaged by MAMPs in the microorganisms used to stimulate by the cells. It is important to notice that NF-kB activation was assessed only by the activation of p65 (RelA), whereas other NF-kB signaling subunits relevant for osteoclast formation, such as p50 and p52 (31) were not assessed. Moreover, short-term JNK MAPK activation, which is capable of activating AP-1/c-Fos was not inhibited in Nod1-deficient macrophages. Moreover, the different stimulation periods for the RT-qPCR arrays and cytokine ELISAs may be partially involved in this discrepancy between the regulation at mRNA and protein level.

We do not have an explanation for these intriguing results; however since Nod1 has been show to interact with NLRP3 (32), leading to caspase-1 activation, it is possible that the reduced activation of caspase-1 in Nod1-deficient macrophages inhibited the post-translational processing of IL-1 and thus its detection by ELISA (which only detects active cytokines) and the corresponding uncoupling of cytokine mRNA and protein levels. TNF-α and IL-12 are also known to be regulated by post-translational

mechanisms (33, 34), although the participation of caspase-1 in the regulation of these cytokines has not been shown. Inhibition of caspase-1 blocks LPS-induced production of IL-1β and IFN-γ in vitro, and the effect on IFN-γ production is indirectly mediated by the inhibition of IL-18 (35). This information supports our observations of inhibition of IL-1β production by ELISA and IFN-γ gene expression by RT-qPCR array in Nod1-deficient macrophages, with a potential role for the decreased caspase-1 activation in these cells as a direct and indirect mechanism affecting cytokine gene expression at mRNA and protein post-translational processing levels. Recent evidence indicates that Nod1 may bind/interact with different intracellular molecules, and the effects associated with Nod1 deficiency may be derived from the interference with the positive or negative regulatory function of these interacting partners, such as BID (36) and CENTβ1 (37). Future studies will assess the role of other interacting partners of Nod1, besides Rip2, that may play a role in mediating its functions.

In summary, we report on the aggravation of inflammatory-driven bone resorption in Nod1KO mice subjected to a Gram-negative microorganism-induced model of experimental periodontal disease, suggesting that Nod1 may have a role in modulating osteoclast differentiation in vivo. We also show that association of a Gram-positive microorganism significantly attenuated bone resorption induced by Gram-negative bacteria in Nod1KO mice. Lack of Nod1 renders macrophages less responsive to microbial stimulation and markedly inhibits the activation of p38 MAPK, NF-kB/p65 and STAT3. The discrepancy between in vivo and in vitro in our results was similarly reported for *Chlamydia trachomatis* infection, which was increased in vitro in MEFs from Nod1KO mice, whereas vivo Nod1KO mice were not more susceptible to the infection (38). We conclude that Nod1 is an important asset for activation of macrophages by microbial stimulation, however this attenuation of macrophage response does not translate into reduced inflammation and bone resorption associated with host-microbial interactions in the experimental periodontal disease in vivo.

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5 DISCUSSÃO

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Neste estudo, demonstramos que Nod1 e Nod2 tem efeitos distintos na inflamação e reabsorção óssea associadas à doença periodontal experimental. Este é o primeiro estudo utilizando camundongos geneticamente modificados, com deleção genômica de Nod1, Nod2 ou Rip2 em um modelo experimental de doença periodontal induzida por injeção de bactérias. No estudo in vitro, utilizamos macrófagos derivados da medula óssea das tíbias e fêmurs destes animais onde avaliamos o papel de Nod1, Nod2 e Rip2 na resposta dos macrófagos à bactérias Gram-negativas e Gram-positivas utilizando arrays focados nas plataformas de Elisa e PCR em tempo real. Optamos por utilizar microrganismos inativados por calor para evitar problemas de colonização e também o viés introduzido por produtos secretados em caso de bactérias vivas. Evidências indicam que a diferenciação de osteoclastos induzida pela injeção de bactérias vivas ou mortas por calor em camundongos é semelhante[63] assim como podem ativar a sinalização por Nod[46].

Nossos resultados mostraram que, no modelo de indução de doença periodontal por injeção de bactérias Gram-negativas, enquanto que nos camundongos Nod1 KO a reabsorção óssea alveolar aumentou significantemente, a deleção de Nod2 causou efeito contrário, diminuindo a reabsorção óssea. Mudanças significativas em relação ao volume ósseo não foram observadas nos animais Rip2 KO. A mudança no padrão de reabsorção óssea em comparação aos camundongos selvagens, refletiu diretamente no número de osteoclastos e no agravamento da inflamação observada histologicamente (apenas nos camundongos Nod1 KO). Estes dados sugerem que tanto Nod1 quanto Nod2 podem ter efeitos sobre a osteoclastogênese, afetando diretamente as células percussoras de osteoclastos ou indiretamente modulando a expressão de citocinas que são importantes para a diferenciação do osteoclastos. Como alternativa, Nod pode ter efeito sobre a função dos osteoclastos maduros, interferindo na expressão de genes envolvidos com o processo de reabsorção.

A atenuação da reabsorção óssea em camundongos Nod2 KO é suportada por estudos recentes em que a utilização in vivo de agonistas de Nod2 causou aumento da reabsorção óssea[30]. Inesperadamente, nos camundongos Nod2-/- a diminuição na reabsorção óssea alveolar ocorreu sem efeito marcante sobre a inflamação avaliada histologicamente. Isso pode estar relacionado com a modulação de citocinas inflamatórias (TNF-α, IL-6, IL-10, IFN-δ, IL-12, IL1r1) e das vias de sinalização (NF-

kB e MAPK), como indicado pelo estudo in vitro, o que pode ter ocorrido na ausência de mudanças óbvias no aspecto histológico. Também é possível que a falta de Nod2 tenha afetado a osteoclastogênese ou ainda atividade de osteoclastos.

O efeito protetor de Nod1 em relação a doença periodontal induzida experimentalmente encontrado neste estudo, diverge dos resultados encontrados na literatura. Em nosso estudo a deleção de Nod1 levou ao aumento da reabsorção óssea in vivo e ao aumento da expressão de RNAm de citocinas inflamatórias (IL-1α, IL-1β, TNF-α e IL-12a) em macrófagos. Utilizando modelo de doença periodontal induzida por ligadura em camundongos, Jiao et al. mostrou que a perda óssea alveolar em camundongos Nod1 KO foi reduzida. Os autores relataram uma diminuição na expressão de citocinas inflamatórias e no infiltrado de neutrófilos[26]. A diferença óbvia no modelo experimental (ligadura durante 10 dias, contra estimulação sustentada com bactérias inativadas por calor durante 4 semanas) pode explicar as conclusões diametralmente opostas.

Entretanto, dados da literatura demostram que a ativação de Nod1 por seu ligante específico (meso-DAP) induz resposta adaptativa predominantemente do tipo Th2[12]. IL-4 e IL-13, citocinas do tipo Th2, afetam o sistema RANK/RANKL/OPG e inibem a reabsorção óssea e a diferenciação de osteoclastos[43]. Além disso, em fibroblastos de gengiva humana, IL-4 e IL-13 inibem a produção LIF e IL-11, importantes mediadores no processo de osteoclastogênese[52]. Estes dados suportam com os resultados encontrados em nosso estudo in vivo. O fato da deleção de Nod1 afetar negativamente a resposta imune adaptativa do tipo Th2 pode ter levado a um aumento da reabsorção óssea. Além disso, nossos resultados in vitro utilizando macrófagos demonstraram aumento na expressão de Csf2G-CSF nas células do animais KO após estimulação bacteriana. Evidências na literatura mostram que a administração in vivo de G-CSF aumenta a diferenciação de osteoclastos[17].

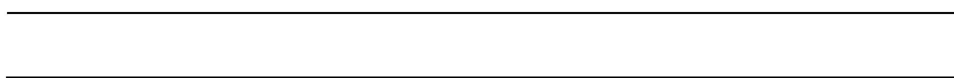
É importante ter em mente que o fato dos animais serem KO para Nod1, não exclui a presença dos demais PRRs (incluindo TLRs e NLRs). Dados da literatura demonstram que fibroblastos de gengiva expressam todos os tipos de TLRs e NODs[56]. Em nosso modelo de estudo, caracterizado pela estimulação bacteriana sustentada por período de 4 semanas e envolvimento de múltiplos tipos celulares (incluindo células estromais, macrófagos, neutrófilos, células dendríticas e linfócitos), a deleção de Nod1, apesar de afetar os diferentes tipos celulares, não afeta os demais PRRs. A reabsorção óssea aumentada nos camundongos Nod1-/- observada em nosso estudo pode ser

resultado de um mecanismo compensatório em resposta a deleção genômica. Alternativamente, Nod1 pode ter efeito inibitório direto na osteoclastogênese. Sendo assim, a exacerbação da reabsorção óssea verificada nos animais Nod1 KO pode ser resultado da ativação de outras vias de sinalização que exercem maior influência neste processo. De fato, a estimulação simultânea com agonista de Nod1 e RANKL em células precursoras de osteoclastos, a diferenciação de osteoclastos in vitro é inibida[31].

Os dados obtidos em nosso estudo in vivo e os dados in vitro foram correspondentes para Nod2 e distintos para Nod1 (ELISA citocinas e vias de sinalização). Em qualquer caso, a correspondência entre os dados in vitro e in vivo deve ser considerada com cautela. Nos estudos in vitro, utilizamos apenas macrófagos, entretanto no modelo in vivo há a participação de diversos tipos celulares que contribuem para a rede de citocinas e vias de sinalização ativadas, o que pode levar a padrões distintos de resposta. Além disso Nod1, Nod2 e Rip2 também podem ter papel importante em outros tipos celulares relacionadas tanto com a imunidade inata como adaptativa.

Mesmo que os resultados obtidos impeçam uma interpretação mecanicista direta dos resultados, ele fornece uma perspectiva in vivo da relevância patológica de Nod1, Nod2 e Rip2 no processo de reabsorção óssea envolvendo a exposição prolongada às bactérias Gram-positivas e Gram-negativas. Além disso fornece informações importantes sobre a influência das proteínas Nod na rede de citocinas e na sinalização intracelular associadas com as doenças periodontais.

6 CONCLUSÃO



6 CONCLUSÃO

Com base nos resultados deste trabalho concluímos que:

1 – Nod1, Nod2 e Rip2 exercem papel importante na expressão gênica de mediadores inflamatórios e na ativação das vias de sinalização p38 MAPK e NF-kB em macrófagos.

2 – As proteínas Nod tem papel relevante na modulação das interações bactéria-hospedeiro associadas à doença periodontal induzida por bactétias inativadas por calor. Enquanto Nod1 parece exercer papel protetor na inflamação e reabsorção óssea, Nod2 atua como amplificador da resposta do hospedeiro.

Ademais, outros estudos são necessários para determinar os mecanismos biológicos pelos quais as proteínas Nod modulam a resposta imune associada as doenças periodontais. O melhor entendimento destes mecanismos pode levar ao desenvolvimento de estratégias terapêuticas.

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*De acordo com o manual da FOAr/UNESP, adaptadas das normas Vancouver.
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APÊNDICE

Material e Métodos

A) – *Experimentos* **in vivo**

***Indução de doença periodontal* in vivo**

Foram utilizados 14 camundongos (*Mus musculus*) da linhagem C57/Bl6 de cada genótipo (deleção de Nod1, Nod2, Rip2/RICK) e 14 camundongos 'selvagens' (sem nenhuma deleção gênica, '*wild-type*' - WT), num total de 42 animais com idades entre 8 e 10 semanas. Estes animais foram mantidos em ambiente Specific Pathogen Free (SPF) em condições ideais de temperatura, umidade e ciclos de luz e escuro, com água e alimento *ad-libitum* em biotério localizado no Departamento de Patologia e Fisiologia da Faculdade de Odontologia de Araraquara-UNESP, segundo condições estabelecidas pelo Colégio Brasileiro de Experimentação Animal (COBEA) e pelo Comitê de Ética em Experimentação Animal (CEEA) da Faculdade de Odontologia de Araraquara (UNESP).

A indução de doença periodontal foi feita ao redor dos primeiros molares superiores bilateralmente, gerando 22 amostras de cada genótipo. Estas 22 amostras foram experimentalmente divididas da seguinte forma: 6 amostras em que foi feita a injeção apenas do veículo (PBS, pH 7.4) utilizado na diluição dos microrganismos inativados pelo calor, 8 amostras em que foi feita a injeção de aproximadamente 10^9 UFC (determinadas por avaliação da absorbância à 660 nm) de *Aggregatibacter actinomycetemcomitans* (Aa, Sorotipo JP1) e 8 amostras em que foi feita a injeção de Aa associado à 10^9 UFC de *Lactobacillus fermentans* (Lf). Tanto Aa quanto Lf foram previamente inativados por calor (incubação das culturas em meio liquido por 30 minutos à temperatura de 60 ^{o}C). Estas bactérias foram selecionadas devido ao fato de representarem periodontopatógenos Gram-negativos (Aa) com grande potência de estímulo de Nod1 e Nod2 (superior, por exemplo, à de *Fusobacterium nucleatum* e *Porphyromonas gingivalis*) [42] e microrganismos comensais Gram-positivos não associados à doença periodontal. As injeções das suspensões microbianas em PBS foram realizadas com microseringas do tipo Hamilton (volume de 3 µL por injeção), sob sedação com anestesia inalatória três vezes por semana, por 4 semanas (período experimental de 30 dias). Ao final do período experimental de 30 dias, os animais foram sacrificados por inalação de CO_2 e a maxila superior, coletada com os tecidos moles

adjacentes, fixada em formol tamponado 4% por 24 horas, transferidas para álcool 70% e mantidas à 4 ^{o}C para as análises subsequentes.

Microtomografia computadorizada (μCT)

As peças foram escaneadas em microtomógrafo (Skyscan, Aastselaar) com resolução de 18 ìm. Após reconstrução das imagens tridimensionais com o software NRecon, estas foram reorientadas espacialmente de forma padronizada no plano sagital com o software DataViewer. A análise foi realizada com o software CTan (Skyscan, Aartselaar), em que foi definida uma área de interesse de 2,5 mm^3 com base em pontos de referência anatômicos e dimensões padronizadas. Nesta área de interesse a análise determinou a fração de volume de tecido mineralizado (o percentual do volume total da área de interesse preenchido com tecido mineralizado – BV%). Estes dados foram combinados segundo o procedimento experimental realizado: injeção de veículo, injeção de Aa ou injeção de Aa+Lf Os resultados foram comparados segundo os diferentes estímulos (PBS, Aa ou Aa + Lf) em cada genótipo; bem como segundo os diferentes genótipos (Nod1 KO, Nod2 KO, Rip2/RICK KO e controle 'WT') após cada estímulo. Imagens tridimensionais das peças reorientadas no plano sagital foram obtidas com o software CTVol. Todos os softwares utilizados fazem parte do pacote disponível no microtomógrafo (Skyscan, Aartselaar).

Análise histológica e estereométrica

Após a realização da microtomografia, as peças foram desmineralizadas em solução de EDTA (0.5M, pH 8.0) sob agitação à temperatura ambiente durante 8 semanas (com troca da solução 2x/semana), para posterior inclusão em parafina. Cortes semi-seriados de 5 µm de espessura foram obtidos na direção vestíbulo-lingual, montados em lâminas e corados apropriadamente (H/E). As lâminas foram submetidas à análise qualitativa do processo inflamatório através da descrição e atribuição de escores para severidade (0-não inflamado, 1-leve; 2-moderada e 3-severa) por examinador experiente e cego para os grupos experimentais.

Imunohistoquímica (TRAP)

A detecção e localização da expressão de TRAP como indicador do processo de reabsorção óssea foi realizada através de imunohistoquíma. Cortes seriados (5 µM de espessura) das peças incluídas em parafina foram obtidos no plano sagital (sentido vestíbulo-lingual) e subsequentemente montados em lâminas silanizadas. Após otimização para o anticorpo/proteína-alvo, a detecção da proteína-alvo foi feita pelo sistema DAB-streptavidina (LSAB-2, Dako Cytomation). As lâminas receberam lamínulas de vidro coladas com permount e após 24 horas foram observadas e fotografadas em 100X de aumento com microscópio óptico. Nas imagens digitalizadas foi feita a contagem do número total de osteoclastos.

B) – *Experimentos* **in vitro**

Culturas primárias de macrófagos

Os macrófagos foram obtidos a partir de células estromais da medula óssea (‘*bone marrow stromal cells*’) da tíbia e fêmur diferenciadas em macrófagos e expandidos in vitro[35]. Neste protocolo, após a eutanásia por deslocamento cervical, os camundongos foram pulverizados com etanol 70% e os fêmures e tíbias foram dissecados com tesoura e lâmina de bisturi. Os ossos foram colocados em um tubo de polipropileno contendo meio de cultura RPMI-1640 estéril e, em seguida, as duas epífises foram removidas usando tesouras e pinças esterilizadas. A porção medular destes ossos foram lavados com uma seringa com 5 mL RPMI-1640 para expulsar da medula óssea em tubos de polipropileno de 15 mL estéril. Pipetas de plástico de 1 mL foram utilizadas para homogeneizar delicadamente a medula óssea e foi obtida uma suspensão de células da medula óssea fresca. Para diferenciação das células derivadas da medula óssea fresca em macrófagos o seguinte procedimento foi realizado: as células foram ressuspendidas em 10 mL de meio de diferenciação (RPMI-1640 com 20% de soro fetal bovino, 30% de sobrenadante de culturas de células L929 – produtoras de M-CSF, 100 U/mL de penicilina, 100 mg/mL de estreptomicina, e 2 mM L-glutamine). As células foram semeadas em placas cultura não-tratadas (para baixa aderência celular, Optilux - BD Biosciences) e incubadas em estufa a 37 ^{0}C e 5% de CO_2. Quatro dias após a semeadura das células, foi adicionado um volume extra de 10 mL do meio de diferenciação por placa e incubados por um período adicional de três dias. Para obter os

macrófagos, os sobrenadantes foram descartados e as células aderidas foram lavadas com 10 mL de PBS estéril. Dez mL de PBS gelado foram adicionados a cada placa e incubados a 4 ^{0}C por 10 minutos. Os macrófagos foram destacados da placa por pipetagem. As células foram centrifugadas a 200 x g por 5 minutos e ressuspendidas em 10 mL de meio de cultura RPMI-1640 contendo 10% de soro fetal bovino, 5% de sobrenadante de L929, 100 U/mL de penicilina, 100 mg/mL de estreptomicina, e 2 mM L-glutamina. As células foram contadas em hemocitômetro e plaqueadas para o experimento.

Estímulo das células* in vitro *e coleta das amostras

Macrófagos de animais controle (WT) e geneticamente modificados (Nod1-/-, Nod2-/- e Rip2/RICK-/-) obtidos da forma descrita anteriormente foram plaqueados em placas de 12 poços (1 x 10^{6} células/poço) em meio RPMI-1640 suplementado com 10% de soro fetal bovino inativado por calor e antibióticos (penicilina/estreptomicina) (Gibco-Invitrogen Corp). Como rotina, 24 horas após o plaqueamento, as células foram desinduzidas por 6 horas por meio de incubação em meio de cultura com reduzida (0.2%) concentração de FBS para sincronização do ciclo celular e eliminação dos efeitos associados a presença de substâncias presentes no soro fetal bovino (por ex., fatores do sistema complemento) que podem induzir a ativação e expressão de citocinas por estas células. O estímulo foi feito pela adição de 1 µL de uma suspensão de microrganismos inativados pelo calor, contendo de 10^{9} UFC de *A.actinomycetemcomitans* em PBS ou 1 uL de uma suspensão de 10^{9} UFC de *A.actinomycetemcomitans* e 1 uL de uma suspensão de 10^{9} UFC de *Lactobacillus fermentans.* Nas amostras-controle, foram acrescentados apenas 1 uL de PBS sem a presença dos microrganismos. A coleta de RNA total foi realizada após 6 h e a coleta do meio de cultura após 24 horas. As amostras foram armazenadas em freezer à -80 ^{0}C até o momento de sua utilização nos ensaios de array focados nas plataformas RT-qPCR e ELISA.

Para avaliação da ativação das vias de sinalização, os macrófagos foram plaqueados em placas de 35 mm de diâmetro (1 x 10^{6} células/placa) e estimulados com 2 µL de uma suspensão contendo de 10^{9} UFC de Aa em meio PBS ou com 2 uL de uma suspensão de 10^{9} UFC de Aa e 2 uL de uma suspensão de 10^{9} UFC de Lf. A coleta dos lisados celulares foi feita por raspagem (ou centrifugação do meio de cultura e

resuspensão, no caso dos linfócitos não aderentes às placas de cultura) em 300 µL de tampão M-Per (Pierce, Thermo Fisher Scientific) suplementado com coquetel de inibidores de protease e fosfatase (Complete e PhosStop, respectivamente, Roche Applied Science) nos seguintes períodos: 10, 30 e 60 minutos após o estímulo. Estas amostras foram armazenadas em freezer -80 ^{0}C para posteriormente serem utilizadas nos ensaios utilizando os kits ELISA-sanduíche.

Array de citocinas em formato RT-qPCR

Nestes experimentos, utilizamos arrays focados para a detecção de RNAm de 84 diferentes genes envolvidos na sinalização via TLRs (RT2 ProfilerTM PCR Array Mouse Toll-Like Receptor Signaling Pathway, SABiosciences). As amostras de RNA utilizadas neste experimento foram obtidas de 3 experimentos independentes utilizando um sistema de colunas de afinidade para o isolamento e purificação do RNA total, incluindo tratamento com DNAse (RNeasy Micro kit, Qiagen). Estas amostras foram combinadas em um 'pool' de quantidades iguais de cada um dos três experimentos (300ng de cada experimento, totalizando 900ng por amostra). A reação de transcriptase reversa para conversão de 900ng de RNA de cada amostra em cDNA, foi feita utilizando o kit recomendado pelo fabricante do array (RT^2 First Strand cDNA kit, SABiosciences). Estão representados neste array os genes de efetores da sinalização via TLR (incluindo TLRs e proteínas adaptadoras que interagem com estes receptores), dos membros das vias de sinalização ativadas por TLRs (incluindo NF-kB, MAPKinases, IRF e NFAT) e também genes-alvo destas vias de sinalização (incluindo diversas citocinas e interferons), além de um painel com 5 diferentes genes constitutivos para normalização. Uma única amostra foi avaliada em cada placa do array (este é o motivo de utilizarmos o 'pool' de amostras), assim foram necessárias 8 placas de array. A reação de PCR em tempo real foi feita utilizando o equipamento StepOne Plus (Applied Biosystems) nas condições de ciclagem otimizadas pelo fabricante (10 min à 95°C para desnaturação inicial seguido de 40 ciclos de 15 segundos à 95°C e 1min à 60°C). Os valores cycle threshold (Ct) de cada poço foram calculados pelo software da termocicladora segundo instruções do fornecedor dos arrays, exportados para uma planilha de dados fornecida pelo fabricante do array e analisados pelo método $\Delta\Delta$Ct. O objetivo deste array foi demonstrar a regulação da expressão dos genes-alvo de forma relativa, assim, foram realizadas as seguintes comparações: controle WT x controle

geneticamente modificado (Nod1 -/-, Nod2 -/-, Rip2/RICK -/-); e em cada genótipo utilizado as comparações controle x estímulo com Aa e controle x estímulo com a associação Aa+Lf.

Array de citocinas em formato ELISA

Foram utilizados ELISArrays, que permitem a detecção, numa mesma placa, de 6 citocinas e 6 quimiocinas (Mouse TLR-induced Cytokines II: Microbial-induced Multi-Analyte ELISArray Kit, SABiosciences) envolvidas na resposta à patógenos microbianos por meio da ativação de TLRs: TNFa, IL1b, IL6, IL12, IL17A, SDF-1, RANTES, MCP-1, MIP-1a, MIP-1b, MDC, Eotaxin. Amostras provenientes de três experimentos independentes foram analisadas. A quantidade total de proteína presente foi determinada pelo método de Bradford para normalização da concentração das proteínas-alvo. O procedimento foi realizado segundo instruções do fabricante, e as leituras realizadas em leitor de placas a 450nm com correção a 570nm.

Avaliação da ativação das vias de sinalização

As amostras de proteína total de macrófagos e linfócitos coletadas após, 10, 30 e 60 minutos do estímulo com Aa ou Aa+Lf foram quantificadas pelo método de Bradford. Quantidades iguais de cada um dos três experimentos independentes (num total de 10 ìg/'pool') foram combinadas e utilizadas no experimento. Para avaliação da ativação das principais vias de sinalização associadas à expressão de mediadores inflamatórios, caracterizando as diferenças devido aos diferentes estímulos e também determinação da influência de Nod1, Nod2 de forma independente e combinada, utilizamos ensaios ELISA-sanduíche multi-análise (PathScan Inflammation Multi-Target Sandwich ELISA kit, Cell Signaling). Este ensaio em formato ELISA utiliza placas de 96 poços e possibilita a avaliação simultânea da ativação de NF-kB (p65 e IkB-á), p38, JNK e STAT3 numa mesma amostra. A normalização dos resultados foi feita pela quantidade de proteína total utilizada (10 ìg/'pool' de amostra). O procedimento foi realizado segundo as instruções do fornecedor do kit e a leitura à 450 nm. O perfil de ativação destas vias de sinalização, representando pontos de convergência e proteínas-chave na sinalização de estímulos de estresse e inflamação, foi comparado segundo os diferentes estímulos (Aa ou Aa+Lf) em cada genótipo; bem

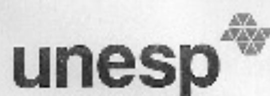
como segundo os diferentes genótipos *(Nod1-/-, Nod2-/-, Rip2/RICK -/- e controle 'WT')* após cada estímulo.

Forma de análise dos resultados

A análise estatística teve ênfase na comparação dos resultados entre diferentes genótipos, segundo o tipo de estímulo, considerando as amostras de forma independente. Para isso foram utilizados métodos paramétricos (teste t com Welch's correction para amostras independentes). O nível de significância adotado foi de 95% (p=0.05).

Os dados do array de citocinas em formato qPCR obtidos com amostras em pool foram analisados utilizando uma ferramenta online de bioinformática, DAVID (Database for Annotation, Visualization and Integrated Discovery - http://david.abcc.ncifcrf.gov)[19, 20], de forma exploratória consistente com a hipótese do estudo. A finalidade destas análises foi avaliar se os clusters funcionais dos genes que foram regulados em macrófagos estimulados com bactérias inativadas de calor foram afetados pela deleção de genes Nod1, Nod2 ou Rip2.

ANEXO



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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.

Profª Drª ELENY ZANELLA BALDUCCI
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