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Papel das proteínas intracelulares Nod e da proteína adaptadora MyD88 na regulação da expressão de RANKL e modulação da resposta inflamatória induzidos por antígenos bacterianos in vitro. Estudo em células relevantes do periodonto.

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Orientador: *Prof. Dr. Carlos Rossa Junior*
Co-Orientador: *Prof. Dr. Joni Augusto Cirelli*

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FÁBIO RENATO MANZOLLI LEITE

PAPEL DAS PROTEÍNAS INTRACELULARES NOD E DA PROTEÍNA ADAPTADORA MYD88 NA REGULAÇÃO DA EXPRESSÃO DE RANKL E MODULAÇÃO DA RESPOSTA INFLAMATÓRIA INDUZIDOS POR ANTÍGENOS BACTERIANOS IN VITRO. ESTUDO EM CÉLULAS RELEVANTES DO PERIODONTO.

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Sumário

Lista de abreviaturas

AP-1	*activating protein 1*
BMSC	*bone marrow stromal cell*
CARD	*caspase-activating and recruitment domain*
cDNA	*complementary DNA*
CXCL	*chemokine (C-X-C motif) ligand*
DNA	*deoxyribonucleic acid*
E. coli	*Escherichia coli*
ERK	*extracellular-regulated kinases*
FBS	*fetal bovine serum*
GAPDH	*glyceraldehyde-3-phosphate dehydrogenase*
IFNAR	*interferon-alpha/beta receptor*
IFN-b	*interferon beta*
IkB	*inhibitor of kappa B*
IKK	*inhibitor of the nuclear factor kappa B*
IL	*Interleukin*
IL-1R	*interleukin-1 receptor*
IRAK	*interleukin-1 receptor-associated kinase*
IRF	*interferon regulatory transcription factor*
ISGF3	*interferon-stimulated gene factor 3*
JNK	*c-Jun N-terminal kinase*
LBP	*LPS-binding protein*
LPS	*Lipopolysaccharide*
LRR	*leucine-rich repeats domain*
MAPK	*mitogen-activated protein kinase*
M-CSF	*macrophage colony-stimulating factor*
MDP	*muramyl dipeptide*
Meso-DAP	*meso-diaminopimelate acid*
mPDL	*Murine periodontal ligament fibroblast*
MyD88	*myeloid differentiation factor 88*
NBS	*nucleotide-binding site*
NF-κB	*nuclear factor kappa B*
NLR	*nod-like receptor*

NOD	*nucleotide-binding oligomerization domain*
OPG	*Osteoprotegerin*
P. gingivalis	*Porphyromonas gingivalis*
PGN	Peptidoglicano
PAMP	*pathogen-associated molecular patterns*
RANK	*receptor activator of nuclear factor-kappa B*
RANKL	*receptor activator of nuclear factor-kappa B ligand*
RAW264.7	linhagem de macrófagos de camundongos leucêmicos
RIP	*receptor interacting protein*
RNA	*ribonucleic acid*
RNAm/mRNA	RNA mensageiro / *messenger RNA*
ROS 17/2.8	*rat osteosarcoma cell line*
RT-PCR	*reverse transcription-polymerase chain reaction*
STAT	*signal transducers and activators of transcription protein*
TAB	*TAK-1 binding protein*
TAK	*TGF-activated kinase*
TIR	*Toll/Interleukin-1Receptor*
TIRAP	*TIR domain-containing adaptor protein*
TLR	*toll-like receptor*
TNF	*tumor necrosis factor*
TRAF6	*TNF receptor-associated kinase*
TRAM	*TRIF-related adaptor molecule*
TRIF	*TIR domain-containing adaptor-inducing IFN*
WT	*wild-type*
WT1	*wild-type P. gingivalis strain grown at 1µg/mL hemin*
WT10	*wild-type P. gingivalis strain grown at 10µg/mL hemin*

Leite FRM. Papel das proteínas intracelulares Nod e da proteína adaptadora MyD88 na regulação da expressão de RANKL e modulação da resposta inflamatória induzidos por antígenos bacterianos in vitro. Estudo em células relevantes do periodonto [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2009.

RESUMO

A reabsorção do osso alveolar é uma das principais características associadas à progressão da doença periodontal. Apesar da enorme complexidade da microbiota envolvida, considera-se que bactérias Gram-negativas tenham um papel relevante em sua etiopatogênese. Um dos fatores de virulência destes microrganismos é representado por um componente de sua parede externa denominado lipopolissacarídeo (LPS). A presença de LPS na proximidade dos tecidos periodontais é capaz de induzir a produção de diversos mediadores inflamatórios que levam à degradação tanto do tecido conjuntivo quanto ósseo. Atualmente acredita-se que a interação do ligante do receptor-ativador do fator nuclear kappa-B (RANKL) com seu receptor (RANK) presente em precursores hematopoiéticos é necessária e suficiente para a indução da diferenciação de osteoclastos. Por outro lado, a ligação de RANKL com seu falso-receptor, denominado osteoprotegerina (OPG), reduz sua biodisponibilidade e inibe, desta forma, a osteoclastogênese. Assim, a razão da expressão de RANKL e OPG é considerada como o principal determinante do "turnover" do tecido ósseo. A produção de RANKL e OPG depende das vias de sinalização ativadas, as quais são influenciadas pela natureza do estímulo extracelular. Atualmente, a família de receptores NLRs (*nod-like receptors*) foi identificada como receptor intracelular para componentes bacterianos e agentes moduladores de diferentes vias de sinalização. Considerando a relevância do LPS bacteriano na patogênese da doença periodontal, o papel do RANKL no processo de reabsorção óssea e a possível implicação das proteínas Nod na transdução de sinais regulando a expressão de RANKL, o objetivo geral deste projeto foi estudar os mecanismos de regulação da expressão de RANKL induzido por LPS bacteriano em células relevantes do periodonto (macrófagos, osteoblastos e fibroblastos). Os objetivos específicos propostos para este projeto foram avaliar, após estímulo com LPS e interleucina-1 beta, o papel das vias de sinalização intracelulares dependentes e não-dependentes de MyD88 e das proteínas Nod 1 e Nod2 na expressão de RANKL, OPG, IL-10 e IFN-b. A expressão de RANKL, OPG, IL-10 e IFN-β nos níveis de RNA mensageiro (RNAm) foram avaliados por RT-PCR; já os níveis protéicos de RANKL,

Nod1, Nod2, NF-κB p50 e p38 MAPK foram analisados por meio de Western blot. O papel das proteínas Nod1, Nod2 e MyD88 na expressão de RANKL e ativação de p38 MAPK e NF-κB foram avaliados em culturas de células do estroma ósseo (BMSC) de camundongos *knockout* para os genes dessas proteínas intracelulares. Ainda que ambos LPS e IL-1β tenham induzido significativo aumento na produção de RNAm de RANKL, a ausência de MyD88, Nod1 e Nod2 resultou em significativa inibição deste efeito, indicando que estas proteínas têm um papel relevante na expressão de RANKL. MyD88 e Nod1 estão envolvidas na inibição da expressão de OPG após ativação de TLRs e IL1R, enquanto Nod2 parece exercer um papel de bloqueio ou repressão parcial da expressão de OPG induzida pelo estímulo dos mesmos receptores. O tratamento de culturas primárias de BMSC com qualquer um dos agonistas utilizados não resultou em regulação importante da expressão de RNAm de IFN-b. A ausência de Nod1 não levou a efeitos significativos sobre a expressão de IFN-b, no entanto MyD88 e Nod2 são necessários para a repressão da expressão de IFN-b por BMSC. Esses achados demonstram que as proteínas MyD88, Nod1 e Nod2 exercem papel distinto na regulação da expressão de RANKL e OPG, sendo um possível alvo para novas drogas no tratamento de distúrbios ósseos.

Palavras-chave: NF-kappa B; Receptor Ativador de Fator Nuclear kappa-B; Proteínas Adaptadoras de Sinalização Nod; Fator 88 de Diferenciação Mielóide; Sistema de sinalização das MAP Quinases.

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ABSTRACT

Bone resorption is one of the major characteristics of destructive periodontal disease. Despite the great number of different bacterial species in the dental biofilm, Gram-negative microorganisms were demonstrated to have a very important role on periodontal disease pathogenesis. Lipopolysaccharide (LPS) is a bacterial cell wall component, which is acknowledged as one of the main virulence factors of these microorganisms. The mere presence of LPS in proximity with the periodontal tissues initiates the expression and production of inflammatory mediators and other cytokines which can culminate in degradation of both soft and hard tissues. It is currently accepted that the interaction between receptor-activator of nuclear factor kappa-B ligand (RANKL) and its receptor (RANK) is both necessary and sufficient to induce osteoclast differentiation and activation. However, RANKL can interact with its soluble decoy receptor osteoprotegerin (OPG) inhibiting osteoclastogenesis by decreasing the bioavailability of RANKL. Production of RANKL/OPG is the result of the signaling pathways activated by external stimuli. Recently, the NLR (nod-like receptors) family was identified as cytosolic receptors for bacterial components and also, as capable of modulating different signaling pathways. Considering the relevance of LPS and RANKL in bone resorption and the possible implication of Nod proteins in signal transduction regulating RANKL expression, the aim of this study was to evaluate the influence of different intracellular signaling pathways on the regulation of RANKL expression induced by LPS in relevant cells of the periodontium (macrophages, osteoblasts and fibroblasts). The specific objectives proposed were to determine after LPS and interleukin-1 beta stimulation the role of MyD88-dependent and independent signaling pathways, Nod1 and Nod2 on the expression of RANKL, OPG, IL-10 and IFN-beta. Expression of RANKL, OPG, IL-10 and IFN-beta at mRNA level was evaluated by reverse transcription polymerase chain reaction; moreover proteic levels of RANKL, Nod1, Nod2, NF-κB p50 and p38 MAPK were analyzed by Western blot. The role of Nod1, Nod2 and MyD88 proteins on RANKL expression and on the activation of p38 MAPK and NF-κB were determined in primary cultures of bone marrow stromal cell cultures (BMSC) isolated from genetically-modified mice lacking expression of these

proteins. Though both LPS and IL-1β induced a significant increase on RANKL mRNA level, lack of MyD88, Nod1 and Nod2 resulted in a remarkable inhibition on RANKL transcription. Thus, all of these proteins have a relevant role on RANKL expression. MyD88 and Nod1 are related to the inhibition of OPG expression after cellular activation by LPSs and IL-1β, on the other hand Nod2 seems to block or repress the inhibition of OPG expression induced by the stimulation with the three agonists. Expression of IFN-beta mRNA by BMSCs was not affected by any of the agonists used. Nod1 was not required for IFN-beta mRNA expression; however MyD88 and Nod2 are essential for the repression of IFN-beta expression in BMSCs. These findings demonstrate that MyD88, Nod1 and Nod2 have distinct roles on RANKL and OPG regulation and may be a target for new drugs to treat bone disorders.

Keywords: Receptor Activator of Nuclear Factor-kappa B; NF-kappa B; Nod Signaling Adaptor Proteins; Myeloid Differentiation Factor 88; MAPKinases signaling system.

INTRODUÇÃO

Introdução

As doenças periodontais são condições nas quais microrganismos Gram-negativos têm papel fundamental (Genco[27], 1992; Moore, Moore[68], 1994; Slots, Genco[90], 1984). Um dos principais fatores de virulência destes microrganismos é o lipopolissacarídeo (LPS), um constituinte da parede microbiana capaz de induzir uma resposta inflamatória. As conseqüências desta resposta induzida pelos antígenos microbianos, como o LPS, incluem destruição de tecido conjuntivo e reabsorção óssea (Ekuni et al.[17], 2003; Garlet et al.[25], 2003; Rossa et al.[85], 2006).

Acreditava-se que o efeito do LPS era mediado exclusivamente pelo receptor CD14, expresso primariamente por monócitos/macrófagos como receptor protéico de membrana (Wright[114], 1995). As células que não expressavam CD14 constitutivamente, responderiam ao LPS por meio de uma forma solúvel de CD14 presente no soro (Frey et al.[21], 1992). Ambas as formas de CD14 reconheceriam um complexo formado por ligação entre LPS e proteína ligante ao LPS (LPS-binding protein, ou LBP), uma glicoproteína encontrada tanto no plasma de indivíduos sadios quanto no plasma de pacientes que apresentam resposta inflamatória aguda.

No entanto, tanto o CD14 ligado à membrana quanto o complexo formado por CD14 solúvel-LPS/LBP não apresentam uma extensão, porção ou domínio citoplasmático. Assim, a ativação de vias de sinalização intracelulares dependeria de um co-receptor. Esta necessidade foi evidenciada pela observação de camundongos nocauteados para a expressão de CD14, os quais ainda respondiam ao estímulo com LPS por vias de sinalização alternativas (Perera et al.[82], 1997).

Recentemente, uma nova classe de receptores de membrana foi identificada, denominada Toll-like receptors (TLR). Até o momento, mais de 10 membros desta família de proteínas foram identificados (Wang, Ohura[110], 2002). TLR são proteínas transmembranas com porções ricas em leucina nos domínios extracelulares, enquanto a porção intracelular é similar a do receptor de interleucina-1, e por isso denominado domínio Toll/IL-1R (TIR). Atualmente, os TLR são considerados os efetivos receptores de LPS (Chow et al.[15], 1999; Kirschning et al.[51], 1998; Poltorak et al.[84], 1998).

Cinco membros desta família, TLR2, TLR4, TLR5, TLR6 e TLR9 medeiam a resposta a padrões moleculares associados a patógenos (PAMP) que são expressos em componentes microbianos, incluindo LPS, lipopolipeptídeos, peptidoglicanos (PGN), ácido lipotéico, flagelina e motivos CpG no DNA. TLR2 e TLR4 são os principais receptores ativados frente à agressão bacteriana via LPS (Inohara et al.[40], 2001). Na tabela 1 abaixo foram destacados alguns dos principais receptores do tipo toll (TLR) relacionados a processos inflamatórios e/ou infecciosos, seus respectivos ligantes e tipos celulares nos quais estão presentes (Akira[3], 2009; Chiron et al.[14], 2008; Himmel et al.[35], 2008; McGettrick, O'Neill[63], 2007; Pollanen et al.[83], 2009; Uehara, Takada[107], 2007; Uehara et al.[108], 2007).

Tabela 1 – Relação dos principais tipos celulares e ligantes dos receptores do tipo toll (TLR):

Receptor	Ligante	Localização do ligante	Tipo celular
TLR1	Lipopeptídeos	Bactérias	Monócitos/macrófagos, linfócitos B, células dendríticas, células epiteliais orais, fibroblastos gengivais
TLR2	Glicolípidios, lipopeptídeos, ácido lipoteicóico, lipoproteínas Zimozan	Bactérias Fungos	Monócitos/macrófagos, mastócitos, células dendríticas, células epiteliais orais, fibroblastos gengivais, osteoblastos, osteoclastos, linfócitos B e T, neutrófilos
TLR3	RNA dupla-fita, Poli I:C	Vírus	células epiteliais orais
TLR4	Lipopolissacarídeo, proteínas de choque térmico Fibrinogênio, heparam sulfato, ácido hialurônico	Bactérias Mamífero	células epiteliais orais, fibroblastos gengivais, osteoblastos, osteoclastos, linfócitos T, neutrófilos
TLR5	Flagelina	Bactéria	células epiteliais orais, fibroblastos gengivais, osteoblastos, linfócitos B e T, neutrófilos
TLR6	Lipopeptídeos	Micoplasma	células epiteliais orais, fibroblastos gengivais, linfócitos B e T, neutrófilos
TLR7	Imidazoquinolina, bropirimina RNA simples-fita	Fármacos Vírus	células epiteliais orais, fibroblastos gengivais, linfócitos B, neutrófilos
TLR8	RNA simples-fita	Vírus	células epiteliais orais, fibroblastos gengivais, linfócitos B e T, neutrófilos
TLR9	CpG DNA	Bactéria	células epiteliais orais, fibroblastos gengivais, osteoblastos, linfócitos B, neutrófilos

Alguns tipos celulares são importantes na homeostase tecidual do periodonto, por exemplo, osteoblastos, macrófagos e fibroblastos. Os osteoblastos mantêm o "turnover" tecidual participando do equilíbrio entre os processos anabólicos e catabólicos (Takayanagi[96], 2005). Os macrófagos são altamente prevalentes no periodonto acometido por doença periodontal, representando o protótipo do tipo celular envolvido na resposta à agressão microbiana, além de representarem os progenitores diretos dos osteoclastos (Boyle et al.[10], 2003). Por outro lado, os fibroblastos são o tipo celular mais abundante, sendo responsáveis pela síntese e degradação do tecido conjuntivo (Hassell[32], 1993). Estes tipos celulares secretam diversas citocinas imunoreguladoras, podendo exercer papel importante na reabsorção óssea associada à inflamação causada pela agressão microbiana (Hefti[34], 1993; Kwan Tat et al.[56], 2004).

Fibroblastos do tecido gengival e do ligamento periodontal e osteoblastos expressam TLR2 e TLR4 constitutivamente (Rossa et al.[87], 2007). Assim, tanto fibroblastos quanto osteoblastos apresentam os receptores necessários para responder ao estímulo por LPS (Hatakeyama et al.[33], 2003; Kikuchi et al.[48], 2001). Estudos em animais mostraram que injeções de LPS purificado ou a introdução de uma ligadura ao redor de molares são capazes de induzir perda óssea alveolar, principal característica das doenças periodontais destrutivas (Garcia de Aquino et al.[24], 2009; Patil et al.[81], 2006).

Em relação ao processo de reabsorção do tecido ósseo, o paradigma atual é baseado num sistema de citocinas identificado na década de 90. O ligante do receptor ativador do fator nuclear kappa-B (RANKL) foi descoberto durante a busca de um ligante para osteoprotegerina (OPG), uma

proteína capaz de inibir a reabsorção óssea (Aubin, Bonnelye[6], 2000). RANKL pode ser expresso em duas formas moleculares: um polipeptídio ancorado na membrana celular, e uma forma solúvel/secretada resultante de uma modificação enzimática pós-traducional (Hofbauer, Heufelder[36], 2001). Segundo o entendimento atual do sistema de citocinas controlando a osteoclastogênese, a ligação de RANKL ao RANK (receptor ativador do fator nuclear kappa-B), seu receptor expresso na membrana de células precursoras da linhagem hematopoiética, inicia uma cascata de sinalização intracelular e expressão gênica que levará à diferenciação, maturação e/ou ativação de osteoclastos. A osteoprotegerina funciona como receptor falso para RANKL, impedindo sua interação com RANK e, conseqüentemente, inibindo a osteoclastogênese e reabsorção óssea (Figura 1).

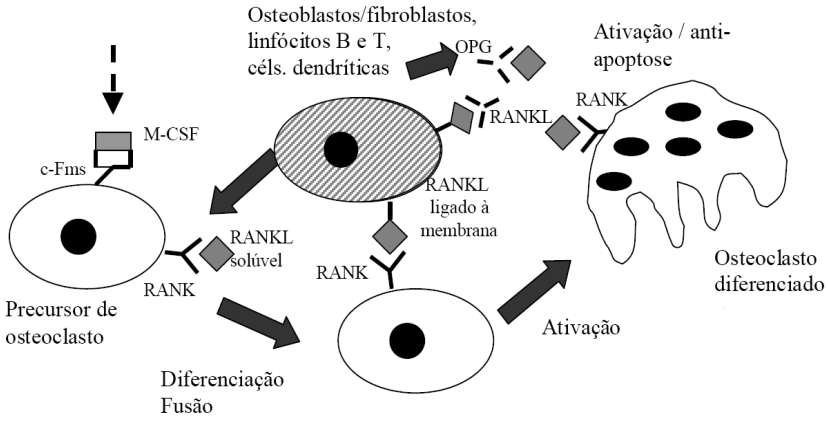

FIGURA 1 – Modelo de regulação da biologia celular de osteoclastos pelo sistema RANKL, seu receptor RANK e seu antagonista OPG (Hofbauer, Heufelder[36], 2001).

Embora a expressão tanto de RANK quanto de RANKL ao nível de RNAm seja amplamente distribuída em diversos tipos celulares, a produção

da proteína RANKL, real agente desencadeador de resposta, é restrita às linhagens de osteoblastos, osteoclastos, células dendríticas, linfócitos T e B e fibroblastos (Hsu et al.[38], 1999). Fibroblastos dos tecidos periodontais expressam RANKL, em especial na presença de outros mediadores inflamatórios (Kanzaki et al.[44], 2001) e também em resposta a LPS bacteriano. Além disso, a expressão de mediadores inflamatórios como IL-1, IL-6 e IL-8 pode ser induzida por LPS bacteriano nos tipos celulares acima citados, podendo representar um mecanismo indireto pelo qual o estímulo com LPS induz à expressão de RANKL (Kent et al.[46], 1999; Takada et al.[95], 1991; Tamura et al.[99], 1992; Yamaji et al.[116], 1995).

Os diferentes tipos de respostas imunológicas observadas no curso da doença periodontal ainda são controversos. De acordo com alguns estudos, a expressão de citocinas pró-inflamatórias como IL-1β, IL-6, TNF-α e IL-8 está aumentada na doença periodontal, caracterizando um perfil de citocinas relacionado ao padrão Th2 de resposta (Matsuki et al.[61], 1992; Tokoro et al.[104], 1997). Por outro lado, outros estudos demonstram que a doença periodontal em progressão tende a apresentar níveis mais elevados de citocinas relacionadas ao padrão Th1 de resposta imunológica e em quando crônica pode resultar em padrão Th2 (Berglundh, Donati[9], 2005; Garlet et al.[25], 2003).

Outro ponto ainda a ser esclarecido seria a expressão de citocinas com capacidade de modulação negativa da resposta inflamatória, como IL-2, IL-4 e IL-10, com relatos de maior e menor expressão em situações clínicas de doença periodontal (Fujihashi et al.[22], 1993; Tokoro et al.[104], 1997). Em relação ao metabolismo ósseo, a associação entre expressão aumentada de RANKL e menor expressão de OPG na doença periodontal é demonstrada em

estudos com modelos animais e também humanos (Mogi et al.[67], 2004; Teng et al.[103], 2000).

As vias de sinalização intracelular envolvidas após o estímulo por LPS são complexas e apenas parcialmente compreendidas. A similaridade entre as porções intracelulares dos TLR e do receptor da IL-1 é evidenciada pela ativação de algumas proteínas kinase em comum, por exemplo, IRAK (*IL-1 receptor-associated kinase*) e TRAF6 (*TNF receptor-associated kinase*), assim como pela ativação dos mesmos fatores de transcrição (como NF-κB). Desta forma, neste estudo a utilização de IL-1β como um agente estimulante extracelular foi devido a sua ativação de IL-1R permitindo uma avaliação comparativa do papel de mediadores inflamatórios derivados da resposta do hospedeiro na ativação das vias de sinalização e expressão de RANKL/OPG. Além disso, o uso de IL-1β permitiria avaliar o comportamento de cada tipo celular frente a um estímulo inflamatório atuando como “controle inflamatório”.

Existe grande complexidade nas proteínas adaptadoras envolvidas na sinalização intracelular de TLRs (Figura 2). 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.

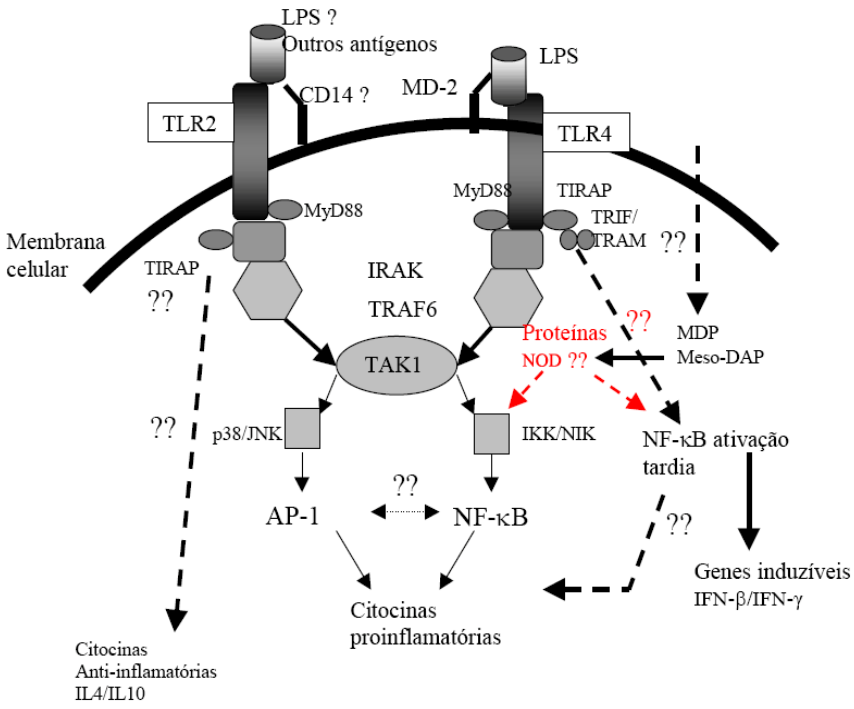

FIGURA 2 – Vias de sinalização intracelular envolvidas após a estimulação por LPS e outros antígenos bacterianos (Kopp, Medzhitov[54], 1999).

As proteínas Nod, ou *Nucleotide-binding Oligomerization Domain*, representam uma família de proteínas citoplasmáticas recentemente identificadas, 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*) (Chamaillard et al.[11], 2003; Murray[70], 2005). Duas destas proteínas, denominadas Nod1 e Nod2 vêm sendo intensamente estudadas, e são produtos de genes de cópia única altamente conservados e amplamente expressos (Iwanaga et al.[42], 2003; Ogura et al.[79],

2001). Ainda que a função destas proteínas não seja completamente conhecida, sua similaridade estrutural com as proteínas R de plantas sugere um papel relacionado ao reconhecimento de antígenos e de patógenos intracelulares. Nas plantas, cada célula funciona como uma unidade autônoma devido à falta de resposta imune adaptativa, as proteínas R são produzidas tanto na superfície celular como no citoplasma (Holt et al.[37], 1988). Esta observação, juntamente com evidências de que as proteínas Nod efetivamente reconhecem antígenos derivados da parede celular microbiana, somado à identificação de mutações no gene da NOD2 em pacientes com doença de Crohn, uma doença inflamatória intestinal associada à alteração na resposta imune aos microrganismos do trato intestinal, levou a proposição das proteínas Nod como análogas citoplasmáticas dos TLR (Inohara et al.[40], 2001; Ogura et al.[78], 1994).

No entanto, existe também a hipótese de que Nod1 e Nod2 atuem como modificadores da transdução de sinais iniciada por antígenos microbianos, modulando a resposta celular quanto à expressão de citocinas ou indução de apoptose. Esta modulação ocorreria especialmente na presença dos supostos ligantes específicos de Nod. Nod1 reconhece peptidoglicanos contendo fragmentos de ácido meso**-**diaminopimélico (meso-DAP) presente na maioria das bactérias Gram-negativas e algumas Gram-positivas (Girardin et al.[29], 2003), enquanto Nod2 reconhece muramildipeptídeo (MDP) encontrado em peptidoglicanos de bactérias Gram-positivas e –negativas (McDonald et al.[62], 2005). Dados recentes indicam que as proteínas Nod, especificamente Nod1 e Nod2, estão involvidas na ativação da expressão de genes inflamatórios (Franchi et al.[20], 2009) e até no reconhecimento de LPS independente de TLR (Inohara et al.[40], 2001). Atualmente, a interação entre as vias de sinalização induzidas após

ativação de TLR e o papel das proteínas Nod na ativação da resposta celular permanecem desconhecidos. Tanto Nod1 como Nod2 ativam uma molécula sinalizadora comum, Rip2 (*receptor interacting protein* 2, também conhecida como RICK ou CARDIAK), a qual leva à ativação de NF-κB pela interação com a subunidade reguladora do IKK (IKK ou NEMO). Esta interação resulta na fosforilação de IκB-α, subseqüente liberação do NF-κB e degradação pelo proteassomo. O NF-κB é translocado ao núcleo para ativação da transcrição (Inohara et al.[40], 2001). Além disso, outras vias de sinalização podem ser ativadas pelas proteínas Nod, como JNK (Girardin et al.[28], 2001).

Os modelos de sinalização associados a TLR2 e TLR4 estão apresentados resumidamente na Figura 2, e a ativação de kinases como TRAF6 e TAK1 que são ativadores *upstream* comuns a diversas vias de sinalização sugere que outras vias podem estar implicadas após a ligação de antígenos microbianos a estes receptores. Além disso, a sinalização pelos TLRs pode incluir o recrutamento de diferentes proteínas adaptadoras, o que pode modular a cinética e/ou intensidade da ativação das proteínas kinase ativadas por mitógenos (mitogen-activated protein kinases, MAPK) como ERK (extracellular-regulated kinases), JNK (c-Jun N-terminal kinase) e p38 (Wright et al.[115], 1990).

A sinalização intracelular induzida por LPS via TLR apresenta dois possíveis pontos de ramificação: o primeiro é no início da via, representado pela proteína adaptadora MyD88 (myeloid differentiation factor 88). A via MyD88-dependente leva à ativação de IRAK, TRAF6 e finalmente NF-κB, sendo essencial na indução da expressão de citocinas (Medzhitov et al.[64], 1998). De fato, a ativação de NF-κB é relevante para diversas condições inflamatórias crônicas, como aterosclerose, artrite reumatóide e as doenças periodontais

(Nichols et al.[76], 2001). Já a via MyD88-independente não ativa IRAK, mas leva à ativação tardia de NF-κB. Esta via alternativa requer proteínas adaptadoras diferentes, como TIRAP (TIR *domain-containing adaptor protein*), TRIF (TIR *domain-containing adaptor-inducing IFN*) e TRAM (TIR *domain-containing adaptor-inducing IFN-related adaptor molecule*) e supõe-se que não seja relevante para a expressão de citocinas pró-inflamatórias (Sato et al.[88], 2004; Takeuchi, Akira[98], 2001; Yamamoto et al.[117], 2002; Yamamoto et al.[118], 2003; Yamamoto et al.[119], 2003). Na verdade, sugere-se que esteja relacionada à secreção de interferon (IFN)-beta e ativação indireta de outros genes IFN-dependentes, os quais podem agir como reguladores indiretos da expressão de RANKL (Mirosavljevic et al.[66], 2003; Muzio et al.[71], 1998; Swantek et al.[94], 2000; Takayanagi[96], 2005). O segundo ponto de ramificação ocorre na etapa seguinte à ativação da MAP kinase-kinase-kinase (MAPKKK), também conhecida como TAK-1 (TGF-activated kinase 1). Seguindo o ramo da via que envolve as MAPKs, deve ocorrer a ativação preferencial de AP-1 (activating protein 1), enquanto seguindo a via do inibidor do fator nuclear kappa B (IKK) ocorrerá a ativação preferencial de NF-κB. Não se sabe a relevância e/ou a razão da ativação de diversas vias de sinalização intracelulares pelo mesmo receptor: pode ser um mecanismo redundante/compensatório, ou uma forma de especificar a resposta celular em termos de modulação da resposta biológica de diferentes tipos celulares pelo balanço na produção de citocinas pró-inflamatórias ou anti-inflamatórias (Netea et al.[74], 2004; Wang, Ohura[110], 2002). A interação e participação das proteínas Nod na sinalização intracelular iniciada por agonistas de TLR pode resultar em ativação sinérgica de NF-κB (Marriott et

al.[60], 2005; Ogura et al.[78], 1994). Esta ativação sinérgica pode aumentar a expressão dos genes regulados por NF-κB, acentuando a resposta inflamatória.

O papel do LPS na osteoclastogênese é complexo, evidências recentes indicam que seu efeito modulador pode ser positivo ou negativo. Assim, LPS pode aumentar a reabsorção óssea pelo favorecimento da osteoclastogênese, por exemplo, por meio da indução de RANKL e/ou inibição de OPG (Taubman et al.[102], 2005). Trabalhos envolvendo análise do eixo RANKL/OPG utilizam LPS de *E. coli* como agonista de TLR4 representando o LPS da maior parte dos microrganismos Gram-negativos associados tanto a doenças sistêmicas quanto a periodontais. Nos estudos a seguir também foi avaliado o efeito do LPS de *P. gingivalis* devido à sua capacidade de ativar tanto TLR2 quanto TLR4 e por sua associação com a presença de doença periodontal em humanos.

O LPS pode inibir a atividade do RANKL em células precursoras de osteoclastos pela redução da expressão tanto de RANK quanto do receptor para o fator estimulador de colônia de macrófagos (M-CSF) (Zou, Bar-Shavit[124], 2002). É interessante notar que o uso de LPS como único agonista em fibroblastos do tecido conjuntivo gengival não induziu a expressão de RNAm para RANKL. Pelo contrário, o estímulo com LPS nestas células induziu a expressão e secreção de OPG, como evidenciado pela inibição da diferenciação de osteoclastos, quando os precursores de osteoclastos foram cultivados na presença do sobrenadante obtido de culturas de fibroblastos estimulados por LPS (Nagasawa et al.[72], 2002). Por outro lado, LPS estimulou a expressão de RANKL em fibroblastos do ligamento periodontal (Rossa et al.[86], 2008; Wada et al.[109], 2004). Estes fatos ressaltam a importância de avaliar a expressão de

RANKL induzido por LPS em diferentes tipos celulares. Uma melhor compreensão das vias de sinalização ativadas por LPS, incluindo o papel das proteínas Nod na amplificação e/ou ativação sinérgica da expressão gênica, e suas conexões com a expressão das moléculas relacionadas ao “turnover” do tecido ósseo (RANKL, OPG) e à regulação da resposta inflamatória (IL-10 e IFN-β) ainda se faz necessária. Este conhecimento pode proporcionar alvos potenciais para o desenvolvimento de novas estratégias terapêuticas com objetivo de inibirem/reduzirem a reabsorção óssea e modulação da resposta inflamatória. Uma destas estratégias pode ser baseada no uso de compostos bioquímicos para o bloqueio ou inibição de vias de sinalização ativadas pela interação do LPS aos seus potenciais receptores (TLR2, TLR4 e CD14).

PROPOSIÇÃO

Os objetivos deste trabalho foram:

1 - Avaliar, após estímulo com LPS e IL-1β, o papel das vias de sinalização intracelulares dependentes e não-dependentes de MyD88 na expressão de RANKL, do seu antagonista OPG, do regulador negativo da inflamação interleucina 10, e também de interferon beta.

2 - Estudar o papel das proteínas Nod1 e Nod2 na expressão de RANKL, do seu antagonista OPG, do regulador negativo da inflamação interleucina 10, e também de interferon beta.

3 – Verificar a influência da modulação estrutural hemina-dependente do lípide A do lipopolissacarídeo de *Porphyromonas gingivalis* sobre a regulação da expressão de IL-6, IL-12 e CXCL10 em cultura de monócitos humanos.

CAPÍTULO 1

TLR2 and TLR4 signaling modulate RANKL expression differentially in relevant periodontal cells*

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Running title: TLR2 and -4 activation in periodontal cells

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ABSTRACT

Resident, non-immune cells have been suggested to play a role in innate immune response, since these cells express various pattern-recognition receptors and produce inflammatory cytokines in response to microbial antigens. Alveolar bone resorption is the hallmark of destructive periodontitis and it is due to the immune system reaction to bacteria and their mediators present on the biofilm. The balance between the expression levels of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) is pivotal for osteoclast differentiation and activity and have been implicated in the progression of bone loss in periodontitis. To assess the contribution of resident cells to the bone resorption mediated by innate immune signaling, we stimulated fibroblasts and osteoblastic cells with LPS from *Escherichia coli* (TLR4 agonist), *Porphyromonas gingivalis* (TLR2 and -4 agonist), and interleukin-1 beta (as a control for cytokine signaling through Toll/IL-1receptor domain). Expression of RANKL and OPG mRNA was studied by RT-PCR, whereas the activation of p38 MAPK and NF-kB signaling pathways, two signaling pathways activated downstream of TIR-containing receptors that are involved in inflammatory gene expression, was analyzed by western blot. In periodontal ligament cells and osteoblastic cells p38 MAPK pathway activation was biphasic with peaks occurring earlier in IL-1β-stimulated cells (at 10 and 60 minutes) than in those LPS-stimulated (20 and 120 minutes). LPS activated NF-κB in the later time points (60 and 120 min). *E.coli* LPS was a better inducer of RANKL; however OPG expression was affected only in osteoblasts. *P. gingivalis* downregulated RANKL expression in periodontal ligament cells but increased its expression in osteoblasts. IL-1β induced higher levels of RANKL especially in osteoblasts. Thus, the evaluated cellular types responded differently to the same agonist suggesting different roles in the beginning and progression of periodontal disease.

INTRODUCTION

In recent years, the essential role of RANKL/ RANK/ OPG axis in osteoclast differentiation/activation in inflammatory bone diseases, including periodontal disease was confirmed (Kawai et al.[8], 2006). In the inflamed periodontal microenvironment, various cell types are capable of expressing RANKL, including periodontal ligament fibroblasts, leading to tissue destruction through activation of RANK on pre-osteoclasts (Kanzaki et al.[7], 2001). In fact, increased RANKL levels with lower expression of OPG were observed in both animal experimental models (Taubman et al.[23], 2005) and human periodontally-diseased sites (Kawai et al.[8], 2006). Specifically in periodontal disease, bacterial lipopolysaccharide (LPS) of Gram-negative microorganisms, has shown both in vitro (Rossa et al.[18], 2006) and in vivo (Garlet et al.[4], 2003) an important role in this damage process by inducing the expression of various inflammatory mediators including IL-1, IL-6 and IL-8 (Kent et al.[9], 1999) which can promote an indirect induction of RANKL and ultimately result in alveolar bone loss through Toll-like receptors (TLRs) signaling (Qureshi et al.[17], 1999). We have previously shown that TLR4 stimulation in periodontal ligament fibroblasts result in increased RANKL expression, which is at least partially dependent on p38 MAPK signaling (acrescente Rossa Jr et al., J Perio Res; v.43: 2012, 2008). In the periodontal tissues, gingival fibroblasts (GF) (Tabeta et al.[21], 2000), periodontal ligament fibroblasts (PDL) (Hatakeyama et al.[6], 2003) and osteoblasts (Kikuchi et al.[10], 2001) were shown to express TLR2 and TLR4 constitutively, which indicates that these cells are responsive to bacterial LPS (Kent et al.[9], 1999). In this context, these cell types that are classically thought of as contributing to periodontal tissue homeostasis (Takayanagi[22], 2005) can also participate in bone resorption during microbial aggression through secretion of many immunoregulatory cytokines (Kwan Tat et al.[11], 2004). Some of the cytokines produced by these resident cell types, such as IL-6 (Patil et al., 2006), may not only act synergistically with the pathogen-associated molecular patterns in the activation of dendritic cells and modulation of the adaptive immune

response; but can also have direct effects contributing to the process of tissue degradation and bone resorption. Other inflammatory mediators and cytokines produced by the resident cell types in response to TLR signaling may have primarily direct effects on the modulation of tissue degradation, including matrix metalloproteases (Rossa et al., 2005; ref), PGE_2 (Kim et al.,) and RANKL (Rossa Jr et al., J Perio Res; v.43: 2012, 2008). Thus, the cytokine network associated with diseased periodontal tissues is very complex, and it is currently thought that the cytokine profile ultimately determines disease activity (Bickel et al.[1], 2001). This cytokine profile is the consequence of the activation of different signaling pathways by a multitude of external signals present in the periodontal microenvironment, resulting in a signaling network that modulates cytokine production. Considering the infectious nature of periodontal disease and the crucial role of bacterial LPS as a ubiquitous and chronically present microbial stimulus, we studied LPS-induced activation of signaling pathways that are especially relevant for expression of inflammatory genes. LPS stimulation may have a positive role in the modulation of the osteoclastogenesis process by inducing RANKL and/or inhibiting OPG expression or a negative role by reducing the expression of RANK or M-CSF receptor on pre-osteoclasts (Zou e Bar-Shavit[27], 2002). In human gingival fibroblasts, LPS had a bone-protective effect by inducing the production of OPG (Nagasawa et al.[12], 2002).

TLR signaling is very complex because it can involve the recruitment of different protein adaptors and/or the activation of *mitogen-activated protein kinases* (MAPK), including ERK (*extracellular-regulated kinases*), JNK (*c-Jun N-terminal kinase*) and p38 (Patil et al.[16], 2006) in addition to the activation of the transcription factor NF-κB. These signaling pathways have an essential role in cytokine expression during many chronic inflammatory conditions such as atherosclerosis, rheumatoid arthritis and periodontal diseases (Garcia de Aquino et al.[3], 2009; Nichols et al.[13], 2001). Recently, our research group showed that activation of p38 and ERK MAPKinases, as well as of NF-κB during the course of LPS- and ligature-induced

experimental periodontitis was associated with the severity of inflammation, but the kinetics of the activation of these signaling pathways was different in each experimental model (Garcia de Aquino et al.[3], 2009). These results suggest that the activation of signaling pathways and ultimately the cytokine profile varies depending on the nature of the extracellular stimuli and the cellular type. In this regard, it is especially interesting to evaluate the LPS-induced RANKL and OPG expression, as well as to know the kinetics of activation of p38 MAPK and NF-κB signaling pathways by TLR signaling in resident cell types that are relevant to periodontium, such as periodontal ligament fibroblasts and osteoblasts.

MATERIAL AND METHODS

Cells and materials

Mouse periodontal ligament fibroblasts (mPDL), immortalized with simian vírus 40 large T antigen, were originally obtained from Dr Martha Somerman (University of Washington, Seattle, WA, USA). These cells were previously characterized for the expression of genes normally expressed by primary periodontal ligament cells, including bone sialoprotein, osteopontin, osteocalcin and type I collagen (30). Osteoblastic rat osteosarcoma cells ROS 17/2.8 (ROS) were obtained from Dr. Laurie McCauley (University of Michigan, Ann Arbor, MI, USA). Osteoblast phenotypic mRNAs, including bone sialoprotein and osteocalcin, were routinely assayed to verify osteoblastic phenotype expression in these cells. These cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 100 IU/ mL of penicillin, 100 lg/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO2. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide from *Escherichia coli* (serotype O55:B5) was purchased from Sigma-Aldrich (St Louis, MO, USA) and *Porphyromonas gingivalis*

lipopolysaccharide was purchased from Invivogen (San Diego, CA, USA). Both *E. coli* and *P. gingivalis* lipopolysaccharide were diluted in RNAse-free water to 5 mg/mL. Recombinant human Interleukin-1β protein was from R & D systems (Minneapolis, MN, USA). All primer pairs were purchased from Invitrogen (Carlsbad, CA, USA). Mouse RANKL monoclonal antibody was obtained from Imgenex (San Diego, California, USA). Phosphorylated p38 monoclonal antibody was from Cell Signaling (Danvers, MA, USA), and monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as phosphorylated p50 antibodies were from Santa Cruz Biotecnology (Santa Cruz, CA, USA)

Semi-quantitative RT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3 (Thermo Electron Corporation) spectrophotometer. Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 μM Oligo (dT) 12-18 primers and 1.25 U/uL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM MgCl2, 2 mM dNTPs and 0.8 U/μL of RNAse inhibitor, according to the manufacturer's protocol (Improm II – Promega). The PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 25 uL total volume PCR reaction mix (Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for RANKL, OPG and GAPDH genes yielding products of 467, 140 , 503 and 418 bp for RANKL (mouse - mPDL) , RANKL (rat - ROS) OPG and GAPDH, respectively. The primer pair used for RANKL – mouse - (accession no.: NM011613) was: sense 5'-CAGCACTCACTGCTTTTATAGAATCC-3';antisense 5'-AGCTGAAGATAGTCTGTAGGTACGC- 3'; for RANKL – rat - (accession no.: NM057149) was: sense 5'- TCGGGTTCCCATAAAGTCAG-3', antisense 5'-CTGAAGCAAATGTTGGCGTA, for osteoprotegerin (accession no.: NM008764) was:

sense 5'- TGTAGAGAGGATAAACGG - 3'; antisense 5'- CTAGTTATATGCAGCTTAT-3'; and for GAPDH (accession no.: BC083065) was: sense 5'-CACCATGGAGAAGGCCGGGG- 3'; antisense 5'- GACGGACACATTGGGGTAG- 3'. Optimyzed cycling conditions used for RANKL (from mouse) and OPG were: initial denaturation at 95°C for 2 min and 30 cycles (35 cycles for OPG) of: 95°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 7 min in the presence of 2.5 mM MgCl2 whereas for RANKL (from rat) the cycling conditions were as follows: initial denaturation at 95°C for 2 min and 40/36 cycles of: 95°C for 15 sec, 58°C for 30 sec, 72°C for 30 sec and a final extension step at 72°C for 7 min in the presence of 2.5 mM MgCl2. For GAPDH, RT thermocycler conditions were as follows: initial denaturation at 95°C for 2 min and 25 cycles of: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl2. The PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5µg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using Image J 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to RANKL and OPG mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Western Blot

A total of 10^5 mouse periodontal ligament cells (as well as of ROS osteoblastic cells) were grown for 24h in each well of six-well plates, de-induced by incubation for 12h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* LPS (100 ng/ mL or 1ug/mL), *P. gingivalis* LPS (1ug/mL or 10 ug/mL) or IL-1β (1ng/mL or 10 ng/mL) for 10, 20, 60 and 120 min for assessing activation of NF-κB and p38 MAPK, and also for 24h and 48h for RANKL protein expression. Whole cell lysates were prepared by scraping the cells of each well of six-

well plates with 100 ul of sodium dodecyl sulfate sample buffer (62.5 mM Tris HCl buffer, pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) on ice, followed by centrifugation at 9,000 RPM at 4°C for 5 minutes. 40ul of this content was heat-denatured at 95°C for 5 minutes and separated on 10% Tris-Cl polyacrylamide gels run at 100 V for 90 min and subsequently electro-transferred to 0.2 μM nitrocellulose membranes (300 mA constant current for 60 minutes). The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies. The presence of the primary antibodies was detected on radiographic film by using HRP-conjugated secondary antibodies and a chemiluminescence system (Lumi-Glo, Cell Signaling). The membranes were stripped off both primary and secondary antibodies by 10-minute washing in dH2O (2x) followed by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 10 minutes at room temperature and a final 10-minute wash in Tris-buffered saline containing 0.1% Tween-20. After stripping, the membranes were blocked as described previously and incubated with antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to verify equal loading of the wells. Digitalized images of the radiographic films were obtained in a gel documentation system (ImageQuant 100, GE Lifesciences) and densitometric measurements were obtained using ImageJ 1.37v software (NIH, Bethesda, MD, USA).

Statistical analysis

Pairwise comparisons between experimental groups were performed using the t-test with Welch's correction for unequal variances. Comparison between fold changes on mRNA expression between lipopolysaccharide-stimulated and untreated cells was performed with the one-sample t-test. The significance level was set to 5% and all calculations were performed using PRISM 4 software (GraphPad, Inc., San Diego, CA, USA).

RESULTS

TLR and IL-1R signaling results in differential activation of NF-κB and p38 MAPK in murine osteoblastic cells and periodontal ligament fibroblasts. TLR4, TLR2 and 4 and IL1R signaling was induced by stimulating both osteoblastic and fibroblastic cells with LPS from *E. coli*, LPS from *P. gingivalis* and with rhIL-1β, respectively. In periodontal ligament fibroblasts, activation of p38 MAPK was biphasic using either LPS or IL-1β. However TLR signaling resulted in delayed activation of p38 in comparison to IL-1R signaling (10 and 60 minutes for IL-1R versus 20 and 120 minutes for TLR signaling; fig. 1A). Interestingly, activation of p38 MAPK was more intense with the lower concentration of LPS (100 ng/mL), whereas the higher concentration of LPS (1 µg/mL) resulted in a time-dependent activation with less evident peaks. In osteoblastic cells, TLR2 signaling was a more potent activator of p38 and NF-kB than TLR4 signaling. Stimulation of IL-1R was of intermediary potency in comparison to TLR2 and TLR4, but also resulted in more rapid and transitory activation, in particular of NF-kB (fig. 1B).

The same stimuli result in differential induction of RANKL and OPG expression in periodontal ligament fibroblasts and osteoblastic cells. Activation of TLR4 signaling in periodontal ligament fibroblasts resulted in a concentration-dependent increase on RANKL mRNA expression in later experimental periods (fig. 2A), whereas OPG mRNA levels were not affected. In contrast, TLR2 signaling significantly inhibited RANKL mRNA expression in these cells (fig. 2B). In fact RANKL mRNA expression only returned to the basal levels 24 hours after P.gingivalis LPS treatment. There was also a trend to decrease OPG mRNA expression with TLR2 signaling, but this inhibition was not as obvious. In IL-1β-stimulated periodontal ligament fibroblasts a more rapid induction of RANKL mRNA occurred with both IL-1β concentrations (fig. 2C), followed by a decrease in later experimental periods (18 and 24h). A discrete and rapid increase of OPG mRNA expression was observed in earliest

experimental periods, especially with the higher concentration of IL-1β. Regulation of RANKL at the protein level supported *E.coli* LPS as an inducer of RANKL, and this expression was sustained for 48h after the stimulation. Consistently with the inhibitory effect on mRNA expression, *P.gingivalis* LPS failed to induce RANKL protein at 24h; however a slight, but significant, increase was observed after 48h. In contrast to the regulation of mRNA, IL-1β was a more potent inducer of RANKL protein expression than *E.coli* LPS; however IL-1β-mediated induction of RANKL protein was observed at 24h, with a return to the basal levels of expression at 48 hours (Fig. 4A).

In osteoblastic cells, TLR4 stimulation by *E.coli* LPS (Fig. 3A) also proved to be the most potent inducer of RANKL mRNA, with a concentration-dependent increase. In contrast to the periodontal ligament fibroblasts, induction of RANKL occurred at early experimental periods, returning to the basal levels after 24 hours. Interestingly, induction of OPG mRNA was inversely proportional to the concentration of *E.coli* LPS. As opposed to the inhibitory effect on periodontal ligament fibroblasts, TLR2 activation by *P.gingivalis* LPS in osteoblastic cells also increased RANKL mRNA expression, with approximately the same potency as IL-1β (Fig. 3B). OPG mRNA levels fluctuated over time, but there was a trend of increased expression, especially after stimulation with *E.coli* LPS and IL-1β. In another contrast with the periodontal ligament fibroblasts, *E. coli* LPS was the most potent inducer of RANKL at the protein level, and this induction was sustained for 48h after the stimulation. IL-1β and *P.gingivalis* LPS also resulted in slight, albeit significant, increases on RANKL protein expression in osteoblastic cells (Fig. 4B).

DISCUSSION

Innate immunity is not ‘turned off’ once an adaptive immune response is elicited, and in fact these different aspects of host response can complement each other. In support to this concept, there is evidence that innate immune signaling

through pattern-recognition receptors (PRRs) may actually have a co-stimulatory effect in adaptive immune cells (Raffeiner et al., Arthritis Res Ther; v.7: R1412, 2005). In the periodontal disease microenvironment, the ubiquitous presence of various PAMPs and the widespread expression of PRRs by immune and non-immune cell types suggest that innate immune signaling play a role modulating the host response. However, cell type and differentiation state can influence both the expression of PRR, and also the signaling pathways activated by the same PAMPs (Schaeffer e Weber[20], 1999).

Our research group has previously shown that p38 MAPK and its upstream activators MKK3 and MKK6 play an important role on lipopolysaccharide-induced RANKL expression by periodontal ligament cells (Rossa et al.[19], 2008), and in this study we report that signaling through TLR2, TLR4 and IL1R results in biphasic activation of p38 MAPK, with a delayed kinetics of activation being associated with TLR signaling. The delayed activation of p38 upon TLR4 signaling is associated with a delayed induction of RANKL mRNA in comparison to IL1R signaling. A previous study suggested that RANKL and OPG expression in periodontal ligament cells was indirectly induced by the production of IL-1β and TNF-α 6 hours after LPS stimulation (Wada et al.[25], 2004). Our data show induction of RANKL mRNA in most of the groups 8 hours or more subsequent to stimulation, so it could be influenced by autocrine/paracrine IL-1β and TNF-α. When stimulated with IL-1β both periodontal ligament fibroblasts and osteoblasts showed a more rapid increase of RANKL mRNA. However, at the protein level these different agonists showed varying potencies in each cell type. *E. coli* LPS potently induced RANKL protein in osteoblastic cells, whereas IL-1β was a stronger inducer in periodontal ligament fibroblasts. It is tempting to speculate on potentially different roles for these cell types in periodontal disease based on their differential response to the same agonists: osteoblasts were more responsive to TLR signaling and periodontal ligament cells more sensitive to inflammatory mediators, e.g. IL-1, that may be produced in response to the initial bacterial challenge.

Activation of nuclear factor-kappa B (NF-κB) followed a similar pattern regardless of cell type, suggesting it is receptor-dependent with distinct results for TLR or IL1R stimulation. In both periodontal ligament fibroblasts and osteoblastic cells only IL1R signaling resulted in biphasic activation of NF-kB (especially with the lower concentration of IL-1β), whereas TLR2 and TLR4 signaling was associated with a more sustained activation over time. LPS and IL-1β induce IkB phosphorylation by IKKa, IKKb, and IKKg and its subsequent ubiquitination and degradation. IL-1β signaling through IL1R may also follow an alternative pathway where the adapter molecule MyD88 mediates the formation of the complex IL-1R-MyD88-IRAK4-IRAK-TRAF6 which activates IL-1 receptor-associated kinase 4 (IRAK4) leading to IRAK phosphorylation. Subsequently the complex IRAK-TRAF6-TAK1-TAB2-TAB3 leads to transforming growth factor β-activated kinase (TAK1) and TAK1-binding protein 2 and 3 (TAB2 and TAB3) phosphorylation on the membrane (Xiao et al.[26], 2008). The second peak of activation of NF-kB observed with IL1R signaling may be caused by a delayed NF-κB activation through TAK1, which, together with TRAF6, is also an upstream activator of MAPKinases.

We do not have an explanation of the inhibitory effect of *P.gingivalis* LPS on RANKL mRNA, but it is possible it might be the result of other signaling pathways activated by simultaneous TLR2/TLR4 stimulation. Moreover, this was a cell-type specific effect, as this inhibition was not observed in osteoblastic cells stimulated with *P. gingivalis* LPS. In the osteoblastic cells, we could not observe a clear difference on the time course of p38 activation after stimulation pf TLR and IL1R; however TLR4 stimulation was associated with a delayed activation of NF-kB in comparison to IL1R. As observed for the fibroblasts, stimulation of TLR4 was a more potent inducer of RANKL mRNA than IL1R, but in contrast to the fibroblasts, induction of RANKL mRNA was more rapid. Also, in osteoblastic cells there was no inhibition of RANKL mRNA after simultaneous stimulation of TLR2 and TLR4 by *P. gingivalis* LPS. In fact, there was a slight but significant increase on RANKL mRNA and *P.gingivalis*

LPS had approximately the same potency as IL-1β in inducing RANKL mRNA. Downregulation of RANKL mRNA and increased OPG expression was already observed in cementoblasts stimulated with *P. gingivalis* LPS (Nociti et al.[14], 2004). The authors suggest that cells attached to the root surface could have a protective mechanism to avoid tooth resorption, since progression of untreated periodontitis is associated with bone loss, but rarely present root resorption. We did not found consistent changes of great magnitude on OPG mRNA expression on fibroblasts and osteoblasts, suggesting that the agonists used to activate TLR and IL1R signaling did not evoke a protective feedback mechanism to maintain bone homeostasis. The relatively minor changes in OPG expression further suggests that the ultimate outcome of bone turnover induced by these agonists might be determined by the changes on the expression of RANKL.

These results illustrate how gene regulation induced by the same agonists may vary depending on cell type, as well as the differential utilization of intracellular signaling pathways in these different cell types. Periodontal ligament fibroblasts have been shown to be relevant for bone formation in periodontal regenerative procedures and also to express RANKL in response to different interleukin-1, lipopolysaccharide, prostaglandin E2 and mechanical stress (Fukushima et al.[2], 2005; Gottlow et al.[5], 1984; Tiranathanagul et al.[24], 2004) by the activation of mitogen-associated protein kinases (MAPKs). In osteoblasts exposed to the whole bacteria *Porphyromonas gingivalis*, RANKL mRNA levels were dependent on activation of activator protein 1 (AP-1); but, surprisingly, this activation did not require p38, ERK MAPKs or the phosphoinositol-3-kinase (PI3K) pathway (Okahashi et al.[15], 2004).

NF-κB and p38 MAPK are involved in the expression of various inflammatory mediators that play a role in the tissue degradation associated with periodontal diseases. We show that both LPSs and IL-1β were able to activate these signaling pathways in periodontal ligament fibroblasts and osteoblastic cells and also modulate the expression of RANKL and, to a lesser extent, of OPG. Among the

limitations of this study are the use of murine cell lines instead of primary cells, and also the fact that we did not assess the role of the signaling pathways on RANKL and OPG expression, which is currently being addressed by subsequent studies. The varying responses to TLR signaling by different cell types present in the periodontal tissues suggest different roles in the response to bacterial challenge. This concept is supported by our results showing that each cell type responds differently to the same agonists. The understanding of the potential role of different cell types and their activation mechanisms can contribute to the development of future strategies in periodontal disease treatment based on interfering with signal transduction.

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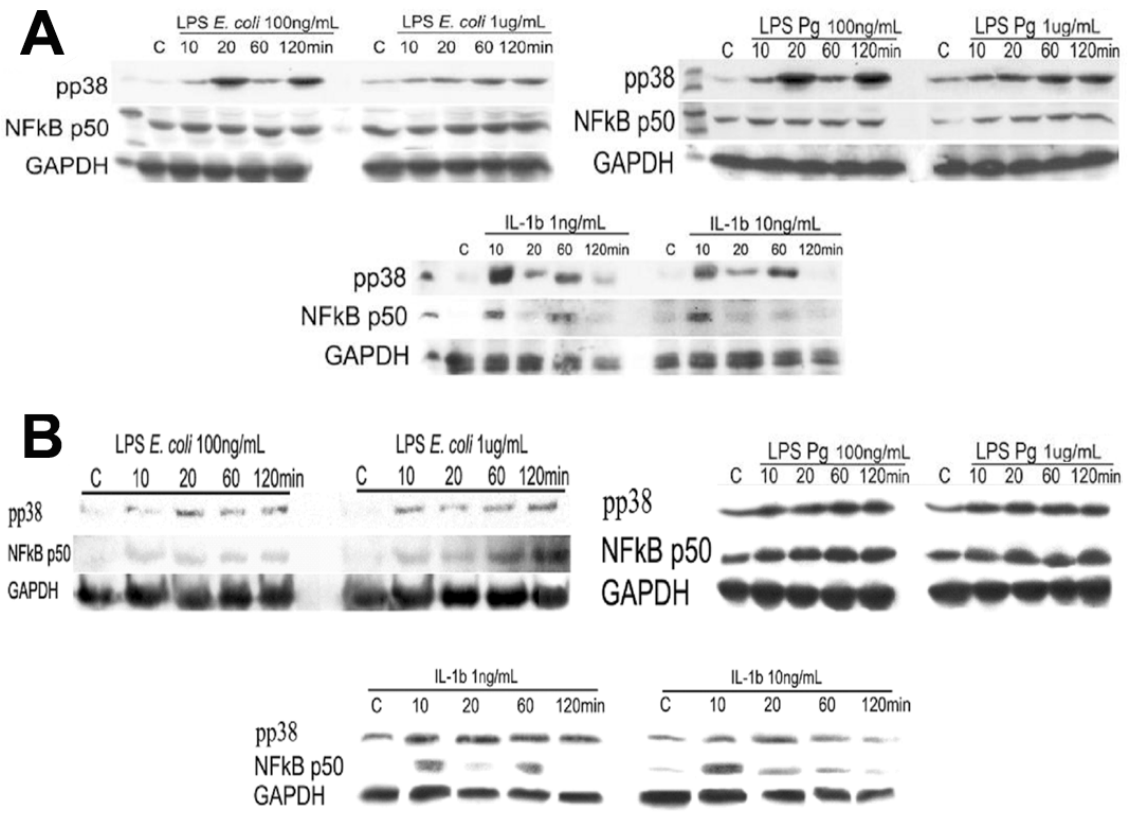

Figure 1 – Representative image of three independent western blot experiments for nuclear factor-kappa B p50 (NF-κB) and p38 mitogen-activated protein kinase (MAPK) evaluation. Periodontal ligament cells (A) and osteoblastic rat cells (B) grown on six-well plates were de-induced for 12 h in culture medium containing 0.3% fetal bovine serum and then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* (100 ng/mL and 1 µg/mL) or IL1β (10 ng/mL and 100 ng/mL) for 0 (control – C), 10, 30, 60 and 120 minutes. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; IL-1b, interleukin-1 beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

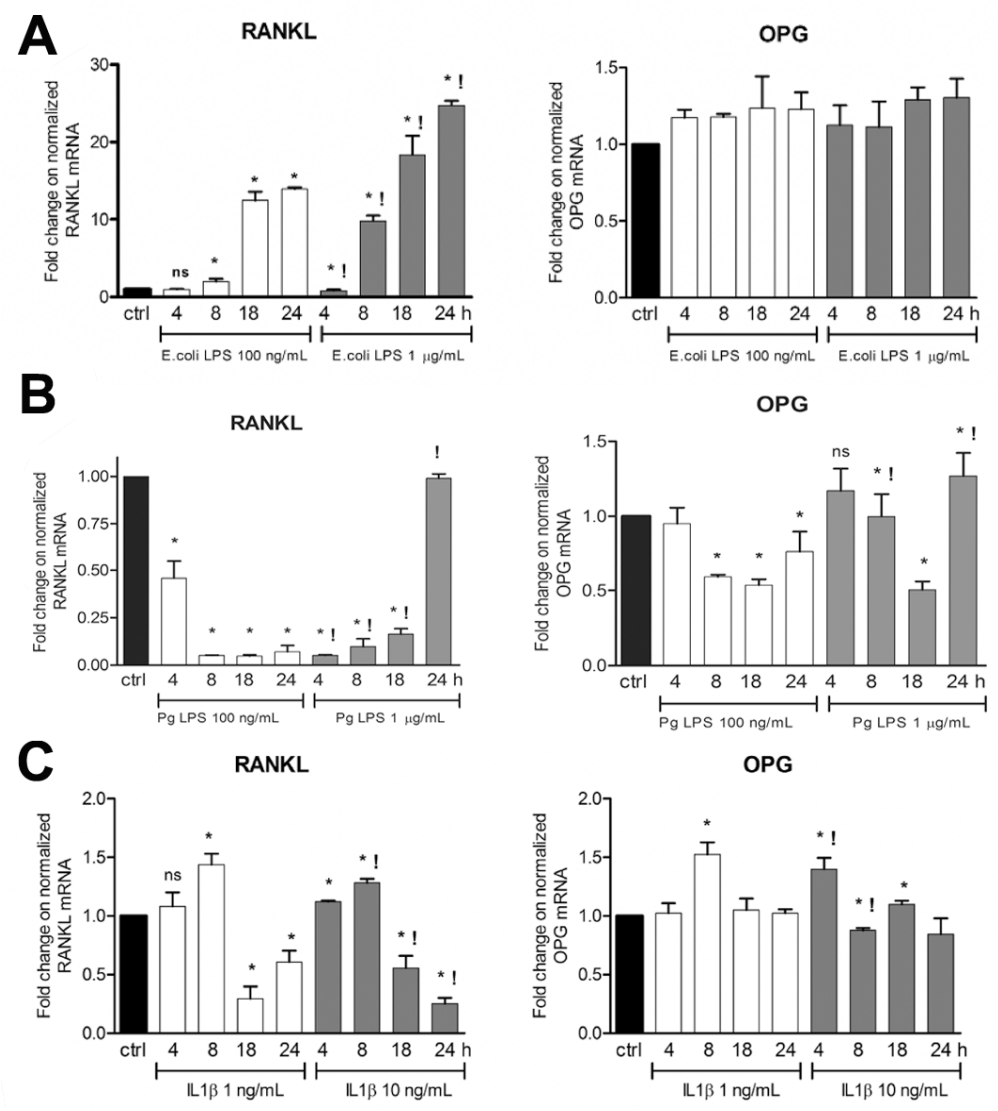

Figure 2 – Time course of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) mRNA expression induced by *Escherichia coli*, *Porphyromonas gingivalis* and interleukin-1 beta in periodontal ligament cells (mPDL). Bars indicate mean and standard deviation of density analysis of three independent experiments. Data are expressed as fold change in unstimulated control group (ctrl) after normalization to GAPDH. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control (ctrl) by one-sample t test. Exclamation mark (!) indicates significant difference ($p<0.05$) within the same time point with the other dose of the same agonist by unpaired t test. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; IL-1b, interleukin-1 beta; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

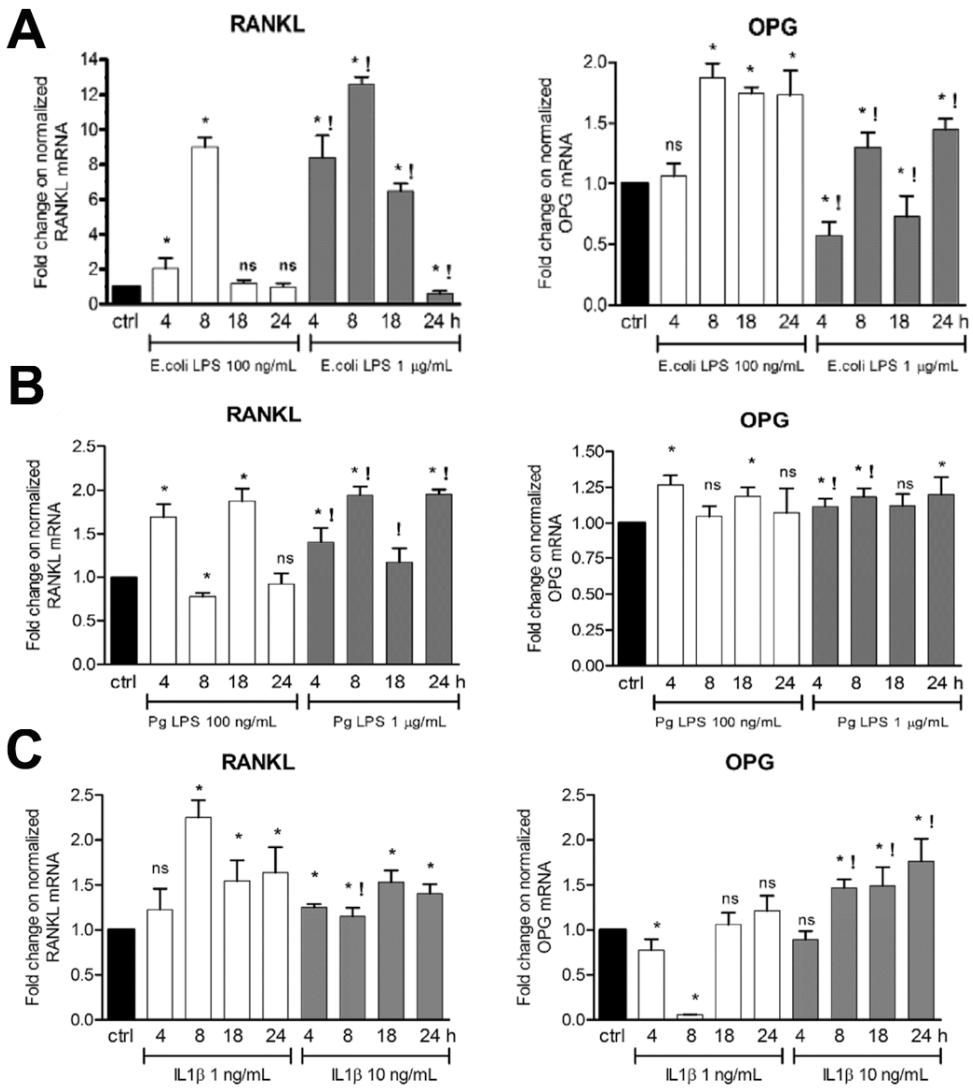

Figure 3 – Time course of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) mRNA expression induced by *Escherichia coli*, *Porphyromonas gingivalis* and interleukin-1 beta in osteoblastic cells (Ros). Bars indicate mean and standard deviation of density analysis of three independent experiments. Data are expressed as fold change in unstimulated control group (ctrl) after normalization to GAPDH. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control (ctrl) by one-sample t test. Exclamation mark (!) indicates significant difference ($p<0.05$) within the same time point with the other dose of the same agonist by unpaired t test. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; IL-1b, interleukin-1 beta; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

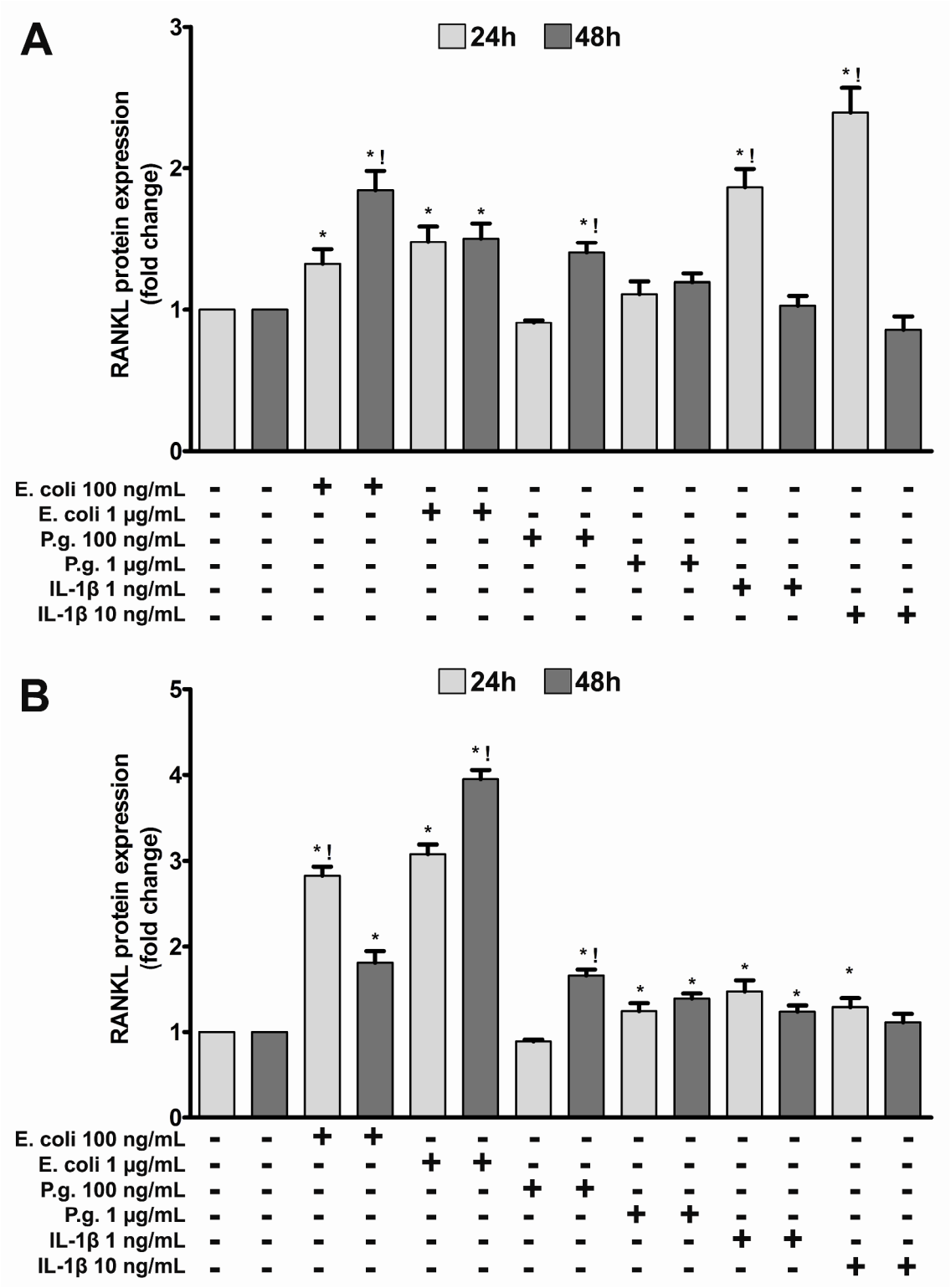

Figure 4 – Receptor activator of nuclear factor-kappa B ligand (RANKL) protein expression induced by *Escherichia coli* lipopolysaccharide, *Porphyromonas gingivalis* lipopolysaccharide and interleukin-1 beta in periodontal ligament cells (A) and osteoblastic cells (B). Bars indicate mean and standard deviation of density analysis of

three independent experiments. Data are expressed as fold change in unstimulated control group after normalization to GAPDH. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to ‘1’ attributed to unstimulated control by one-sample t test. Exclamation mark (!) indicates significant difference ($p<0.05$) within the same dose comparing 24h and 48h stimulation with the same agonist by unpaired t test. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; IL-1β, interleukin-1 beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CAPÍTULO 2

Role of MyD88, Nod1 and Nod2 on p38 MAPK and NF-kB activation and RANKL/OPG expression induced by TLR and IL-1R signaling in bone marrow stromal cells*

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ABSTRACT

Nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) were initially described as cytosolic receptors for bacterial components while toll-like receptors mediated bacterial recognition at the cell surface. Mutations in *NOD1* and *NOD2* genes cause hyperinflammatory responses to bacterial components and stimulation of Nod2 in osteoblasts synergistically enhances osteoclast formation induced by LPS, IL-1α, and TNF-α; suggesting that Nod proteins play an important role modulating signaling pathways associated with the immune response. We examined the role of Nod1, Nod2 and MyD88 proteins on p38 MAPK and NF-κB activation induced by *Escherichia coli* and *Porphyromonas gingivalis* lipopolysaccharide (LPS) and interleukin-1 beta and also, their effect on receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) production in bone marrow stromal cells. Bone marrow stromal cells exposed to *Escherichia coli* (TLR4 agonist) and *Porphyromonas gingivalis* (TLR2 and -4 agonist) lipopolysaccharide and to interleukin-1beta were analyzed for p38 MAPK and NF-κB pathways activation and MyD88, Nod1 and Nod2 levels by western blot and production of RANKL and OPG mRNA by RT-PCR. MyD88, Nod1 and Nod2 were all required for LPS-induced RANKL expression. Inhibition of OPG mRNA induced by LPS and IL-1 required MyD88 and Nod1, but not Nod2. MyD88 -/- cells showed decreased activation of NF-κB, which was completely abrogated in cells lacking either Nod1 or Nod2. Activation of p38 MAPK in MyD88 -/- BMSC was preserved, but was impaired in Nod1 -/- and Nod2-/- cells. Our results suggest that MyD88, Nod1 and Nod2 modulate activation of NF-kB and p38 MAPK and may play important roles in regulation of bone turnover in inflammatory and infectious conditions.

INTRODUCTION

Bone marrow stromal cells and osteoblasts have been implicated in osteoclastogenesis by the production of macrophage colony-stimulating factor (M-CSF) which is essential for osteoclast formation (Yoshida et al.[39], 1990). More recently, the receptor activator of NF-κB ligand (RANKL), another osteoclastogenesis-associated cytokine, was found to be expressed in the membrane of osteoblasts (Boyle et al.[4], 2003). RANKL stimulates differentiation, activation and survival of osteoclasts and its expression is modulated by different stimuli e.g. 1α,25-dihydroxyvitamin D3, parathyroid hormone, PGE2, IL-1, and IL-11 (Suda et al.[29], 1999). Osteoprotegerin (OPG) is the natural endogenous inhibitor of RANKL, produced by osteoblasts and fibroblasts. OPG functions by acting as a soluble decoy receptor that prevents RANKL from interacting with its receptor (RANK) on osteoclast precursor cells. Thus, the RANKL/OPG expression ratio is thought to ultimately determine outcome of bone turnover: resorption or neoformation.

Lipopolysaccharides (LPSs) induce production of cytokines and other inflammatory mediators by a usually tightly-controlled process through toll-like receptors (TLRs). Proinflammatory gene expression induced by LPS-activated TLRs is mediated by the activation of downstream intracellular signaling pathways which results in the transcription of DNA into pre-mRNA (Garcia de Aquino et al.[8], 2009). NF-kB and p38 MAPK are two signaling pathways activated downstream of TLR that play important roles in the regulation proinflammatory cytokines and RANKL (Chae et al.[5], 2005; Leibbrandt e Penninger[19], 2008; Rossa et al.[25], 2008).

Most lipopolysaccharides from Gram-negative bacteria interact with host cells by activating TLR4, and interestingly the LPS from the periodontopathogen *Porphyromonas gingivalis* LPS is the only one shown to activate also TLR2 (Bainbridge e Darveau[3], 2001). Members of TLR family present a cytoplasmic tail known as Toll/IL-1 receptor (TIR) which is associated with the myeloid differentiation factor 88 (MyD88) in a homophilic interaction (Adachi et al.[1], 1998; Akira[2], 2003). However, other two

intracellular proteins called TIR domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) were shown to be involved in a MyD88-independent signaling pathway also associated with TLR4 (Yamamoto et al.[35], 2003; Yamamoto et al.[37], 2002). Mice deficient for both TRIF and TRAM showed that both MyD88-dependent and –independent pathways are essential for LPS-induced proinflammatory cytokine production in macrophages (Yamamoto et al.[36], 2003). MyD88-dependent signaling leads activation of p38 and extracellular signal-regulated kinase (ERK) MAPkinases and production of proinflammatory cytokine in human osteoblastic cells (Kondo et al.[18], 2001), while TRIF/TRAM activates NF-κB (Fitzgerald et al.[7], 2003).

Nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) are involved in the cytosolic recognition of bacterial components and induction of innate immune response. Gram-negative and some Gram-positive bacteria produce the amino acid meso-diaminopimelic acid, which is the ligand for Nod1. Nod2, on the other hand, recognizes muramyl-dipeptide (MDP) present in both Gram-negative and Gram-positive bacteria (Kim et al.[17], 2008). The relevance of Nod proteins for the immune response is demonstrated by the association of mutations in *NOD2* gene and in Crohn's disease, a hyperinflammatory condition (Hugot et al.[11], 2001). Specifically in the regulation of bone turnover, activation of Nod2 by muramyl dipeptide in osteoblasts enhanced osteoclast formation concomitantly induced by LPS and IL-1α through increased RANKL expression (Yang et al.[38], 2005). Since there is paucity of information on the role of NLRs on the regulation of RANKL expression induced by LPS and proinflammatory cytokines, we examined the role of MyD88, Nod1 and Nod2 proteins on p38 MAPK and NF-κB activation and the effect on RANKL/OPG production in bone marrow stromal cells.

MATERIAL AND METHODS

Cells and materials

Bone marrow stromal cells (BMSCs) were collected from the femurs and tibias of C57/BL6 wild-type and MyD88, NOD1 and NOD2 deficient (¯/¯) mice. The proximal and distal ends of the bones were removed and the marrow cavity washed with 10 mL of α-MEM growth medium containing 10% fetal calf serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin through a 5-gauge needle. The cells were plated on 100-mm dishes and incubated at 37^{o}C and 5% CO_2. After 24h, in order to remove the nonadherent cells growth medium was discarded, plates washed with PBS and medium replaced. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide from *Escherichia coli* (serotype O55:B5) was purchased from Sigma- Aldrich (St Louis, MO, USA) and *P. gingivalis* lipopolysaccharide was purchased from Invivogen (San Diego, CA USA). Both *E. coli* and *P. gingivalis* LPSs were diluted in RNAse-free water to 5 mg/mL. Interleukin-1β recombinant protein was from R&D systems (Minneapolis, MN, USA). All primers were purchased from Invitrogen (Carlsbad, CA, USA). Mouse RANKL monoclonal antibody was obtained from Imgenex (San Diego, California, USA). Phosphorylated p38 monoclonal antibody was from Cell Signaling (Danvers, MA, USA), and monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH), nuclear factor NF-kB, Nod1 and Nod2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Semi-quantitative RT-PCR

A total of 10^5 bone marrow stromal cells were grown for 48h in six-well plates, de-induced by incubation for 8h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* or *P. gingivalis* LPSs (1 ug/mL) or with IL-1β (1ng/mL) for 18 hours for RANKL, OPG and GAPDH mRNA expression.

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a

Biomate3 (Thermo Electron Corporation) spectrophotometer. Complementary DNA (RT-PCR) was synthesized by reverse transcription of 500 ng of total RNA using 2.5 µM Oligo (dT) 12-18 primers and 1.25 U/uL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM MgCl2, 2 mM dNTPs and 0.8 U/µL of RNAse inhibitor, according to the manufacturer's protocol (Improm II – Promega). PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 25 uL total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for RANKL, OPG and GAPDH genes yielding products of 467, 503, 290 and 418 bp, respectively. The primer pair used for RANKL (accession n.: NM011613) was: sense 5'-CAGCACTCACTGCTTTTATAGAATCC-3', antisense 5'-AGCTGAAGATAGTCTGTAGGTACGC-3'; osteoprotegerin (accession n.: NM008764) sense 5'-TGTAGAGAGGATAAACGG-3', antisense 5'-CTAGTTATATGCAGCTTAT-3'; and for GAPDH (accession n.: BC083065) was: sense 5'-CACCATGGAGAAGGCCGGGG-3', antisense 5'-GACGGACACATTGGGGTAG-3'. PCR conditions for RANKL and OPG were as follow: 95°C for 2 min; 35 cycles of 95°C, 58°C and 72°C for 30 s and extension at 72°C for 7 min in the presence of 2.5 mM $MgCl_2$; and GAPDH 95°C for 2 min; 25 cycles of 95°C, 52°C and 72°C for 1 min; and extension at 72°C for 7 min in the presence of 1.5 mM $MgCl_2$. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5µg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using ImageJ 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to RANKL and OPG mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Western Blot

A total of 10^5 bone marrow stromal cells were grown for 48h in six-well plates, de-induced by incubation for 8h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* or *P. gingivalis* LPSs (1 ug/mL) or with IL-1β (1ng/mL) for 15 and 45 min for NF-κB and p38 MAPKinases evaluation, and also 24h for RANKL, Nod1 and Nod2 expression. Protein detection was evaluated in total protein extracts from whole-cell lysates by scraping the cells of each well with 100 µl of sodium dodecyl sulfate sample buffer (62.5 mM Tris HCl buffer, pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) on ice, followed by centrifugation at 9,000 RPM at 4°C for 5 min. Forty µl of this content was heat-denatured at 95°C for 5 min and separated on 10% Tris-Cl polyacrylamide gels run at 100 V for 90 min and subsequently electro-transferred to 0.2 µM nitrocellulose membranes (300 mA constant current for 60 minutes). The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies. The presence of the primary antibodies was detected on radiographic film by using HRP-conjugated secondary antibodies and a chemiluminescence system (Lumi-Glo, Cell Signaling). The membranes were stripped off both primary and secondary antibodies by 10-minute washing in dH_2O twice followed by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 10 min at room temperature and a final 10-min wash in Tris-buffered saline containing 0.1% Tween-20. After stripping, the membranes were blocked as described and incubated with antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to verify equal loading of the wells. Digitalized images of the radiographic films were obtained in gel documentation system (ImageQuant 100, GE Lifesciences).

Statistical analysis

Pairwise comparisons between experimental groups were performed using the t-test with Welch's correction for unequal variances. Comparison between

fold changes on mRNA expression between lipopolysaccharide-stimulated and untreated cells was performed with the one-sample t-test. The significance level was set to 5% and all calculations were performed using PRISM 4 software (GraphPad, Inc., San Diego, CA, USA).

RESULTS

MyD88, Nod1 and Nod2 play important roles in the activation of NF-kB and p38 MAPK induced by LPS and IL-1β. BMSC from wild-type animals showed a similar pattern of activation of NF-κB after stimulation with all agonists. Activation of NF-κB was sustained up to 45 min after stimulation with all agonists, although there was an initial delay after stimulation with *E. coli* LPS (Fig. 1A). MyD88-independent pathway resulted in weaker activation of NF-kB after TLR4 and TLR2/TLR4 stimulation with LPS. Even though the initial activation induced by IL-1β was preserved in MyD88 -/- cells, as opposed to WT cells the activation was not sustained at 45 min (Fig. 1B). On the other hand, both Nod1 -/- and Nod2 -/- cells failed to activate NF-κB in comparison to unstimulated control, except for IL-1β-stimulated Nod2 -/- cells, which showed slight activation of NF-κB. In wild-type cells, phosphorylation of p38 MAPK was better induced by LPSs than IL-1β. *P. gingivalis* and *E. coli* LPSs showed different kinetics of activation, peaking at 15 and 45 min, respectively (Fig. 1A). MyD88 was not required for activation of p38 MAPK (Fig. 1B), whereas similarly to what was observed for NF-kB, Nod1 -/- failed to show activation of p38 upon stimulation. In Nod2 -/- BMSC, activation of p38 MAPK upon stimulation was attenuated but preserved in comparison to Nod1 -/- cells (Fig. 1B, 1C, 1D).

MyD88, Nod1 and Nod2 are required for the induction of RANKL mRNA expression, but only MyD88 and Nod1 are necessary for the negative regulation of OPG mRNA. Since Nod1, Nod2 and MyD88 played important roles in the activation

of NF-kB and p38 MAPK, which are signaling pathways that have a significant impact on the direct or indirect modulation of bone tissue turnover, we evaluated their role on the regulation of steady-state levels of RANKL and OPG mRNA induced by TLR and IL-1R signaling. RANKL mRNA was significantly ($p<0.05$) induced after stimulation with all agonists in WT BMSC cells, but this induction was markedly inhibited in MyD88 -/- cells and completely abrogated in Nod1 -/- and Nod2 -/- cells. Interestingly, Nod2 was not required for IL-1β-induced RANKL (Fig. 2A). As opposed to the regulation of RANKL, OPG mRNA expression was decreased after stimulation with all agonists in WT BMSC cells, indicating that these external stimuli may effectively alter bone coupled turnover processes towards resorption. This agonist-induced inhibition of OPG mRNA was absent in Nod1-/- BMSC and actually reversed in Myd88-/- cells; which showed significantly ($p<0.05$) increased expression of OPG mRNA upon stimulation (Fig. 2B). This suggests that MyD88-dependent signaling plays a dominant role on negative regulation of OPG mRNA by TLR and IL-1R signaling. On the other hand, Nod2 had an opposite role in comparison to MyD88, since Nod2 -/- cells showed an even more marked inhibition of OPG mRNA expression upon stimulation with all agonists. Thus, Nod2 may have an inhibitory role on the negative regulation of OPG induced by TLR and IL-1R signaling.

Induction of RANKL protein expression requires Nod1 and Nod2, but not MyD88.

To confirm that influence of Nod1, Nod2 and MyD88 in the regulation of RANKL may have biological significance, we evaluated the levels of RANKL protein by Western blot. As opposed to the regulation at the mRNA level, MyD88 -/- cells still showed induction of RANKL protein upon stimulation with all agonists. On the other hand, in agreement with the mRNA data, RANKL protein was not noticeably induced in Nod1-/- and Nod2-/- BMSC cells after stimulation with all agonists. Particularly interesting was the finding that RANKL protein induction was preserved in IL-1β-treated Nod2 -/- cells; which also

agrees with the regulation at the mRNA level and further supports the concept that Nod2 is not required for IL-1β-induced RANKL expression in BMSC cells (Fig. 3).

TLR and IL-1R signaling regulate Nod1, but not Nod2, expression in BMSC. Since Nod1 and Nod2 had important roles on the regulation of RANKL induced by TLR and IL-1R signaling in BMSC cells, we decided to investigate if the same agonists modulated their own expression levels. In general, only Nod1 expression was modulated in BMSC, whereas Nod2 protein levels remained fairly constant. The only exception was with IL-1β stimulation in MyD88 -/- and Nod1-/- cells. On the other hand, Nod1 expression was upregulated by all agonists in MyD88 -/- cells, but only *P.gingivalis* LPS (TLR2/TLR4 agonist) induced Nod1 in WT BMSC (Fig. 3). Actually, in WT cells *E. coli* LPS and IL-1β decreased Nod1 protein expression, which is interesting since Nod1 is required for expression of RANKL mRNA and protein induced by these same agonists.

DISCUSSION

Nod proteins and the nuclear factor-κB (NF-κB) were recently implied as the main therapeutic targets for human chronic inflammatory bowel disease, Crohn's disease, and ulcerative colitis. Anti-inflammatory drugs used during the course of these diseases aim at NF-κB and p38 MAPK activation; which might be influenced by NOD/RICK signaling pathways (Hollenbach et al.[10], 2005). This indicates the relevance of both Nod proteins and p38 and NF-kB signaling in inflammation and immune response. Nod-like receptors act as intracellular receptors for different bacterial peptidoglycans (e.g., meso-diaminopimelate and muramyl dipeptide) contributing for the activation of in the innate immune system (Inohara et al.[13], 2003). In spite of the demonstration that oral epithelial cells express high levels of Nod1 and Nod2 (Sugawara et al.[30], 2006) and the recent reports of synergistic role for Nod proteins with TLR signaling for the induction of inflammatory mediators (Uehara et al.[32], 2008; Uehara e Takada[33], 2008; Uehara et al.[34], 2005), there is a paucity of information on

the role of Nod1 e Nod2 in modulating signaling pathways involved in the expression of cytokines that are important for bone tissue turnover.

Our data indicates that Nod1, Nod2 and MyD88 are all required for RANKL mRNA expression induced by TLR signaling in bone marrow stromal cells (BMSC). We also observed that MyD88 and Nod1 are involved in the inhibition of OPG mRNA production induced by LPS and IL-1β. Furthermore, their role in this regulation is not redundant, as we used cells obtained from single-gene knockout mice. Interestingly, IL-1R induction of RANKL was not dependent on Nod2, and transcriptional-translation coupling of RANKL gene expression was preserved in BMSC, except in MyD88 -/- cells. We speculate that the preservation of p38 MAPK signaling in MyD88-/- cells, as opposed to Nod1 -/- and Nod2-/- cells, may play a role in post-transcriptional mechanisms regulating the expression of RANKL at the protein level. This data is in agreement with the literature demonstrating that activation of Nod proteins has a synergistic effect in LPS- and cytokine-induced RANKL expression in osteoblasts, indicating that Nod proteins have a positive regulatory role in microbial antigen-induced RANKL expression. However, to our knowledge this is the first report of the role of Nod1 and Nod2 on RANKL expression using cells from gene-deficient animals.

Nod1 is primarily expressed by epithelial cells (Uehara et al.[31], 2007), whereas Nod2 expression is more restricted to cells of the myeloid lineage (Ogura et al.[21], 2001); however their expression can vary depending on the cell type. For example, monocytes express Nod2 in the absence of stimuli (Gutierrez et al.[9], 2002) while in primary osteoblasts Nod2 mRNA levels are detectable only upon stimulation with LPS, IL-1α, or TNF-α (Yang et al.[38], 2005). In osteoblasts, LPS- and IL-1β-induction of Nod2 was reported to be dependent on MyD88, whereas TNF-α induction of Nod2 was not (Yang et al.[38], 2005). We show that BMSC express both Nod1 and Nod2, but we did not observe significant regulation of Nod2 protein in BMSC, except for IL-1β stimulation of MyD88 -/- and Nod1 -/- cells. On the other hand, we did not

observe significant regulation of Nod1 in MyD88 -/- cells, which as induced by all agonists. Since both Nod1 and Nod2 interact with Rip2/RICK/CARDIAK kinase through their CARD domains, resulting in activation of NF-kB (Inohara et al.[12], 2000; Ogura et al.[21], 2001), a speculative interpretation of these results suggest that the upregulation of Nod1 might represent a compensatory mechanism for the impaired activation of NF-kB in MyD88-/- cells. In support to this, Nod1 expression was recently shown to be upregulated in TLR-deficient adipocytes and Nod1 was also shown to activate NF-kB upon stimulation with its specific ligand and also with LPS (Stroh et al.[28], 2008). We did not observe a compensatory increase of Nod proteins in Nod1-/- or Nod2-/- deficient cells, suggesting that, besides their ability to recognize different ligands, they may have non-redundant roles in TLR and IL-1R signaling. In support to this concept, in contrast to Nod1, Nod2 was not required for IL-1β-induced RANKL or for the inhibition of OPG mRNA upon stimulation with LPS and IL-1β.

The NF-κB family of transcription factors can be activated by various signaling pathways downstream of multiple receptors involved in inflammation and immune response, including TNF receptor family members, the IL-1 receptor (IL-1R)/TLR superfamily, the Nod-like receptor (NLR) family, IL-17 family members, and B cell and T cell receptors (O'Neill[20], 2006). NF-kB plays a crucial role for the expression of early-immediate genes and, thus, in innate immunity and inflammation. Expression of these inflammatory mediators has been shown to require MyD88 (Adachi et al.[1], 1998; Kawai et al.[16], 1999). Moreover, inflammatory cytokine and LPS-induced expression of RANKL, as well as osteoclastogenesis are highly dependent on MyD88 (Sato et al.[26], 2004). We confirmed the relevance of MyD88-dependent pathway for RANKL mRNA expression induced by TLR and IL-1R signaling; however the expression of RANKL at the protein level was still inducible. This may suggest that osteoclastogenesis process induced by IL-1R and TLR signaling could be somewhat preserved in the absence of MyD88, which is in sharp contrast with the results of Sato et al. using co-culture models of osteoclastogenesis (Sato et al.[26], 2004). It is important

to note that even though these authors reported a significant decrease on the number of osteoclasts in the MyD88 knockout mice, bone resorption was not completely abrogated and the MyD88-deficient animals did not have a noticeable skeletal phenotype. Moreover, as opposed to more purified cultures of calvarial osteoblasts, we used primary BMSC, which contain other cell types in various stages of differentiation, including adipocytes and dendritic cells which do express TLR and Nod proteins as well (Stroh et al.[28], 2008; Zeuthen et al.[40], 2008). This might account for differences on the cytokine and signaling networks established upon stimulation of TLR and IL-1R; which may result in direct or indirect induction of RANKL through autocrine/paracrine stimulation by other cytokines.

Activation of NF-kB was severely impaired in Nod1-/- and Nod2-/- BMSC, and this might explain the decrease on the induction of RANKL by TLR and IL-1R signaling in these cells. Downstream of Nod activation, Rip2 can phosphorylate ERK1/2 and enhance RANKL expression in osteoblasts (Yang et al.[38], 2005). We chose to evaluate the effects of MyD88, Nod1 and Nod2 on p38 MAPK activation because of previous data from our research group demonstrating the predominant role of this signaling pathway for RANKL expression induced by cytokines in BMSC (Rossa et al.[24], 2006) and by LPS in periodontal fibroblasts (Rossa et al.[25], 2008); as well as due to the important role of this signaling pathway for inflammation-mediated bone resorption in vivo (Rogers et al.[23], 2007). Our data indicates that besides NF-kB activation, TLR- and IL-1R-induced p38 MAPK signaling was also inhibited in Nod1-/- and Nod2-/- BMSCs, suggesting that Nod proteins may be required for the activation of an upstream signaling intermediate important for these pathways. One possibility is the regulation of the pre-formed membrane-bound complex of TAB1/TAK1/TAB2. This complex is activated by the binding of TRAF6, followed by its ubiquitination and phosphorylation of TAB2 and TAK1. TAK1 phosphorylates the IKK complex, ultimately resulting in activation of NF-kB (Doyle e O'Neill[6], 2006), but TAK1 is also an upstream activor of MAPKinases. Another possibility is the regulation of MKK3/MKK6 complexes,

downstream of TAK1. MKK6 phosphorylates all p38 isoforms while MKK3 phosphorylates only the p38α isoform (Shi e Gaestel[27], 2002), and MKK6 was also shown to activate BMK1 (also known as ERK5), RSK1, Ικκα/Ικκβ and finally the NF-κB complex (Kato et al.[14], 2000; Kato et al.[15], 1997; Pearson et al.[22], 2001). In this paper we show that MyD88, Nod1 and Nod2 are all involved in the regulation of RANKL and OPG expression induced by TLR and IL-1R signaling in BMSC. This suggests that these proteins may play an important role in the modulation of bone turnover in inflammatory/infectious conditions. We also observed that in addition to NF-kB, p38 MAPK signaling downstream of TLR and IL-1R is affected by Nod and MyD88.

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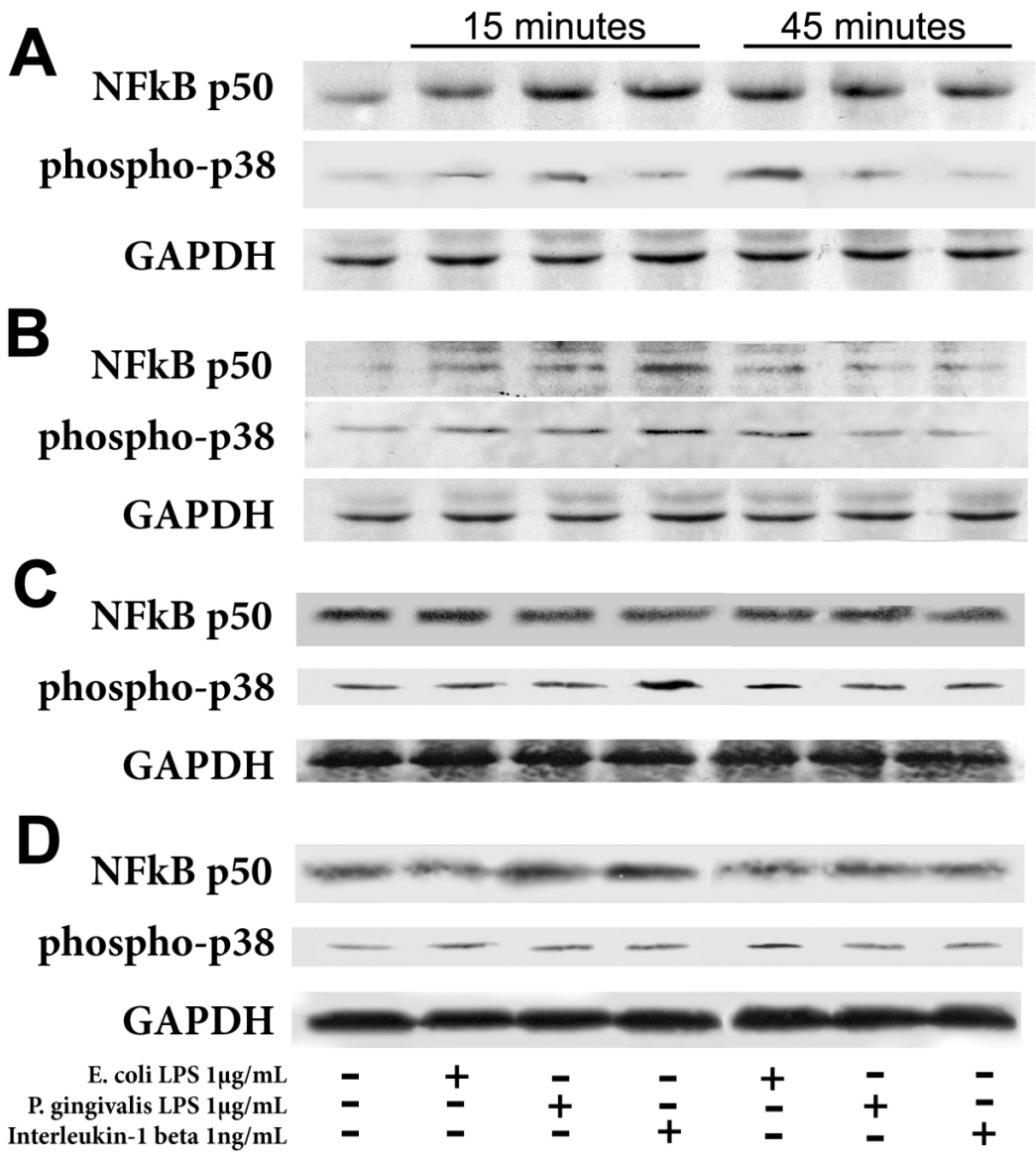

Figure 1 - Representative image of at least three independent western blot experiments for nuclear factor-kappa B p50 (NF-κB) and p38 mitogen-activated protein kinase (MAPK) evaluation. Bone marrow stromal cells (BMSCs) grown on six-well plates were de-induced for 8h in culture medium containing 0.3% fetal bovine serum and then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* (1 µg/mL) or IL1β (1 ng/mL) for 15 and 45 minutes. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide. (A) BMSCs obtained from wild-type animals – control; (B) MyD88 knockout cells; (C) NOD1 knockout; and (D) NOD2 knockout.

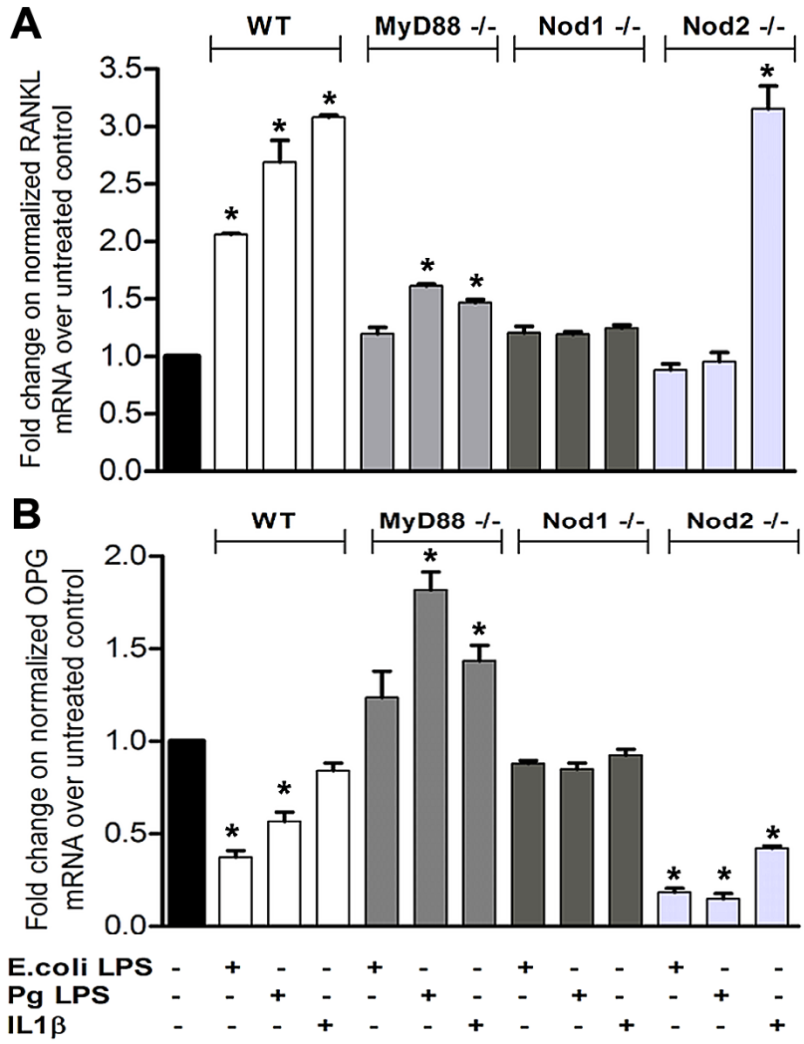

Figure 2 - Receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) mRNA expression induced by 18h-stimulation with *Escherichia coli* and *Porphyromonas gingivalis* LPS and interleukin-1 beta in bone marrow stromal cells. Bars indicate mean and standard deviation of density analysis of three independent experiments. Data are expressed as fold change in unstimulated control group after normalization to GAPDH. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. WT, wild-type; *E. coli*, *Escherichia coli*; *Pg*, *Porphyromonas gingivalis*; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

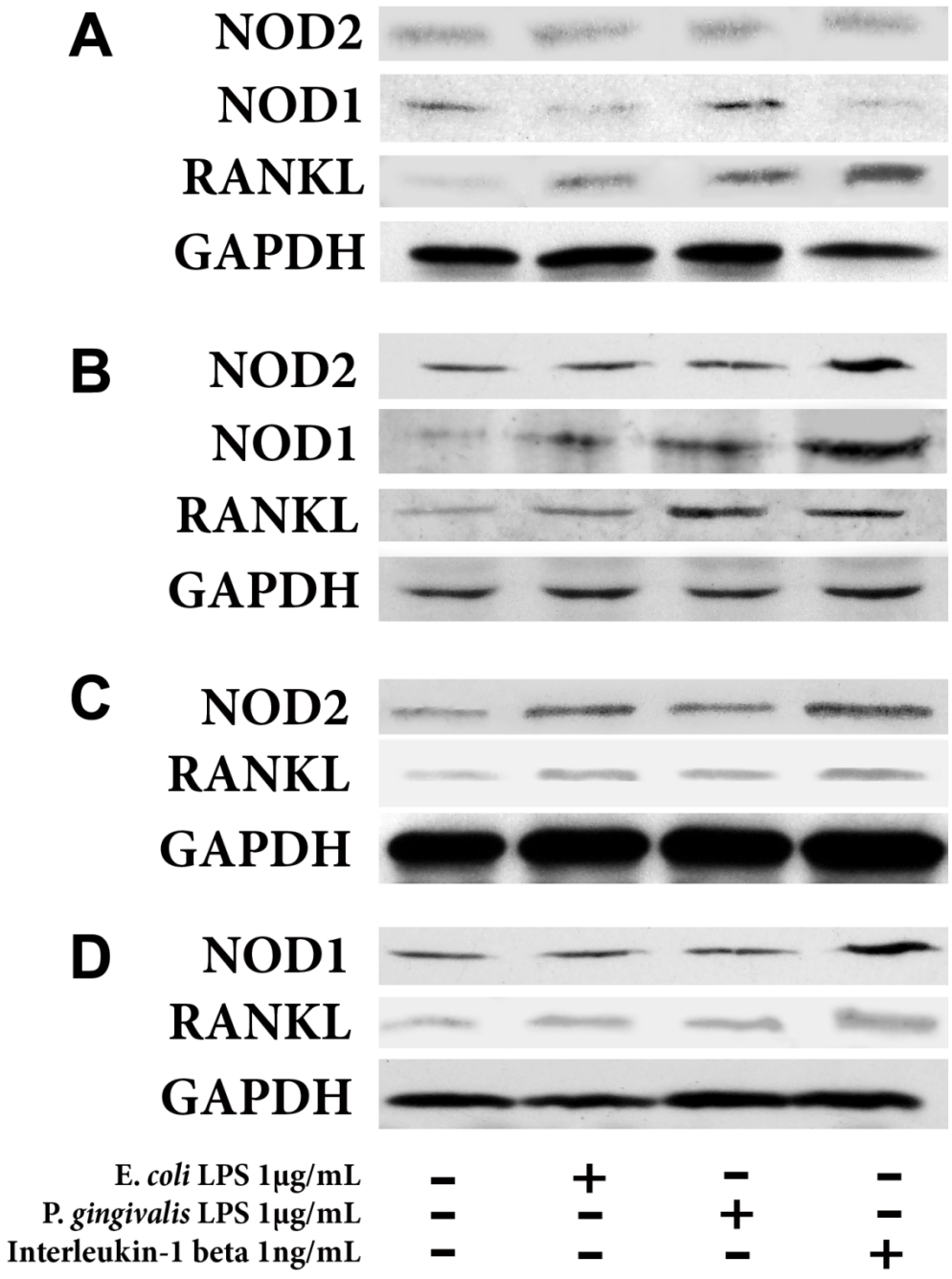

Figure 3 - Receptor activator of nuclear factor-kappa B ligand (RANKL) and nucleotide-oligomerization domain 1 and 2 (Nod1 and Nod2) protein expression in bone marrow stromal cells induced by *Escherichia coli* lipopolysaccharide, *Porphyromonas gingivalis* lipopolysaccharide and interleukin-1 beta after 24h stimulation. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (A) BMSCs obtained from wild-type animals – control; (B) MyD88 knockout cells; (C) NOD1 knockout; and (D) NOD2 knockout.

CAPÍTULO 3

Relevance of the myeloid differentiation factor 88 (MyD88) on RANKL, OPG and Nod expression induced by TLR and IL1R signaling in bone marrow stromal cells

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Running title: MyD88 and Nod proteins influence on OPG and RANKL expression

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ABSTRACT

The myeloid differentiation factor 88 (MyD88) plays a pivotal role in TLR- and IL1R-induced osteoclastogenesis. Also, Nod2 stimulation is known to synergistically enhance osteoclast formation through RANKL production. We examined the role of MyD88 protein on p38 MAPK and NF-κB activation and Nod induction by *Escherichia coli* and *Porphyromonas gingivalis* LPS and IL-1 beta and also, their effect on receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) production in bone marrow stromal cells. Expression of RANKL and osteoprotegerin mRNA in bone marrow stromal cells stimulated with *E. coli* and *P. gingivalis* LPSs and IL-1β was studied by reverse transcription-polymerase chain reaction. RANKL, Nod1, Nod2, NF-κB and p38 protein levels were determined by western blot. Nod2 was stimulated with muramyl dipeptide (MDP) one hour prior to TLR4 stimulation with *E. coli* LPS. MyD88 deficiency markedly inhibited RANKL expression after LPS stimulation and increased OPG mRNA production. Also, MyD88 was necessary for NF-κB p50 and p38 MAPK activation. MDP alone did not induce RANKL and OPG expression, however when used combined with *E. coli* LPS their expression was significantly increased ($p < .05$). Our results support that MyD88 signaling has a pivotal role in osteoclastogenesis thought NF-κB and p38 activation. Nod2 and especially Nod1 protein levels were influenced by MyD88.

INTRODUCTION

Toll-like receptors (TLRs) and interleleukin-1 receptors (IL-1Rs) have similar intracellular domains and activate common signaling cascades after stimulation (Doyle *et al.*, 2006). The nuclear factor kappa B (NF-κB) is one of the shared pathways and it is responsible for the expression of proinflammatory proteins (Doyle *et al.*, 2006). The transcription factor NF-κB p65/p50 is a dimer found in the cytoplasm of mammalian cells and its activity is controlled by the inhibitory protein IκB. When this pathway is activated, an IκB kinase complex (IKK) phosphorylates IκB with immediate ubiquitination and IκB degradation by the proteasome (Janssens *et al.*, 2002). Thus, the active p65-p50 heterodimer translocates to the nucleus to begin the transcription for different cytokines and chemokines (Janssens *et al.*, 2002).

The mitogen-activated protein kinase (MAPK) pathway is also shared by both families of receptors. This kinase cascade is responsible for the activation of p38, ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) (Sato *et al.*, 2004). The initial step of the cascade activation is the recruitment of different adapter proteins because TLRs and IL-1Rs do not present and intrinsic kinase activity (Janssens *et al.*, 2002). One of these adapters is the myeloid differentiation factor 88 (MyD88) which is directly related to these receptors. Previous studies showed that the overexpression of MyD88 causes a potent activation of NF-κB but its absence reduces not only NF-κB activation but also JNK (Medzhitov *et al.*, 1998, Burns *et al.*, 1998). The role of MyD88 as a universal adapter was proven by the induction of cytokines release using of different membrane receptors, such as lipopolysaccharide, interleukin-1 beta, interleukin 18, CpG-DNA and poly(I:C) (Adachi *et al.*, 1998, Schnare *et al.*, 2000, Alexopoulou *et al.*, 2001). Although signaling through different receptors including TLRs and IL-1R, all of them required MyD88 recruitment. The underlying mechanisms involved in MyD88 activation especially with regards to the recruitment of adapter molecules are still being investigated. To the best of our knowledge, MyD88 phosphorylates IRAK that subsequently interacts with TNF-receptor-associated factor 6

(TRAF6) and transforming growth factor-β activated kinase 1 binding protein (TAB2) (Doyle *et al.*, 2006, Janssens *et al.*, 2002). Both TRAF6 and TAB2 translocates to cytosol activating TAK1 that phosphorylates IKKβ and MAPKinase kinase 6 (MKK6) (Deng *et al.*, 2000, Wang *et al.*, 2001). This way, MyD88-dependent pathway culminates in NF-κB, JNK and p38 MAPK pathways activation (Doyle *et al.*, 2006, Janssens *et al.*, 2002).

The activation of a receptor and the expected result is not linear and an overlapping among different pathways is perfectly possible. The interpretation of results have to consider the specific effects resulting from the activation of a given signaling pathway depending on the nature of the external stimuli; the cell-specificity of signaling pathways activated by the same external signal; and the cross-talk and interaction among different signaling pathways that are simultaneously activated by the same external signal (Garcia de Aquino *et al.*, 2009). For example, TRAF6 can activate parallel pathways involving different adapter proteins (such as TAB2, ECSIT and p62) and kinases (TAK1, MAP/ERK kinase kinase-1 and atypical protein kinase C) (Takaesu *et al.*, 2000, Kopp *et al.*, 1999, Sanz *et al.*, 2000).

The production of cytokines and/or the presence of LPSs next to bone marrow stromal cells and osteoblasts have been implicated in osteoclastogenesis induced by TLR/IL-1R activation (Yoshida *et al.*, 1990, Boyle *et al.*, 2003). In sum, the receptor activator of NF-κB ligand (RANKL) binds to the receptor activator of NF-κB (RANK) at the membrane of osteoclast precursors cells leading to osteoclasts differentiation, increased activity and survival in the presence of M-CSF (Boyle *et al.*, 2003). On the other hand, osteoprotegerin (OPG) was found to be a soluble decoy receptor for RANKL produced mostly by osteoblasts. This way, osteoclastogenesis blockage is possible due to OPG/RANKL combination reducing RANKL/RANK interaction. MyD88-deficient mice present an increased resistance to respond to LPSs and IL-1 and their macrophages showed a delayed activation of NF-κB and MAPK cascades in response to LPS (Adachi *et al.*, 1998, Kawai *et al.*, 1999).

Lipopolysaccharides interact with host cells by activating TLR4 and until this moment only the LPS from a periodontopathogen called *Porphyromonas gingivalis* is able to use TLR2 as well (Bainbridge *et al.*, 2001). It has been suggested that nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) are responsible for cytosolic recognition of bacterial components and induction of innate immune responses acting as intracellular TLRs (Kim *et al.*, 2008). Gram-negative and part of Gram-positive bacteria produce the amino acid meso-diaminopimelic acid responsible for Nod1 activation, on the other hand Gram-negative and -positive bacteria present muramyl-dipeptide (MDP) which is recognized by Nod2 (Kim *et al.*, 2008). Mutations in *NOD2* gene cause hyperinflammatory responses to bacteria components such as in Crohn's disease (Hugot *et al.*, 2001). In cases of bone resorption, osteoblast stimulated by MDP (Nod2 agonist) synergistically enhances osteoclast formation induced by LPS, IL-1α, and TNF-α through RANKL expression (Yang *et al.*, 2005).

The relevance of the MyD88 pathway on RANKL and OPG production and consequently on bone resorption-related diseases is still under investigation. There is lack of information regarding possible differences in RANKL and OPG induction using TLR2 and TLR4. Also, we suppose that Nod1 and Nod2 protein levels might be influenced by MyD88 presence to increase cellular response to LPS and IL-1 stimulation.

MATERIAL AND METHODS

Cells and materials

Bone marrow stromal cells (BMSCs) were collected from the femurs and tibias of C57/BL6 wild-type mice and MyD88, NOD1 and NOD2 deficient (¯/¯). The proximal and distal ends of the bones were removed and the marrow cavity washed with 10 mL of growth medium containing α-MEM, 10% fetal calf serum (FBS), 100 U/mL penicillin, and 10 µg/mL streptomycin through a 5-gauge needle. The cells were plated on 100-mm dishes and incubated at 37^{o}C and 5% CO_2. After 24 h, in order to remove the

nonadherent cells growth medium was discarded, plates washed with PBS and medium replaced. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide from *Escherichia coli* (serotype O55:B5) was purchased from Sigma-Aldrich (St Louis, MO, USA) and *Porphyromonas gingivalis* lipopolysaccharide was purchased from Invivogen (San Diego, CA, USA). Both *E. coli* and *P. gingivalis* lipopolysaccharide were diluted in RNAse-free water to 5 mg/mL. Recombinant human Interleukin-1β protein was from R & D systems (Minneapolis, MN, USA). All primer pairs were purchased from Invitrogen (Carlsbad, CA, USA). Mouse RANKL monoclonal antibody was obtained from Imgenex (San Diego, California, USA). Phosphorylated p38 monoclonal antibody was from Cell Signaling (Danvers, MA, USA), and monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphorylated p50, Nod1 and Nod2 antibodies were from Santa Cruz Biotecnology (Santa Cruz, CA, USA).

Semi-quantitative RT-PCR

A total of 10^5 cells were grown for 48h in six-well plates, de-induced by incubation for 8h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* or *P. gingivalis* LPSs (1ug/mL) or with IL-1β (1ng/mL) for 18 hours for RANKL, OPG and GAPDH mRNA expression.

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate3 (Thermo Electron Corporation) spectrophotometer. Complementary DNA (RT-PCR) was synthesized by reverse transcription of 500 ng of total RNA using 2.5 µM Oligo (dT) 12-18 primers and 1.25 U/uL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM MgCl2, 2 mM dNTPs and 0.8 U/µL of RNAse inhibitor, according to the manufacturer's protocol (Improm II – Promega). PCR reaction was

performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 25 uL total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for RANKL, OPG and GAPDH genes yielding products of 467, 503, 290 and 418 bp, respectively. The primer pair used for RANKL (accession no.: NM011613) was: sense 5'-CAGCACTCACTGCTTTTATAGAATCC-3', antisense 5'-AGCTGAAGATAGTCTGTAGGTACGC-3'; osteoprotegerin (accession no.: NM008764) sense 5'-TGTAGAGAGGATAAACGG-3', antisense 5'-CTAGTTATATGCAGCTTAT-3'; and for GAPDH (accession no.: BC083065) was: sense 5'-CACCATGGAGAAGGCCGGGG-3', antisense 5'-GACGGACACATTGGGGTAG-3'. PCR conditions for RANKL and OPG were as follow: 95°C for 2 min; 35 cycles of 95°C, 58°C and 72°C for 30 sec and extension at 72°C for 7 min in the presence of 2.5 mM $MgCl_2$; and GAPDH 95°C for 2 min; 25 cycles of 95°C, 52°C and 72°C for 1 min; and extension at 72°C for 7 min in the presence of 1.5 mM $MgCl_2$. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5µg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using ImageJ 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to RANKL and OPG mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Western Blot

A total of 10^5 cells were grown for 48h in six-well plates, de-induced by incubation for 8h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* or *P. gingivalis* LPSs (1 ug/mL) or with IL-1β (1ng/mL) for 15 and 45 min for NF-κB and p38 MAPKinases evaluation, and also 24h for RANKL, Nod1 and Nod2 expression. Protein detection was evaluated in total protein extracts from whole-cell

lysates by scraping the cells of each well with 100 µl of sodium dodecyl sulfate sample buffer (62.5 mM Tris HCl buffer, pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) on ice, followed by centrifugation at 9,000 RPM at 4°C for 5 minutes. Forty µl of this content was heat-denatured at 95°C for 5 minutes and separated on 10% Tris-Cl polyacrylamide gels run at 100 V for 90 min and subsequently electro-transferred to 0.2 µM nitrocellulose membranes (300 mA constant current for 60 minutes). The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies. The presence of the primary antibodies was detected on radiographic film by using HRP-conjugated secondary antibodies and a chemiluminescence system (Lumi-Glo, Cell Signaling). The membranes were stripped off both primary and secondary antibodies by 10-minute washing in dH_2O twice followed by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 10 minutes at room temperature and a final 10-minute wash in Tris-buffered saline containing 0.1% Tween-20. After stripping, the membranes were blocked as described and incubated with antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to verify equal loading of the wells. Digitalized images of the radiographic films were obtained in a gel documentation system (ImageQuant 100, GE Lifesciences).

Statistical analysis

Pairwise comparisons between experimental groups were performed using the t-test with Welch's correction for unequal variances. Comparison between fold changes on mRNA expression between lipopolysaccharide-stimulated and untreated cells was performed with the one-sample t-test. The significance level was set to 5% and all calculations were performed using PRISM 4 software (GraphPad, Inc., San Diego, CA, USA).

RESULTS

MyD88 is important for NF-κB p50 and p38 MAPK activation induced by LPS and IL-1β. Control wild-type BMSC cells (WT) presented a similar pattern of activation of NF-κB after stimulation with all agonists. NF-κB activation after stimulation with all agonists was sustained up to 45 minutes, although there was an initial delay after stimulation with *E. coli* LPS (Fig. 1A). MyD88-independent pathway resulted in weaker activation of NF-kB after TLR4 and TLR2/TLR4 stimulation with both LPSs. Even though an initial activation induced by IL-1β was observed in MyD88 -/- cells it was not sustained at 45 minutes (Fig. 1B). NF-κB p50 activation was highly influenced by MyD88; although all agonists increased NF-κB levels at 15 minutes it was remarkably inferior to wild-type levels (Fig. 1B). IL-1β results were less affected than LPSs by MyD88 deficiency at 15 minutes however analyzing the 45 minutes results, NF-κB p50 levels almost returned to basal (Fig. 1B).

Comparing the results for p38 MAPK phosphorylation in MyD88 deficient cells and WT cells significant differences were observed. In WT cells, p38 MAPK phosphorylation was better induced by LPSs than IL-1β. *P. gingivalis* and *E. coli* LPSs demonstrated different kinetics of activation, peaking at 15 and 45 minutes, respectively (Fig. 1A). On the other hand, IL-1β barely activated p38 MAPK at 15 minutes (Fig. 1A). In the absence of MyD88, LPSs tended to induce a little p38 activation which was stronger with IL-1β stimulation (Fig. 1B) and *E. coli* LPS induced delayed p38 activation with peak at 45 minutes. MyD88 was not required for activation of p38 MAPK (Fig. 1B)

MyD88 is required for the induction of RANKL mRNA expression and downregulate OPG mRNA. Since NF-kB and p38 MAPK have a significant impact on the direct or indirect modulation of bone tissue turnover (Rossa *et al.*, 2008) and were influenced by MyD88, we evaluated their role on the regulation of steady-state levels of RANKL and OPG mRNA induced by TLR and IL1R signaling. In WT BMSC cells RANKL mRNA was significantly ($p < .05$) induced after stimulation with all agonists, but

it was markedly inhibited in MyD88 -/- cells (Fig. 2A). The opposite to the regulation of RANKL was observed for OPG mRNA expression that was decreased after stimulation with all agonists in WT BMSC cells, indicating that these external stimuli may effectively alter bone coupled turnover processes towards resorption. This agonist-induced inhibition of OPG mRNA actually reversed in Myd88-/- cells; which showed significantly ($p<0.05$) increased expression of OPG mRNA upon stimulation (Fig. 2B). Thus, MyD88-dependent pathway has a dominant role on negative regulation of OPG mRNA by TLR and IL1R signaling.

RANKL expression at protein level is induced by a MyD88-independent pathway. In order to confirm that MyD88 influence in the regulation of RANKL may have biological significance, RANKL protein levels were evaluated by Western blot. As opposed to the regulation at the mRNA level, MyD88 -/- cells still showed RANKL protein expression upon stimulation with all agonists probably induced by a MyD88-independent pathway (Fig. 3).

TLR and IL1R signaling regulate Nod1, but not Nod2, expression in BMSC. Since Nod1 and Nod2 were suggested to have an important role on the regulation of RANKL induced by TLR and IL1R signaling in BMSC cells (Yamashita *et al.*, 2006, Yang *et al.*, 2005), we decided to investigate if the same agonists modulated Nod1 and Nod2 expression levels. In general, only Nod1 expression was modulated in BMSC, whereas Nod2 protein levels remained fairly constant with an exception with IL-1β stimulation in MyD88 -/-. On the other hand, Nod1 expression was upregulated by all agonists in MyD88 -/- cells, but only *P. gingivalis* LPS (TLR2/TLR4 agonist) induced Nod1 in WT BMSC (Fig. 3). Actually, in WT cells *E. coli* LPS and IL-1β decreased Nod1 protein expression.

Nod2 synergistically enhances RANKL and OPG expression after TLR4 activation in BMSC cultures. Although Nod2 protein levels seemed not to be influenced by TLR activation (Fig. 3), we decided to analyze its possible isolated and synergic role after TLR4 and Nod2 stimulation with *E. coli* LPS and MDP, respectively as previously suggested (Yamashita *et al.*, 2006, Yang *et al.*, 2005). The Nod2 agonist MDP was employed alone and 1 hour before LPS stimulation. The use of MDP as unique stimulation did not influence RANKL and OPG expression when compared to control group (not stimulated) (Fig. 4). Even though *E. coli* LPS increased RANKL mRNA levels, MDP exerted a synergistic effect.

DISCUSSION

Microbial virulence factors or microbial-associated molecular patterns are able induce immune/inflammatory responses that result in bone resorption in different diseases, such as rheumatoid arthritis, osteoporosis and periodontitis (Soory, 2007, Falgarone *et al.*, 2005). Many molecular mechanisms are involved in coupled bone turnover and their understanding can help the development of therapeutic approaches in order to control infection-associated bone resorption. In the present study, we examine the effect of the MyD88 protein on RANKL and OPG levels in bone marrow stromal cells, a source of osteoblast and osteoclast precursor cells (Basak *et al.*, 2009).

According to our results, MyD88 is required for RANKL mRNA expression induced by TLR signaling in bone marrow stromal cells (BMSC). Also, it is involved in the inhibition of OPG mRNA production induced by LPS and IL-1β. The use of cells from single-gene knockout mice illustrates that this regulation is not redundant. It is important to emphasize that MyD88 -/- BMSC cells lost the transcriptional-translation coupling of RANKL gene expression that is observed in WT BMSC cells. Further studies are necessaries but maybe the preservation of p38 MAPK signaling in MyD88-/- cells may play a role in post-transcriptional mechanisms regulating the expression of RANKL at the protein level.

RANKL expression was proven to be induced by LPS- and IL-1β-stimulated osteoblasts though MyD88 and Nod2 activation (Yang *et al.*, 2005). Our results show that BMSC express both Nod1 and Nod2, but the latter is not significantly regulated by TLR and IL1R activation, except for MyD88 -/- cells stimulated with IL-1β. On the other hand, significant regulation of Nod1 in MyD88 -/- cells is observed induced by all agonists. Since both Nod1 and Nod2 interact with Rip2/RICK/CARDIAK kinase through their CARD domains, resulting in activation of NF-kB (Ogura *et al.*, 2001, Inohara *et al.*, 2001), a speculative interpretation of these results suggest that the upregulation of Nod1 might represent a compensatory mechanism for the impaired activation of NF-kB in MyD88-/- cells. Supporting this idea, in TLR-deficient adipocytes Nod1 expression was shown to be upregulated and to be able to activate NF-kB when stimulated with its specific ligand and LPS (Stroh *et al.*, 2008).

This way, the NF-κB family of transcription factors can be activated by various signaling pathways downstream of multiple receptors involved in inflammation and immune response, including TNF receptor family members, the IL-1 receptor (IL-1R)/TLR superfamily, the Nod-like receptor (NLR) family, IL-17 family members, and B cell and T cell receptors (O'Neill, 2006). Early-immediate genes related with innate immunity and inflammation are expressed with MyD88 and consequently, NF-kB activation (Adachi *et al.*, 1998, Kawai *et al.*, 1999). Moreover, inflammatory cytokine and LPS-induced expression of RANKL, as well as osteoclastogenesis are highly dependent on MyD88 (Sato *et al.*, 2004). We confirmed the relevance of MyD88-dependent pathway for RANKL mRNA expression induced by both TLR and IL1R signaling; even so, RANKL expression at protein level was still inducible. This may suggest that osteoclastogenesis process induced by IL1R and TLR signaling could be somewhat preserved in the absence of MyD88, which is in evident contrast with the results of Sato et al. using co-culture models of osteoclastogenesis (Sato *et al.*, 2004). We stress that even though these authors showed a significant decrease on the number of osteoclasts in MyD88 knockout mice, it is important to note that bone resorption was

not completely abrogated and the MyD88-deficient animals did not have a noticeable skeletal phenotype. Besides, instead of using purified cultures of calvarial osteoblasts, we employed primary BMSC, which contain other cell types in various stages of differentiation, including adipocytes and dendritic cells which do express TLR and Nod proteins as well (Stroh *et al.*, 2008, Zeuthen *et al.*, 2008). This might account for differences on the cytokine and signaling networks established upon stimulation of TLR and IL1R; which may result in direct or indirect induction of RANKL through autocrine/paracrine stimulation by other cytokines.

The choice to evaluate the effects of MyD88 on p38 MAPK activation was due to previous data from our research group demonstrating the predominant role of this signaling pathway for RANKL expression induced by cytokines in BMSC (Rossa *et al.*, 2006) and by LPS in periodontal fibroblasts (Rossa *et al.*, 2008); as well as due to the important role of this signaling pathway for inflammation-mediated bone resorption in vivo (Rogers *et al.*, 2007). Summarizing, MyD88 is involved in the regulation of RANKL and OPG expression induced by TLR and IL1R signaling in BMSC. This way, MyD88 play an important role in bone turnover in inflammatory/infectious conditions and Nod2 activation has a synergistic effect in LPS- and cytokine-induced RANKL expression. We also observed that in addition to NF-kB, p38 MAPK signaling downstream of TLR and IL1R is affected by MyD88 in BMSC.

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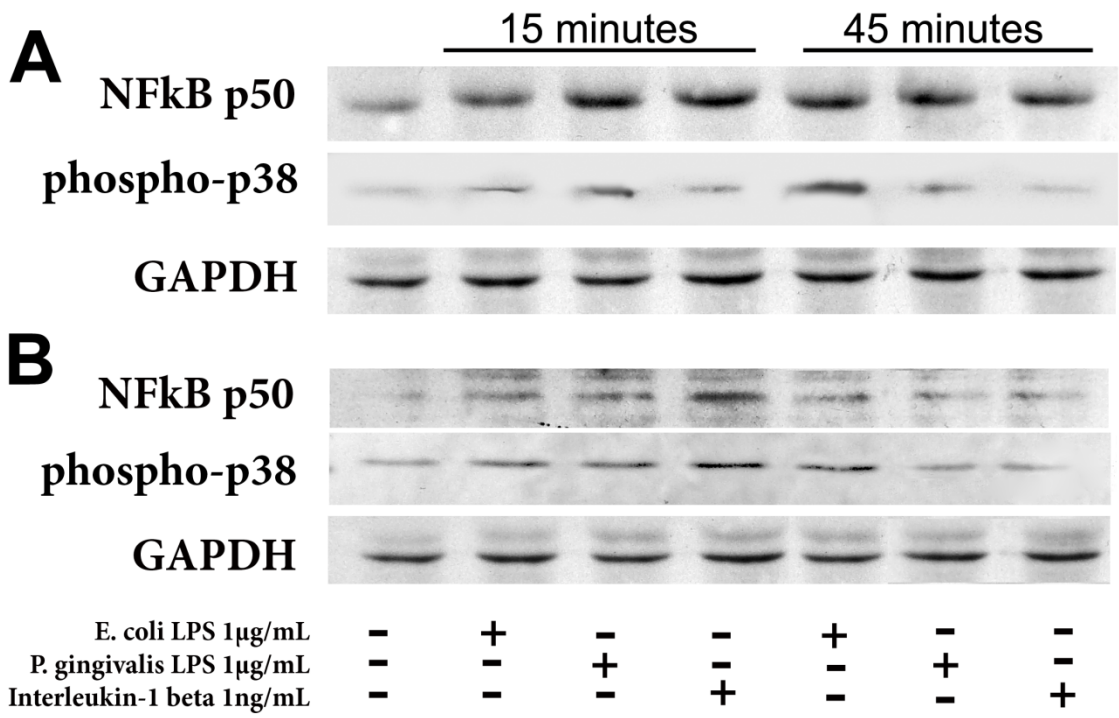

Figure 1 - Representative image of at least three independent western blot experiments for nuclear factor-kappa B p50 (NF-κB) and p38 mitogen-activated protein kinase (MAPK) evaluation. Bone marrow stromal cells (BMSCs) grown on six-well plates were de-induced for 8h in culture medium containing 0.3% fetal bovine serum and then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* (1 µg/mL) or IL1β (1 ng/mL) for 15 and 45 minutes. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide. (A) BMSCs obtained from wild-type animals – control; and (B) MyD88 knockout cells.

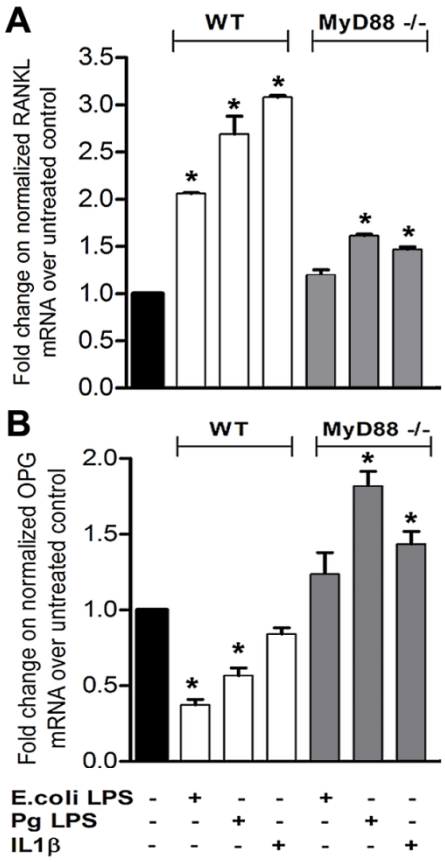

Figure 2 - Receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) mRNA expression induced by 18h-stimulation with *Escherichia coli* and *Porphyromonas gingivalis* LPS and interleukin-1 beta in bone marrow stromal cells. Bars indicate mean and standard deviation of density analysis of three independent experiments. Data are expressed as fold change in unstimulated control group after normalization to GAPDH. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. WT, wild-type; *E. coli*, *Escherichia coli*; *Pg*, *Porphyromonas gingivalis*; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

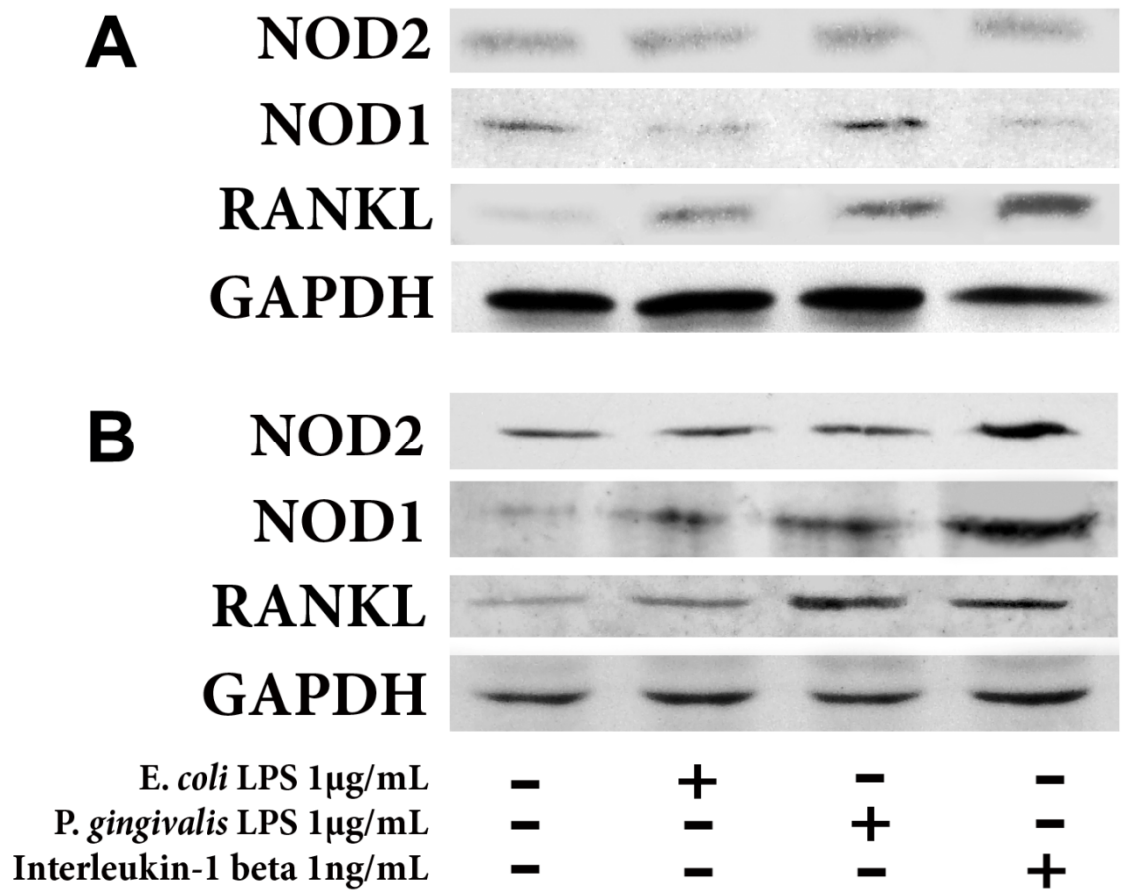

Figure 3 - Receptor activator of nuclear factor-kappa B ligand (RANKL) and nucleotide-oligomerization domain 1 and 2 (Nod1 and Nod2) protein expression in bone marrow stromal cells induced by *Escherichia coli* lipopolysaccharide, *Porphyromonas gingivalis* lipopolysaccharide and interleukin-1 beta after 24h stimulation. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (A) BMSCs obtained from wild-type animals – control; and (B) MyD88 knockout cells.

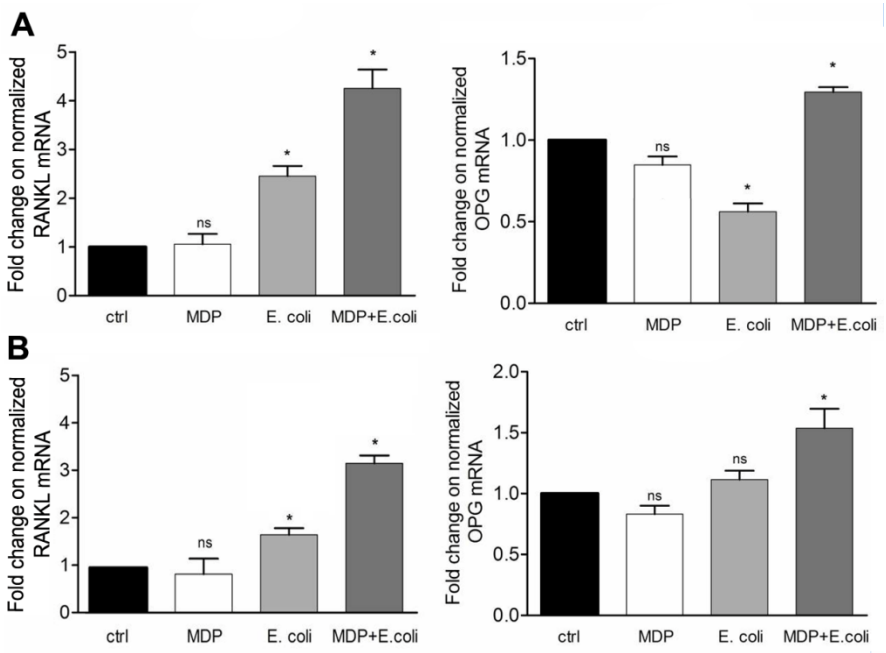

Figure 4 - Receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) mRNA expression induced by 18h-stimulation with *Escherichia coli* LPS and muramyl dipeptide in bone marrow stromal cells. Bars indicate mean and standard deviation of density analysis of three independent experiments. Data are expressed as fold change in unstimulated control group after normalization to GAPDH. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control (ctrl) by one-sample t test. WT, wild-type; *E. coli*, *Escherichia coli*; *MDP*, *muramyl dipeptide*; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CAPÍTULO 4

Porphyromonas gingivalis lipopolysaccharide grown at 1 and 10 ug ml^{-1} hemin induce a significantly different monocyte response*

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Running title: Monocyte response to different *P. gingivalis* LPS

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Summary

Lipid A in LPS molecule modulates host innate immune system which may influence the course of infectious conditions. Hemin concentration in growth media can alter lipid A structure. LPS from *P. gingivalis* grown at 1 µg ml^{-1} (WT1) hemin presents more penta-acylated lipid A structures and at 10 µg ml^{-1} (WT10) have more tetra-acylated structures. ELISA accessed differences in monocytes IL-6, IL-12 and CXCL10 production using WT1 and WT10 LPS. Also, the use of TLR2 and TLR4 and the activation of the MAPK pathway and NF-kB were analyzed. WT1 induced higher production of IL-6, IL-12 and CXCL10 than WT10, however both used TLR2 and TLR4 to activate monocytes. ERK levels were similar in both groups with peak at 10 minutes, the same peak was observed with NF-kB after WT10 stimulation. The same way, JNK was rapidly activated with decrease in activation until 60 minutes. On the other hand, p38 activation was sustained until 60 minutes after stimulation. All these pathways were individually inhibited and affected in different levels IL-6, IL-12 and CXCL10 production. Thus, IL-6, IL-12 and CXCL10 levels are inversely proportional to hemin concentration probably due to differences in lipid A structure.

Introduction

Porphyromonas gingivalis is a gram-negative anaerobic periodontopathogen, associated with adult periodontitis and subsequently with tooth loss (Park et al.[21], 2007; Silva et al.[24], 2008). Lipopolysaccharide (LPS) is one of the major components of its outer membrane amongst other virulence factors including proteases and fimbria (Bainbridge et al.[3], 2008). LPS is able to modulate host innate immune system which influence the beginning and course of infectious conditions. Most of this activity is known to be contained in the lipid portion of the LPS molecule known as lipid A (Liu et al.[13], 2008). *P. gingivalis* LPS shows different lipid A structures which are less acylated and phosphorylated than *E. coli* LPS, a known inflammatory response inducer, also its fatty acids are more branched and the chain longer. Most of the lipid A structures consist of di- and mono-phosphoryl penta-acylated lipid A and mono-phosphoryl tetra-acylated lipid A forms.

Some microenvironmental conditions contribute to alter the virulence of different bacteria. Hemin concentration in growth media regulates the expression of many virulence factors, such as gingipains, lipid A and extracellular vesicle formation (Olczak et al.[20], 2005). *P. gingivalis* evolved to use different hemin-containing compounds, i.e. hemoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin, and cytochrome c. Interestingly, *P. gingivalis* has the ability to store hemin on its cell surface a probable nutritional advantage for its survival in healthy periodontal pockets, an iron-limited environment (Genco[11], 1995). It's been suggested that *P. gingivalis* hemagglutinin and hemolysin helps in pocket colonization by aiding in the acquisition of hemin/iron by the lyse of erythrocytes and liberation of hemin (Genco[11], 1995). So, it is presumable that the increase in vascular ulceration during periodontitis, the higher is the local concentration and the better will be the conditions for *P. gingivalis* growth and lipid A modification.

In terms of inflammation, interleukin-6 is an acute phase response mediator and acts as a procoagulant cytokine released by monocytes (Willerson e Ridker[28], 2004). It has been implicated in different diseases from periodontitis to cardiovascular disease because increases fibrinogen and plasminogen activator inhibitor type-1 concentration in plasma which induces the expression of C-reactive protein amplifying the inflammatory response (Marcaccini et al.[17], 2009). Another highly expressed cytokine by monocytes was interleukin-12 (IL-12) which is also called T cell stimulating factor because it may be implied in the differentiation of naive T cells into Th1. IL-12 plays different roles in inflammation enhancing the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes and also blocking the formation of new blood vessels though increase on interferon gamma levels (Tellides e Pober[26], 2007). In fact, the anti-angiogenic effect is attributed to the chemokine (C-X-C motif) ligand 10 (CXCL10) also known as IP-10 which is inducible by IFN-gamma. CXCL10 shows chemoattractive characteristics for monocytes/macrophages, T cells, NK cells and dendritic cells and also contributes for T cell adhesion to endothelial cells (Angiolillo et al.[2], 1995; Dufour et al.[9], 2002).

Monocytes and macrophages have been employed to study the mitogen-activated protein kinase pathway (MAPK) role on LPS-induced proinflammatory responses. The activation of the pattern recognition receptors by LPS initiates innate immunity by triggering the activation of signaling pathways (such as MAPK) involved in immediate response. Activation of MAPK pathways occurs by a cascade of phosphorylation events that will lead to the phosphorylation of downstream kinases that can translocate to the nucleus and phosphorylate their substrates (transcription factors, RNA-binding proteins or other kinases); which, ultimately will affect gene expression. There are three well-described MAPK signaling pathways, named after their downstream effector kinases: extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK)(Krishna e Narang[12], 2008).

Until now the presence of multiple lipid A structures in *P. gingivalis* LPS is a confounding factor in the interpretation of host response, specially the role of *P. gingivalis* LPS in the pathology of periodontitis and in cardiovascular diseases (Liu et al.[13], 2008). To the best of our knowledge, this is the first report that *P. gingivalis* grown in different hemin concentrations induce distinct cell responses due to alterations on the lipid A structure.

Results

***P. gingivalis* grown at 1 µg mL^{-1} hemin induces higher production of IL-6, IL-12 and CXCL10 than *P. gingivalis* at 10 µg mL^{-1} hemin.** The first experiment determined whether both types of *P. gingivalis* LPS were able to activate MM6 cells and induce cytokine and chemokine expression. Tests were performed stimulating cells with *P. gingivalis* WT1 and WT10 LPS and *E. coli* LPS at a concentration of 100 ng mL^{-1}; which was determined according to previous experiments (Coats et al.[5], 2007; Coats et al.[6], 2005). Cell culture supernatants were examined for the presence of target proteins by ELISA. In unstimulated MM6 cells (negative control) no detectable expression of IL-6, IL-12 and CXCL10 was observed. As shown in Fig. 1, all LPS preparations stimulated the production of IL-6, IL-12 and CXCL10 in MM6 cultures after a 24-hour challenge. *E. coli* was a significantly more potent inducer of IL-6 and CXCL10 than both *P. gingivalis* ($P < .05$) however *E. coli* and *P. gingivalis* grown at 1µg ml^{-1} hemin were more potent inducers of IL-12 than *P. gingivalis* grown at 10 µg ml^{-1} hemin ($P < .05$).

***P. gingivalis* activates both TLR-2 and TLR-4 at both 1 and 10 µg ml^{-1}.** To identify whether *P. gingivalis* grown in different hemin concentrations activated toll-like receptor (TLR) -2 or -4 and confirm whether *E. coli* activated only TLR-4; anti-TLR-2 and -4 antibodies were employed. Preliminary experiments (data not shown) determined that the best concentration of anti-TLR antibodies for optimum blocking effect was 2 µg ml^{-1},

added to the culture media 1 h before LPS stimulation. Fig. 2 illustrates that *E. coli* LPS activated TLR-4, while *P. gingivalis* at 1 and 10 μg ml^{-1} activates both TLR-2 and -4. Apparently TLR2 and TLR4 are equally important for the expression of IL-6 and IL-12 induced by LPS.

Different intracellular signaling pathways are involved on IL-6, IL-12 and CXCL10 production and show different kinetics. Because of TLR activation, we inferred that NF-kB and MAPK signaling pathways would be involved in the regulation of gene expression. On Fig. 3 a significant activation of NF-κB, p38, ERK and JNK pathways was observed on MM6 cells after LPS stimulation. ERK MAPK activation showed a similar pattern among the three groups with peak at 10 minutes ($P < .05$) (Fig. 3A). However, p38 MAPK activation was sustained for 60 minutes after stimulation with *P. gingivalis* LPS; whereas 60 minutes after E.coli LPS stimulation p38 activity showed some decrease in comparison to the 30 minutes time point indicating a possible constitutive activation (Fig. 3B). NF-κB was significantly activated after 10 minutes of stimulation with *E. coli* and *P. gingivalis* WT10 LPS; which may represent the classical activation of NF-kB through TLR signaling (Fig. 3C) (Bonizzi e Karin[4], 2004). The same way, JNK was shown to be rapidly activated in all groups, with progressive decrease in activation until 60 minutes (Fig. 3D). E.coli LPS was, however, obviously more potent in activating both NF-kB and JNK MAPKinase in MM6 cells.

Different signaling pathways are required for production of IL-6, IL-12 and CXCL10 by human monocytes upon stimulation with *P. gingivalis* and *E. coli* LPS. IL-6 and IL-12 production were similarly influenced by the different inhibitors suggesting either cross-talk or a synergistic effect of these signaling pathways; in spite of the use of TLR-2 or TLR-4 by the different LPSs. Actually, according to figure 4, when TLR2 is also activated by P.gingivalis LPS stimulation of the cells p38 MAPK plays a smaller role on IL6 and IL-12 expression. On the other hand, for expression of

CXCL10, NF-kB seems to play a smaller role when only TLR4 is activated by *E. coli* LPS; however CXCL10 was p38-dependent (Fig. 4).

IL-6 and CXCL10 mRNA levels correlates with protein levels. *E. coli* LPS was a more potent activator of MM6 cells than *P. gingivalis* LPS especially in comparison to the WT10 strain; but mRNA levels correlated with protein levels for IL-6 and CXCL10, indicating that transcription-translation coupling of these genes is preserved in MM6 cells. IL-12 p70, however, can be induced indirectly by the production of interferon-gamma; which surprisingly was highly expressed on MM6 cells (Fig. 5). WT10 induced a less potent expression of IL-6, IL-12 and CXCL10 mRNA in spite of the overall similar pattern of activation of the signaling pathways studied (p38, JNK, ERK and NF-kB) suggesting that other signaling pathways might play an important role on gene regulation.

Discussion

MonoMac 6 cells have been employed as a mature monocyte model to understand the role and response of monocytes in different diseases. Pure MonoMac 6 cultures express NaF-sensitive non-specific esterases, reactive oxygen specimens, staining for My4 and other monocyte-specific mAb, and promote phagocytosis in 80% of cells (Erl et al.[10], 1995). Monocytes exert two basic functions in the immune system the replenish of macrophages cells in healthy tissues and quick chemotaxis to infected sites contributing for macrophages and dendritic cells differentiation to initiate the inflammatory response (Proost et al.[22], 1996).

A previous study (Diya et al.[8], 2008) already showed the activation of TLR-2 and TLR-4 and the downstream intracellular signaling pathways activated by *P. gingivalis* LPS; however this is the first report illustrating differences on cell activation using two distinct *P. gingivalis* LPS preparations obtained from bacteria grown in low (1 μg ml^{-1}) and high (10 μg ml^{-1}) hemin concentrations. Lipid A synthesis is highly

regulated in gram-negative bacteria and usually maintains the same basic structure showing small variations in number, type, and placement of fatty acids and phosphates (Wang et al.[27], 2006). Differences in lipid A structure can be seen with changes in the substrate of synthetic enzymes or with addition of lipid A modifying enzymes that add or remove components auxiliary to normal synthesis (Bainbridge et al.[3], 2008). The reason for such change in lipid A structure is yet under study, different mechanisms have been suggested for this occurrence. It is known that the sensing of hemin concentration by hemin acquisition proteins Kgp and HmuR increase hemin uptake which may lead to two different ways, at high hemin concentrations, deacylase activity may be induced causing the loss of the major penta-acylated lipid A structures at *m/z* 1,690 and the formation of the major tetra-acylated lipid A structures found at *m/z* 1,449. On the other hand, the increase in hemin concentration may lead to LPS biosynthesis appearing in the form of penta-acylated diphosphorylated lipid A structures at *m/z* 1,770 (Al-Qutub et al.[1], 2006).

P. gingivalis can gain access to a large supplement of hemin in periodontitis because of epithelium and vascular ulcerations which may alter the lipid A form and guarantee the infection of bloodstream. Lately, *P. gingivalis* was suggested to be involved in the etiology of cardiovascular disease since it has been found in biopsies taken from the aortas patients during open-heart surgery (Stelzel et al.[25], 2002), also chronic *P. gingivalis* infection may facilitate monocyte recruitment to vascular endothelium through sustained upregulation of ICAM-1 and VCAM-1 contributing to atherogenesis (Nakamura et al.[18], 2008).

Hemin stored on *P. gingivalis* cell surface may be an advantage for its survival in periodontal pockets (Genco[11], 1995). *P. gingivalis* contains hemagglutinin and hemolysin which help the acquisition of hemin/iron by the lyse of erythrocytes and liberation of hemin (Genco[11], 1995). This way, it can be assumed that periodontal pockets at early stages of adult periodontitis present less hemin due to epithelium integrity and *P. gingivalis* LPS is more penta-acylated inducing a more aggressive

response, however with the increase in vascular ulceration during disease progression, the higher local hemin concentration contributes for lipid A shifting to tetra-acylated structures and reduction of TLR2 and -4 activation.

The differences observed on IL-6 and IL-12 p70 levels in MM6 cells comparing the LPS from *P. gingivalis* grown at low and high hemin concentrations may be due to the presence of penta-acylated lipid A structural forms at low concentration in contrast with the tetra-acylated structural forms found at high hemin concentrations (Coats et al.[6], 2005; Reife et al.[23], 2006). In the future, the mechanism of interaction between these two types of *P. gingivalis* LPS with TLR-2 and TLR-4 should be investigated, since if by altering the LPS structure, *P. gingivalis* can affect its recognition by different TLR receptors. This can have an immunomodulatory effect and may represent adaptive mechanisms aimed at facilitating its survival in different environments, especially in human body.

One interesting point was the production of IFN-gamma by MM6 because this cytokine has distinct properties such as antiviral, and immunoregulatory (Maher et al.[14], 2007). Also, it enhances antigen presentation with activation of lysosomes in macrophages, supress activity of Th2 cells and increase activity of NK and Th1 cells. Another IFN-gamma function is the stimulation of NF-κB activity by the release of nitric oxide and degradation of TRAF6 in RANK-RANKL signaling pathway (Zheng et al.[29], 2006). Increased NF-kB activation would result in more pro-inflammatory cytokine production, but degradation of TRAF6 and reduced signaling in RANK-RANKL pathway would reduce osteoclastogenesis and osteoclast activity. TRAF6 is also upstream of JNK/p38 MAPKinases, so its degradation would probably affect signaling by these pathways as well.

This way, according to our results all signaling pathways were activated after stimulation with *E. coli* LPS however p38 and ERK showed a sustained activation pattern in all time-points (10, 30 and 60 minutes) while NFkB p65 and JNK displayed a quick profile with peak at 10 minutes. This result suggests a possible role of MyD88-

dependent and -independent pathways in TLR2 and -4 activation. The activation of MyD88-independent pathway by TLR4 recruits the adapter-inducing interferon-β (TRIF) which potentiates the secretion of proinflammatory cytokines, TRIF leads to NF-kB activation and consequently secretion of IFN-β and indirect up-regulation of IFN-dependent genes such as CXCL10 and inducible nitric oxide synthase (iNOS)(Netea et al.[19], 2004). LPS from *P. gingivalis* grown in high hemin concentrations has less penta-acylated clusters and resulted in weaker induction of IL-6 and IL-12. Thus, IL-6, IL-12 and CXCL10 levels are inversely proportional to hemin concentration probably due to differences in lipid A structure. All three MAPKinase pathways and NF-kB play equally important roles on CXCL10 expression induced by bacterial LPS, and p38 MAPK seems to play a lesser role on IL-6 and IL-12 expression. We could not find striking differences on the role of these signaling pathways in the expression of IL-6, IL-12 and CXCL10 induced by the different types of LPS used.

Experimental procedures

Bacterial strains and growth conditions. *P. gingivalis* strains ATCC 33277, W83, 381, A7436 and WS15 were obtained from our stock collection. Bacterial culture media included both enriched Trypticase soy broth (ETSB) and Trypticase soy broth-yeast extract-hemin-vitamin K (menadione) (TYHK) with variations described in the text. The ETSB consisted of Trypticase soy broth (TSB) (30 g/950 ml), yeast extract (Difco, Detroit, MI, USA) (1 g/950 ml), glucose (1 g/950 ml), and potassium nitrate (0.5 g/950 ml), pH 7.1. This basal media was autoclaved and filter-sterilized. Supplements added included, anhydrous sodium carbonate (0.4 g), cysteine-HCl (0.4 g), hemin (Sigma-Aldrich, St. Louis, MO, USA) stock solution (solution a) (10 ml), vitamin K (Sigma-Aldrich) stock solution (solution b) (0.2 ml), and distilled water (40 ml). Solution a was made by dissolving 50 mg of hemin (Sigma-Aldrich) in 1.0 ml of 1.0 N NaOH, adding 99 ml of distilled water, and storing the solution at 4°C. Solution b was made by dissolving 250 mg of vitamin K (Sigma-Aldrich) in 50 ml of 95% ethanol and storing it at

4°C. TYHK was Trypticase soy broth (30 g/liter), yeast extract (5 g/liter) (Difco), hemin (Sigma-Aldrich), and vitamin K3 (menadione; Sigma-Aldrich) (0.001 g/liter), pH 7.2, and the media was subjected to autoclaving. Two different hemin concentrations were employed 1 and 10 µg ml^{-1} (labeled WT1 and WT10 respectively). Bacterial growth was monitored by optical density (600 nm), cells were harvested in the stationary phase of growth (Al-Qutub et al.[1], 2006).

Purification and characterization of LPS. *P. gingivalis* LPS was prepared by the cold $MgCl_2$-ethanol (EtOH) procedure followed by lipid extraction (Darveau et al.[7], 2004). *E. coli* 0111:B4 LPS (Sigma-Aldrich) was subjected to Folch extraction to remove contaminating phospholipids. Traces of endotoxin protein were removed from all LPS preparations by Manthey and Vogel method (Manthey e Vogel[16], 1994) with the following modification. After the final EtOH precipitation, LPS was lyophilized and resuspended in distilled water to 1 mg ml^{-1}. LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained for protein by the enhanced colloidal gold procedure. The presence of nucleic acid was determined by ethidium bromide fluorescence quantification of the amount of double-stranded DNA by using the plastic wrap method (Maniatis[15], 1982).

GC/MS analysis of LPS fatty acids. Each LPS sample (0.25 mg dry weight) was analyzed as previously described (Darveau et al.[7], 2004), Tuning and mass calibration were performed using perfluorotributylamine with repetitive scan. Matrix assisted laser desorption–time-of-flight (MALDI-TOF) MS was performed (Darveau et al.[7], 2004) and two separate extractions of *P. gingivalis* LPS were produced WT1 (more penta-acylated structures) and WT10 (tetra-acylated) as shown previously (Al-Qutub et al.[1], 2006).

Cell culture. The established human monocytic cell line MonoMac 6 (MM6) was grown in RPMI 1640 media supplemented with 2 mM L-glutamine, 5 ml/L non-essential amino acids, penicillin (50 IU ml^{-1}), streptomycin (100 µg ml^{-1}), 1 mM sodium pyruvate, 0.2 U ml^{-1} bovine insulin and 10% heat-inactivated fetal bovine serum (FBS). All supplies were obtained from Gibco (Grand Island, NY, USA). Before addition of FBS, the media was ultra-filtrated to eliminate LPS. Cells were cultured at a density of 1 to 3 x 10^6 cells ml^{-1} in humidified 5% CO_2 at 37°C. Fresh media was added to the cultures twice weekly. For the experiments, cells were harvested, washed with phosphate buffered saline (PBS), pH 7.4, and seeded on 12-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 0.8x10^6 cells ml^{-1}.

Determination of cytokines in culture supernatants. MM6 cells stimulated with 100 ng ml^{-1} *P. gingivalis* LPS grown at 1 (WT1) and 10 µg ml^{-1} hemin (WT10), 100 ng ml^{-1} *E. coli* LPS, or without LPS (negative control) were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Culture supernatants were collected and quantitatively analyzed by enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-12 p70 (eBioscience, San Diego, CA, USA) and CXCL10 levels (RayBio, Norcross, GA, USA) according to the protocol suggested by the manufacturer.

Intracellular signaling pathways activation. Phospho-NF-κB p65, phospho-p38MAPK (T180/Y182), phospho-ERK1/2 (T202/Y204) and phospho-JNK (T183/Y185) sandwich ELISA kits (SABiosciences, Frederick, Maryland, USA) were used to determine the kinetics and phosphorylation levels of NF-κB, p38MAPK, ERK1/2 and JNK. For these tests, MM6 cultures were stimulated with LPS for 10, 30 and 60 minutes, lysed and assayed according to the ELISA protocol. After that, specific biochemical inhibitors were used for each pathway to determine the role of the NF-κB, p38MAPK, ERK and JNK signaling pathways on the production of IL-6, IL-12 and CXCL10. MM6 cells (1 x 10^5 cells/well) were incubated with 10 µM of NF-κB inhibitor

481407, 10 µM of p38MAPK inhibitor SB203580, 1 µM of ERK inhibitor peptide I and 10 µM of JNK1-3 inhibitor SP600125 (Calbiochem Bioscences, Los Angeles, CA, USA) for 1 h before stimulation with LPS. Culture supernatants of MM6 cells stimulated with *P. gingivalis* WT1 and WT10 LPS or *E. coli* LPS for 24 h were collected and assayed for IL-6, IL-12 p70 and CXCL10 by ELISA. MM6 stimulation with LPS without signaling pathway inhibitors were used as positive controls and the absence of both LPS and signaling pathway inhibitors were used as negative controls. All experiments were performed in triplicate.

TLR blocking using antibodies. To determine the activation of TLR2 and TLR4 by *P. gingivalis* LPS grown in 1 and 10 µg ml^{-1} hemin and its influence on cytokine production, MM6 cells were incubated with 2 µg ml^{-1} of anti-TLR2 (Invivogen, San Diego, CA, USA) and anti-TLR4 polyclonal antibody (USBiological, Swampscott, MA, USA) for 1 h before stimulation with 100 ng ml^{-1} *P. gingivalis* WT1 or WT10 LPS or *E. coli* LPS. Culture supernatants were assayed for IL-6 and IL-12 p70 by ELISA after stimulation for 24h. Supernatants from cells incubated with LPS without anti-TLRs were used as positive controls, and pure cultures without both LPS and anti-TLRs were used as negative controls. All experiments were performed in triplicate.

Semiquantitative reverse transcription-polymerase chain reaction. Reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate mRNA expression. Briefly, total RNA was harvested using Trizol (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions. A Promega RT-PCR (Promega, Madison, WI, USA) was employed and RT-PCR and PCR were done in the same reaction as recommended by manufacturer: 500 ng of total RNA was employed using 1 µM Oligo (dT) 15 primers, 0.1 U/µL of Avian Myeloblastosis Virus reverse transcriptase, 0.1 U/µL of *Tfl* DNA Polymerase, 1 mM $MgSO_4$, 0.2mM dNTPs, 0.4 U/µL of RNAse inhibitor, 1X *Tfl* reaction buffer and 1µM of upstream and downstream

primers according to manufacturer's protocol. The primer pair used for IL-6 was: sense 5'-GCAAAGAGGCACTGGCAGAA-3'; antisense 5'-CTCAGGCTGGACTGCAGGAA-3'; IL-12 sense 5'-TGTAGAGAGGATAAACGG-3'; antisense 5'-CTAGTTATAAGCAGCTTAT-3'; CXCL10 sense 5'-GGCTGCCTCTCCCATCACTT-3'; antisense 5'- AAAGAATTTGGGCCCCTTGG-3'; and beta-actin was: sense 5'-AGCCCTGGCTGCCTCCAC-3'; antisense 5'-GTCGGTTGGAGCGAGCATC-3'. Reverse transcription was carried out at 48°C for 50 min. Amplification with all primers sets was performed for 28 cycles at 94°C for 30 s, 58°C for 45 s, and 68°C for 5 min. PCR products were resolved on 1.0% agarose gels, stained, and visualized. Images were stored with the Eagle-eye gel documentation system (Stratagene, La Jolla, CA, USA). Images of PCR products were quantified with ImageJ software (NIH, Bethesda, MD, USA). The densitometric values obtained for IL-6, IL-12 and CXCL10 PCR products were normalized to the values obtained for beta-actin PCR products, and the resulting values were analyzed with GraphPad Prism software (GraphPad Software Inc., Los Angeles, CA, USA).

Statistical analysis. All data are presented as the mean ± SD of at least three independent experiments. Pairwise comparisons between experimental groups were performed using the t-test with Welch's correction for unequal variances. Comparison between fold changes on mRNA expression between lipopolysaccharide-stimulated and untreated cells was performed with the one-sample t-test. The significance level was set to 5% and all calculations were performed using PRISM 4 software (GraphPad, Inc., San Diego, CA, USA).

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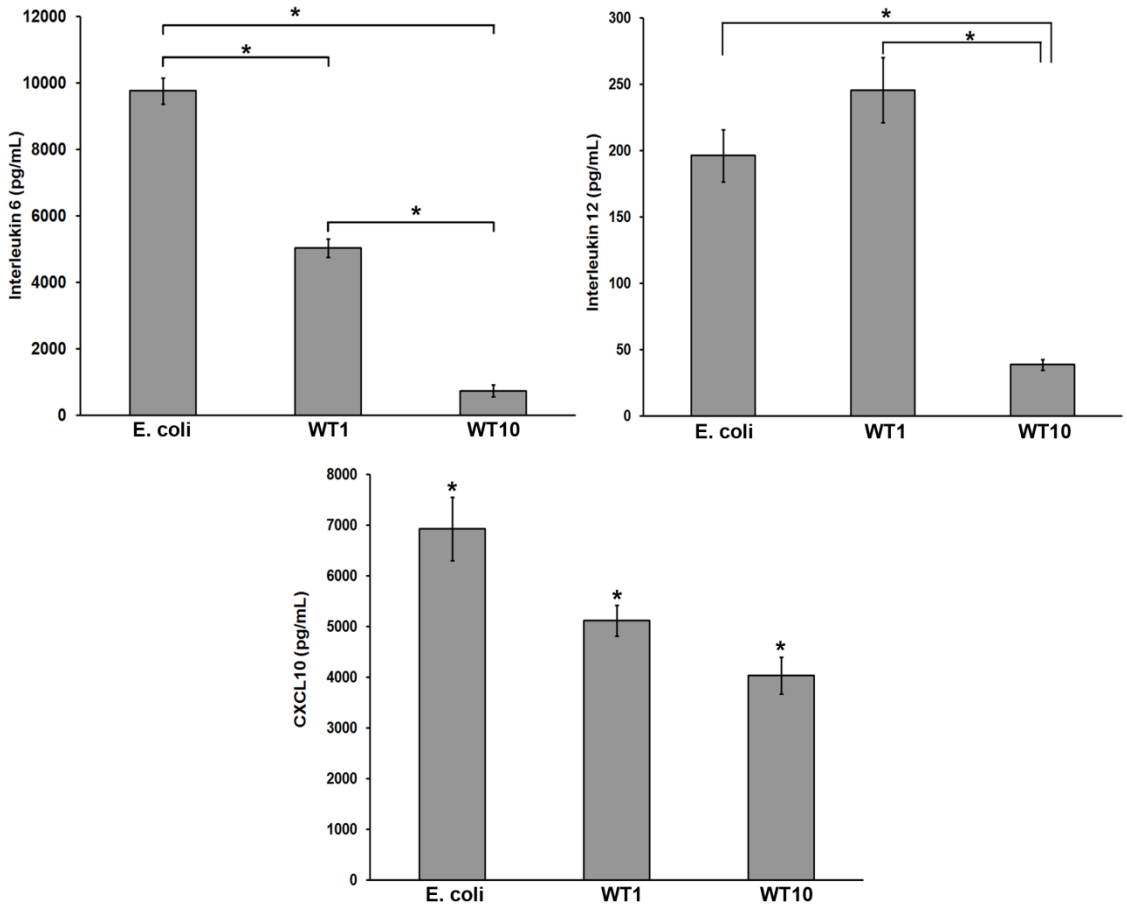

Figure 1 – Interleukin-6, interleukin-12 and CXCL10 protein expression by lipopolysaccharide-stimulated MonoMac6 cells is significantly different between *P. gingivalis* grown in low and high hemin concentration. MonoMac 6 cells grown on six-well plates were de-induced for 12 h in culture media containing 0.3% fetal bovine serum and were then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* grown in low (WT1) and high hemin (WT10) for 24 h. Illustration of ELISA analysis of IL-6, IL-12 and CXCL10 expression from MonoMac6 supernatants. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. Bars indicate mean and standard deviation of three independent experiments. *E. coli*, *Escherichia coli*; WT1, *Porphyromonas gingivalis* grown media containing 1 µg ml^{-1} hemin; WT10, *Porphyromonas gingivalis* grown media containing 10 µg ml^{-1} hemin; IL, interleukin.

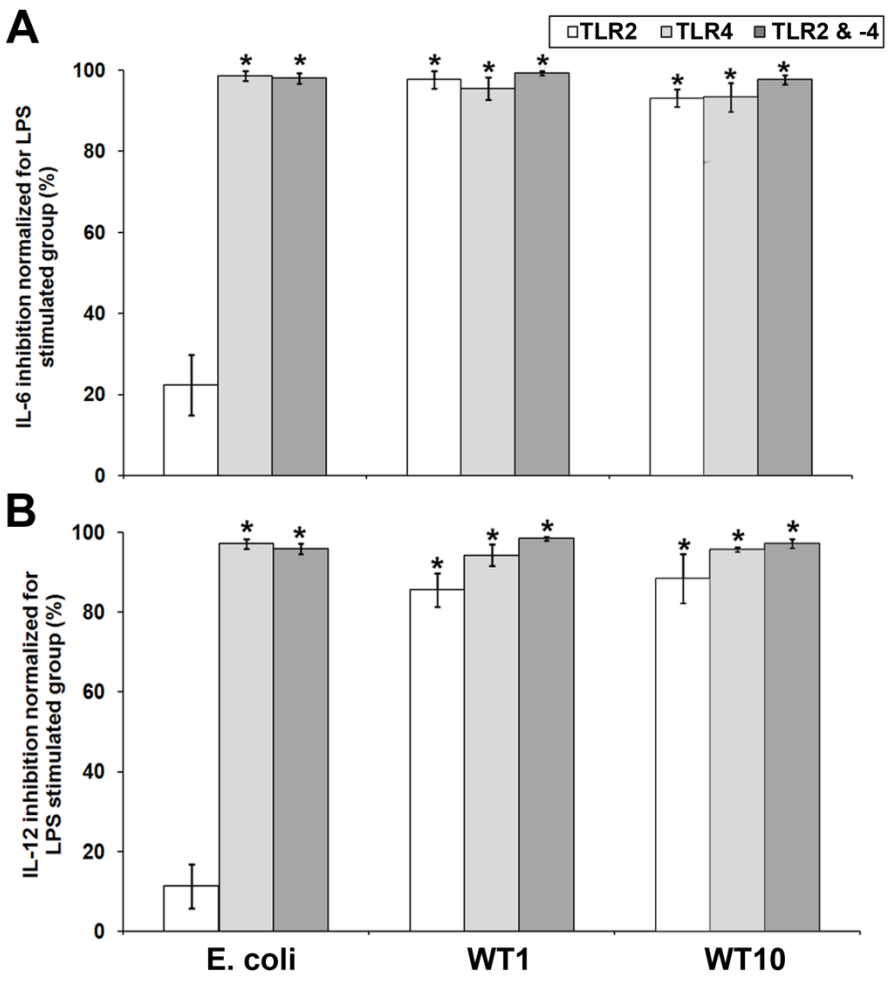

Figure 2 - Interleukin-6 and interleukin-12 protein expression by lipopolysaccharide-stimulated MonoMac6 cells is significantly influenced by TLR2 or TLR4 stimulation. MonoMac 6 cells grown on six-well plates were de-induced for 12 h in culture media containing 0.3% fetal bovine serum and were then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* grown in low (WT1) and high hemin (WT10) for 24 h. Illustration of ELISA analysis of IL-6 and IL-12 expression from MonoMac6 supernatants. Anti-TLR2 and anti-TLR4 antibodies (2 µg ml^{-1}) were added to the culture media 1 hour before stimulation with lipopolysaccharide (100 ng ml^{-1}). In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. Bars indicate mean and standard deviation of three independent experiments. *E. coli*, *Escherichia coli*; WT1,

Porphyromonas gingivalis grown media containing 1 µg ml^{-1} hemin; WT10, *Porphyromonas gingivalis* grown media containing 10 µg ml^{-1} hemin; IL, interleukin.

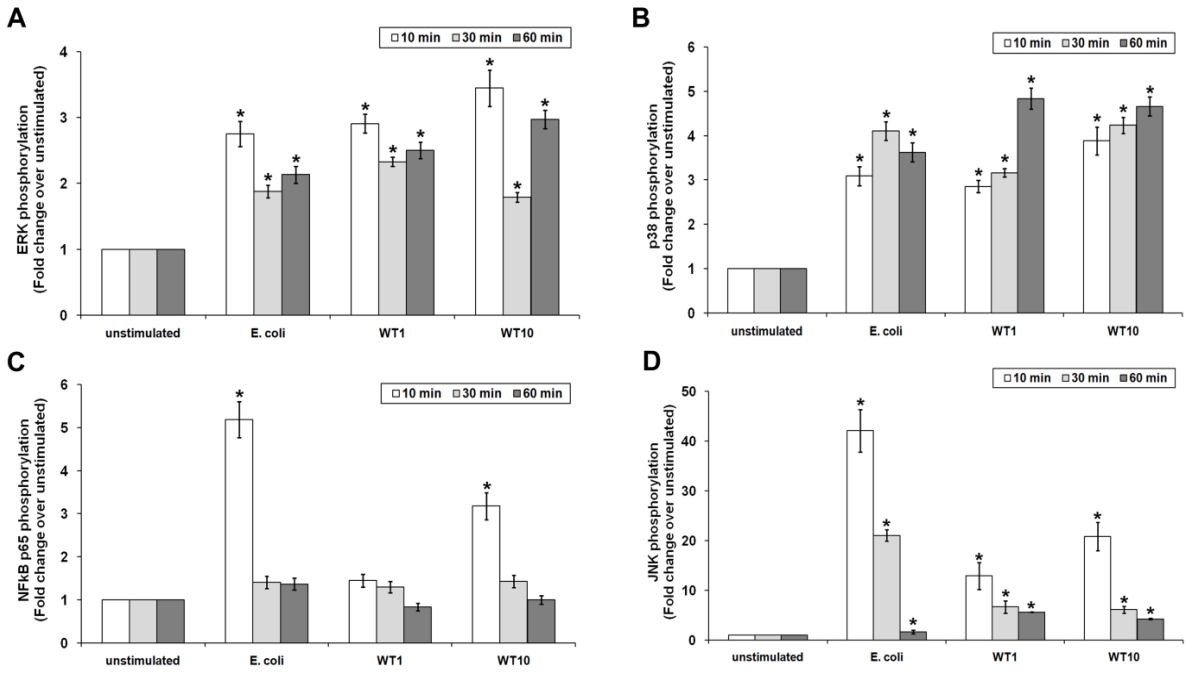

Figure 3 - ERK, JNK and p38 MAPKs and NF-kB phosphorylation is induced by all LPS preparations but shows different kinetics. MonoMac 6 cells grown on six-well plates were de-induced for 12 h in culture media containing 0.3% fetal bovine serum and were then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* grown in low (WT1) and high hemin (WT10) for 24 h. ERK, JNK, p38, and NF-kB phosphorylation levels from MonoMac6 whole-cell lysates 0 (unstimulated), 10, 30 and 60 minutes after LPS stimulation. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. Bars indicate mean and standard deviation of three independent experiments. *E. coli*, *Escherichia coli*; WT1, *Porphyromonas gingivalis* grown media containing 1 µg ml^{-1} hemin; WT10, *Porphyromonas gingivalis* grown media containing 10 µg ml^{-1} hemin; IL, interleukin.

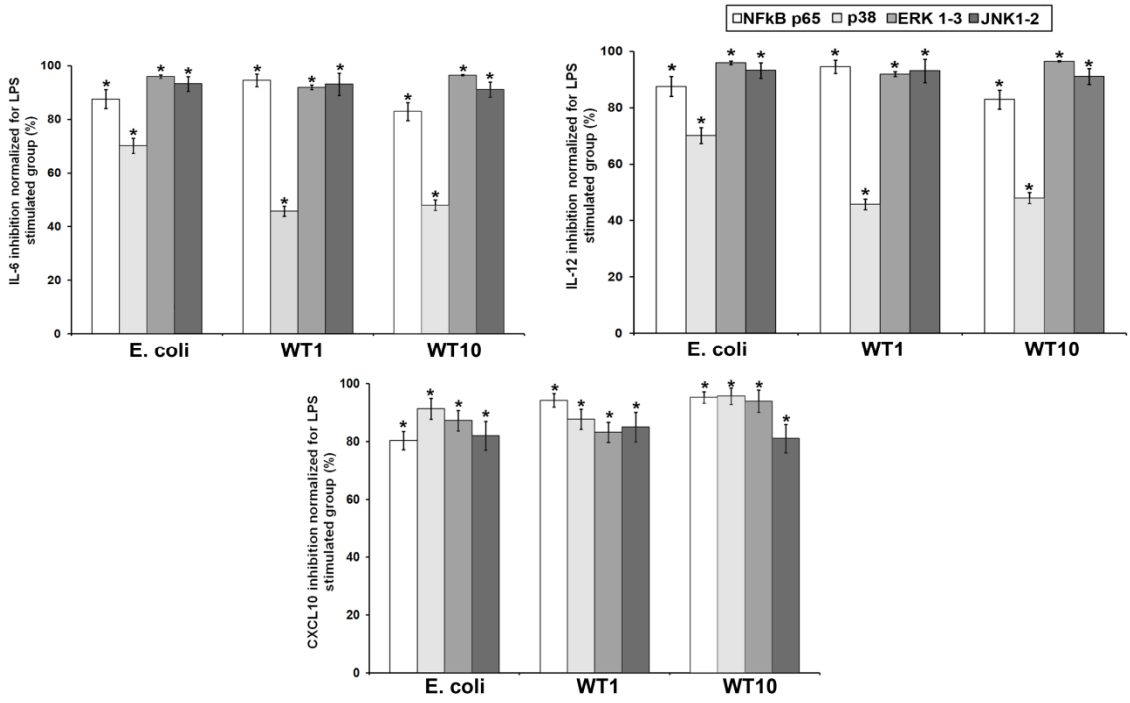

Figure 4 - ERK, JNK and p38 MAPKs and NF-kB activation are involved in IL-6, IL-12 and CXCL10 production. MonoMac 6 cells grown on six-well plates were de-induced for 12 h in culture media containing 0.3% fetal bovine serum and were then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* grown in low (WT1) and high hemin (WT10) for 24 h. One hour prior to LPS stimulation 10 µM of NF-κB inhibitor 481407, 10 µM of p38MAPK inhibitor SB203580, 1 µM of ERK inhibitor peptide I and 10 µM of JNK1-3 inhibitor SP600125 (Calbiochem Bioscences, Los Angeles, CA, USA) were added to the culture media for 1 h. IL-6, IL-12 and CXCL10 levels were analyzed in MonoMac6 culture supernantants 24 h after LPS stimulation. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. Bars indicate mean and standard deviation of three independent experiments. *E. coli*, *Escherichia coli*; WT1, *Porphyromonas gingivalis* grown media containing 1 µg ml^{-1} hemin; WT10, *Porphyromonas gingivalis* grown media containing 10 µg ml^{-1} hemin; IL, interleukin.

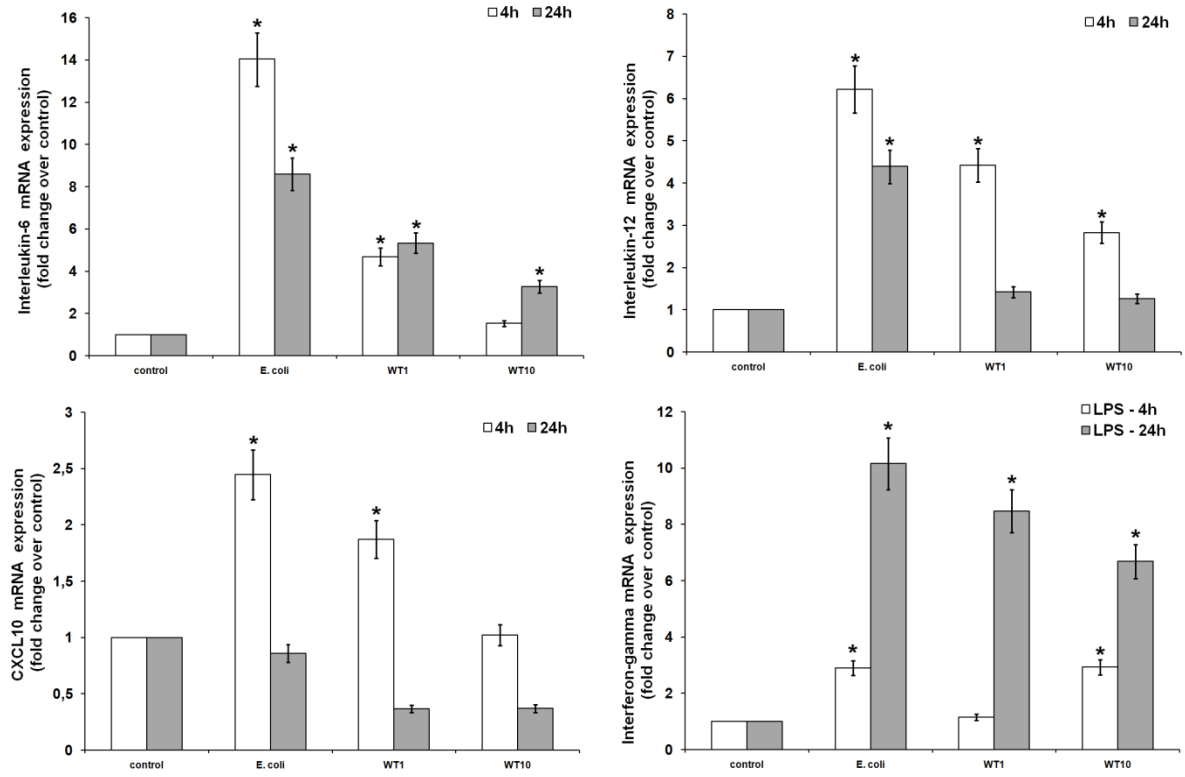

Figure 5 - mRNA levels show differences but some correlation between *P. gingivalis* grown in 1 and 10 ug ml^{-1} hemin. MonoMac 6 cells grown on six-well plates were de-induced for 12 h in culture media containing 0.3% fetal bovine serum and were then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* grown in low (WT1) and high hemin (WT10) for 4h or 24 h. Illustration of RT-PCR gel analysis of IL-6, IL-12, CXCL10 and interferon-gamma expression from MonoMac6 whole-cells lysis. In all graphs asterisk (*) indicates significant difference ($p<0.05$) in relation to '1' attributed to unstimulated control by one-sample t test. Bars indicate mean and standard deviation of three independent experiments. *E. coli*, *Escherichia coli*; WT1, *Porphyromonas gingivalis* grown media containing 1 µg ml^{-1} hemin; WT10, *Porphyromonas gingivalis* grown media containing 10 µg ml^{-1} hemin; IL, interleukin.

DISCUSSÃO

Discussão

O aumento da prevalência de doenças afetando direta ou indiretamente o tecido ósseo, incluindo osteoporose, osteoartrite, câncer e doenças periodontais tem estimulado o estudo dos mecanismos moleculares relacionados à homeostase do tecido ósseo, sendo que a descoberta do sistema de citocinas RANKL/OPG década de 90 representa um grande avanço na área. A alteração no equilíbrio entre o número de osteoclastos e osteoblastos pode ocorrer devido a inúmeros fatores como o aumento da produção de citocinas inflamatórias e alterações hormonais (Leibbrandt, Penninger[58], 2008). O excesso de tecido ósseo, osteopetrose, é uma condição rara e geralmente herdada geneticamente, por outro lado a perda de estrutura óssea (osteoporose) é prevalente e associada a diversas doenças como infecções crônicas, artrite reumatóide, periodontite, leucemia, entre outras (Leibbrandt, Penninger[58], 2008). Assim, diversas terapias para alterar o curso dessas doenças têm sido procuradas para interferir no metabolismo ósseo, requerendo o estudo aprofundado de interações celulares e mecanismos intracelulares de transdução de sinais, no caso do tecido ósseo, essa ciência é chamada osteoimunologia.

A osteoclastogênese é mediada pela interação entre três moléculas RANKL e seus receptores RANK e OPG. OPG foi a primeira proteína descoberta deste eixo metabólico e descrita como capaz de inibir a diferenciação de osteoclastos (Simonet et al.[89], 1997; Tsuda et al.[105], 1997). Pouco tempo depois, RANKL foi descrita como uma proteína da família dos fatores de necrose tumoral capaz de aumentar a capacidade de células dendríticas na indução da proliferação de células T (Anderson et al.[5], 1997; Wong et al.[113], 1997). Ao mesmo tempo, outros dois grupos procurando um ligante para OPG relataram a

presença de um fator de diferenciação de osteoclastos (ODF) e um ligante de OPG (OPGL) in vitro (Lacey et al.[57], 2000; Yasuda et al.[121], 1999). Após análises do lócus gênico codificador para a proteína concluiu-se que RANKL, ODF e OPGL eram a mesma proteína. Já RANK foi clonado de uma biblioteca de cDNA de células dendríticas mielóides por mostrar-se capaz de aumentar a sobrevida de células dendríticas quando ativado (Anderson et al.[5], 1997), concomitantemente, verificou-se que estava envolvido na sinalização para diferenciação de osteoclastos in vitro (Nakagawa et al.[73], 1998).

RANKL pode ser encontrado em duas formas nos tecidos, ligada a membrana celular ou solúvel, decorrente de clivagem proteolítica ou 'splicing' alternativo. A primeira forma é de uma proteína transmembrana tipo II contendo 40 a 45 kDa, já a forma solúvel ativa apresenta 31 kDa. A expressão de RANKL é detectada em ossos, tecido linfóide, queratinócitos, tecido epitelial de glândulas mamárias, coração, músculo esquelético, pulmão, estômago, placenta, tireóide e cérebro (Fata et al.[18], 2000; Kartsogiannis et al.[45], 1999; Lacey et al.[57], 2000; Loser et al.[59], 2006; Wong et al.[112], 1997; Yasuda et al.[121], 1999). O receptor RANK pode ser detectado em células dendríticas, células T CD4+ e CD8+ e células de Langerhans (Anderson et al.[5], 1997; Josien et al.[43], 1999; Williamson et al.[111], 2002; Wong et al.[112], 1997). Resumidamente, o eixo RANK/RANKL/OPG funciona pela interação RANKL–RANK regulando positivamente a osteoclastogênese e é contrabalanceada por OPG a qual é um falso-receptor natural para RANKL, impedindo sua ligação a RANK (Hofbauer, Heufelder[36], 2001).

Diversos estímulos podem induzir ao aumento da expressão de RANKL principalmente em osteoblastos, sendo eles paratormônio, 1,25-

dihidroxivitamina D, interleucina 1 e lipopolissacarídeo (Gallagher[23], 2008). Neste estudo ficou claro que RANKL é induzido tanto em osteoblastos quanto fibroblastos do ligamento periodontal e células da medula ósseo após estimulação com lipopolissacarídeo e interleucina 1 beta. A sinalização intracelular de TLRs por estimulação bacteriana ou via IL-1R induzida por interleucina 1, requer a ativação de proteínas intracelulares semelhantes especialmente p38 MAPK e NF-κB (O'Neill, Bowie[77], 2007; Stone et al.[92], 1988). O papel da proteína adaptadora MyD88 na expressão de genes pró-inflamatórios induzidos via ativação de TLRs envolve o recrutamento de diversas proteínas sinalizadoras comuns a ambas as vias localizadas *upstream*, como as IRAKs (IL-1 receptor-associated kinases), as quais por sua vez ativam TRAF6 (Tumor necrosis factor-associated receptor-associated factor 6), que subseqüentemente recruta TAK1 (Transforming growth factor β-activated-kinase-1) e TABs (TAK1 binding proteins), que podem então, ativar as duas vias de sinalização distintamente, por meio do recrutamento e ativação do complexo IKK (IκB kinase) e das MAP3Ks (como MKK3 e MKK6, ativadores *upstream* de p38 MAPK) (Adhikari et al.[2], 2007; Kobayashi et al.[52], 2002).

Por outro lado, a ativação de p38 MAPK e NF-κB também pode ser induzida por outras duas proteínas adaptadoras: TRIF (TIR domain-containing adapter protein inducing IFN-b) e TRAM (-related adapter molecule) (Krishnan et al.[55], 2007). Desta forma, as vias de sinalização intracelular por meio de ramificação e plasticidade possibilitam que a ativação de uma mesma via de sinalização resulte na regulação de genes distintos em diferentes tipos celulares ou, alternativamente, na regulação distinta de um mesmo gene em distintos tipos celulares.

Além disso, condições do microambiente podem influenciar as vias de sinalização ativadas. Entre as bactérias orais a *Porphyromonas gingivalis* tem sido muito estudada, pois apesar de ser um patógeno oportunista, em alguns estudos relatam baixa atividade pró-inflamatória e em outros, mostra-se altamente agressiva (Al-Qutub et al.[4], 2006; Bainbridge, Darveau[7], 2001; Bainbridge et al.[8], 2002; Champagne et al.[12], 1996). A progressão da doença periodontal gera muitas mudanças no ambiente crevicular, incluindo o potencial de redução (Mettraux et al.[65], 1984), aumento no sangramento (Tanner et al.[101], 1996) e aumento nos níveis de ferro no fuido crevicular (Mukherjee[69], 1985). Acredita-se que a maior parte do ferro encontrado no fluido crevicular provém da molécula de hemina e este é transportado ao interior da bactéria pelo anel de protoporfirina IX (Genco[26], 1995).

A alteração no suplemento de hemina a *Porphyromonas gingivalis* é capaz de provocar alterações estruturas no lípide A presente no LPS da bactéria. A presença de diferentes estruturas de lípide A torna difícil a interpretação do papel dessa bactéria na patogênese da doença periodontal. Por exemplo, as formas penta-aciladas da estrutura do lípide A são agonistas de TLR4 e, em alguns casos, a estrutura tetra-acilada atua como antagonista (Coats et al.[16], 2003). No conjunto geral dos dados, mostramos que ambas as estruturas de lípide A presente em *P. gingivalis* é capaz de ativar tanto TLR2 quanto TLR4, algumas diferenças foram observadas tanto em termos de ativação das vias da MAPKinase, quanto em produção de citocinas inflamatórias. Demonstramos que as possíveis diferenças observadas em estudos comparando o papel da bactéria *P. gingivalis* na produção de citocinas pró-inflamatórias podem ser devidas a presença de diferentes formas do lípide A no tecido periodontal. O LPS de *P.*

gingivalis do fabricante Invivogen, utilizado nos demais trabalhos é caracterizado por diversas estruturas de lípide A englobando tetra- e penta-aciladas segundo contato pessoal com o fabricante e análise no laboratório do Prof. Richard Darveau, a bactéria é mantida em meio de cultura contendo aproximadamente 5µg/mL de hemina. Desta forma, temos uma reprodução mais fiel dos diferentes tipos de LPS contidos em uma bolsa periodontal proveniente de *P. gingivalis*.

Embora tanto em fibroblastos quanto em osteoblastos o estímulo via TLR4 com LPS de *E. coli* tenha sido capaz de induzir a expressão de RNAm para RANKL, os efeitos da estimulação de TLR2 utilizando LPS de *P. gingivalis* foram claramente distintos, segundo o tipo celular: fibroblastos apresentaram inibição da expressão de RANKL, enquanto em osteoblastos o estímulo levou à indução de RANKL, sendo este efeito independente da dose ou concentração do estímulo em ambos tipos celulares. Estes resultados são particularmente interessantes em se considerando que a ativação de p38 MAPK e NF-κB ocorreu de forma similar tanto em osteoblastos quanto em fibroblastos após estímulo com agonistas de TLR4 e TLR2.

Dessa forma, as diferenças podem estar relacionados à expressão diferencial de proteínas adaptadoras em cada tipo celular, como demonstrado, por exemplo, pela não expressão da proteína adaptadora TRAM em osteoblastos e osteoclastos (Udagawa et al.[106], 2007). Outra possível explicação é a ativação diferencial de outras vias de sinalização, além de p38 e NF-kB, em fibroblastos e osteoblastos estimulados com LPS não analisadas neste trabalho. Além disso, a inibição de RNAm de RANKL após estímulo de TLR2 em fibroblastos é um resultado oposto ao reportado para sinoviócitos humanos semelhantes a fibroblastos (Kim et al.[50], 2007). As razões para esta

discrepância podem ser atribuídas ao fenótipo celular ou ao fato das células serem originárias de diferentes espécies animais (humanos x camundongos).

Por outro lado, a osteoprotegerina é um gene de regulação complexa e para o qual a quantidade de informações disponíveis na literatura é escassa. De forma interessante, a expressão de RNAm para OPG em células fibroblásticas não foi afetada por agonistas de TLR2 e TLR4, enquanto em osteoblastos concentrações menores de agonista de TLR4 resultaram em estímulo à produção de OPG, o que pode estar relacionado a uma modulação do *turnover* do tecido ósseo como parte de um mecanismo de tolerância, uma vez que concentações maiores deste mesmo agonista resultaram em discreta inibição da expressão de RNAm para OPG. As diferenças detectadas neste projeto em termos da regulação diferencial de RANKL e OPG em células com fenótipo de fibroblastos e osteoblastos podem ser atribuídas à utilização diferencial das vias de sinalização como resultado do processo de diferenciação celular. Com relação aos nossos estudos é importante ressaltar que apesar dos níveis de OPG mostrarem-se elevação após estímulo de TLR4, estes foram inferiores aos níveis de RANKL, desta forma, a razão RANKL/OPG indicaria possível aumento da reabsorção óssea mesmo com o aumento da expressão de OPG.

Embora entre os objetivos deste projeto não estivesse o estudo dos mecanismos moleculares envolvidos na utilização diferencial destas vias de sinalização por distintos tipos celulares, esta é uma questão interessante, uma vez que os resultados sugerem que a modulação da atividade de uma mesma via de sinalização pode resultar em efeitos “específicos” sobre a expressão de RANKL e OPG ou, ao menos, efeitos restritos a um determinado tipo celular.

Diversos mecanismos podem estar envolvidos, incluindo o conjunto de fatores de transcrição ativados em cada tipo celular e também de fatores de repressão da transcrição ativados por estas vias de sinalização, além de possíveis interações e sobreposições das vias de sinalização p38 MAPK e NF-κB com outras vias relevantes para a expressão destes genes, como por exemplo, JNK MAPKinase e PI3-kinase.

Um objetivo deste projeto foi a avaliação comparativa da importância da via dependente e independente da proteína adaptadora MyD88 e também das proteínas Nod na regulação da expressão destes mesmos genes-alvo (RANKL e OPG), definindo um papel da resposta imune inata na modulação do turnover do tecido ósseo. Desta forma, foram obtidas culturas primárias de células do estroma ósseo (*bone marrow stromal cells*, ou BMSC) a partir de animais 'knockout' para estas proteínas em comparação ao mesmo tipo de células (BMSC) obtidas de animais *wild-type*, com níveis endógenos normais de expressão destas proteínas. Ainda que os resultados obtidos nestes experimentos não sejam diretamente aplicáveis às linhagens celulares de fibroblastos e osteoblastos utilizadas nos demais experimentos deste projeto, as BMSC representam uma população heterogênea de células do estroma em diversos estados de diferenciação, incluindo fibroblastos e osteoblastos, capazes de suportar a osteoclastogênese.

Considerando estas limitações, os resultados indicam que todas as proteínas são necessárias para a máxima indução de RNAm de RANKL após o estímulo ambos TLRs. A redução da expressão de RANKL na ausência de MyD88 era esperada baseada no fenótipo dos camundongos knockout (Sato et al.[88], 2004; Udagawa et al.[106], 2007). A expressão de RNAm de RANKL após

estímulo de IL-1R também se mostrou dependente de MyD88 e Nod1; porém não dependente de Nod2. Estes resultados concordam com dados recentes da literatura, e pode-se especular que Nod2 liga-se à serina/treonina quinase RIP2 (também conhecida por RICK ou CARDIAK), a qual está envolvida na transdução de sinais via NF-κB (Hasegawa et al.[31], 2008). Estes resultados indicam que a sinalização via Nod1 e Nod2 é necessária para a expressão de RANKL induzido por agonistas de TLR2 e 4, porém apenas Nod1 é necessária para a expressão de RANKL após estímulo por IL-1β. A relevância das proteínas Nod já foi demonstrada anteriormente em osteoblastos (Yang et al.[120], 2005). Estes resultados são especialmente interessantes, em se considerando que a regulação negativa por Nod2 pode envolver *cross-talk* com TAK1 (ativador *upstream* das vias NF-κB e MAPK), via interação entre estas proteínas por meio do domínio rico em leucina (*leucine-rich domain*, LRR), a qual é capaz de regular negativamente a ativação de NF-κB (Chen et al.[13], 2004), podendo causar redução da expressão de RANKL.

Na ausência de Nod2, este mecanismo regulatório negativo deixaria de atuar, assim seria esperado um aumento da expressão de RANKL. Podemos especular que no caso dos estímulos via TLR2 e TLR4 a proteína Nod1 pode ter o mesmo efeito regulatório que Nod2, no entanto permanece a ser esclarecida a razão pela qual a expressão de RANKL via IL-1R independe da presença de Nod2. Uma mutação na proteína Nod2 que leva à diminuição da ativação de NF-κB está associada a um fenótipo hiperinflamatório que leva à maior susceptibilidade à doença de Crohn em humanos (Inohara et al.[41], 2003), o que juntamente com evidências demonstrando que aumento na expressão de Nod2 por citocinas pró-inflamatórias e LPS bacteriano (Gutierrez et al.[30], 2002),

demonstram o importante papel desta proteína na transdução de sinais inflamatórios e regulação da expressão de citocinas pró-inflamatórias.

É importante destacar que as informações relativas ao papel das proteínas Nod como mediadores inflamatórios da sinalização envolvida na resposta imune inata ainda são escassas, porém existem evidências destacando seu papel na expressão de diversas citocinas inflamatórias, incluindo IL-1β, IL-6, CXCL8/IL-8 e TNF-α, tanto em células monocíticas quanto células epiteliais (Kim et al.[49], 2004; Kobayashi et al.[53], 2005; Uehara et al.[108], 2007) e na modulação da expressão de citocinas via ativação de TLRs (Netea et al.[75], 2005). Os dados deste projeto indicam um importante papel de Nod1 e Nod2, uma vez que mesmo na presença de MyD88, a expressão de RNAm de RANKL via TLR2 e TLR4 é inibida na ausência de Nod1 e/ou Nod2. Estudos iniciais demonstraram que a ativação de NF-κB por Nod1 e Nod2 pode ocorrer de forma independente da sinalização via TLRs (Inohara et al.[40], 2001), e que a ativação do substrato das proteínas Nod (RIP2) é essencial para o recrutamento de TAK1, a qual é um ativador upstream comum necessário para a ativação tanto de p38 MAPK quanto de NF-κB (Hasegawa et al.[31], 2008; Park et al.[80], 2007). Não encontramos informações na literatura em relação à relevância das proteínas Nod na expressão de RANKL e OPG.

Outra forma de regulação dos níveis de RANKL é através da produção de interferon beta (Takayanagi et al.[97], 2002). A elevação dos níveis de RANKL induz a transcrição do gene que codifica IFN-b em células precursoras de osteoclastos, o qual por sua vez inibe a diferenciação de osteoblastos em osteoclastos pela inibição de c-Fos (Takayanagi et al.[97], 2002). A ativação de c-Fos mostrou-se essencial para a formação de osteoclastos em camundongos

deficientes para o receptor de interferon IFNAR1, exibindo severa osteopenia decorrente de elevada osteoclastogênese. Aparentemente, tanto IFN-a quanto IFN-b iniciam suas respostas celulares pela ativação de fatores de transcrição como ISGF3 (um complexo heterotrímero formado por Stat1, Stat2 e fator regulador de interferons 9 – IRF9), GAF/AAF (homodímero de STAT1) e IRF1 (Stark et al.[91], 1998; Taniguchi et al.[100], 2001). Esse efeito inibitório é anulado em células de medula óssea (BMSC) deficientes em Stat1 ou IRF9, mas não IRF1 indicando que essa ação é mediada por ISGF3. Tanto c-Fos quanto NF-κB estimulam a produção de IFN-b após interação entre RANK e RANKL como parte da regulação negativa da osteoclastogenesis (Abraham et al.[1], 2009). A sinalização por IFN-b via receptor IFNAR inclui uma série de eventos de fosforilação seguida por transcrição gênica que inibe a transcrição de c-Fos (Feng[19], 2005). Interferon beta foi inicialmente descrita como uma proteína capaz de interferir com a replicação viral de vertebrados e amplamente produzida por fibroblastos, leucócitos, macrófagos e osteoblastos (Abraham et al.[1], 2009; Zhang et al.[122], 2008).

Na década de 90 descobriu-se que a indução de IFN-b poderia ser estimulada por diversos agentes externos como microorganismos e citocinas inflamatórias (por exemplo, interleucina-1) (Zhang et al.[122], 2008). Atualmente é utilizado como imunomodulador devido às suas propriedades antinflamatórias inibindo a proliferação de leucócitos, a apresentação de antígenos, a migração de linfócitos T e a atividade de metaloproteinases de matriz (MMPs) (Kieseier et al.[47], 2008). Dessa forma, o uso de IFN-b como agente antinflamatório tem mostrado resultados positivos no tratamento de doenças auto-imunes como esclerose múltipla, lúpus, encefalomielite, entre outros (Kieseier et al.[47], 2008).

No presente estudo, o tratamento de culturas primárias de BMSC com qualquer um dos agonistas (LPSs ou IL-1β) não resultou em regulação importante da expressão de RNAm de IFN-b. Da mesma forma, a ausência de Nod1 não levou a efeitos significativos sobre a expressão de IFN-b, no entanto MyD88 e Nod2 são necessários para a repressão da expressão de IFN-b por BMSCs, uma vez que na ausência destas proteínas significativo aumento na atividade transcricional de IFN-b foi observado após estímulo com todos os agonistas. De acordo com alguns estudos a produção de IFN-b também é importante na indução de iNOS que a partir do óxido nítrico inibe também a atividade de osteoclastos (Hukkanen et al.[39], 1995; Sunyer et al.[93], 1996; Zheng et al.[123], 2006).

Coletivamente, os resultados deste projeto demonstram a regulação diferencial da expressão de RNAm de RANKL por agonista de TLR2 em fibroblastos e osteoblastos, em que pese tanto p38 MAPK quanto NF-κB serem ativadas pelo mesmo agonista nos dois tipos celulares. Também observamos o papel fundamental das proteínas MyD88 e Nod1 na expressão de RNAm de RANKL após estímulo de TLR2, TLR4 e IL-1R. Por outro lado, Nod2 não é necessária para a expressão de RNAm de RANKL após estímulo de IL-1R.

CONCLUSÃO

Conclusão

De acordo com as limitações deste estudo, pode-se concluir que:

- O lipopolissacarídeo de *Porphyromonas gingivalis* é capaz de ativar tanto TLR2 quanto TLR4 e torna-se tetracilado em altas concentrações de hemina;
- As diferenças na expressão de RANKL observadas entre os tipos celulares e agonistas de TLR e IL1R podem estar relacionadas às especificidades das vias de sinalização ativadas em cada uma destas situações;
- MyD88 e Nod1 parecem exercer função importante na expressão de RANKL após estímulo de TLR2, TLR4 e IL-1R. Por outro lado, Nod2 não parece ser necessária para a expressão de RNAm de RANKL após estímulo de IL-1R;
- A ativação de Nod2 tem efeito sinérgico na expressão de RANKL induzida por estimulação com LPS e IL-1β.

Ademais, outros estudos são necessários para determinar o nível de atuação das proteínas Nod nas vias dependente e independente de MyD88 na indução de RANKL.

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ANEXOS

Linhagens celulares

Foram empregadas linhagens celulares de ratos e camundongos devido à maior estabilidade da resposta celular entre passagens sucessivas e também maior facilidade de obtenção e cultivo. A utilização de células provenientes desses animais proporciona maior facilidade de avaliação/expansão dos resultados in vitro para futuros estudos em um modelo in vivo, considerado adequado ao estudo das doenças periodontais e com a vantagem da possibilidade de utilização de animais geneticamente manipulados (*knockout*).

Fibroblastos do ligamento periodontal de camundongos (mPDL) imortalizados pela infecção com vírus (*SV40 large T antigen*) foram empregados nesse experimento. Esta linhagem celular mantém o fenótipo de culturas primárias, com a expressão de sialoproteína óssea, osteocalcina, osteopontina e colágeno tipo I. Além disso, estas células expressam TLR-2 e TLR-4, além de serem responsivas ao estimulo por lipopolissacarídeo (LPS) expressando RANKL (Patil et al.[81], 2006).

As linhagens celulares de osteoblastos e macrófagos (ROS 17/2.8 e RAW264.7, respectivamente) foram obtidas comercialmente e provenientes de ratos. Estas linhagens são amplamente utilizadas na literatura, responsivas ao LPS e viáveis para serem utilizadas em experimentos de co-cultura para a diferenciação de osteoclastos indicando a validade da utilização da linhagem de macrófagos como precursoras de osteoclastos. Todas as linhagens celulares foram mantidas de forma apropriada em DMEM suplementado com 10% de soro fetal bovino (FBS) inativado por calor, 100 U/mL de penicilina e 100 U/mL estreptomicina, a 37^{o}C em atmosfera umedecida de ar e 5% de CO_2.

Previamente a estimulação (com LPS, IL1-β, MDP, entre outros), as células foram rotineiramente desinduzidas em meio de cultura DMEM suplementado com 0.3% de FBS por um período mínimo de 8h. Esta desindução teve a finalidade de evitar possíveis influências de componentes do soro fetal bovino nos resultados dos experimentos e também proporcionar melhor sincronização do ciclo celular.

Reação de transcriptase reversa seguida por reação de polimerase em cadeia (RT-PCR)

RNA total foi coletado com o reagente Trizol (isoticianato de guanidina), segundo o protocolo do fornecedor (Invitrogen Corp.). A pureza e quantidade do RNA coletado foram determinadas em espectrofotômetro, respectivamente, pelo valor da relação entre as absorbâncias a 260 e 280 nm, e pelo valor da absorbância a 260 nm. A síntese de DNA complementar (cDNA) foi realizada subseqüentemente utilizando 500 ng de RNA total e 200 unidades da enzima de transcriptase reversa na presença de OligodT (12-18) primers, dNTP e $MgCl_2$, segundo as instruções do fabricante.

A reação de PCR foi feita num volume total de 25 µL, utilizando 2 µL do produto da reação de transcriptase reversa na presença de 100 pmol/µL de primers de cada gene (50 pmol/µL de cada primer, *sense* e *antisense*). As seqüências dos primers utilizados são apresentados na Tabela 1. Os produtos da reação de PCR foram resolvidos por meio de eletroforese em gel de agarose a 1.5% e corados com brometo de etideo (0.5 µg/mL). Para documentação foram obtidas imagens digitalizadas destes géis, as quais foram submetidas à análise densitométrica. A expressão dos genes-alvo foi normalizada para a expressão

do gene constitutivo gliceraldeido fosfato desidrogenase (GAPDH) e expressas como *fold change* em relação ao controle negativo.

Tabela 1 – Seqüência dos primers e amplicons esperados.

Gene	Primers (5' – 3') sense (S) e antisense (AS)	Acession number	Amplicon
GAPDH	CACCATGGAGAAGGCCGGGG – S GACGGACACATTGGGGGTAG – AS	BC083065	418 bp
mRANKL	CAGCACTCACTGCTTTTATAGAATCC – S AGCTGAAGATAGTCTGTAGGTACGC – AS	NM011613	462 bp
mOPG	ACCTCACCACAGAGCAGCTT – S TTGTGAAGCTGTGCAGGAAC – AS	NM008764	264 bp
rRANKL	TCGGGTTCCCATAAAGTCAG – S CTGAAGCAAATGTTGGCGTA – AS	NM057149	140 bp
rOPG	CACTGCACAGTCAGGAGGAA – S TGCTTTCGATGACGTCTCAC – AS	NM012870	318 bp
Nod1	CTTGCATTCAATGGCATCTC – S ACATCGGTGTGCACTGTGGA – AS	NM172729	469bp
Nod2	AGCAGAACTTCTTGTCCCTGA – S TCACAACAAGAGTCTGGCGT – AS	NM45857	515bp
IL4	ATGGGTCTCAACCCCCAGCTAGT – S GCTCTTTAGGCTTTCCAGGAAGTC – AS	NM021283	399bp
IFN-b	GCACTGGGTGGAATGAGACTATTG – S TTCTGAGGCATCAACTGACAGGTC – AS	NM010510	290bp
IFN-γ	TGAACGCTACACACTGCATCTTGG – S CGACTCCTTTTCCGCTTCCTGAG – AS	NM008337	460bp
IL-10	CTTGCACTACCAAAGCCACA – S AAGTGTGGCCAGCCTTAGAA – AS	M37897	950bp

Western Blot

As células foram lavadas com PBS resfriado a 4^0C, mantidas sempre sobre o gelo e os lisados celulares preparados por raspagem mecânica

em tampão de amostra SDS (62.5 mM tampão Tris-HCl pH 6.8, 10% glicerol, 50 mM DTT, 2% SDS, 0.01% azul de bromofenol na presença dos inibidores de proteases leupeptina, aprotinina e ortovanadato de sódio). Após dois ciclos de congelamento a -70^{0}C/descongelamento a 37^{0}C para rompimento das membranas celulares, as amostras foram desnaturadas por fervura (95^{0}C por 5 minutos) seguida de resfriamento em gelo por 10 minutos. A concentração de proteína total nas amostras foi feita pelo método de Bradford (RC-DC assay, BioRad Lab.). Quarenta microgramas de cada amostra foram submetidos à eletroforese vertical em gel de acrilamida 10% SDS-PAGE (Ready-gel, BioRad Lab) e subseqüentemente eletrotransferidos para membranas de nitrocelulose (BioRad Lab).

Após o bloqueio em tampão Tris-NaCl (TBS) contendo 5% de leite desnatado liofilizado (*blocking buffer*), as membranas foram incubadas com os anticorpos primários para as proteínas de interesse (Nod1, Nod2, RANKL, p38MAPK, NF-κB e GAPDH). Tanto estes anticorpos primários quanto os anticorpos secundários conjugados a *horseradish peroxidase* foram obtidos comercialmente. A detecção da presença das proteínas foi realizada por um sistema de quimiluminescência (LumiGlo, Cell Signaling). Filmes radiográficos expostos as membranas foram processados e digitalizados.

Cultura de células de animais *knockout*

Células de camundongos *knockout* para os genes MYD88, NOD1 e NOD2, foram obtidas por meio de lavagem da medula óssea de ossos longos (fêmur e tíbia). Após dissecção de fêmures e tíbias dos camundongos, as epífises foram removidas por meio de tesouras e o canal medular lavado com

meio de cultura alfa-MEM suplementado com 10% de soro fetal bovino (FBS), 100 U/mL de penicilina e estreptomicina. O material foi coletado em tubos do tipo Falcon, homogeneizado e mantido em placas de cultura a 37^{o}C em atmosfera umedecida de ar e 5% de CO_2 por 24 horas. Em seguida as células não aderentes foram removidas por meio de lavagem com PBS 1X e o meio de cultura renovado. Estas células foram obtidas por meio de contato com os professores Dario S. Zamboni e João Santana da Silva, ambos da Faculdade de Medicina de Ribeirão Preto – USP.

Extração de RNA total de cultura de células

1. Homogeneizar a amostra.
2. Adicionar 200µL clorofórmio para cada 1mL de trizol.
3. Agitar vigorosamente e aguardar 2 minutos.
4. Centrifugar a 13.000 rpm por 15 minutos a 4ºC.
5. Transferir sobrenadante incolor para outro eppendorf.
6. Acrescentar 500µL de isopropanol.
7. Homogeneizar e aguardar 10 minutos.
8. Centrifugar a 13.000 rpm por 10 minutos a 4ºC.
9. Descartar o isopropanol sem tocar no pellet.
10. Acrescentar 500µL de etanol a 75%.
11. Agitar 2 segundos em vortex.
12. Centrifugar a 9.000 rpm por 5 minutos a 4 ºC
13. Remover o etanol sem tocar no pellet.
14. Levar o eppendorf a capela para evaporar o etanol.
15. Adicionar entre 15 e 40µL de TE 1X.
16. Incubar em banho seco a 55 ºC por 5 minutos.
17. Centrifugar por 5 segundos a 13.200 rpm.

Extração de proteínas de lisados celulares

1. Colocar a placa de cultura sobre gelo por 5 minutos.
2. Aspirar o sobrenadante.
3. Lavar a placa duas vezes com PBS 1X.
4. Remover todo o PBS.
5. Adicionar 30µL de T-PER com inibidor de protease.
6. Raspar as células das placas.
7. Aspirar o tampão e centrifigar 9.000 rpm por 5 min a 4ºC.
8. Coletar apenas o sobrenadante.
9. Congelar em freezer a -20ºC

Transcrição reversa com kit Improm II – Promega

Para 20µL de reação

1. Diluir todas as amostras de RNA a mesma concentração.
Utilizar no total da reação entre 300 a 1000 ng de RNA.
2. Acrescentar a cada tubo sobre gelo:
a- 1ul de Oligo(dT) a 0,5 ug/mL.
b- 'X' µL de amostra.
c- Completar para 5µL com água livre de DNAse e RNAse.

3. Levar ao termociclador a 70ºC por 5 minutos.
4. Resfriar amostras em gelo durante 5 minutos.
5. Adicionar a cada tubo:
a. 5,3µL de água livre de DNAse e RNAse.
b. 4µL de tampão Improm 5x.
c. 3,2µL $MgCl_2$.
d. 1µL de dNTP mix.
e. 0,5µL de RNAse OUT.
f. 1µL da enzima trancriptase reversa.

6. Retornar as amostras ao termociclador:
a. 25ºC – 5 min
b. 3- 42ºC – 60 min
c. 4- 70ºC – 15 min

7. Armazenar em freezer a -20ºC

Western blot - transferência em sistema de imersão

Inicialmente, lavar todo o aparato com água destilada e álcool 70%, montá-lo e checar vazamento com água destilada. Se o aparato não vazar entornar a água e secar com papel absorvente.

1- Preparar gel de acrilamida 10% (“resolving gel”):

a- 4,2mL água destilada

b- 2,5mL de solução Tris.Cl 1,5M pH 8,8

c- 100µL de solução aquosa de SDS 10%

d- 3,33mL de 30% Acrylamide/Bis solution 37.5:1

Suficiente para 2 géis de 1.0mm de espessura.

2- Adicionar 50µL de persulfato de amônia (APS) em solução aquosa a 10% e 5µL de TEMED.

3- Homogeneizar a solução e aplicar rapidamente no aparato deixando 2cm entre a borda da placa de vidro menor e o topo do gel.

4- Imediatamente, acrescentar 50µl de água saturada com butanol sobre a solução.

5- Aguardar 40 minutos para polimerização e retirar a água saturada com butanol utilizando papel filtro.

6- Preparar o segundo gel (“stacking gel”) de acrilamida a 4%:

a- 6,1 mL de água destilada

b- 2,5 mL de solução Tris.Cl 0,5M pH 6,8

c- 100µL de solução aquosa SDS 10%

d- 1,33 mL de 30% Acrylamide/Bis solution 37.5:1

7- Adicionar 50µL de APS 10% e 10µL de TEMED.

8- Aplicar a solução no aparato e inserir o pente.

9- Aguardar polimerizar por 40 minutos e remover o pente.

10- Acoplar o gel no aparato para corrida.

11- Equilibrar o gel em tampão de corrida em corrente elétrica constante de 100V por 10 minutos.

12- Aplicar a amostra e correr a 100V.

13- Transferir a proteína do gel para a membrana em tampão de transferência por 1 hora em corrente de 300mA constante sob refrigeração.

14- Bloquear a membrana por 1h com tampão de bloqueio.

15- Lavar a membrana três vezes com TBS-T por 5min.

16- Incubar a membrana com anticorpo primário diluído em PBS 1X a 4°C sob agitação durante 8-12 horas (“overnight”).

17- Lavar a membrana três vezes com TBS-T por 5min.

18- Incubar com anticorpo secundário e anti-biotina diluídos em tampão de bloqueio por 1 hora.

19- Lavar a membrana três vezes com TBS-T por 5 min.

20- Revelar a membrana.

21- Expor em sistema de detecção de quimiluminescência ou filme radiográfico.

Remoção de anticorpos (“stripping”) da membrana:

- Lavar a membrana três vezes por 5 minutos com TBS-T.
- Imergir a membrana em ‘stripping buffer’ por 10 minutos.
- Lavar a membrana três vezes por 5 minutos com TBS-T.
- Bloquear a membrana por 1h com ‘blocking buffer’.
- Encubar com novo anticorpo primário.

Tampão de remoção de anticorpo (“Stripping buffer”):

- 8.35mL de água destilada
- 2.5mL solução aquosa com SDS (sodium dodecyl sulphate) 10%
- 1.56mL de solução Tris.Cl 0,5M pH 6,8
- 87.5µl 2-mercaptoethanol

Solução de Tris.Cl 1.5M pH 8.8

- 18.17 g de tris base
- 70mL de água MilliQ
- Ajustar o pH para 8.8 com HCl ou NaOH
- Completar volume para 100mL com água milliQ

Solução de Tris.Cl 0.5M pH 6.8

- 6.05g de tris base
- 70mL de água MilliQ
- Ajustar o pH para 6.8 com HCl ou NaOH
- Completar volume pra 100mL com água MilliQ

Tampão TBS 10X

- 24.2g de tris base
- 80g de $NaCl_2$ (cloreto de sódio)
- Completar para 1 litro com água destilada
- Ajustar o pH para 7.6 com HCl ou NaOH
- Para TBS 1x diluí-lo 1:10 em água destilada

Tampão TBS–T (tampão para lavagem ou "wash buffer")

Adicionar ao tampão TBS 1X, 0.1% de Tween 20

Tampão de corrida concentrado 5X ("running buffer")

- 15g de tris base
- 72g de glicina
- 5g de SDS
- Completar com água destilada para 1 litro
- Diluir na proporção 1:5 no momento de utilizar

Tampão de transferência (“transfer buffer”)

- 6.06g de tris base
- 28.80g de glicina
- 1.33 litros de água destilada
- Adicionar 400mL de metanol
- Completar para 2 litros com água destilada

Tampão de bloqueio (“blocking buffer”)

- 135mL de água destilada
- 15mL de tampão TBS 10X
- 7.5g de leite em pó desnatado
- Adicionar 150µl de Tween 20 sob agitação

Tampão de bloqueio (“blocking buffer”) com BSA 3%

- 1.5g de BSA (“bovine serum albumine”)
- 25uL de Tween 20

Araraquara, 06 de agosto de 2009.

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