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"JÚLIO DE MESQUITA FILHO"
FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Adriana Alicia Cabrera Ortega

Sinalização via Akt1 em células dendríticas modula a interação microbiota-hospedeiro e a reabsorção óssea inflamatória

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RESUMO

Células dendríticas têm papel crucial na patogênese das doenças periodontais por orquestrarem a resposta imune adaptativa e por seu papel como precursoras de osteoclastos. A sinalização via Akt tem importante papel em processos como metabolismo, proliferação, apoptose e também na resposta imune. Evidências indicam que Akt1 tem papel de regulador endógeno negativo da resposta inflamatória; porém pode tanto estimular quanto inibir a osteoclastogênese. Considerando que as células dendríticas participam tanto da inflamação/resposta imune quanto do *turnover* do tecido ósseo como células precursoras de osteoclastos, propusemos avaliar através de um estudo in vivo o papel da atividade de Akt1 na inflamação associada a interações microbiota-hospedeiro, bem como investigar in vitro os efeitos desta via de sinalização sobre os diferentes eventos biológicos das células dendríticas. Para o estudo in vivo foi utilizado um modelo de doença periodontal experimental induzida por *P. gingivalis* e *Fusobacterium nucleatum*, em um modelo de animais transgênicos com deleção gênica condicional. Os desfechos avaliados foram: inflamação (morfometria), reabsorção óssea (μCT), osteoclastogênese (IHC), anticorpos específicos para *P.gingivalis* e *Fusobacterium nucleatum* (ELISA). No estudo in vitro foi avaliado o papel da via de sinalização Akt1 sobre as seguintes atividades das células dendríticas: proliferação, apoptose, atividade fagocitária, migração, apresentação de antígeno e osteoclastogênese. Resultados do estudo in vivo demonstraram que a sinalização via Akt1 em células dendríticas parece ter um papel importante na patogênese da doença periodontal, podendo modificar a intensidade da resposta imune adaptativa assim como sua diferenciação osteoclástica, modificando assim o *turnover* fisiológico do osso alveolar. Adicionalmente, é possível concluir que a sinalização via Akt é necessária para o desenvolvimento das funções biológicas (proliferação, fagocitose, apresentação de antígeno e diferenciação osteoclástica) das células dendríticas.

Palavras-chave: Doenças periodontais. Células dendríticas. Proteínas proto-oncogênicas c-Akt. Imunidade inata. Imunidade adaptativa.

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ABSTRACT

Dendritic cells play a crucial role in the pathogenesis of periodontal diseases by orchestrating the adaptive immune response and their role as osteoclasts precursors. Akt signaling plays an important role in processes such as metabolism, proliferation, apoptosis and also in the immune response. Evidence indicates that Akt1 plays a role of negative endogenous regulator of the inflammatory response; but can both stimulate and inhibit osteoclastogenesis. Considering that dendritic cells participate in both inflammation/immune response and turnover of bone tissue as osteoclast precursor cells, we have proposed to evaluate in a vivo study the role of Akt1 activity in inflammation associated with microbiota-host interactions, as well as to evaluate in vitro the role of the Akt1 signaling pathway in dendritic cell biology. In vivo study was employed a model of experimental periodontal disease induced by *P. gingivalis* and *Fusobacterium nucleatum* in transgenic animals model with conditional gene deletion. The outcomes evaluated were: inflammation (morphometry), bone resorption (μCT), osteoclastogenesis (IHC), antibodies specific for *P.gingivalis* and *Fusobacterium nucleatum* (ELISA). In vitro study was evaluated the role of Akt1 signaling pathway on the following outcomes related to dendritic cell biology: proliferation, apoptosis, phagocytic activity, migration, antigen presentation and osteoclastogenesis The results showed that signaling via Akt1 in dendritic cells seems to play an important role in the pathogenesis of periodontal disease, modifying the intensity of the adaptive immune response as well as its capacity in osteoclastic differentiation, thus modifying the physiological turnover of the alveolar bone. Futhermore, it is possible to conclude that Akt signaling is necessary for the development of biological functions (proliferation, phagocytosis, antigen presentation and osteoclast differentiation) of dendritic cells.

Keywords: Periodontal diseases. Dendritic cells. Proto-oncogene proteins c-Akt. Innate immunity. Adaptive immunity.

LISTA DE ABREVIATURAS E SIGLAS

Akt: Protein kinase B / Proteína quinase B

APCs: Antigen-presenting cells / células apresentadoras de antígeno

Bcl-2: B-cell lymphoma 2/ Célula-B de linfoma 2

BMDCs: Bone marrow derived dendritic cells / Células dendríticas derivadas da medula óssea

BSA: Bovine serum albumin / Albumina do soro bovino

CCL-19: Chemokine (C-C motif) ligand 19/ Ligando de quimiocina (motivo C-C) 19

CCR10: C-C chemokine receptor type 10 / C-C recetor de quimiocinas tipo 10

CCR5: C-C chemokine receptor type 5 / C-C recptor de quimiocinas tipo 5

CCR6: C-C chemokine receptor type 6 / C-C recetor de quimiocinas tipo 6

CCR7: C-C chemokine receptor type 7 / C-C recetor de quimiocinas tipo 7

CCR9: C-C chemokine receptor type 9 / C-C recptor de quimiocinas tipo 9

CD34: Cluster of Differentiation 34 / Conjunto de Diferenciação 34

CXCL8: Interleukin 8 / Interleucina 8

DAMPs: Damage-associated molecular patterns / Padrões moleculares associados ao dano

DAPI: 4′,6-diamidino-2-phenylindole / 4 ', 6-diamidino-2-fenilindole

DCs: Dendritic cells / Células dendríticas

DMSO: Dimethyl sulfoxide/ Dimetilsulfóxido

DNA: Deoxyribonucleic acid / Ácido desoxirribonucleico

DTT: Dithiothreitol / Ditiotreitol

EDTA: Ethylenediamine tetraacetic acid / Ácido etilenodiaminotetracético

ELISA: Enzyme-linked immunosorbent assay / Ensaio de imunoabsorção enzimática

FasL: Fas Ligand / Ligante Fas

FBS: Fetal bovine serum / Soro fetal bovino

***Fn*:** *Fusobacterium nucleatum*

GM-CSF: Granulocyte-macrophage colony-stimulating factor / Fator estimulador de colônias de granulócitos e macrófagos

H&E: Hematoxylin-Eosin / Hematoxilina-Eosina

HC: Histochemistry /Histoquímica

HRP: Horseradish peroxidase / Peroxidase obtida a partir da raiz forte

IL-1: Interleukin 1 / Interleucina 1

IL-10: Interleukin 10 / Interleucina 10

IL-4: Interleukin 4 / Interleucina 4

IL-6: Interleukin 6 / Interleucina 6

iNOS: inducible isoform of nitric oxide synthase / Isoforma induzível da sintase do óxido nítrico

JCE: Cement-enamel junction / Junção cemento-esmalte

KO: Knockout / Nocaute

LPS Lipopolysaccharide / Lipopolissacarídeo

MAPKinase: Mitogen-activated protein kinase / Proteína quinase ativada por mitógenos

mDCs: Myeloid dendritic cells / Células dendríticas mielóides

MHC: Major histocompatibility complex / complexo principal de histocompatibilidade

NF-kB: Factor nuclear kappa B / Fator de transcrição nuclear kappa B

NK: Natural killer cells / Células exterminadoras naturais

OPG: Osteoprotegerin / Osteoprotegerina

PBMCs: peripheral blood mononuclear cells / células mononucleares no sangue periférico

PBS: Phosphate buffered saline / Solução salina tamponada com fosfato

pDCs: Plasmocytoid dendritic cells / Células dendríticas plasmocitóides

Pg*: *Porphyromonas gingivalis

PI3-kinase: Phosphatidylinositol-4,5-bisphosphate 3-kinase / Fosfatidilinositol-4,5-bifosfato 3-kinase

PMNs: Polymorphonuclear neutrophils / Neutrófilos polimorfonucleares

PVDF: Polyvinylidene difluoride / Difluoreto de polivinilideno

RANKL: Receptor activator of nuclear factor kappa-B ligand / Ligante do receptor do ativador do fator nuclear Kappa B

ROI: Region of interest / Região de interesse

Runx2: Runt-related transcription factor 2 / Fator 2 de transcrição relacionado com Runt

TBS: Tris-buffered saline / Solução salina tamponada com Tris

TGF-beta: Transforming growth factor beta/ Fator de transformação do crescimento beta

TLRs: Toll like receptors / Receptores do tipo toll

TNF-alfa: Tumor necrosis factor alpha / Fator de necrose tumoral alfa

TNF: Tumor necrosis factor / Fator de necrose tumoral

TRAP: Tartrate-resistant acid phosphatase / Fosfatase ácida resistente ao tartarato

Treg: Regulatory T cell / células T regulatórias

UFC: Colony forming unit / Unidade de formação de colônias

µCT: Microcomputed tomography / Microtomografia computadorizada

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1 INTRODUÇÃO

Até os anos 60, o desenvolvimento da resposta imune era atribuído basicamente aos macrófagos devido às suas propriedades de fagocitar antígenos. Já se reconhecia a relevância dos linfócitos como mediadores da resposta imune tardia ou adaptativa, no entanto não se sabia como estes linfócitos localizados nos linfonodos eram ativados pelos antígenos fagocitados pelos macrófagos. Na década de 70, os trabalhos de Steinman e Cohn[1-4] identificaram células distintas de macrófagos na função e aparência, com expressão de níveis elevados de moléculas MHC classe II (major histocompatibility complex) necessárias à apresentação de antígenos extracelulares, apresentando projeções citoplasmáticas semelhantes à dendritos, e com uma quantidade relativamente menor de lisossomos e atividade fagocitária menos intensa.

Células dendríticas (DCs) tem origem hematopoiética e representam o exemplo prototípico de células apresentadoras de antígeno (Antigen-presenting cells, APCs), tendo papéis fundamentais no reconhecimento de antígenos e na resposta imune inata, bem como na ativação e modulação da resposta adaptativa[5]. Atualmente, especialmente com base em suas características fenotípicas, são reconhecidas diversas populações distintas de células dendríticas, dependendo de sua localização ou "nicho" nos quais exercem função imunomodulatória. Assim, são reconhecidas populações de células dendríticas circulantes (periféricas), cutâneas, intestinais, pulmonares e hepáticas. Porém, apenas no início da década de 90 começaram a surgir evidências indicando que, além de sua função estimulatória da imunidade, as células dendríticas também podem ter função inibitória na resposta imune por meio de mecanismos de tolerância[6]. Esta função tolerogênica/inibitória das células dendríticas pode ter implicações importantes tanto na patogênese quanto na terapia de algumas doenças e condições, como a artrite reumatoide, asma e o diabetes tipo I.

Existem evidências sugerindo a existência de células precursoras comuns (CD34+) derivadas do sangue/medula óssea que podem gerar células dendríticas, granulócitos e macrófagos. Conceitualmente, as células dendríticas tem origem hematopoiética a partir de precursores localizados na medula óssea, os quais originam as células dendríticas periféricas que 'semeiam' diferentes órgãos e tecidos, inicialmente como células imaturas[7,8]. Alguns autores descrevem mais de um subtipo de células dendríticas em um mesmo 'nicho', como células dendríticas cutâneas[9], e até mesmo células dendríticas periféricas (que são raras entre as células mononucleares no sangue periférico (PBMCs – peripheral blood mononuclear

cells), perfazendo menos de 1% dos números totais de leucócitos) que podem ser 'mielóides' (mDC, alta atividade fagocítica, e expressão de diversos receptores do tipo toll (TLRs – toll like receptors), sendo capazes de reconhecer antígenos bacterianos e virais)[10,11], e as células dendríticas plasmocitóides (pDC, derivadas de precursores linfóides e que expressam predominantemente TLR-7 e -9, reconhecendo primariamente antígenos virais e com elevada produção de intérferon)[12,13]. Monócitos CD14+ também podem gerar DCs nas condições adequadas (na presença do fator estimulador de colônias de granulócitos e macrófagos (GM-CSF) e Interleucina 4 (IL-4)), o que implica que a população de células monocíticas pode servir como fonte de precursores de células dendríticas, que se diferenciam em condições externas / de microambiente apropriadas. Além destas possibilidades, também existem evidências indicando que DCs possam se originar de precursores linfocíticos, os quais poderiam originar também células T, B e natural killer (NK), dependendo das condições e estímulos externos (microambiente). É possível que estas diferentes origens resultem em distintos 'subtipos' de DCs que podem apresentar diferenças fenotípicas e funcionais. Por exemplo, DCs de origem linfóide expressam o marcador CD8 e podem exercer efeito supressor sobre células T por meio da indução de apoptose via ligante Fas (FasL – Fas Ligand), diferentemente de DCs de origem mielóide (CD8-) que não exercem este efeito supressor[14]. Mesmo quando derivadas do precursor mielóide comum (caracterizado pela expressão do marcador CD34) pelo estímulo com GM-CSF e TNF, aquelas diferenciadas a partir de células monocíticas (CD14+) são capazes de suportar a proliferação e diferenciação de linfócitos B, enquanto as DCs originadas de células CD14- não tem esta capacidade[15].

Independentemente de sua origem, as DCs usualmente apresentam um fenótipo inicial denominado 'imaturo', no qual apresentam grande capacidade fagocitária, porém baixa capacidade de estimular linfócitos. A exposição e fagocitose de antígenos, juntamente com condições específicas do microambiente em que se encontram (exposição a mediadores inflamatórios e fatores de crescimento) induzem o processo de maturação das DCs, caracterizado por profundas mudanças fenotípicas, incluindo a sua migração de tecidos não-linfóides para tecidos linfóides ou corrente sanguínea (por meio do aumento da expressão de alguns receptores de quimiocinas como CCR7, relacionada à localização/atração das células aos linfonodos e inibição da expressão de outros receptores de quimicionas, como CCR5 e CCR6, relacionados à localização/atração à sítios com inflamação ativa), drástica redução da capacidade fagocítica e marcante capacidade estimulatória de linfócitos, incluindo a apresentação de antígenos via MHC classe II, de moléculas co-estimulatórias e de citocinas

associadas à polarização fenotípica de células T. O Quadro 1 apresenta os marcadores específicos da polarização fenotípica das DCs.

Quadro 1 - Marcadores mais comuns e genéricos associados à maturação e polarização fenotípica/ativação de células T por DCs (dados de humanos e camundongos, incluindo diversos subtipos de DCs)[5, 16]

Marcadores / Fenótipo	**Maturação**	**CD8/CTL**	**Th1**	**Th2/Treg (DC regulatórias)**
MHC II	+++	+	+++	+++
MHC I	+	+++	+/-	+/-
CD80	+++	+++	+++	+++
CD86	+++	+++	+++	+++
CD40	+++	+	+++	+++
CCR7	+++	++	+++	+++
CD1a, b, c	+		+/-	+
CD11c	+		+	++
CD11b	+		+++	+
CD8	+/-	+++		
CD4	+/-	-	+/-	+/-
IL-12	++	+++	+++	+
IL-10	++	+/-	+	+++

Fonte: Elaboração própria.

Interessante notar que além destes sinais “comuns” à ativação da resposta imune adaptativa, evidências mais recentementes indicam que as células dendríticas expressam um “4o sinal” de ativação de células T, representado pela “orientação” ou “direcionamento” ("homing") da célula T presente no linfonodo ao tecido ou órgão de interesse. Por exemplo, no intestino TGF-beta e ácido retinóico produzido pela células dendríticas induzem a expressão de CCR9 nas células T, o qual é importante para o “homing” destas células ao intestino[17]. De forma similar, células dendríticas cutâneas liberam metabolitos de vitamina D que induzem a expressão de CCR10 pelas células T, o qual é importante para o 'homing' destas células para a pele[18].

De forma geral as informações e evidências apresentadas nesta seção implicam as células dendríticas como um tipo celular fundamental na regulação da resposta imune adaptativa.

2 REVISÃO DE LITERATURA

2.1 Células Dendríticas e Regulação da Resposta Imune nas Doencas Periodontais

A patogênese das doenças periodontais destrutivas envolve a imunidade inata e adaptativa e sua característica é o aumento da osteoclastogênese, levando à reabsorção óssea inflamatória do osso alveolar. As células dendríticas (DCs) podem ter um papel central nas doenças periodontais devido ao seu papel tanto como células da resposta imune (envolvidas na fagocitose de microrganismos, secreção de mediadores inflamatórios, ativação e modulação de células da imunidade adaptativa) como precursores de osteoclastos (possivelmente, contribuindo diretamente na reabsorção óssea).

Condições inflamatórias crônicas são, em geral, prejudiciais à homeostase dos tecidos/órgãos devido à destruição associada do tecido conjuntivo, como a observada nas doenças periodontais, asma e artrite reumatóide. A cronicidade da inflamação sugere uma condição auto-imune ou a persistência do agente desencadeante/antígeno. Um mecanismo que pode contribuir para esta condição é a presença de DCs mielóides (que não são indutoras eficientes da morte de microrganismos fagocitados), atuando como um nicho "protetor" ou "acumulador" destes antígenos[19,20]. Alternativamente, a subversão da função imunoestimuladora das DCs pela inibição das funções efetoras ou pela indução de propriedades inibitórias ou "reguladoras negativas" (DCs reguladoras) também pode favorecer a perpetuação do processo inflamatório[5].

Em relação à primeira possibilidade (DCs como "nicho" de acumulação de antígenos/agentes patogênicos), Carrion et al.[21] relataram a expansão de DCs mielóides (CD1c+DC-SIGN+) em pacientes com doença periodontal crônica. Foi demonstrado que as células carregavam o organismo periodontopatógeno *Porphyromonas gingivalis* assim como outras espécies microbianas. Essas células também não expressaram marcadores e proteínas co-estimuladoras que são necessárias para a ativação da resposta imune adaptativa. Outras alterações funcionais relacionadas com a "segunda possibilidade" (subversão da função imunoestimuladora das DCs) observadas em DCs podem estar associadas à doença periodontal crônica, como falta de maturação adequada, comprometimento da migração/quimiotaxia e da sinalização/apresentação de antígenos à outras células imunes[22].

Na patogênese das doenças periodontais (semelhante a outros nichos de vigilância imunológica com DCs "residentes", como a pele, pulmões e intestino), as DCs geralmente são expostas a uma variedade de antígenos derivados de espécies microbianas não patogênicas

(organismos comensais), associados à "saúde clínica" ou ao estado homeostático. Para estes estímulos não patogênicos deve ser gerada uma resposta de tolerância, em oposição a resposta imunoestimulante efetiva que resulta da exposição a microrganismos/antígenos patogênicos e a antígenos e/ou produtos derivados do dano tecidual do hospedeiro (Padrões Moleculares Associados ao Dano, DAMPs – *Damage-associated molecular patterns*) para 'limpar' a agressão/danos e restaurar a homeostase.

Assim, o microambiente em que as DCs estão localizadas e sua exposição a diferentes moléculas ligantes de TLR, ou à mediadores inflamatórios como IL-10, IL-1, TNF, Interferon e outros decidirão o padrão de ativação das DCs. Por exemplo, a exposição da DCs semi-maduras à IL-10 induz a polarização de células Treg (CD25+/FoxP3+)[23]. De forma similar, a ativação preferencial de TLR2 por LPS de *P. gingivalis* (microorganismo anaeróbio Gram-negativo, periodontopatógeno) induz a maturação de DCs com baixo potencial imunoestimulatório[24]. DCs derivadas de indivíduos portadores de periodontite mostraram-se mais imaturas, porém com expressão de um perfil de citocinas pró-inflamatórias (e baixa expressão de IL-10), em comparação à DCs de pacientes periodontalmente saudáveis. Estes dados indicam uma desregulação importante da função de DCs em pacientes com periodontite crônica[25].

DCs imaturas e maduras são encontradas próximas ao osso alveolar na doença periodontal[26]. Por meio de seu efeito direto e indireto em células T, as DCs podem estimular a osteoclastogênese e reabsorção óssea, além da possibilidade de se diferenciarem diretamente em osteoclastos, dependendo das condições do microambiente[27]. Assim as DCs podem, potencialmente, exacerbar a inflamação e induzir ou agravar a reabsorção óssea indiretamente, por meio da modulação da imunidade inata e adaptativa, ou diretamente servindo como células precursoras de osteoclastos. Como comentado anteriormente, LPS de *P. gingivalis* promove a maturação de DCs[24], e existem evidências de que o estímulo microbiano do ligamento periodontal resulta em aumento no número de DCs e de seu contato direto com linfócitos T, indicativo da ativação/apresentação de antígeno, o que é suportado pelo aumento na expressão de MHC-II e do receptor de célula apresentadora de antígeno semelhante à lectina A1 (antigen-presenting cell-lectin-like receptor A1)[26,28]. Na periodontite agressiva localizada, DCs estimulam a produção de interferon-gama que agrava a inflamação[29]. Além disso, o número de DCs presentes na gengiva e ligamento periodontal é reduzido após o tratamento clínico, indicando seu papel na etiopatogênese da periodontite crônica[30].

Coletivamente, estas informações indicam que DCs são encontradas no tecido gengival e no ligamento periodontal, são ativadas pela interação com microrganismos do biofilme dental e participam da resposta imune e reabsorção óssea associadas à patogênese das doenças periodontais.

2.2 Sinalização Via Akt1 na Resposta Imune e na Função de Células Dendríticas

A proteína-quinase Akt foi originalmente identificada como um oncogene retroviral[31] e seu papel em processos metabólicos e de diferenciação celular, incluindo proliferação celular e apoptose é extensamente estudado. Em mamíferos são conhecidas três isoformas de Akt: Akt1, Akt2 e Akt3, as quais são produtos de genes distintos. Em geral, a ativação de Akt é induzida pela ativação de PI3-kinase, seu principal ativador upstream. Por sua vez, PI3-kinase pode ser ativada por diversos receptores, incluindo receptores de fatores de crescimento e citocinas.

Recentemente, evidências tem demonstrado que Akt tem papel crítico em processos imunes e auto-imunes, como a artrite reumatoide[32], psoríase[33], asma[34] e aterosclerose[35]. Assim, a atividade de Akt está diretamente envolvida na regulação da resposta inflamatória, sendo a maior parte da evidência disponível relacionada à imunidade inata devido à sua expressão em neutrófilos, macrófagos e DCs[36]. De fato, a sinalização via Akt é crucial no desenvolvimento e diferenciação de DCs, influenciando primariamente a proliferação e sobrevivência das células hematopoiéticas precursoras. Além disso, DCs diferenciadas com supressão da sinalização via Akt apresentavam deficiência funcional. A inibição da sinalização via Akt afeta a sobrevivência de DCs circulantes (plasmacytoid DCs, pDCs) e a ativação de Akt é diretamente correlacionada aos números de DCs circulantes, sugerindo influência na proliferação destas células in vivo[37,38].

Akt tem papel inibidor da apoptose em neutrófilos[39], macrófagos[40] e células dendríticas[37], além de participar em outras funções biológicas destas células como a migração/quimiotaxia, produção de mediadores inflamatórios e polarização fenotípica[36]. O envolvimento direto da sinalização via Akt na resposta imune e, especificamente, na interação microbiota-hospedeiro é demonstrado pelo envolvimento do eixo PI3K-Akt na expressão de TLR4 em macrófagos[41] e também na ativação de neutrófilos por TLR2, em que desempenha papel de intermediário ativador de NF-kB[42]. As diferentes isoformas de Akt podem ter funções biológicas distintas, dependendo do tipo celular. Por exemplo, Akt2 (mas não Akt1) tem papel relevante na migração, fagocitose, produção de radicais oxigenados e degranulação de neutrófilos

polimorfonucleares[43].

Em células classicamente reconhecidas como apresentadoras de antígenos, Akt1 parece ter um papel regulador negativo na inflamação. Em macrófagos de animais knockout para Akt1, a polarização fenotípica ocorre para o perfil de ativação clássica (M1, 'pró-inflamatório'), com elevada produção de iNOS, TNF-alfa e IL-6, enquanto que macrófagos de animais knockout (KO) para Akt2 apresentam polarização preferencialmente para o perfil de ativação alternativa (M2, "anti-inflamatório"), caracterizado por reduzida produção de citocinas anti-inflamatórias (como IL-10) e arginase-1. O papel de Akt1 como "regulador negativo endógeno da inflamação" é suportado pelo fato de que a deleção do gene Akt1 impede o desenvolvimento de tolerância à endotoxina em macrófagos, e também pelo fato de que animais KO para Akt1 são mais suscetíveis ao choque séptico induzido por LPS[41, 44]. Por meio de abordagem genética para inibição ou hiperativação da sinalização via PI3K-Akt em macrófagos, observou-se que a atividade de Akt inibe a ativação de MAPKinases (EKR, JNK e p38), mas não a ativação de NF-kB, após estímulo com LPS. Esta inibição de MAPKinases mediada pela ativação de Akt resultou na inibição da produção de IL-6 e TNF por macrófagos estimulados com LPS, demonstrando o papel regulador negativo do eixo PI3K-Akt na sinalização de LPS em macrófagos[45].

A deficiência de Akt1 inibe a sobrevivência e ativação de DCs tanto na imunidade inata (ativação por LPS) quanto adaptativa (ativação por CD40), por meio da modulação da expressão da proteína pró-sobrevivência Bcl-2[46,47]. O tratamento de pDCs com extrato de fumaça de cigarro inibiu a sinalização via PI3K-Akt e induziu aumento da produção de CXCL8 e inibição da produção de TNF-alfa, IL-6 e interferon-alfa, indicando que a sinalização via Akt pode influenciar a função de DCs por meio de modulação da expressão de citocinas [48].

DCs, como macrófagos, são células precursoras de osteoclastos, assim podem participar diretamente do *turnover* fisiológico do tecido ósseo e também em processo de reabsorção óssea patológica. Este aspecto da biologia das DCs é de particular importância no contexto de condições inflamatórias crônicas associadas à reabsorção de tecido ósseo, como artrite reumatoide ou as doenças periodontais. Existem evidências sugerindo um papel direto de Akt1 na diferenciação e função de osteoblastos e de um papel indireto de Akt1 na osteoclastogênese. A inibição da expressão gênica de Akt1 estimula a diferenciação de células estromais da medula óssea em osteoblastos, fenômeno associado ao aumento da

expressão do fator de transição Runx2 (Cbfa1, “master switch” da diferenciação osteoblástica). Em animais geneticamente deficientes para Akt1, além da maior diferenciação osteoblástica, observa-se inibição de formação e atividade de osteoclastos, fenômeno que está correlacionado com a inibição da produção de citocinas RANKL e OPG, essenciais para a osteoclastogênese, pelos osteoclastos[49,50]. Assim, Akt1 (mas não Akt2) tem papel relevante para a diferenciação osteoblástica e também, indiretamente, na osteoclastogênese [51]. Desta forma, a ativação de Akt1 pode estar relacionada a menor formação e maior reabsorção óssea, sugerindo que a inibição seletiva de Akt1 pode ser uma estratégia terapêutica para diminuir a perda óssea/favorecer o ganho ósseo.

As informações desta seção indicam o envolvimento da sinalização via Akt na resposta imune, os efeitos biológicos diferenciais das isoformas de Akt segundo o tipo celular associado à imunidade inata, e a possibilidade da sinalização via Akt estar envolvida direta e indiretamente na regulação do tecido ósseo.

3 PROPOSIÇÃO

3.1 Hipótese

A hipótese primária deste projeto é que a sinalização via Akt1 é crucial na regulação da função de células dendríticas e, subsequentemente, para a resposta imune e reabsorção óssea inflamatória associadas às doenças periodontais.

3.2 Objetivos Específicos

O objetivo geral do projeto é verificar a relevância da sinalização via Akt1 especificamente em células dendríticas na patogênese da doença periodontal experimental.

Portanto, os objetivos específicos do trabalho incluem:

- Determinar, in vitro, a influência da sinalização via Akt1 nos seguintes aspectos da biologia de células dendríticas: proliferação, apoptose, migração celular, atividade fagocitária, apresentação de antígeno, e osteoclastogênese;

- Verificar in vivo, usando abordagem de deleção gênica condicional em camundongos, o efeito da sinalização via Akt1 em células dendríticas em modelo de doença periodontal de inoculação com *P.gingivalis* e *Fusobacterium nucleatum*. Os desfechos avaliados serão: inflamação (morfometria), reabsorção óssea (μCT), osteoclastogênese (HC), anticorpos específicos para *P.gingivalis* e *Fusobacterium nucleatum* (ELISA)

4 PUBLICAÇÕES

4.1 Publicação 1

The Role of Forkhead Box 1 (FOXO1) in the Immune System: Dendritic Cells, T Cells, B Cells, and Hematopoietic Stem Cells· *

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The Role of Forkhead Box 1 (FOXO1) in the Immune System: Dendritic Cells, T Cells, B Cells, and Hematopoietic Stem Cells

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ABSTRACT: Forkhead box-O (FOXO) transcription factors have a fundamental role in the development and differentiation of immune cells. FOXO1 and FOXO3 are FOXO members that are structurally similar and bind to the same conserved consensus DNA sequences to induce transcription. FOXO1 has been studied in detail in the activation of dendritic cells (DCs), where it plays an important role through the regulation of target genes such as ICAM-1, CCR7, and the integrin $\alpha_v\beta_3$. FOXO1 is activated by bacteria challenge in DCs and promotes DC bacterial phagocytosis, migration, homing to lymph nodes, DC stimulation of CD4+ T cells and resting B cells, and antibody production. Deletion of FOXO1 in DCs enhances susceptibility to bacteria-induced periodontal disease. FOXO1 and FOXO3 maintain naive T cell quiescence and survival. FOXO1 and FOXO3 enhance the formation of regulatory T cells and inhibit the formation of T-helper 1 (Th1) and Th17 cells. FOXO1 promotes differentiation, proliferation, survival, immunoglobulin gene rearrangement, and class switching in B cells, but FOXO3 has little effect. Both FOXO1 and FOXO3 are important in the maintenance of hematopoietic stem cells by protecting them from oxida- tive stress. This review examines FOXO1/FOXO3 in the adaptive immune response, key target genes, and FOXO inhibition by the phosphoinositide 3-kinase/AKT pathway.

KEY WORDS: protein kinase B (AKT), antibody, acquired immunity, forkhead, inflammation, lymphocyte

ABBREVIATIONS: AKT, protein kinase B; **DC**, dendritic cell; **EBF1**, early B cell factor 1; **FOX**, forkhead box; **HSC**, hematopoietic stem cell; **IFN-γ**, interferon-gamma; **IL**, interleukin; **JNK**, c-Jun N-terminal kinase; **MAPK**, mitogen-activated protein kinase; **mTOR**, mammalian target of rapamycin; **NF-κB**, nuclear factor kappa light-chain enhancer of activated B cells; **PI3K**, phosphoinositide 3-kinase; **RAG**, recombination activating gene; **RORγt**, retinoic acid receptor-related orphan nuclear receptor gamma; **SGK1**, serum/glucocorticoid regulated kinase 1; **Tbet**, T-box protein 21; **TCR**, T cell receptor; **TGF-β**, transforming growth factor beta; **Th1**, T helper-1; **Th17**, T helper-17; **TNFα**, tumor necrosis factor alpha; **Treg**, regulatory T cell

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I. INTRODUCTION

The first forkheadbox (FOX) transcription factor was identified in mammalian cells by homology with the forkhead gene found in *Drosophila*[1] and subsequently cloned and characterized in humans.[2] Since then, many other transcription factors sharing the forkhead DNA-binding domain of approximately 100 amino acids have been identified. The initial discovery was made in the fruit fly and homologs have been found in species ranging from fungi to mammalian cells, suggesting a critical evolutionary-conserved biological role for these proteins.[3] This large family of transcription factors has been grouped into subfamilies according to their structural characteristics and are currently the largest family of transcription factors in humans.[4] The forkhead box "0" subfamily has four members in humans, of which three (FOXO1, FOXO3, and FOXO4) have a high degree of sequence homology[5] and one (FOXO6) is more distantly related with a more restricted expression and distinct regulatory mechanisms.[6] FOXO proteins regulate cell survival, cell cycle, and embryonic pattern formation,[7,8] which are closely related to their involvement in cancer.[9] *In vitro* studies suggest a similar biological activity for FOXO1, FOXO3, and FOXO4 and, in some cases, the regulation of similar target genes by binding to the same conserved DNA sequence. However, disruption of FOXO1 in mice is embryonically lethal at day 10.5, whereas animals lacking either FOXO3 or FOXO4 were viable and grossly similar to wild-type littermates. The primary phenotypes observed in FOXO3-deficient mice are infertility from abnormal ovarian follicular development,[10] abnormal proliferation of lymphatic cells, increased inflammation,[11] and a reduced neural stem cell pool.[12] Deletion of FOXO4 enhances response to inflammatory stimuli[13] and deletion of FOXO6 results in impaired memory and learning.[14] Therefore, the biological functions of FOXOs are complex and sometimes overlapping, but are not completely redundant.

FOXOs may act as transcriptional factors by inducing the expression of target genes with FOXO response elements. FOXO activation is complex, involving not only transcriptional activation, but also various post-transcriptional and post-translational mechanisms, including miRNA-mediated repression;[15] acetylation, phosphorylation, ubiquitination, methylation, and glycosylation;[16] protein–protein interactions; and cytoplasmic–nuclear shuttling.[17] Alterations in FOXO1 affect its nuclear import (activation) or export (inactivation) and DNA- binding activity. FOXOs have four functional motifs, which include a forkhead DNA-binding domain and domains that control nuclear localization,

nuclear export, and transactivation. These domains are highly conserved. FOXOs recognize two different consensus DNA-binding sequences: a Daf-16 binding element (5′-GTAAA(T/C)AA) and an insulin-response element (5′-(C/A)(A/C)AAA(C/T)AA). The core DNA sequence 5′-(A/C)AA(C/T)A is recognized by all FOXO family members. Kinases and acetylases modulate the nuclear localization and nuclear export to control shuttling of FOXOs. The chaperone protein 14-3-3 binds to FOXOs in the nucleus, exports them,[18] and in turn blocks them from returning to the nucleus.[19] FOXOs are phosphorylated by several kinases to modulate FOXO subcellular location, DNA-binding, and transcriptional activity.[20,21] A major negative regulator of FOXOs is the phosphoinositide 3-kinase (PI3K) pathway. PI3K activation induces the recruitment of the kinases AKT and serum/glucocorticoid regulated kinase 1 (SGK1) to the cell membrane, where each is activated by phosphorylation. AKT and SGK1 phosphorylate FOXO transcription factors directly on three different sites to inactivate FOXOs. Phosphorylation of FOXO1 or FOXO3 by AKT or SGK1 decreases FOXO DNA-binding affinity to consensus response elements and also increases their association with 14-3-3 proteins, which leads to inactivation by transport out of the nucleus. In contrast, phosphorylation of FOXOs at different amino acid residues by other kinases can have the opposite effect, demonstrating the complexity of FOXO activation. This alternative phosphorylation can increase nuclear localization to enhance FOXO activity. Kinases that stimulate FOXO activity include c-Jun N-terminal kinase (JNK), p38, 5’ AMP-activated protein kinase (AMPK), and cyclin-dependent kinase 1. Similar to phosphorylation, acetylation has been shown to both promote and decrease FOXO transcriptional activity and to mediate different biological functions of FOXOs.[20,21] The deacetylation of FOXO generally increases FOXO activity, whereas acetylation reduces it. For example, silent information regulator 1 (Sirt-1) and Sirt-2 belong to the sirtuin family of deacetylases and lead to FOXO deacetylation, increasing their binding to DNA.[22] Ubiquitination also regulates FOXO proteins. FOXO undergoes degradation through polyubiquitination, which functionally deactivates FOXOs. However, monoubiquitination of FOXOs can increase nuclear localization, effectively enhancing FOXO activity.[23] FOXOs also interact with β-catenin. When FOXOs bind to β-catenin in osteoblasts, β-catenin is not available to bind to T cell factor, thus diminishing T cell factor activity.[24] In this case, FOXOs act as a transcriptional repressor by ultimately reducing T cell factor activity. In CD8+ T cells, reduced levels of FOXO1 lead to increased stimulatory T cell fac- tor-1 through a similar mechanism.[25]

FOXOs have a fundamental role in the main tenance of organism homeostasis and adaptation to environmental changes,[26] which includes the homeo- stasis and development of immune-relevant cells in higher vertebrates.[27] More recently, the involvement of FOXO1 and FOXO3 in diverse functional aspects of the innate and adaptive immune response such as dendritic cell (DC) activity,[28,29] CD8 T cell response to chronic viral infections,[30] macrophage activation in parasitic[31] and bacterial infections by Gram-negative lipopolysaccharide (LPS),[32,33] and antibody class switching by B cells have begun to be explored.[34] This review focuses on the role of FOXO1 and FOXO3 in the biology of DCs, B and T lymphocytes, and hematopoietic stem cells (HSCs).

II. DCs

DCs express high levels of major histocompatibility complex class II (MHCII), with cytoplasmic projections similar to the dendrites of neurons.[35] Two major DC subsets can be identified in secondary lymphoid tissues: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs).[36] pDCs are recruited to sites of inflammation and are present in lymph nodes,[37] but are not considered to be potent antigen-presenting cells (APCs).[38] mDCs are a heterogeneous population of cells that are associated with activation of adaptive immunity through antigen presentation.[38]

As specialized APCs, DCs connect the innate and adaptive arms of the immune response.[38] The biological activation and function of DCs depends on pattern recognition receptors (PRRs) such as C-type lectin receptors, toll-like receptors (TLRs) and Nod-like receptors, as well as by cytokine and chemokine receptors.[39] DCs can respond to a variety of external antigens of microbial and non-microbial origin (bacterial, viral, fungal, protozoa, allergens, etc.) as well as damage-associated molecule patterns. Ultimately, the combination of PRRs and other stimulated receptors determines subsequent activation of DCs.[39]

DCs are phagocytic in their immature state[40] and acquire enhanced antigen-presenting and migration capacities upon maturation.[41,42] Activated DCs migrate to lymph nodes for effective antigen presentation by up-regulating the expression of MHC and co-stimulatory molecules (CD80 and CD86).[43] DCs from the peripheral circulation and DCs residing in non-lymphoid tissues (e.g., Langerhans cells in the skin) are guided to lymph-node-produced CCL19/ CCL21, which stimulate the chemokine receptor CCR7 on DCs. Activated DCs produce various cytokines, including interleukin (IL)-1, IL-6, IL-10, IL-12, IL-23, IL-27, and

tumor necrosis factor-α (TNFα),[44] which will affect the activation and bio- logical activity of other innate and adaptive immune cells. However, immature (i.e., non-activated) DCs may exert an inhibitory effect.[45] Cytokines such as TNFα and type I interferons (IFNs) and other PRRs induce multiple signaling pathways (e.g., mitogen- activated protein kinase [MAPK], MyD88, Raf/ PI3K, mammalian target of rapamycin [mTOR], and nuclear factor kappa light-chain enhancer of activated B cells [NF-kB]) that induce DC activation.[46] PI3K can be activated by specific stimuli to induce AKT and negatively regulate FOXOs.[47]

mTOR and AKT play a role in controlling the inflammatory response in DCs through regulation of FOXO1. Deletion of Rictor, a key component in mTOR signaling, causes a hyperinflammatory response in DCs. The link between mTOR and FOXO1 is mediated by AKT.[47] FOXO1 is phosphorylated by AKT, which causes FOXO1 to relocate to the cytoplasm. When FOXO1 is overexpressed *in vitro*, DCs produce high levels of IL-12, IL-6, and TNFα and, when FOXO1 is deleted, DCs have reduced capacity to produce inflammatory cyto- kines.[29] Therefore, FOXO1 promotes induction of inflammatory cytokines in DCs; in contrast, activation of mTOR and AKT deactivate FOXO1 to prevent a hyperinflammatory response.[47]

Bacterial infection by anaerobic Gram-negative periodontal pathogens such as *Porphyromonas gingivalis* or LPS stimulates FOXO1 nuclear localization through the MAPK pathway.[28] FOXO1 induces transcriptional activity and stimulates expression of adhesion molecule ICAM-1, integrins αv and β3, as well as CCR7 and matrix metalloproteinase-2, which are needed for DC activity.[28,29] By regulating these downstream target genes, FOXO1 promotes DC migration to lymph nodes and re-circulation to infected non-lymphoid tissue.[28,29] FOXO1 regulation of CCR7 and ICAM-1 is particularly important in homing to lymph nodes because reduced DC homing in *FOXO1* deleted DC is rescued by overexpression of ICAM1 and CCR7.[30] FOXO1 is also needed for DC stimulation of T and B lymphocytes, which can be linked to reduced capacity of DCs to bind to lym- phocytes when FOXO1 is absent. Bacteria-induced stimulation of IFN-γ and IL-13 by CD4+ T cells from lymph nodes is reduced by 50–65% when *FOXO1* is specifically ablated in DCs and FOXO1 ablation reduces the presentation of antigens *in vitro*.[28] There is reduced production of BAFF and APRIL in DCs lacking FOXO1, which accounts for their reduced antibody production and capacity to stimulate B cell proliferation.[28]

Bacterial inoculation stimulates the migration of DCs. When mucosal tissue is inoculated with bacteria, deletion of FOXO1 reduces the number of DCs that migrate to the infected tissue.[29] FOXO1 deletion down-regulates genes that play an important role in DC migration including integrin αv, integrin β3, and matrix metalloproteinase-2.[29] DC deletion of *FOXO1* decreases the formation of plasma cells in the lymph nodes,[29] consistent with reports that FOXO1 is needed for DC activation of B cells.[28] The ultimate effect of reduced FOXO1 in DCs is to attenuate the capacity of mice to generate antibodies in response to bacterial infection by *P. gingivalis*. The functional significance of FOXO1 activation in DCs is shown by increased susceptibility to bacterial infection in mice that lack FOXO1 in DCs, lead- ing to greater inflammation and periodontal bone loss.[29] Therefore, FOXO1 coordinates DC activity by regulating the expression of downstream target genes that are needed for DCs to stimulate T and B lymphocytes and generate an antibody defense to bacteria,[28,29] as shown in Fig. 1.

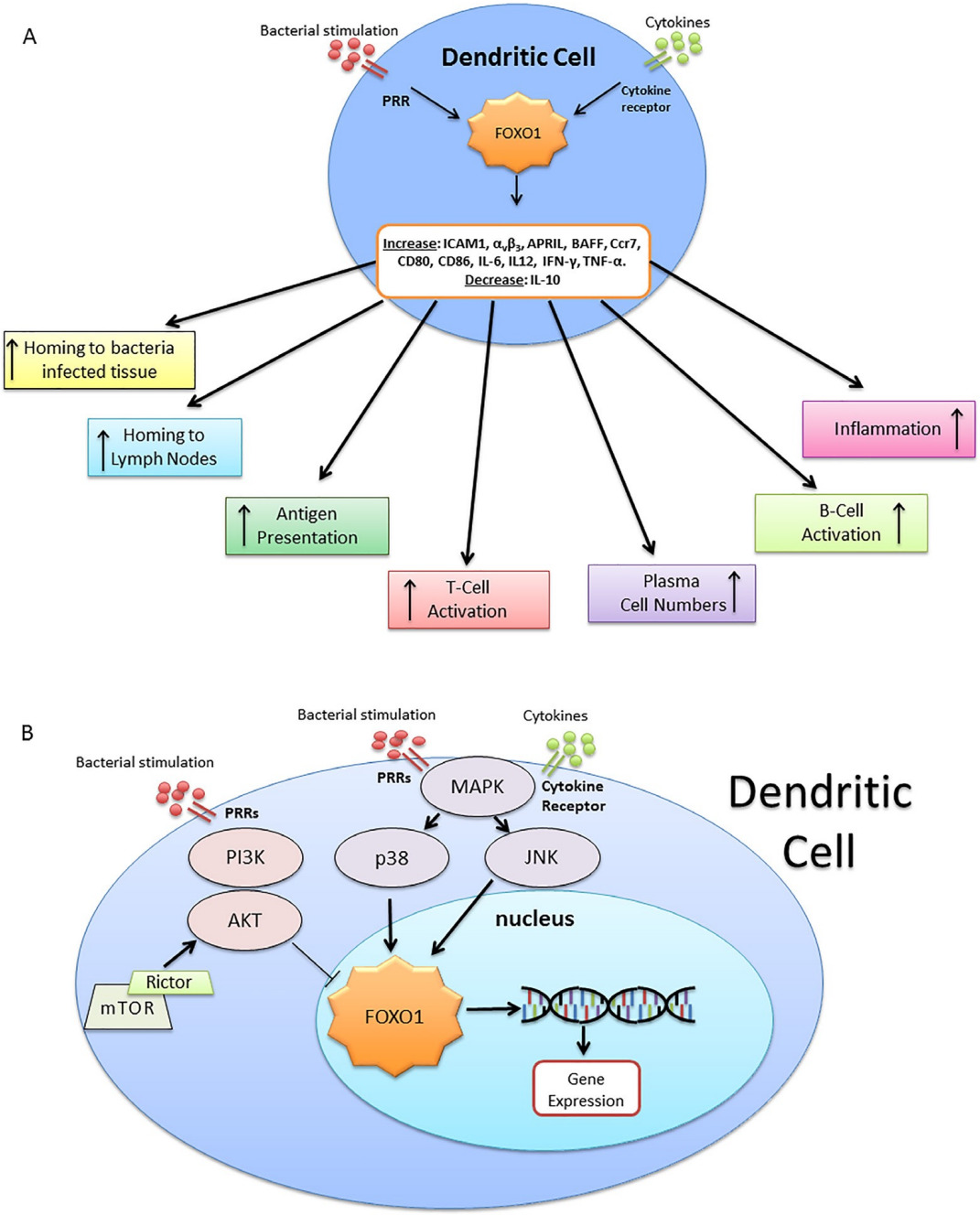

FIG. 1: FOXO1 regulates activation and function of dendritic cells. (A) PRRs such as TLRs and cytokine receptors activate FOXO1 in DCs. FOXO1 up-regulates the expression of target genes ICAM1, $\alpha_v\beta_3$, APRIL, BAFF, Ccr7, CD80, CD86, IL-6, IL12, IFN-γ, and TNF-α while decreasing IL-10. This leads to increased homing of DCs to the lymph nodes and bacteria-infected tissue. It also increases the immune response: antigen presentation, T and B cell activation, plasma cell numbers, and inflammation. The role of FOXO3 in DCs has not been well studied. (B) Pattern recognition receptors and cytokine receptor stimulation initiates a signaling cascade to activation of FOXO1 that involves the MAPK pathway. Activated FOXO1 can bind to the promoter region of target genes and regulate transcription. AKT is a major downstream target of PI3K that functions as a negative regulator of FOXO1. Stimulation of mTOR activates AKT to reduce FOXO1 activity and prevent a hyperinflammatory response.

III. T CELLS

Naive T cells are activated by "professional" APCs and can differentiate into distinct phenotypical subsets such as T helper-1 (Th1), Th2, Th17, Th9, or regulatory T cells (Tregs). FOXO1 plays an important role in the adaptive immune response by promoting the formation of Tregs, the development of the B lymphocytes, and maintaining a pool of HSCs.[48,49] FOXO proteins regulate several aspects of lymphocyte function, including lymphocyte development, cytokine expression, gene recombination, and homing.[50] FOXO1 is needed for the survival of naive T cells and homing to secondary lymphoid organs[51] by regulating the expression of *L-selectin* and sphingosine-1-phosphate receptor 1 (*S1pr1*), which are important for lymphocyte transit to and from lymphatic tissue. Activation of STAT3 inhibits T cell proliferation by up-regulating FOXO1 and FOXO3, which maintain T cells in a quiescent state and enhances their survival.[50] FOXO1 also enhances T cell survival by regulation of IL-7 receptor α (IL- 7ra), a subunit of the survival receptor for naive T cells. Interestingly, although FOXO1 is required for naive T cell survival, FOXO3 appears to have the opposite effect, increasing apoptosis. The basis for this differential response of FOXO1 and FOXO3 on T cell survival is not known. FOXO1 and FOXO3 share the ability to inhibit T cell activation, leading to a decrease in calcineurin/nuclear factor and less hypertrophic cardiomyopathy.[52]

A. Tregs

Tregs are formed in the thymus and play a critical role in deactivating or suppressing the immune response by the reducing activity of effector T cells (Th1, Th17, etc.). The differentiation and function of Tregs is impaired by deletion of *FOXO1* and the addition of *FOXO3* makes this impairment more severe. Tregs are controlled by the transcription factor Foxp3. Mice with T cell-specific combined deletion of *FOXO1* and *FOXO3* have many of the same defects as mice with *Foxp3* deletion, including impaired Treg formation and deficient *Foxp3* expression. FOXO1 stimulates the formation of Tregs by modulating the expression of several different genes. FOXO1 and FOXO3 bind to the promoter region of *Foxp3* and cytotoxic T lymphocyte antigen 4 (*Ctla4*) and regulate directly *Foxp3* and *Ctla4* promoter activity as well as several other genes needed for development of Tregs.[53] Interestingly, FOXO1 appears to regulate a different set of genes compared with Foxp3.[54] Therefore, Foxp3 alone is not sufficient to induce Treg differentiation and requires the participation of FOXO1.

FOXO1 and TGF-β are critical for inducing dif ferentiation and proliferation of Treg

cells.[52] Mice with *FOXO1*$^{-/-}$naive T cells have drastically reduced levels of Treg cells and the Tregs that are produced have reduced function and viability.[55,56] Moreover, in the absence of FOXO1, TGF-β expression leads to the formation of Th1 cells. This indicates that, in addition to driving Treg formation, FOXO1 and FOXO3 prevent naive T cells from acquiring T cell effector function.[57]

Tregs have high levels of FOXO1 and reduced activity of PI3K/AKT. Reduced AKT activity is required to increase FOXO1 activation and enhances Treg formation. The diminished AKT activity may be mediated by PTEN.[58] Interestingly, inflammatory conditions that activate the PI3K/AKT pathway repress Treg differentiation and function.[59] Another critical factor in forming Tregs is the inhibition T-box expressed in T cells (Tbet). Tbet directs naive T cells away from Treg differentiation and is induced by IFN-γ. FOXO1 and TGF-β work together to inhibit IFN-γ and Tbet expression to enhance the formation of Tregs.[60] Without FOXO1, TGF-β is less effective. This relationship is significant because the absence of *FOXO1 results in greater* T cell expression of IFN-γ, which in turn contributes to autoimmunity *in vivo. This phenomenon is further worsened by the combined deletion of both FOXO1* and *FOXO3*.[60] Constitutively active FOXO1 and STAT5 (with high TGF-β and low IL-6) were shown to rescue this autoimmune phenotype.[48] However, it is important to note that, in diabetic conditions, the relationship between FOXO1 and TGF-β1 changes. FOXO1 in a hyperglycemic environment fails to bind to the TGF-β1 promoter and fails to upregulate TGF-β1 transcription.[61] The consequence of having less TGF- β1 is growth-factor-deficient healing, which can be rescued in FOXO1-deficient mice by application of exogenous TGF-β1.[61] In contrast, under normal glucose levels, FOXO1 binds efficiently to the TGF-β1 promoter and upregulates TGF-β1 transcription and expression to promote healing. This change in FOXO1 behavior and the ability to induce TGF-β1 depends on glucose levels.

FOXO1 is also needed for Treg function. For example, an important downstream target of FOXO1 in Tregs is CCR7.[54] As was noted for DCs, FOXO1-induced expression of CCR7 is important in Treg homing.[54] Interestingly, mice with a conditional deletion of *FOXO1*$^{-/-}$ only in Tregs have reduced Treg function and display similar pathology to mice with low Treg populations *in vivo*. These mice have enlarged lymph nodes and a swollen spleen associ- ated with an increase in the number of T cells.[53] Figure 2 describes the role of

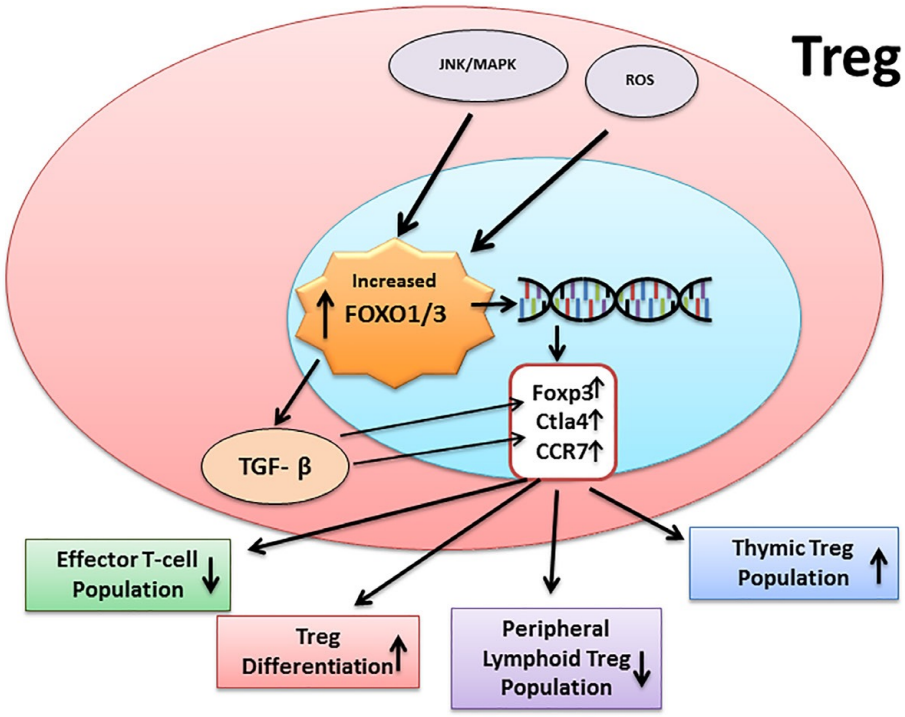

FIG. 2: FOXO1 is highly expressed in Tregs. FOXO1 can induce the expression of Foxp3, Ctla4, and CCR7 to increase the formation of Tregs and enhance Treg function. FOXO1 induces a transcriptome that is distinct from that induced by Foxp3. The effect of FOXO1 is to decrease effector Th cell formation, increase Treg differentiation, and increase the number of Tregs.

FOXO1 and FOXO3 in Treg cell function.

B. Th1

Th cells develop into effector T cells, one of which is Th1. Th1 cells promote inflammation, amplify the innate immune response, and are important in the host response to intracellular pathogens such as viruses. IL-12 signaling stimulates IFN-γ expres- sion to induce T-bet, the master regulator of Th1 cell differentiation. As mentioned above, FOXO1 inhibits T-bet and drives naive T cells to form Tregs. Therefore, the net effect of active

FOXO1 is to inhibit the differentiation of Th1 cells. To form Th1 cells, it is necessary to reduce FOXO1 activity, which is largely accomplished by stimulating AKT. Antigen presentation to the T cell receptor (TCR) activates PI3K, and subsequently, AKT. This leads to FOXO1 phosphorylation and its subsequent inactivation.[52] IL-12 receptor signaling extends and maintains AKT phosphorylation. In addition, Th1 cells produce IL-2, which further activates the AKT pathway to inhibit FOXO1.[62] Therefore, Th1 cell differentiation and proliferation requires the inhibition of FOXO1 (which is active in basal naive T cells). There are multiple signaling pathways that lead to activation of AKT and inhibition of FOXO1. Too much inhibition of FOXO1 via the AKT/PI3K and other pathways can lead to preferential differentiation to Th1 cells, resulting in a higher likelihood of autoimmunity.[52]

C. Th17

Th17 cells are important in the host response at mucosal surfaces.[63] Th17 cells mediate the recruitment of neutrophils and macrophages to infected tissues. They are characterized by production of IL-17 and acquire effector function in the thymus. Like Th1 cells, there is polarization between Tregs and Th17 cells. Differentiation of Th17 cells is controlled by a master regulator of Th17 cell differentiation, the transcription factor retinoic acid receptor-related orphan receptor-gamma-t (RORγt). Th17 cells are induced by IL-23 and IL-1β, which stimulate signal transducer and activator of transcription 3 (Stat3). FOXO1 activity inhibits the formation of Th17 cells and suppresses the expression of IL-17A. FOXO1 inhibits IL-17A and IL-23 receptor expression in part by inhibiting the transcriptional activity of *RORγt* [64] by forming a complex with it. Therefore, FOXO1 acts a repressor of RORγt to inhibit Th17 differentiation.[64] Because of the inhibitory effect of FOXO1 on the formation of Th17 cells, antigen activation of the TCR is needed to reduce FOXO1 activity and permit Th17 cell differentiation. One of the key effector molecules in Th17 cells that inhibits FOXO1 activation is SGK1.[65] Similar to AKT, SGK1 phosphorylates FOXO1 and sequesters it to the cytoplasm for degradation.[65] In addition, FOXO1 suppression via the PI3K/AKT pathway is important in the formation of Th17 cells.[64] Mice with a $FOXO1^{-/-}$ ablation in their T cells have increased numbers of Th17 cells, establishing FOXO1's ability to limit differentiation to Th17 lymphocytes *in vivo*.[64] Figure 3 describes the role of FOXO1 inhibition in the formation of Th17 cells.

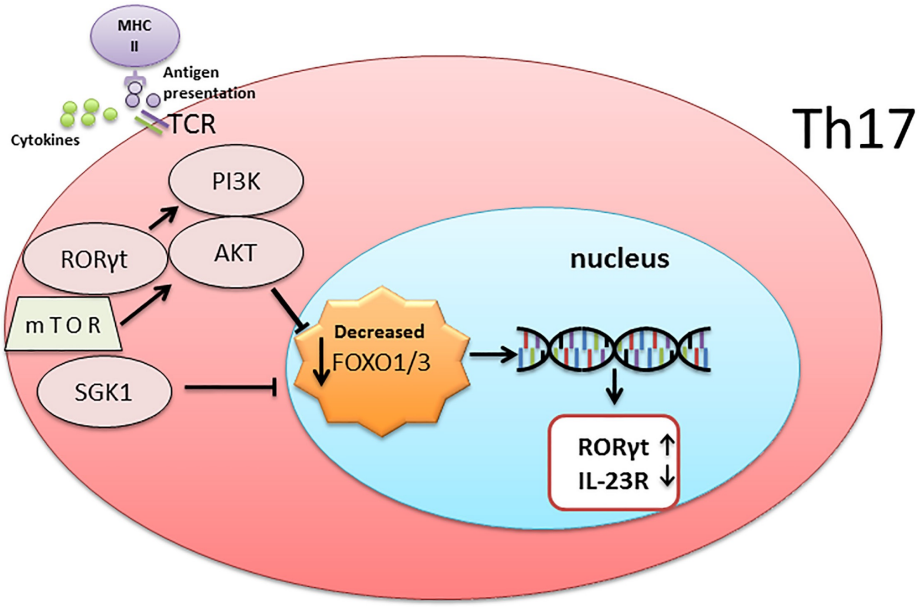

FIG. 3: FOXO1/3 interferes with Th17 effector cell formation. Antigen presentation stimulates the TCR and silences FOXO1/3 through PI3K/AKT and SGK1. FOXO1/3 inhibits IL-23R and RORγt expression to inhibit Th17 cell differentiation. For Th17 cells to form, it is necessary to block FOXO1/3 activity.

D. Memory T Cells

Memory T cells are effector cells that have been exposed previously to a specific antigen and are referred to as "antigen-experienced T cells," which can produce a more rapid and stronger immune response. Memory T cells are produced from CD8 and CD4 effector T cells through a differentiation pathway that requires precise modification of gene expression in the effector cells. When FOXO1 is inhibited by the PI3K/AKT pathway, effector T cell status is preserved at the expense of memory T cell development.[66] FOXO1 becomes active in the formation of memory T cells and reduced FOXO1 can inhibit the formation of memory T cells.[55] In contrast, activation of FOXO1 can inhibit effector T cell function and favor memory T cell formation based in part on its ability to repress Tbet signaling. FOXO1 is also critical for proper functioning and clonal expansion of the memory T cell upon second (repeated) contact with an antigen. In support of this, *FOXO1* deletion reduces the number of memory T cells capable of expanding and differentiating. In contrast, FOXO3 activation enhances the maintenance of memory T cells. Figure 4 describes the role of FOXO1 in memory T cell function.

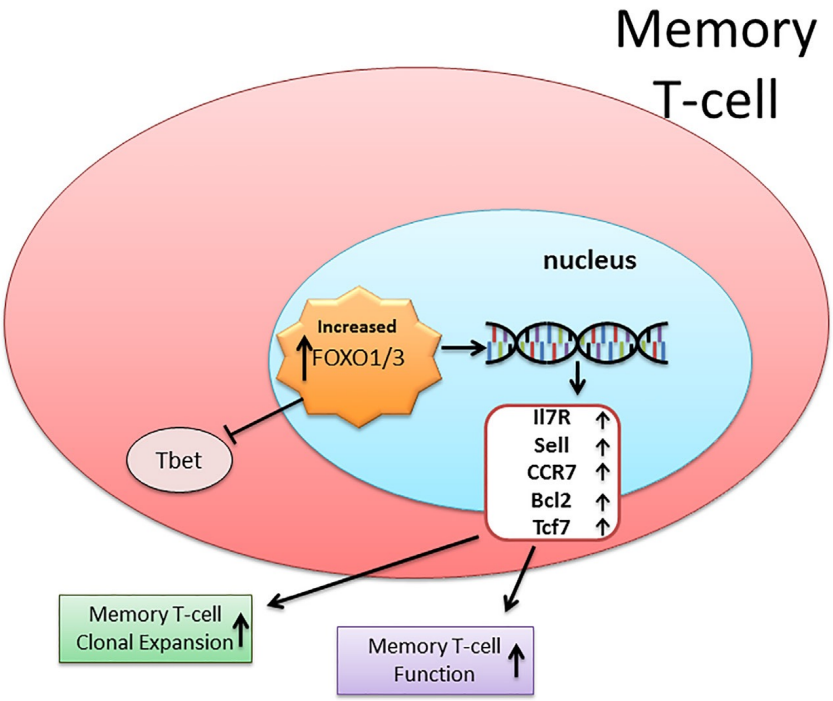

FIG. 4: FOXO1 increases memory T-cell formation. FOXO1 increases expression of CCR7, IL7R, BCL2, Sell, and Tcf7, which leads to increased memory T-cell formation and function. FOXO1 can decrease Tbet, which facilitates memory cell differentiation. FOXO3 plays a role in the maintenance of memory T cells.

IV. B CELLS

B cells produce antibodies, present antigen, and affect inflammation by generating cytokines. In mammals, B cells are produced and mature in the bone marrow and c irculate in the blood and secondary lymphatic organs. B cells that have been stimulated with an antigen undergo class switching and differentiate into plasma cells to produce high-affinity antibodies. B cells express B cell receptors (BCRs) on their cell membranes that bind specific antigens. B and T cells are activated via similar mechanisms: both cells react to antigen binding to the BCR or TCR, respectively, which leads to the activation of a complex signaling cascade.[67] Both BCRs and TCRs initiate signaling that contribute to PI3K activation. PI3K, in turn, activates AKT and inhibits FOXO1 and FOXO3 activity. Mice with a mutation in PI3K that prevents activation of AKT display similar immunodeficiency to $CD19^{-/-}$ mice in that both have reduced capacity to produce antibodies in response to antigen challenge.[68] LPS-challenged primary B cells with constitutively active Foxo1 display decreased proliferation

and increased cell death.[68]

FOXO1 is upregulated at the early pro-B cell stage.[69,70] Deletion of *FOXO1* impairs several stages of B cell development due to FOXO1 regulation of key target genes, particularly IL-17 receptor alpha *(Il7rα)*, recombination activating gene 1 (*RAG1*), and *RAG2*, *L-selectin, Aicda, and* early B cell factor 1 (*EBF1*).[69,70] These findings establish FOXO1 as a critical member of the transcription factor network directing early B cell development and peripheral immune function. Interestingly, deletion of *FOXO3* in B cell progenitors does not have a major effect on B cell differentiation or function.

Genetic deletion of *FOXO1* in lymphoid progenitor cells prevents them from forming mature B cells. FOXO1 activation begins during the pre-pro-B cell phase.[71] A key event in B cell differentiation is the expression of EBF1. EBF1 participates in maintaining the B cell lineage and antagonizing differentiation into other lymphocyte subsets by modulating gene expression. EBF1 expression is stimulated by the binding of transcription factors E2A and FOXO1 to the ir respective consensus response elements to induce EBF1 promoter activity.[71] The binding of both E2A and FOXO1 to the *EBF1* promoter is required because one without the other fails to induce B cell differentiation. Furthermore, E2A and EBF1 both bind to their respective loci on the *FOXO1* promoter to enhance FOXO1 expression, representing feed-forward amplification.

FOXO1 regulates several other important genes needed for B cell function. FOXO1 induction of IL-7Rα prevents apoptosis and maintains B cell viability.[69,70] FOXO1 drives L-selectin expression, which is needed for normal recirculation of B cells. RAG1 and RAG2 expression are necessary for the rearrangement of the immunoglobulin light chain. Knock-down of FOXO1 reduces RAG expression, whereas overexpression of FOXO1 increases it. Therefore, FOXO1 is critical for Ig gene rearrange- ment through its regulation of RAG1 and RAG2.[71] In contrast, knock-down or overexpression of FOXO3 does not modulate RAG levels, which is consistent with reports that FOXO3 does not play an important role in B cell development. Deletion of FOXO1 at later stages of B cell development also has an effect by blocking class-switching recombination. Deletion of FOXO1 in transitional B cells has no effect on the production of antigen-specific IgM, but does block class-switched antibody formation. Blockage of class switching by deletion of FOXO1 is likely due to its dependence on Aicda. Therefore, FOXO1 induces Aicda, which in turn induces class switching.

V. HSCs

HSCs are located in the bone marrow and exist for a lifetime to lead to the production of leukocytes.[72] The development of leukemia and lymphoma is greatly affected by factors that mediate proliferation and survival of HSCs. Activation of the PI3K/AKT pathway and inhibition of FOXOs play an important role in the development of cancers that originate from HSCs.[73] The role of FOXOs in HSCs was investigated in mice with combined lineage-specific deletion of *FOXO1*, *FOXO3*, and *FOXO4*. FOXO4 is related to FOXO1 and FOXO3, but has been less studied than the other two. The combined *FOXO* deletion in HSCs drove these cells from a quiescent state to entry into the cell cycle, indicating that FOXOs inhibit proliferation.[74] FOXOs accomplish this inhibition in HSC by inducing expression of negative cell-cycle regulators p21 and p27 and reducing positive regulators such as cyclin D2, as has been shown in other cell types.[75] The long-term effect of deleting *FOXO1/3/4* in HSCs is due to interfer- ence with HSC self-renewal due to an increase in reactive oxygen species. FOXOs are protective by mediating expression of genes that have anti-oxidant properties.[75] *FOXO1/3/4* combined deficient HSCs have reduced levels of anti-oxidants, including the superoxide dismutase genes *Sod1* and *Sod3*.[74] It is striking that anti-oxidant treatment of mice with lineage-specific deletion of *FOXO1/3/4* in HSCs restores HSC number and function. It also corrects abnormalities in apoptosis and the cell cycle caused by FOXO deletion. Therefore, FOXOs promote qui- escence and survival in the HSC compartment that is required for its long-term regenerative potential.[74]

VI. CONCLUSION

FOXO transcriptions factors, particularly FOXO1 and FOXO3, play important roles in stimulating an adaptive immune response. Through lineage- specific deletion in mice, the role of FOXOs has been elucidated and were found to be involved in the activation and regulation of DCs. Reduced FOXO1 in DCs leads to diminished cytokine production, impaired homing of DCs to lymph nodes, reduced DC stimulation of CD4+ T and B cells, reduced numbers of plasma cells in lymph nodes, and reduced antibody production. In T and B cells, FOXO1 and FOXO3 activation or inhibition is critical for dif- ferentiation and function. FOXOs are important in HSC self-renewal in part by maintaining a quiescent state and protecting HSCs from oxidative stress. Even though FOXO1 and FOXO3 bind to similar conserved consensus DNA sequences, their function may or may not overlap. FOXO1 is required for B cell differentiation, immunoglobulin gene rear-rangement, and class switching, but FOXO3 is not.

In contrast, both FOXO1 and FOXO3 are needed for the maintenance of HSCs. The mechanisms by which FOXOs are regulated are less understood. They are highly regulated by levels of expression; by post-translational modification, which affects nuclear localization and/or DNA-binding activity; and by partnering with specific co-repressors or co-activators.[52] Considerable insight into how FOXOs are modulated and their impact on different cell types comes from studies examining diabetes.[76] For example, in normal wound healing, FOXO1 plays a positive role in promoting keratinocyte behavior to enhance healing, whereas diabetic conditions alter FOXO1-induced gene targets to inhibit healing.[77,78] Therefore, FOXO behavior may depend on the cell type and specific conditions, which is a cautionary tale in not extrapolating FOXO function from one cell type to another or one condition to another. This suggests that FOXOs are tightly regulated by epigenetic considerations, which is certain to be a highly investigated topic.

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4.2 Publicação 2

Deletion of Akt1 in dendritic cells attenuates inflammatory bone resorption in experimental periodontitis·

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Abstract

Periodontitis is characterized by chronic inflammation involving adaptive immunity and osteoclastogenesis. Dendritic cells (DCs) play a key role in the immune response and also are osteoclast precursors. Akt contributes to cell survival, growth and proliferation and has a relevant role in osteoblast differentiation and, indirectly, on osteoclastogenesis. Based on this information we purposed to evaluate how Akt1 activity in dendritic cells influences the immune response and the pathogenesis of periodontal disease. Experimental periodontitis was induced by oral inoculation of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in transgenic CD11c.Cre^{+}/Akt1$^{L/L}$ mice with lineage specific Akt1 deletion in dendritic cells and wild type control CD11c.Cre^{-}/Akt1$^{L/L}$ mice. After 2 and 6 weeks the mice were euthanized, the hemi-mandibles were dissected, scanned on microCT and submitted to histological processing. Outcomes assessed were alveolar bone resorption, osteoclast number, inflammation and specific antibodies for *P.gingi*valis and *Fusobacterium nucleatum*. The analyses showed an inhibition of bone resorption at the 6-week period in Cre+ mice; less loss of attachment was also observed in Cre+ mice than in Cre- mice at 6 weeks; as well as lower number of osteoclasts. The number of infiltrating PMNs in Cre+ mice was also significantly reduced at the 6-week period; and the humoral response to *Pg* was significantly inhibited in the same period. The data indicate that Akt1 signaling in dendritic cells is critically relevant for the late immune response and inflammatory bone resorption in the experimental periodontal disease model.

Key Words: Dendritic Cells, Akt1, Periodontal Disease, Inflammation, Immunity

Introduction

Periodontitis is a highly prevalent condition caused by a chronic exposure of bacteria that stimulate an inflammatory response causing bone loss[1]. Recognition of bacterial pathogens by antigen presenting cells, such as dendritic cells (DCs), that will activate T cells[2,3]. For this reason, DCs are known as "the bridge" between innate and adaptive immune responses[4]; these cells are also capable of differentiating into osteoclasts[5]. There are different subsets of DCs: plasmocytoid dendritic cells (pDCs); myeloid dendritic cells (mDCs) and Langerhans cells (LCs)[3]. mDCs can recognize bacterial and viral antigens[6,7] and express high levels of CD11c[3]. In their immature state, these cells are present in different tissues to detect foreign antigens, whereas in their mature state mDCs migrate to the lymph nodes[8]. LCs are located in the skin and oral mucosa[9], they are also capable to promote activation of antigen-specific T-cells and are characterized by the expression of langerin/CD207[3,10]. LCs localized in oral epithelium and connective tissue are responsive to periodontal pathogens, such as *Porphyromonas gingivalis*, and to the colonized sites during periodontitis[11-13]. It is considered that LCs are related with periodontal disease activity, since increased numbers of LCs are present in the oral mucosa of periodontitis patients in comparison with healthy patients[11,14]. Furthermore, non-surgical periodontal treatment reduced the number of LCs in the gingival tissue[15]. Ablation of LCs in mice with experimental periodontitis aggravated inflammatory bone resorption and was associated with increased production of RANKL and decreased numbers of Treg cells[5].

The biology of DCs has been studied using the CD11c.DTR and CD11c.DOG mouse models that deplete CD11c positive cells. However, these models are limited because of the profound perturbations in the immune response, including the induction of neutrophilia and monocytosis, as well as the depletion of other immune cells[16].

FOXO1 is a transcription factor that regulates many cellular and biological processes, including protection against oxidative stress, apoptosis and progression through the cell cycle[17]. Deletion of FOXO1 in mDCs inhibits their immune-activating properties, such as bacterial phagocytosis, migration, and binding to lymphocytes[18], which indicates that FOXO1 is essential for effective DC homing to lymph nodes and for antigen-presentation to lymphocytes. FOXO1 is a downstream target of the protein kinase B (Akt); which has a critical role in metabolic and cell differentiation processes, including apoptosis and proliferation. Akt phosphorylates FOXO1 inhibiting its transcriptional activity and

contributing to cell survival, growth and proliferation[19]. Transgenic mice genetically deficient for Akt1 present increased osteoblastic differentiation and diminished numbers and activity of osteoclasts, which is associated with inhibition of the expression of RANKL and OPG (major cytokines involved in bone turnover) by osteoblasts[20, 21]. It indicates that Akt1 has a relevant role in osteoblast differentiation and, indirectly, on osteoclastogenesis[22]. Since the activation of Akt1 is associated with diminished bone formation and increased bone resorption, selective inhibition of Akt1 may be an interesting therapeutic approach for the attenuation of bone resorption / stimulation of bone formation. The aim of this study is to evaluate how Akt1 activity in dendritic cells influences the immune response and the pathogenesis of periodontal diseases.

Material and Methods *

Animals

Mice with deletion of Akt1 gene limited to dendritic cells were generated by using the Cre-LoxP system. Transgenic mice expressing Cre recombinase under the control of CD11c promoter, denominated CD11c.Cre, were obtained commercially (from Jackson Laboratory). These mice were bred with transgenic mice with LoxP sequences flanking exons 3 and 4 of the Akt1 gene [23]. The offspring of these breedings were genotyped by PCR and the genotypes of interest for this research are: CD11c.Cre+/Akt1L/L and CD11c.Cre-/Akt1L/L. The animals were kept in an environment with controlled temperature (21 ± 1 C), humidity (65-75%) and light cycles (12h light-12h dark), and fed with water and mouse chow ad libitum. The mice were randomly divided into 6 groups (n = 10/group, 50% female, 50% male): CD11c.Cre+/Akt1L/L with no treatment, CD11c.Cre+/Akt1L/L experimental periodontal disease 2 weeks, CD11c.Cre+/Akt1L/L experimental periodontal disease 6 weeks, CD11c.Cre-/Akt1L/L with no treatment, CD11c.Cre-/Akt1L/L experimental periodontal disease 2 weeks, CD11c.Cre-/Akt1L/L experimental periodontal disease 6 weeks.

*** APÊNDICE A**

Periodontal Disease Model

Periodontal disease was induced using the *Porphyromonas gingivalis* and *Fusobacterium nucleatum* oral inoculation model as described previously[1]. Controls consisted of sham-infected mice that received the same volume of carboxymethyl cellulose (Sigma-Aldrich, St Louis, MO) (100μL) oral inoculation without *P. gingivalis* and *F. nucleatum*. The mice were euthanized after 2 or 6 weeks by overdose of general anesthetics. All regulatory/ethical approval was obtained from IACUC (Institutional Animal Care and Use Committee) and IRB (Institutional Review Board) of the University of Pennsylvania (Anexos A e B).

Bone Resorption Analysis

After euthanasia, the hemi-mandibles of the mice were carefully dissected and fixed in 4% paraformaldehyde at 4°C for 18 h, washed in PBS for 10 min and then transferred to 70% ethanol until they were scanned on the microcomputer tomograph. Scanning was executed using standardized conditions in 18 μm slices using micro-CT-40 (Scanco Medical AG, Bassersdorf, Switzerland). Analysis of the extent of bone resorption was performed after three dimensional reconstruction and standardized re-orientation of these images in the three spatial planes. The three dimensional set of images on the sagittal plane was binarized using a standardized threshold to distinguish between mineralized and soft tissues. The relative quantity/extent of mineralized tissues in each sample was determined using a standardized cuboidal region of interest (ROI) of 750 μm3 positioned on the three dimensional images using pre-defined anatomical landmarks. The fraction of the volume of this ROI occupied by mineralized tissues (Bv/Tv, Bone volume / Total volume) was compared among the groups. These analyses were performed using OsiriX software (Pixmeo SARL, Bernex, Switzerland).

Histomorphometric Analysis

After scanning on the μCT, samples were washed in PBS and demineralized in EDTA (0.5 M, pH 8.0) on gentle and constant shaking at room temperature for 6 weeks (with EDTA exchange 3 times per week). After routine histological processing for paraffin embedding, serial sections of 4 μm thickness were obtained in the mesio-distal direction (anterior-posterior, sagittal plane), and mounted on silanized glass slides. Three serial cuts were selected from the middle portion of the first lower molar (identifying the medial portion by the diameter of the distal root canal) of each mandible. The Hematoxylin and Eosin (H&E)-

stained slides were visualized on a 100X magnification optical microscope to evaluate the bone loss as previously described[24]. Attachment loss was assessed by the measuring the distance from the cementum-enamel junction to the apical extent of epithelium along the root surface at a 400X magnification. Histomorphometrical analyses were conducted by a trained and experienced examiner without knowledge of the experimental conditions.

Osteoclasts

The histological sections were de-waxed in xylenes and rehydrated in a sequence of decreasing concentrations of ethanol, the sections were washed in PBS and stained for 15 min at 37º C with a staining solution composed of 0.1 M sodium acetate trihydrate (Sigma S9513), 50 nM of sodium tartrate (Sigma S4797), 5 mg of fast red violet lb salt (Sigma F3381), 30 mg of naphthol AS-MX phosphate (Sigma N4875), 200 µl dimethylformamide (Sigma D4551) and 200 µl of 0.1 manganese chloride (MnCl2, Sigma M3534). The sections were counterstained with hematoxylin for 10 seconds, air-dried at room temperature for 24 hours, and covered with coverslips. After 24 hours, digital images were obtained on an optical microscope with magnification of 200X, using standardized settings for brightness, contrast and exposure. Osteoclasts were identified by labeling indicative of tartrate-resistant acid phosphatase (TRAP) activity in conjunction with cell morphology (cell size, presence of three or more nuclei) and localization (near the surface of the bone tissue). The region of interest was the coronal 0.35mm of the interproximal alveolar bone between the first and second molars and a trained and experienced examiner counted the osteoclasts without knowledge of the experimental conditions.

Inflammation

The inflammatory condition was estimated by differential counting of polymorphonuclear neutrophils (PMNs) outside of the blood vessels situated in the first 0.05 mm of connective tissue, immediately below the basal membrane of the epithelium, as described[24]. The H&E stained slides were visualized on an optical microscope with a magnification of 600X. A total of 12 sections were assessed for each sample, distributed in 3 groups of 4 serial sections each, spaced by 80 µm. This allows for the assessment of the entire buccal-lingual extension of the lower molars. A trained and experienced examiner without knowledge of the experimental conditions performed the counting.

ELISA

Blood was collected from anesthetized mice immediately prior to euthanasia by cardiac puncture and centrifuged at 3000 RPM for 10 minutes at 4°C to separate the serum. For the detection of IgG specific for *Pg* and *Fn*, ELISA was performed using the Ready-SET-Go! (88-0400, Affymetrix, eBioscience) following the manufacturer's protocol, with the following modifications: Dynatec ELISA, Chantilly antibody plates were used, instead of capture antibodies; we used 50 µl of *Pg* or *Fn* bacterial suspensions (fixed with 1% formaldehyde at 4°C for one hour, OD of 0.3) that were incubated in the plates overnight at 4°C; After two washes with PBS + 0.05% Tween-20 the plate was placed in the oven at 37°C (for drying) for 2 hours. After blocking according to the manufacturer's protocol, an additional blocking step using 2% normal mouse serum for one hour was done to avoid nonspecific binding and calibration of background staining. Standards provided in the kit were used to get the standard curve and results were normalized.

Data analysis

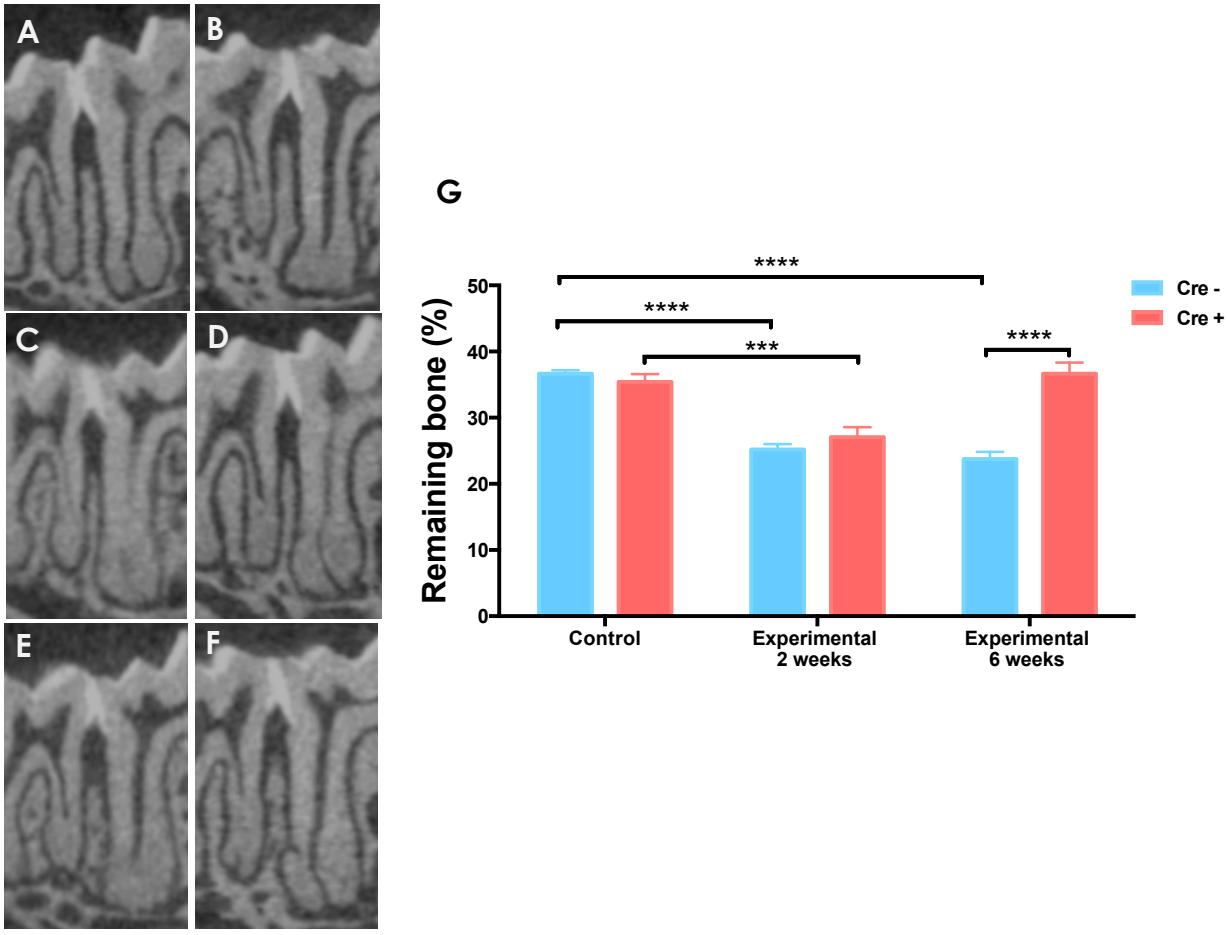
The primary objective of the data analysis was to compare the outcomes of interest considering the presence (gene expression) of Akt1. All data obtained were subjected to a normality distribution test (D'Agostino & Pearson normality test) and statistical analysis performed using the proper methods according to data distribution and number of experimental groups and periods, aimed at verification of dissimilarity (rejection of the null hypothesis) for each of the outcomes of interest. Significance level was set to a minimum of 95% ($p<0.05$) for all analysis; GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used.

Results

Bone Resorption Analysis

The experimental model of periodontal disease induced significant bone resorption in the mice with Akt1 (Cre-), demonstrating the efficacy of this model and the susceptibility of the mouse strain. Inflammatory bone resorption was significantly reduced in the Akt1-deleted mice (Cre+), starting at 6 weeks of the colonization model (Figure 1). This result indicates that Akt1 expression in DCs is required for inflammatory bone resorption in this experimental model of the periodontal disease.

Figure 1: Assessment of inflammatory bone resorption by microCT. – A-F are representative bidimensional images according to the experimental groups and genotype of the mice: **A)** Cre- control group (no treatment); **B)** Cre+ control group (no treatment); **C)** Cre- 2 weeks experimental group; **D)** Cre+ 2 weeks experimental group; **E)** Cre- 6 weeks experimental group; **F)** Cre+ 6 weeks experimental group; **G)** The graph shows the percentage of remaining bone, according to the genotype of the mice and the experimental period; the bars indicate the means and vertical lines the standard errors. Analysis performed by ANOVA, followed by the Tukey post-hoc test for pairwise comparisons (***P < 0.0006, ****P < 0.0001).



Histomorphometric Analysis

In mice with Akt1 (Cre-), the experimental model of periodontal disease induced a significant increase in the length of root surface exposed between the CEJ and the alveolar bone crest, indicating the loss of attachment associated with the colonization model of periodontal disease (Figure 3A, C and E, Figure 2). Similar to the results of the µCT analysis, Akt1-deleted mice (Cre+) presented significantly less loss of attachment than the Cre- mice at 6 weeks only (Figure 3E and F, Figure 2), indicating that expression of Akt1 in DCs has a prominent role in the inflammatory-induced loss of attachment in this model.

Figure 2: Histomorphometric assessment of attachment loss. – The graph shows the length (in µm) of exposed root surface between the cementum-enamel junction and the alveolar bone crest, according to the genotype of the mice and the experimental period. The bars indicate the means and vertical lines the standard error (**P < 0.0005, ***P < 0.0005)

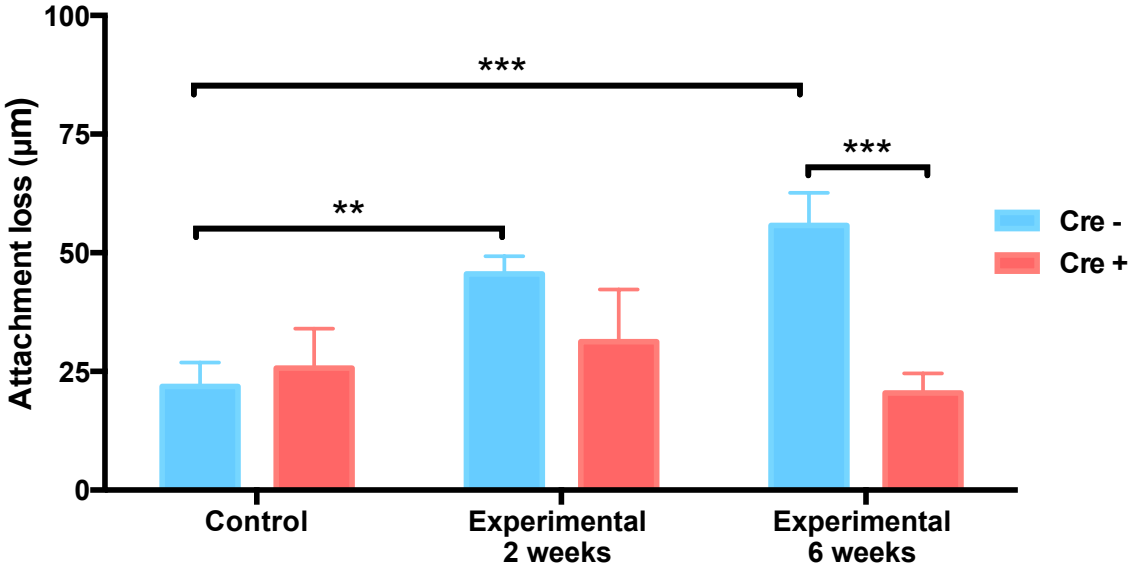
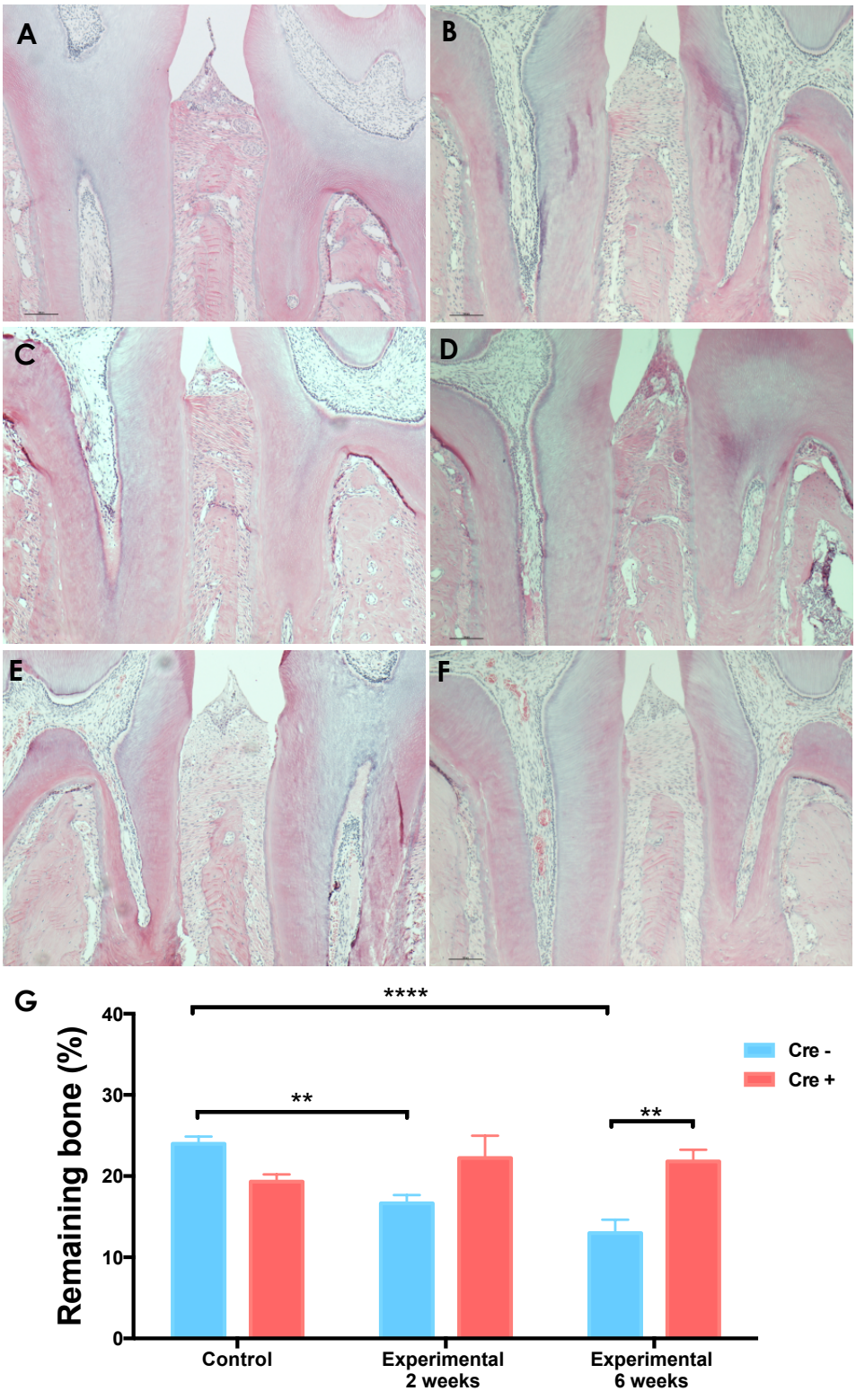


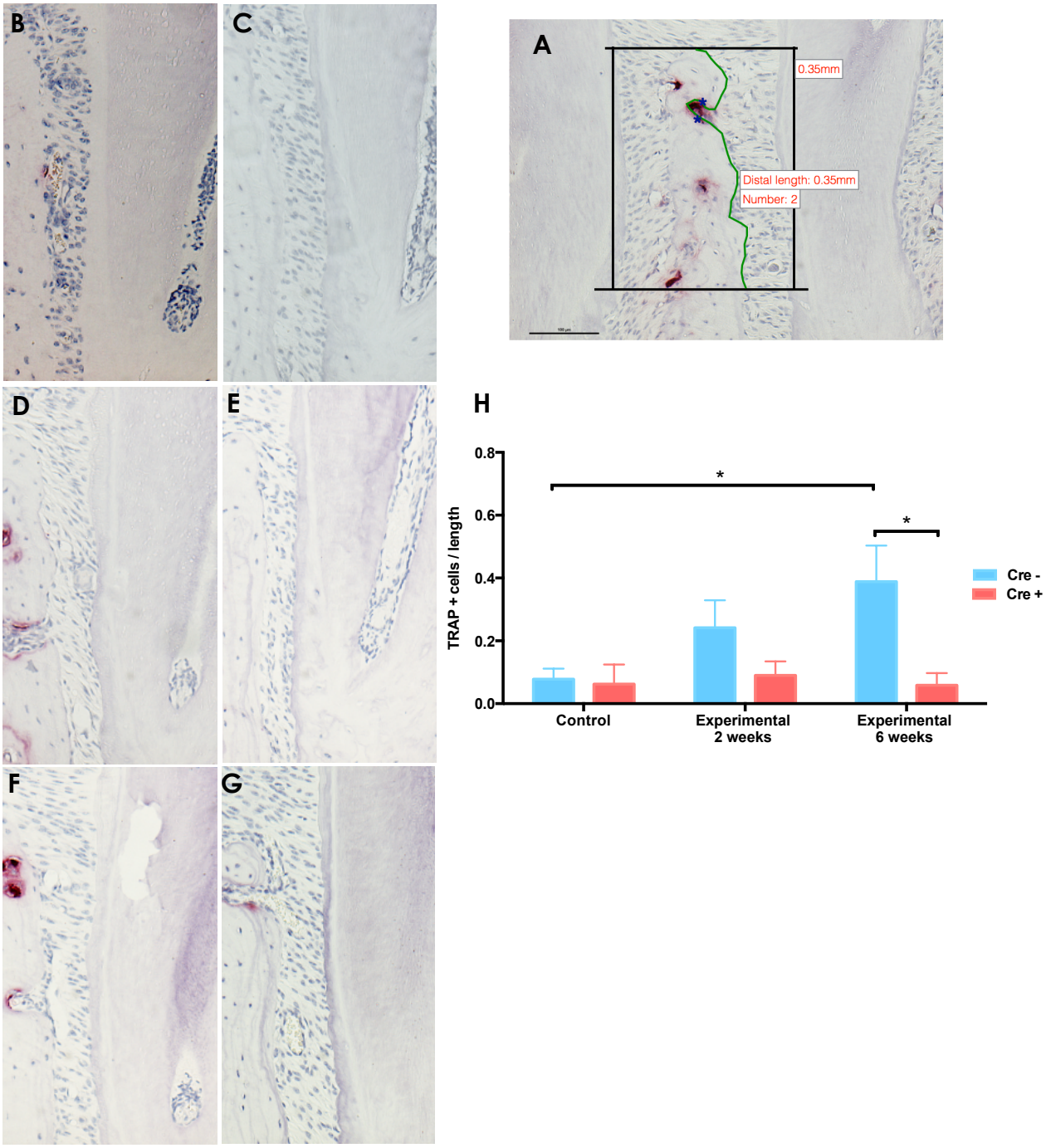
Figure 3: Histological analysis of bone resorption. – A-F are representative images of H&E-stained sections (100X magnification), according to the experimental period and genotype of the mice: **A)** Cre- control group (no treatment); **B)** Cre+ control group (no treatment); **C)** Cre- 2 weeks experimental group; **D)** Cre+ 2 weeks experimental group; **E)** Cre- 6 weeks experimental group; **F)** Cre+ 6 weeks experimental group. The bars indicate the means and vertical lines the standard errors; **G)** The graph shows the percentage of remaining bone, according to the genotype of the mice and the experimental period; the bars indicate the means and vertical lines the standard errors. Analysis performed by ANOVA, followed by the Tukey post-hoc test for pairwise comparisons (**P < 0.0005, ****P < 0.0001).



Osteoclasts

The number of osteoclasts was markedly increased only in the mice with Akt1 (Cre-), with statistical significance reached at the 6-week experimental period (Figure 4A, C, D and G). In contrast, in Akt1-deleted mice (Cre+), the number of osteoclasts remained relatively constant throughout the experiment; however statistically significant difference in the number of osteoclasts between Cre- and Cre+ mice was found only at the 6-week experimental period (Figure 4B, D, F and G). This result indicates that expression of Akt1 in DCs is required for the osteoclastogenesis in the periodontal microenvironment. Significant differences were observed between the Cre- control group and the 6 weeks experimental Cre- group. Significant differences were observed between the Cre- and Cre+ groups from the 6 weeks experimental groups (Figure 4). The results from this experiment confirm the results obtained in the μCT and histomorphometric analyses.

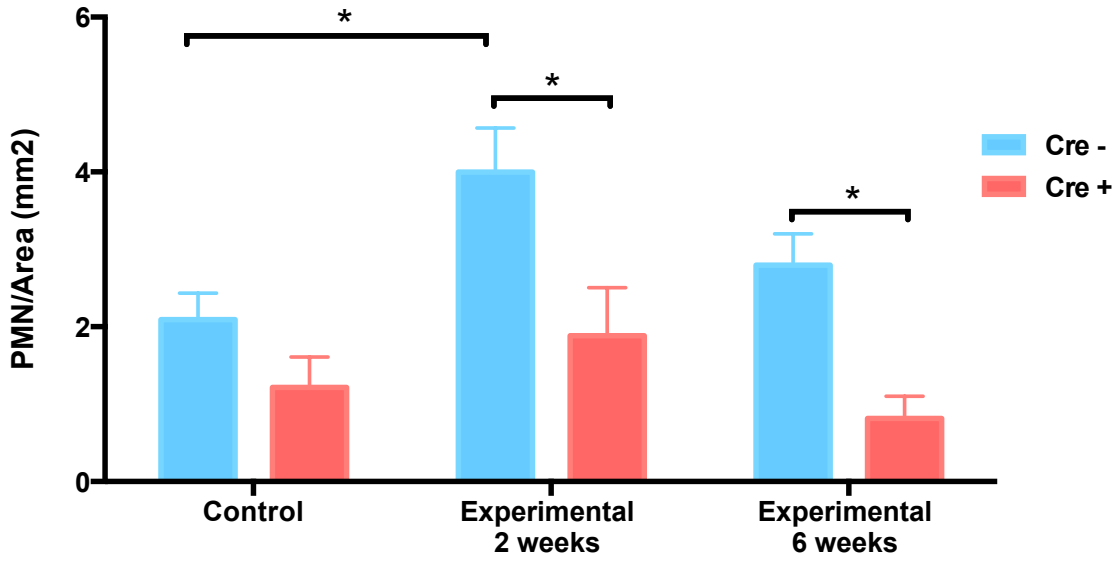
Figure 4: Identification and numbers of osteoclasts on the alveolar bone crest. Osteoclasts were identified as large TRAP+, multinucleated (3 or more nuclei) cells in close proximity with the surface of the alveolar bone crest. (100X magnification), **A)** Representative image of delimited area for counting osteoclasts; A line was drawn at the border of the alveolar crest, between the first and second molar. From this line, 0.35mm was delimited, a line following the surface of bone tissue, in the distal part was traced; The osteoclasts that were present on the surface of the bone were counted. Increase of 20X.– B-G are representative images according to the experimental period and genotype of the mice: **B)** Cre- control group (no treatment); **C)** Cre+ control group (no treatment); **D)** Cre- 2 weeks experimental group; **E)** Cre+ 2 weeks experimental group; **F)** Cre- 6 weeks experimental group; **G)** Cre+ 6 weeks experimental group; **H)** The graph shows the osteoclast number, according to the genotype of the mice and the experimental period; the bars indicate the means and vertical lines the standard errors. The bars indicate the means and vertical lines the standard errors. Analysis performed by ANOVA, followed by the Tukey post-hoc test for pairwise comparisons. (*P < 0.05)



Inflammation

In mice with Akt1 (Cre-), there was a significant increase in the number of PMNs in the periodontal microenvironment at 2 weeks, but not at 6 weeks. The number of infiltrating PMNs in Akt1-deleted mice (Cre+) was slightly (no statistical significance) increased at two weeks; moreover there were significantly less infiltrating PMNs in Cre+ mice in comparison with the Cre- mice at both 2- and 6-week periods (Figure 5). These results indicate that expression of Akt1 in DCs has an important role in the migration of PMNs to periodontal disease sites.

Figure 5: Infiltration of PMNs in the periodontal microenvironment. – Number of PMNs infiltrating the connective tissue adjacent to the junctional epithelium. PMNs were identified by their nuclear morphology in H&E-stained sections of Cre- and Cre+ mice at each experimental period . The bars indicate the means and vertical lines the standard errors. (*$p < 0.05$)

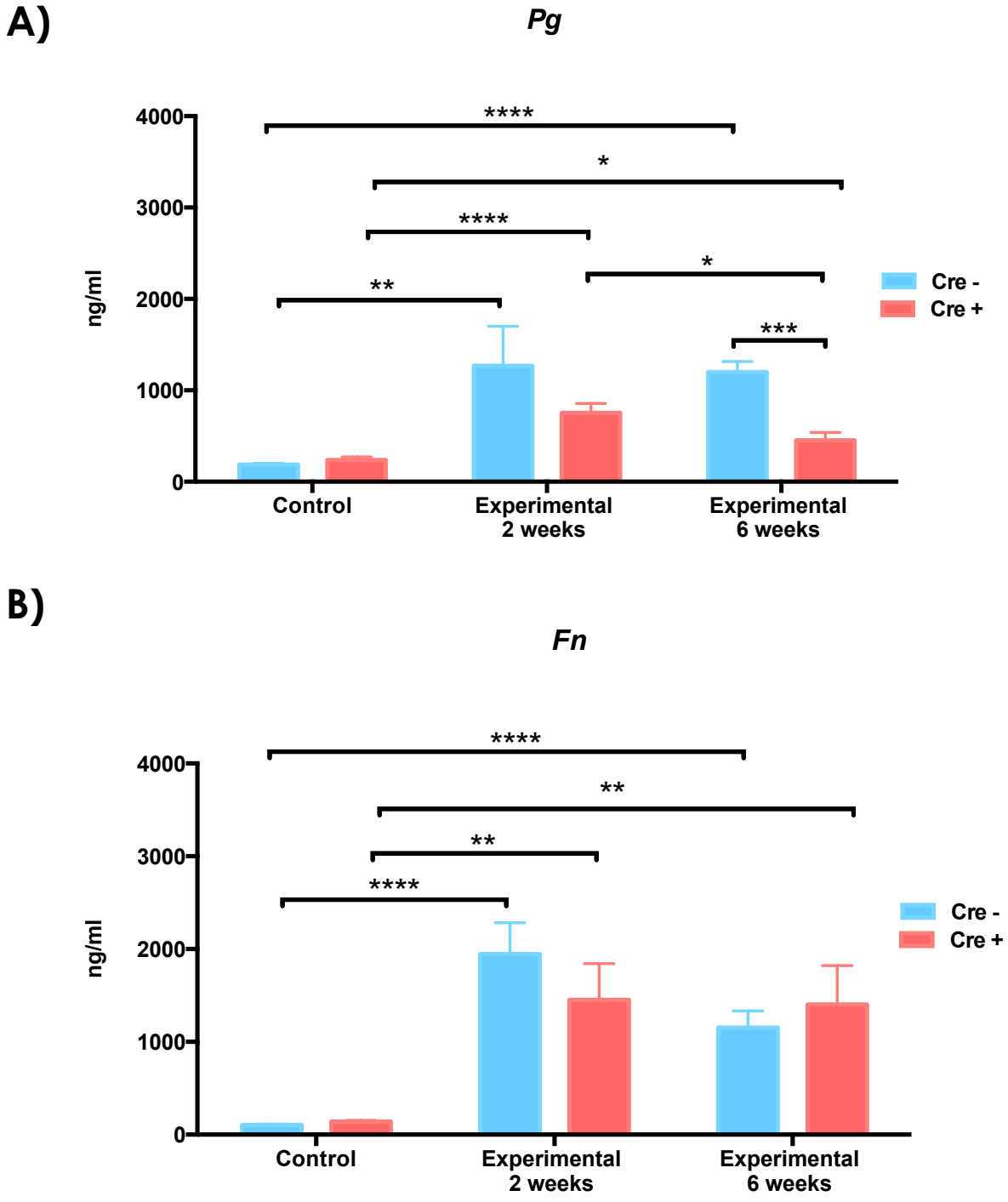


ELISA

There was a significant humoral response to *Pg* in Cre- mice, with significant increase in the concentration of *Pg*-specific antibodies in the serum at 2-week period, which was sustained at the 6-week period (Figure 6A). Interestingly, the humoral response to *Pg* was markedly attenuated at 2-weeks in Cre+ mice in comparison with the Cre- mice and this trend reached statistical significance at 6-weeks. These results indicate a deficient activation of the humoral response to *Pg* by Akt1-deleted DCs.

Cre- mice showed a pronounced and significant increase in the concentration of *Fn*-specific antibodies at the 2-week experimental period, which was attenuated at 6 weeks. In contrast with the results for the *Pg*-specific antibodies, the humoral response to *Fn* in Cre+ mice was preserved (Figure 6B). These results indicate that Akt1 expression by DCs is not required for the activation of humoral response to *Fn*. It is possible that DCs have a different role in the presentation of antigens from *Pg* and *Fn*, which may be compensated by the participation of macrophages in this experiment. It is also possible that *Fn* is more antigenic than *Pg* in the mouse model system. A greater antigenicity of *Fn* would overwhelm the defective activation of humoral response by Akt1-deleted DCs.

Figure 6: Assessment of the humoral response to *Porphyromonas gingivalis* (*Pg*) and *Fusobacterium nucleatum* (*Fn*) periodontopathogens by ELISA. – **A)** Concentration of *Pg*-specific antibodies in the serum of Cre- and Cre+ mice according to the experimental period; **B)** Concentration of *Fn*-specific antibodies in the serum of Cre- and Cre+ mice according to the experimental period. The bars indicate the means and vertical lines the standard errors. (*p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001)



Discussion

A hallmark characteristic of destructive periodontal disease is the inflammatory resorption of alveolar bone due to an imbalance on bone turnover associated with an increase in the osteoclastogenesis. Dendritic cells (DCs) are observed near the alveolar bone in periodontal disease[25] and have a fundamental role in the activation of adaptive immunity. DCs are also osteoclast precursor cells and, as such, may participate directly in the pathological processes associated with bone resorption. Akt signaling has shown to be relevant in various immune cells. For example, Akt has a pro-survival role in neutrophils[26]. The relevance of Akt signaling on immune response and host-microbe interactions is evidenced by the role of phosphotididylinositol-3-kinase (PI3K, the major upstream activor of Akt) in TLR4 expression by macrophages[27], also on the activation of neutrophils by TLR2 ligands, in which Akt role is as an intermediate upstream activator of NF-kB[28]. However, there is scarcity of information concerning the relevance of the PI3K-Akt axis specifically in dendritic cells. This study addresses a gap in the knowledge on Akt signaling in dendritic cells using an experimental periodontal disease model that recapitulates a chronic inflammatory condition of infectious/bacterial origin that is associated with destruction of non-mineralized and mineralized connective tissue.

Akt1 has an important role in osteoblast differentiation and, indirectly, in osteoclastogenesis[22] as Akt1-deficient mice present increased osteoblast differentiation and decreased activity and reduced numbers of osteoclasts. These phenotypical characteristics are associated with an inhibition of RANKL and OPG expression by osteoblasts[20,21]. In this study we used transgenic mice with DC-specific deletion of Akt1 to assess the role of Akt1 signaling in DCs for the progression of periodontal disease. Our results from the μCT and histomorphometric analyses (Figures 1 and 3) demonstrate that the experimental periodontal disease model induces significant bone resorption at 2-weeks in both control mice (Cre-, CD11c.Cre-/Akt1L/L) and mice with DC-specific Akt1-deletion (Cre+, CD11c.Cre+/Akt1L/L).

Destruction of periodontal attachment associated with experimental periodontal disease was significantly reduced at the 6-week period in Cre+ mice (Figure 2). These results are in agreement with the uCT and histomorphometric data showing inhibition of bone resorption at the 6-week period, indicating that loss of Akt in DCs has a greater influence on the mediate/late immune response in this experimental model. In support to this observation,

the number of infiltrating PMNs was also significantly reduced at the 6-week period (Figure 5). The 'late' effect of Akt1 deletion is supported by the finding that humoral response to *Pg* was significantly inhibited only in the 6-week experimental period (Figure 6). Interestingly, the humoral response to *Fn* was not influenced by the deletion of Akt1 in DCs, suggesting that either DC-mediated activation/antigen presentation is not critical for production of immunoglobulins to this bacterium species or that the recognition and response of DCs to *Fn*-derived antigens does not require Akt1 signaling.

Interestingly, inflammatory bone resorption was significantly attenuated in Cre+ mice only at the 6-week period. The results indicate that deletion of Akt1 expression in DCs did not prevent the early inflammatory bone resorption in this model, as the maximum extent of bone resorption assessed by μCT was observed at the 2-week time period, without a significant difference between Cre- and Cre+ mice. It is possible that deletion of Akt1 in DCs affect their response to the cues in the microenvironment and in a manner that promotes a change in the bone turnover only in late periods. This 'delayed bone-sparing effect' observed in Cre+ mice may be related with an effect on inflammation by a change in the 'long-term' (sustained) activation of the adaptive immune cells affecting the microenvironment and osteoclast differentiation and activity indirectly. Alternatively, Akt1-deleted DCs may present a reduced capacity for osteoclastic differentiation; which would alter bone turnover towards bone formation. It is also possible that these two phenomena occur simultaneously, as they are not mutually exclusive.

The fact that inflammatory bone resorption was not affected in Cre+ mice at the 2-week experimental period is particularly intriguing, given that in this same period there was a marked reduction in the number of osteoclasts (Figure 4) and a significant decrease in PMN infiltration (Figure 5). We speculate that other non-professional immune resident cells (e.g., osteoblasts, fibroblasts) capable of responding to *Pg*- and *Fn*-derived antigens are mainly responsible for the initial unbalance in bone turnover by enhancing osteoclast activity through the secretion of biologically-active mediators such as RANKL, IL6, IL1 and TNF. It is also possible that the microenvironment of the periodontal tissues in Cre+ mice reduce osteoblastic anabolic activity in the early stages of periodontal disease induction.

This 'early-stage' microenvironment may have affected osteoclast catabolic activity and not osteoclast differentiation, since the number of osteoclasts in Cre+ mice was significantly reduced only at the 6-week experimental period. This late decrease in osteoclast

numbers is correlated with the attenuation of bone resorption observed in Cre+ mice at the 6-week experimental period (Figures 1 and 3). It is tempting to speculate that part of this decrease in osteoclast numbers is due to a direct effect on the osteoclastic differentiation in Akt1-deleted DCs and deletion of Akt1 may have affected this event directly. The diminished activation of immune and non-immune cells by the Akt1-deleted DCs may have affected the type and/or relative quantity of cytokines produced in the local microenvironment, which in turn could affect the differentiation of macrophages (the other major osteoclast precursor) into osteoclasts.

It is important to consider the limitations of our analysis, as we identified the polymorphonuclear neutrophils (PMNs) based on cellular morphology, which is not as specific as an immunohistochemical approach and, we did not quantified other cell types in the gingival tissues, as lymphocytes. In summary, the results indicate that Akt1 signaling in dendritic cells is critically relevant for the late immune response and inflammatory bone resorption in the experimental periodontal disease model. However this study has some limitations.

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4.3 Publicação 3

Role of Akt signaling in the response of dendritic cells to P. gingivalis· *

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Abstract

DCs participate in the dynamic regulation of innate and adaptive immunity, having an important role in chronic inflammation. Akt is a major downstream target of PI3K and regulates inflammation. Akt has a critical role in immune and autoimmune processes and conditions. PI3K can impact DC cell activation and function, affecting the inflammatory response directly and indirectly. Given the relevance of DCs in chronic inflammation and the critical role of Akt signaling for their function, the aim of this study is to assess the role of Akt1 signaling in the biology of dendritic cells, including their response to *P. gingivalis*. Primary bone marrow-derived dendritic cells (BMDC) were differentiated and Akt signaling was inhibited by biochemical inhibitors. Outcomes assessed (related to dendritic cell biology) were: proliferation, apoptosis, phagocytic activity, migration, antigen presentation and osteoclastogenesis. The assays showed that Akt1-3 and Akt1 inhibitors at the effective concentrations did not affect cell survival; Cell proliferation was inhibited by PI3-kinase, Akt1-3 and Akt1 inhibitors; Inhibition of PI3-Kinase and Akt1-3 significantly reduced chemotaxis of BMDCs, while PI3-kinase inhibitor caused a reduction of the phagocytic activity. Antigen presentation and osteoclastogenesis was affected by PI3-kinase, Akt1-3 and Akt1 inhibitors. The results indicate that Akt1 signaling is required for proliferation, phagocytosis, antigen presentation and osteoclastic differentiation of dendritic cells.

Key Words: Dendritic Cells, Akt , Akt1, PI3-kinase

Introduction

Biological activation and function of dendritic cells (DCs) depend on their high sensitivity to microenvironmental cues, which is mediated by different pattern recognition receptors (PRRs), such as C-type lectin receptors (CLRs), Toll-like receptors (TLRs) and Nod-like receptors (NLRs); as well as by cytokine and chemokine receptors[1]. These cells are able to recognize a variety of external antigens of both microbial (bacterial, viral) and non-microbial origin (fungal, protozoal commensals, allergens, pollutants), as well as damage-associated molecule patterns (DAMPs). Activation and maturation affect the phenotype and biological functions of DCs themselves, including antigen presentation[2] and the production of Th-type-response polarizing cytokines which will continuously and dynamically shape the immune response. Activated DCs produce various cytokines, including interleukin (IL) 1, IL-6, IL-10, IL-12, IL-23, IL-27, and tumor necrosis factor-α (TNFα)[3], which will affect the activation and biological activity of other innate and adaptive immune cells. e.g. IL-12 and IL23 bring on the induction of a Th-1 inflammatory response[4], whereas LPS of oral pathogen *P. gingivalis* stimulates IL-10, promoting a Th-2 effector response[5,6]. On the other hand, immature (i.e., non-activated) DCs are not only poorly stimulatory, but can exert an inhibitory effect associated with partial activation of T cells, such as: deletion of auto-reactive CD8+ T cells[7-9], development of self-tolerance by triggering Foxp3+ regulatory CD4+ T cells, and suppressing auto-reactive T cells[10].

Activation of DCs is also induced by pro-inflammatory cytokines such as TNFα and type I interferons (IFNs)[11-15]. This suggests that signaling via cytokine receptors can substitute PRR signaling and/or act in synergy with PRRs during the activation of DCs[16-18]. The diversity of receptors expressed by DCs that may be activated by the environmental cues indicates that multiple signaling pathways can be involved. MAPK-, MyD88- and Raf/PI3K-medited activation of NF-kB[19] are major signaling pathways activated downstream of PRRs and cytokine receptors; however PI3K-Akt-Nrf2 and PI3K-mTOR-NF-kB axis are also associated with DC activation[20]. PI3K can be activated downstream of TLRs, and since TLRs play an important role in the regulation of pro-inflammatory and anti-inflammatory cytokine production[21-24], PI3K and its downstream targets may also be involved in the regulation of cytokine production by DCs, and thus PI3K can impact DC cell activation and function, affecting the inflammatory response directly and indirectly[25]. Akt is a major downstream target of PI3K, which functions as a negative regulator of FOXO1. Akt has a critical role in immune and autoimmune processes and conditions, such as rheumatoid arthritis[26], psoriasis[27],

asthma[28] and atherosclerosis[29]. Thus, activity of Akt is directly involved in the regulation of inflammation, with most of the available evidence related its function in innate immunity due to its expression in neutrophils, macrophages and DCs[30].

Active periodontal disease is considered a dysbiotic process[31], in which DCs play a significant role in sensing and responding to the microenvironmental cues. DCs participate in the dynamic regulation of innate and adaptive immunity, which directly affects the destruction of non-mineralized connective tissue and bone. Given the relevance of DCs in chronic inflammation and the critical role of Akt signaling for their function, this study assesses the role of Akt1 signaling in the biology of dendritic cells, including their response to the periodontopathogen *P. gingivalis.*

Material and Methods *

Murine primary bone marrow-derived dendritic cells (BMDC) and splenocytes

Bone marrow stromal cells (BMSC) were obtained by flushing the marrow cavity of the femurs of male C57BL/6 mice[32]. Primary bone marrow-derived dendritic cells (BMDC) were differentiated with GM-CSF (20 ng/mL, Peprotech Inc.) following the protocol described by Lutz et al.[32]. Primary splenocytes were obtained from C57BL/6 mouse spleens by mechanical disruption followed by three repeated filtrations of the homogenates through a 50 μm pore cell strain. Differentiation of immature dendritic cells (CD11c+/MHC-II-) was verified by flow cytometry. BMDC and primary splenocytes were grown in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1mM of pyruvate, 1X non essential amino acids, 100 U/mL penicillin and streptomycin 100 μg/mL at 37°C in humidified atmosphere of air and 5% CO2. These experiments were conducted with the approval of the ethics committee (CEUA/FCF/Car:01/2016) (Anexo C).

*** APÊNDICE B**

Biochemical inhibitors

The working concentrations of the biochemical inhibitors LY294002 (cat# 440202, Sigma-Aldrich, 10μM), Triciribine (cat# T3830, Sigma-Aldrich, 1μM) and BML-257 (cat# sc-200666, Santa Cruz Biotechnology, 12.5μM) were adjusted in pilot experiments (data not shown). Controls were prepared by adding DMSO (cat# W387520, Sigma-Aldrich) vehicle at the highest volume used for the inhibitors (1.25 μL/mL). Inhibitors were always added directly to the culture medium 40 min before treatment of the cells. For the proliferation assay, culture medium was replaced every 24 h with fresh medium containing the inhibitors.

P. gingivalis

Porphyromonas gingivalis (ATCC 33277) was cultured in Tryptic Soy Agar supplemented with 5% defibrinated sheep blood, 0.5 mg/mL hemin and 1 mg/mL menadione. After 3 days of culture in an anaerobic chamber (85% N2, 5% CO2 and 10% H2) at 37°C, bacteria was harvested at the logarithmic phase of growth and resuspended in phosphate buffered saline (PBS). To calculate the amount of bacteria, we adjusted an OD of 0.5 in a wavelength of 495 nm, which was determined to correlate to 10 CFU/mL in preliminary experiments.

Immunoblot

Whole cell lysates were prepared with a commercially-available lysis buffer (Cell Lysis Buffer, cat# 9803, Cell Signaling Tech., Inc.) supplemented with protease (Complete Protease Inhibitor Cocktail Tablet, EDTA- free, cat# sc-29131, Santa Cruz Biotech Inc., USA) and phosphatase (PhosSTOP, cat# 04906837001, Roche Ltda., Germany) inhibitors. After quantification (Bradford method - Quick Start Bradford protein dye reagent, cat# 500-0205, Bio-Rad Lab. Inc., USA), 20 μg of total protein were diluted in sample buffer containing SDS and DTT and denatured by heating at 95°C for 5 min. SDS-PAGE electrophoresis was done using 12% polyacrylamide discontinuous gel (constant 100 V, 60 min), followed by wet transfer to PVDF membrane (constant 300 mA, 60 min). After blocking non-specific binding using 5% BSA in TBS/Tween solution for 2 hours, the membrane was incubated with optimized dilution (1:1000) of the primary antibody (anti-phospho Akt (Thr308), cat# 2965S, Cell Signaling Tech.) for 18 hours at 4°C under gentle shaking, followed by washes with TBS/Tween and incubation with HRP-conjugated species-specific secondary antibody for 1 hour at room temperature. Detection of the target proteins

was done with a chemiluminescent development kit (SuperSignal West Pico Chemiluminescent Substrate, cat# PI34077, Pierce Biotech., USA) followed by real-time visualization and digital documentation in a chemiluminescence detection system (Chemi-Doc, Bio-Rad, USA) .

Apoptosis

Detection of Annexin V and incorporation of 7-Aminoactinomycin D in the genomic DNA was used to assess apoptosis by flow cytometry, according to the instructions of the supplier of the reagents (PE Annexin V Apoptosis Detection Kit I, cat# 559763, BD Biosciences). Briefly, cells were exposed to the biochemical inhibitors at the effective concentrations for 24h, collected with a non-enzymatic reagent (Accutase cell detachment solution, cat# 561527, BD Biosciences), resuspended and washed 2 consecutive times in PBS at 4°C, counted in hemocytometer and resuspended in binding buffer at $1x10^6$ cells/mL. This cell suspension was stained with Annexin V conjugated to PE and 7-AAD for 15 minutes in the dark. A minimum of 30,000 events/sample were acquired on a FACSVerse flow cytometer (BD Biosciences). Camptothecin (cat# C9911, Sigma-Aldrich, 8μM) was used as a positive control for apoptosis.

Cell Proliferation

Cell proliferation and viability were determined every 24 h for 3 days by direct cell counting on a hemocytometer using 0.2% trypan blue to detect non-viable cells.. In these experiments, $3x10^4$ cells were plated in each well of 96-well plates (100μL volume). Negative control wells were treated with Mitomycin C (cat # M4287, Sigma-Aldrich, 10μg/mL) added to the culture medium only at the beginning of the experiment.

Chemotaxis

Chemotaxis was assessed by transwell assays (12-well culture plates with 8μm pore polyester membranes). BMDC were counted, resuspended in reduced FBS (1% vol:vol) culture medium and $5x10^4$ BMDCs were seeded on the upper chamber in a volume of 300μL. 600μL of culture medium supplemented with reduced FBS (1%, vol:vol) and 200ng/mL of CCL-19 (cat# 250-27B, Peprotech Inc.) as a chemotactic stimulus were added in the lower chamber. After 18 hours, the cells on the top of the membranes were gently removed with sterile cotton swabs, membranes were cut out from their supports and stained with DAPI (5

min, RT – cat# F6057, Sigma-Aldrich). After mounting on slides with acqueous mounting medium, membranes were visualized and digital images (400X magnification, covering the entire membrane) obtained using standardized exposure settings in an inverted fluorescence microscope (Evosfl, AMG Micro). Cells present in the lower portion of the membrane were counted by a trained examiner, without knowledge of the experimental conditions.

Phagocytic activity

$1.5x10^6$ of live *P. gingivalis* (ATCC 33277) previously labeled with red fluorophore (BacLight red bacterial stain, cat# B35001, Molecular Probes, ThermoFisher Scientific) were added to a 70% confluent culture of BMDCs ($1.5x10^5$ cells, 10:1 MOI) for 2 hours. BMDCs were washed three times with PBS at 4°C, fixed in 4% paraformaldehyde (Cytofix, cat# 554655, BD Biosciences) for 15 min, permeabilized for 15 min in buffer containing saponin (Perm/Wash, cat# 554723, BD Biosciences) and subsequently stained with phalloidin conjugated to AlexaFluor 488 (cat# A12379, Molecular Probes, ThermoFisher Scientific) and DAPI (cat# D9542, Sigma-Aldrich, 0.5 μg/mL) Digital images on phase contrast, red, green and blue fluorescent channels were obtained using an inverted fluorescence microscope (Evos fl, AMG Micro) in 5 random fields of each well (12-well plate) at 200X magnification. A trained examiner without knowledge of the experimental conditions performed the differential count of total cells and the number of phagocytic cells using the overlayed images from the three fluorescent channels. The percentage of phagocytic cells (phagocytic index) was calculated

Antigen presentation

BMDCs were labeled with a fluorescent compound (Cytolabeling Orange, cat # ab176737, Abcam) following the manufacturer's instructions. $1.5x10^5$ cells were plated in each well of a 96-well plate (100 uL volume). Cells were co-cultured with $1.5x10^6$ *P. gingivalis* (MOI 10:1) for 8 hours. After 3 successive washes with PBS, of $5x10^5$ primary splenocytes pre-labeled with 100μl of carboxyfluorescein (CFSE, cat# C34554, Molecular Probes, ThermoFisher Scientific) were added to each well containing BMDCs and co-cultured for 24 hours. At the end of the co-culture period, the cells were washed gently 3 times with PBS and fixed in 4% paraformaldehyde (Cytofix, BD Biosciences). Genomic DNA was labeled with DAPI (cat# D9542, Sigma-Aldrich, 0.5 μg/mL) for 5 min. Analysis was done in digital images obtained an inverted fluorescence microscope (Evos fl, AMG Micro). Images

were obtained using standardized exposure settings and analyzed by a trained examiner without knowledge of the experimental conditions. Total BMDC (stained in red) and BMDCs that were in direct contact with splenocytes (stained in green) were counted in 5 random fields at 200X magnification. The percentage of antigen-presenting cells was calculated.

Osteoclast differentiation

BMDCs were resuspended in α-MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin at $1x10^6$ cells/mL. 5 µL of this cell suspension ($5x10^3$ cells) were plated in the center of each well in a 96-well plate. After 15 min, 95 µL of complete α-MEM supplemented with 30ng/mL of M-CSF (cat# 416-ML, R&D systems) and 4ng/mL of RANKL (cat# 462-TEC, R&D systems) were carefully added to each well. After 4 days, cells were fixed with 4% paraformaldehyde and cytochemical staining for TRAP activity was performed using a staining kit (Acid Phospatase, Leukocyte, TRAP kit, cat # 3871, Sigma-Aldrich), according to the supplier's instructions. TRAP-positive cells with three or more nuclei were identified as osteoclasts. All osteoclasts in each well were counted using a bright field inverted microscope.

Data analysis

Unless noted otherwise in the figure legend, all experiments were repeated independently three times and analyzed in duplicate or triplicate. Continuous and discrete data was checked for normal distribution using the Shapiro-Wilk test. The purpose of the statistical analysis was to assess the differences between each experimental condition and the control group for each outcome. The null hypothesis for all analyses was that there is no difference between each of the experimental conditions and the control group. ANOVA followed by the Tukey post-hoc test for pairwise comparisons or unpaired t-tests were used for the analysis. Significance level was set at 95%. All analyses were performed using GraphPad Prism 5.0 (GraphPad Software).

Results

Differentiation of BMDC

85% of the cells had CD11c expression, and of these cells only 0.05% expressed MHC-II, indicating that BMDC obtained using the differentiation protocol in this study are imature cells (Figure 1).

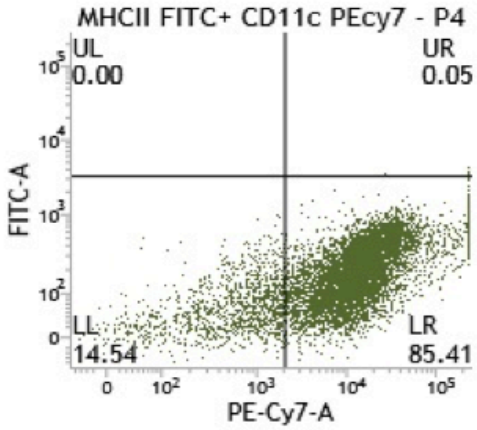

Figure 1: Differentiation of naïve BMDC. – Results from flow cytometry, confirming that differentiated cell are BMDC (85%) in imature state. Representative data from two independent experiments analyzed in triplicate.

Apoptosis

Initially, we assessed the cytotoxicity of the efficient concentrations (determined in preliminary experiments) of each biochemical inhibitor to avoid bias in the subsequent experiment. The effective concentration of the Akt1-3 (Triciribine, 1μM) slightly reduced the percentage of viable cells (58.45% in comparison with 67.24% in the DMSO vehicle). However, since it was the lower effective concentration, we performed the subsequent experiments using this concentration. Both PI3-kinase (LY294002, 10μM) and Akt1 (BML-257, 12.5μM) inhibitors at the effective concentrations did not affect cell survival (Figure 2).

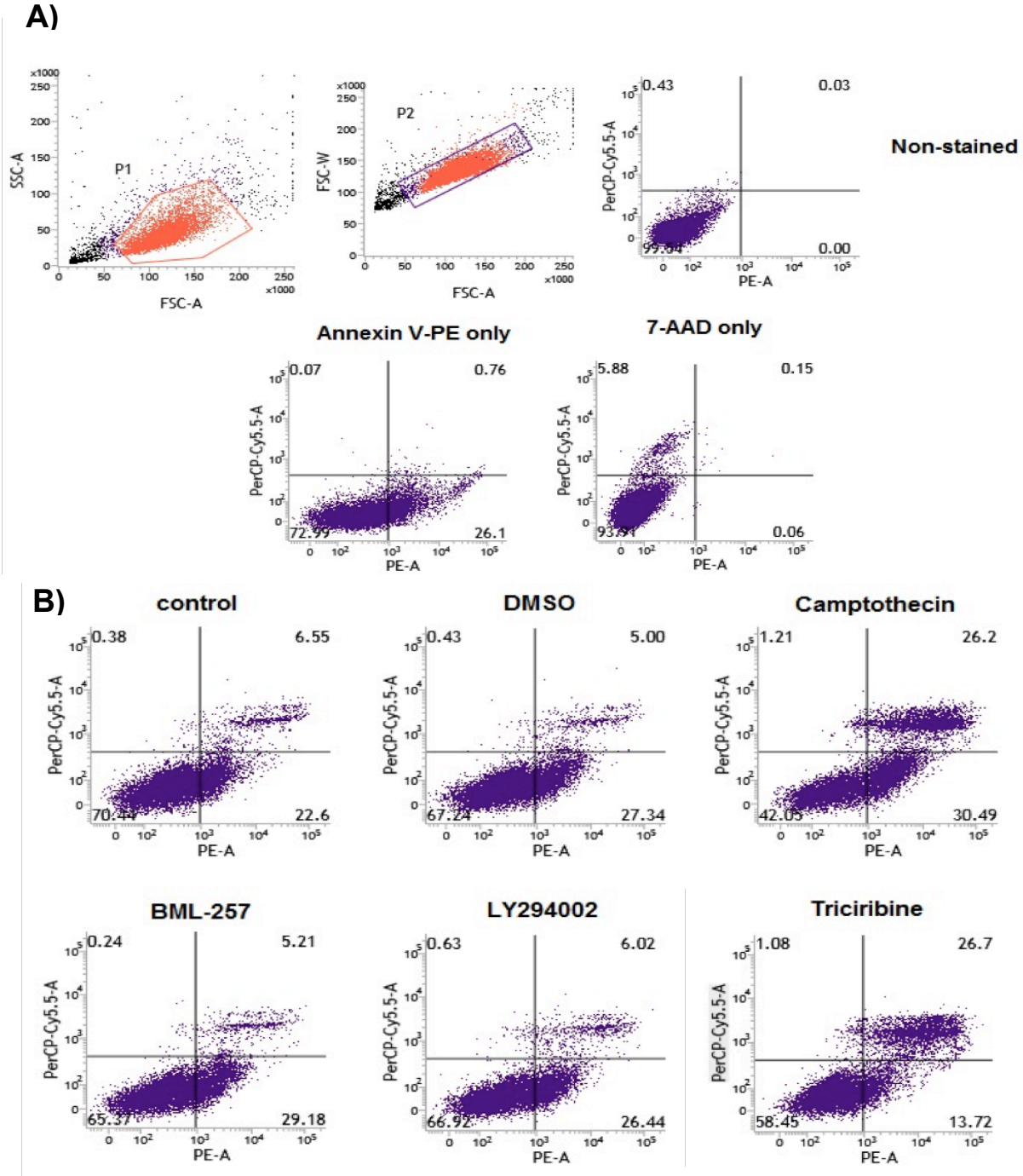

Figure 2: Apoptosis. – Representative dot-plots of three independent experiments, each with acquisition of 10,000 events. **A)** Shows the experimental controls of the apoptosis assay. **B)** Shows that percentage of viable, early and late apoptotic cells after 24 h of treatment with each biochemical inhibitor: Akt1-3 (Triciribine), PI3-kinase (LY294002) and Akt1 (BML-257). Representative data from two independent experiments, analyzed in duplicate.

Proliferation

Cell proliferation was significantly inhibited by all three biochemical inhibitors at both 48 and 72 h, indicating that signaling via PI3-kinase and Akt is required for the proliferation of dendritic cells (Figure 3). Notably, proliferation was significantly inhibited by the Akt1-3 inhibitor (Triciribine) already at 24 h.

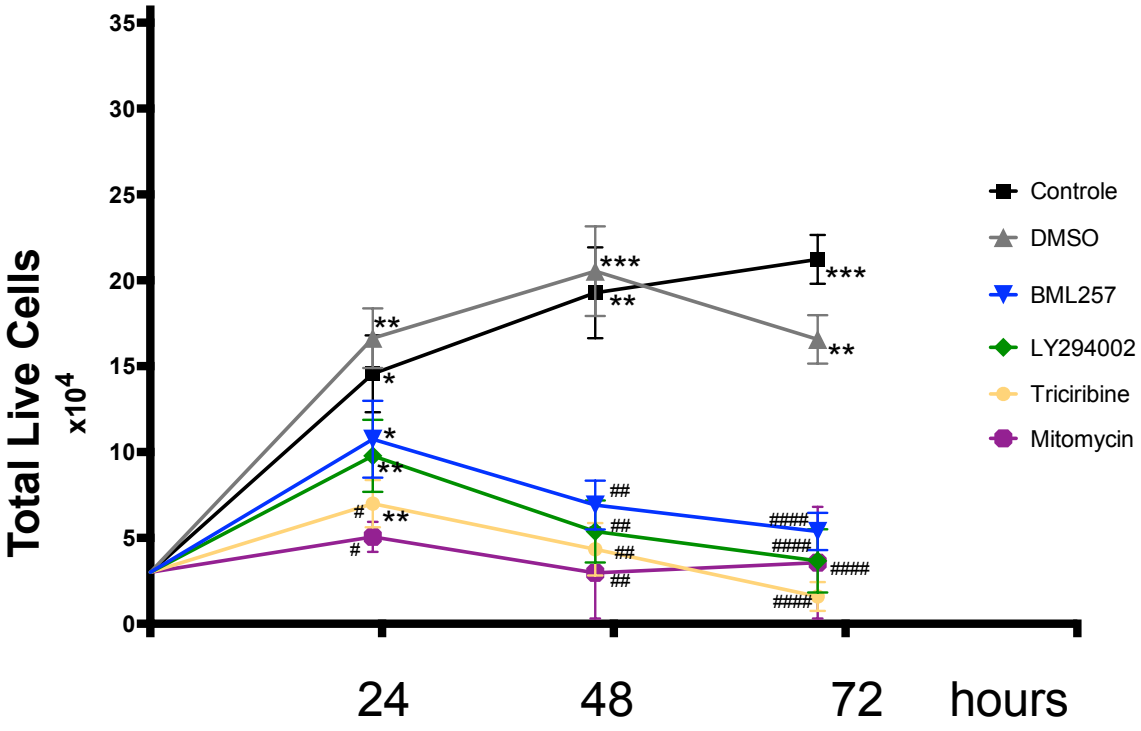

Figure 3: Proliferation. –Treatment with mitomycin C and Triciribine (Akt1-3 inhibitor) significantly inhibited cell proliferation already at the 24 h period. LY294002 (PI3-kinase inhibitor) and BML-257 (Akt1 inhibitor) significantly reduced cell proliferation in comparison to negative control and vehicle control at the 48 and 72 h periods. Analysis was performed by ANOVA, followed by the Tukey test for paired comparisons of the different experimental conditions at each period (24, 48 and 72 h). ANOVA followed by the Tukey post-hoc test was also used for the comparisons between the different periods (24, 48 and 72 h) within the same experimental condition. The asterisk ("*") indicates a significant difference between the indicated period and the baseline (day '0'), for each experimental condition: *p<0.05, **p<0.001, ***p<0.0001. The hashtag symbol ("#") indicates a significant difference between a given experimental condition and the control at each experimental period (24, 48 and 72 hours) (#p <0.05, ## p <0.001, #### p <0, 0001). This experiment was repeated three times and analyzed in triplicate.

Chemotaxis

Inhibition of PI3-Kinase (LY294002) and Akt1-3 (Triciribine), but not of Akt1 (BML-257), significantly reduced chemotaxis of BMDCs (Figure 4).

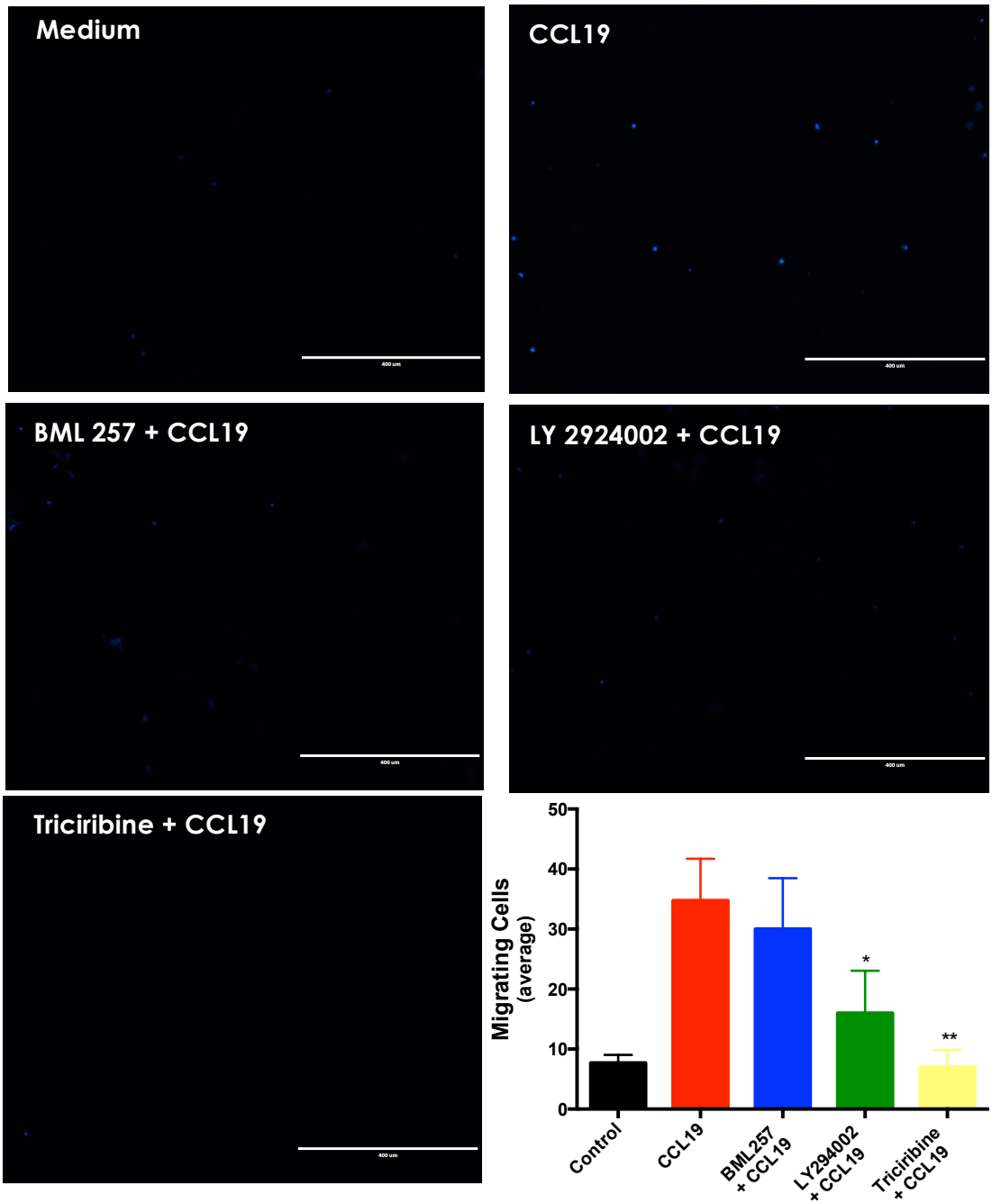

Figure 4: Chemotaxis. – Representative images of DAPI-stained 8µm pore membranes (central portion of membrane) obtained on an inverted fluorescence microscope at 100X magnification. Analysis performed by ANOVA, followed by the Tukey test for paired comparisons. "*" Indicates a significant ($p < 0.05$) difference in comparison with the positive control group (CCL-19/DMSO treatment). Experiment was repeated independently three times and analyzed in duplicate.

Phagocytic activity

BMDC actively phagocytosed *P.gingivalis* in vitro, and this activity was significantly reduced by treatment with all three biochemical inhibitors in comparison with DMSO vehicle treatment ('Control'). The Akt1-3 inhibitor (Triciribine) caused a significantly greater reduction of the phagocytic activity in comparison with PI3-kinase (LY294002) or Akt1 (BML-257) inhibitors (Figure 5).

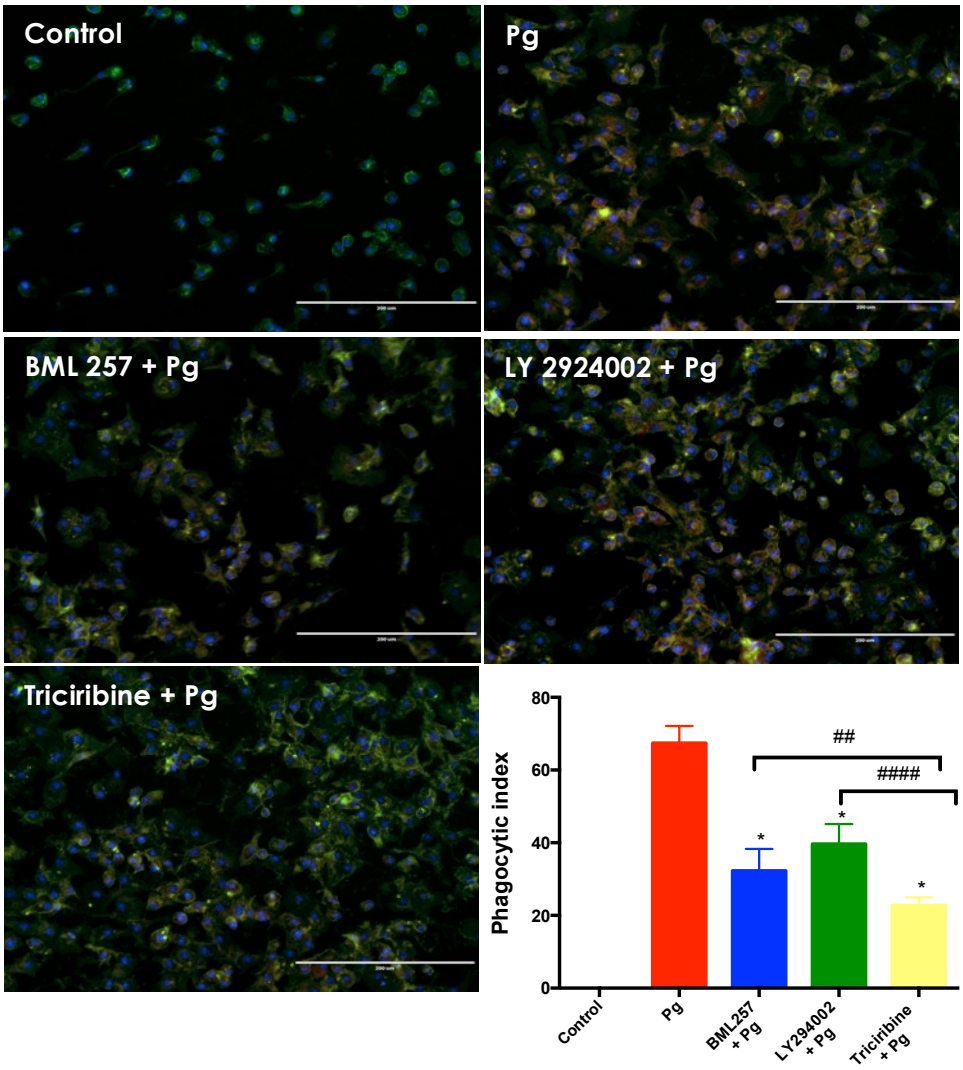

Figure 5: Phagocytic activity. – Representative overlayed (green, red, blue) fluorescent images of the phagocytic activity experiment, obtained at 200X. There was effective phagocytosis of the bacteria (stained in red) by BMDCs (stained with AlexaFluor488-conjugated phalloidin in green, DAPI for the genomic DNA in blue). Pretreatment of BMDCs with the biochemical inhibitors decreased phagocytic activity significantly in comparison with cells treated with the DMSO vehicle. Analysis performed by ANOVA, followed by the Tukey test for paired comparisons. Asterisk ("*") indicates a significant difference between groups when compared with *Pg*/DMSO control group ($p < 0.0005$). Hashtag symbol ("#") indicates a significant difference between the experimental groups and Triciribine group by the unpaired t-test (## $p < 0.005$, #### $p < 0.0001$).

Antigen presentation

Co-culture of BMDCs with live *P.gingivalis* induced a significant increase in the number of cell-to-cell contacts with splenocytes, which is indicative of antigen presentation. Treatment with all three inhibitors significantly reduced the number of direct contact between BMDCs and splenocytes. (Figure 6).

Osteoclastogenesis

Treatment with RANKL effectively differentiated BMDC into osteoclasts. All the biochemical inhibitors significantly reduced the number of differentiated osteoclasts. Inhibition of osteoclast differentiation with BML-257 (Akt1 inhibitor) was less effective than LY294002 (PI3-kinase inhibitor) and Triciribine (Akt1-3 inhibitor) (Figure 7).

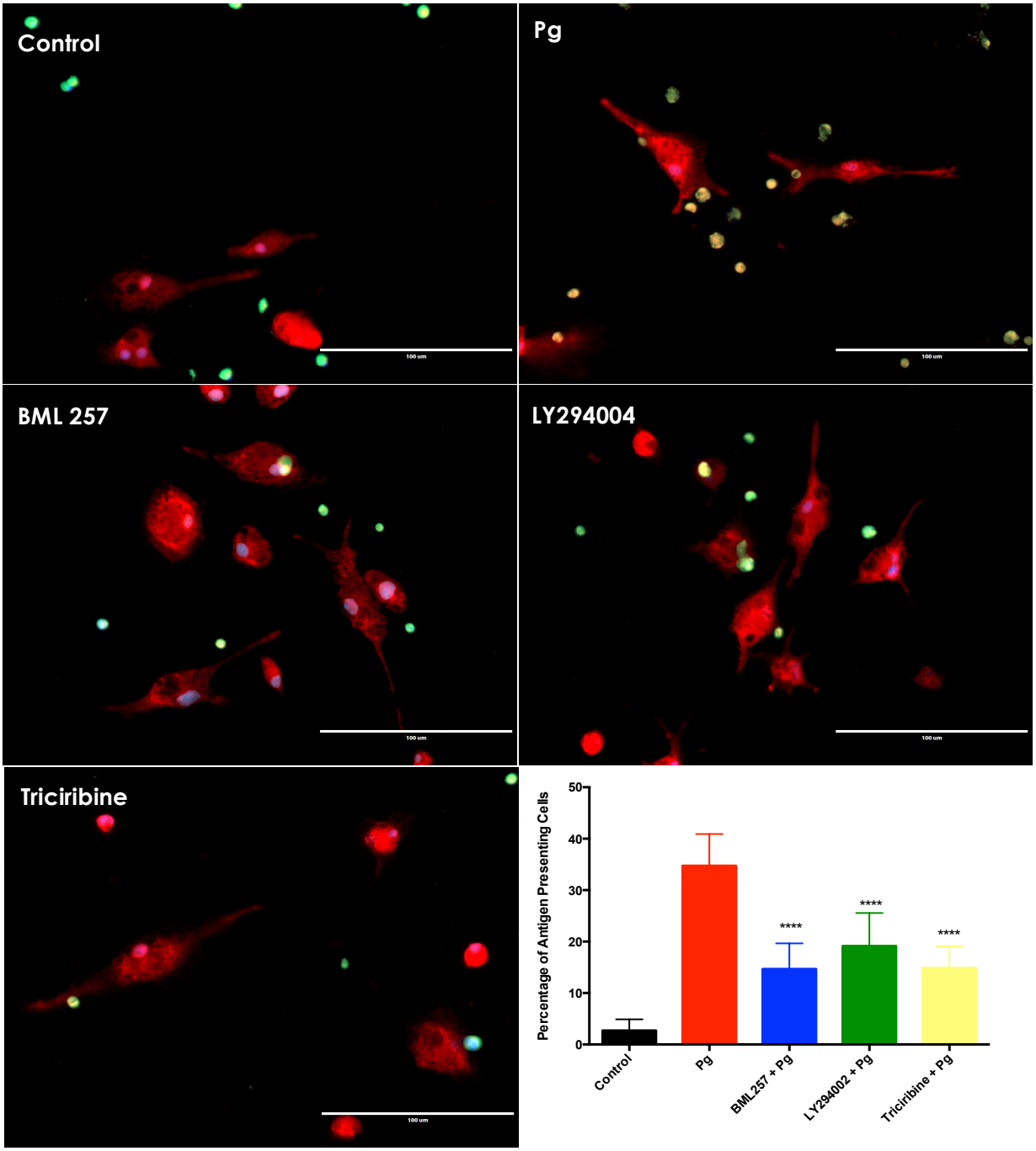

Figure 6: Antigen presentation. – Representative fluorescent images of co-cultures of BMDCs (stained in red) and splenocytes (stained in green) at 400X magnification. Direct contact is indicative of antigen presentation to T cells. Treatment with all three biochemical inhibitors caused a significant reduction in the percentage of dendritic cells in direct contact with splenocytes. Analysis performed by ANOVA, followed by the Tukey post-hoc test for pairwise comparisons. Asterisk ("*") indicates a significant difference between groups in comparison with BMDC treated with the DMSO vehicle alone (*** $p < 0.0005$, **** $p < 0.0001$).

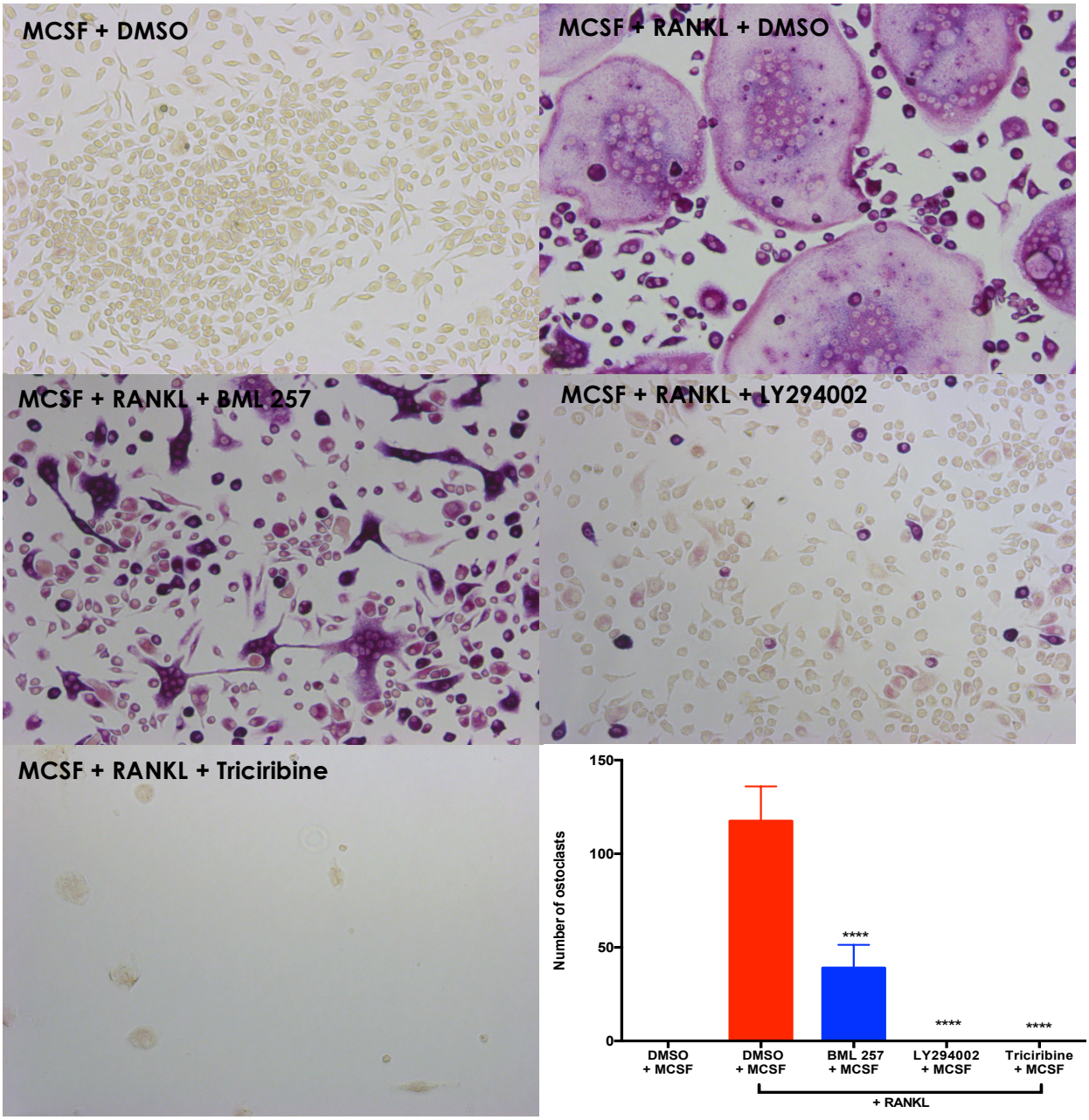

Figure 7: Osteoclastogenesis. – Representative images of RANKL-induced osteoclast differentiation of BMDCs. **A)** BMDC treated with DMSO and M-CSF; **B)** BMDC treated with DMSO, M-CSF and RANKL; **C)** BMDC treated with BML 257, M-CSF and RANKL; **D)** BMDC treated with LY294002, M-CSF and RANKL; **E)** BMDC treated with Triciribine, M-CSF and RANKL. All images obtained at 200X magnification. Graph shows osteoclast numbers and the significant decrease in osteoclastogenesis after treatment with BML257, LY294002 and Triciribine. Analysis performed by ANOVA, followed by the Tukey post-hoc test for paired comparisons. Significant difference between groups when compared to BMDC treated with the DMSO + M-CSF +RANKL (**** $p < 0.0001$).

Discussion

Akt plays an essential role in physiological and pathological mechanisms due to its involvement in cell metabolism, proliferation and survival[33]. Activation of Akt is mediated by phosphatidyl inositol-3-kinase (PI3K), which can be activated by several receptors and external ligands, including molecular pattern receptors (PRRs), cytokine and growth factor receptors. This suggests that Akt-signaling plays a relevant role in the immune response. Most of the evidence on the expression and biological relevance of Akt in immune cells such as neutrophils, macrophages and lymphocytes. There is a lower relative abundance of information regarding the specific relevance of the PI3K-Akt axis in dendritic cells. Evaluation of apoptosis by flow cytometry indicated that inhibition of Akt reduced cell survival (Figure 2). There is evidence showing that pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs) including cytokines and other mediators such as leptin, can activate Akt in dendritic cells, which results in inhibition of apoptosis of these cells by modulating anti-apoptotic cascades (Bcl-2, Bcl-xL)[34]. Specifically, the Akt1 isoform promotes the expression/activation of Bcl-2, but not of Bcl-xL, in response to stimuli of microbial nature (LPS, associated with innate immune function) or host-derived molecules (CD40, associated with adaptive immunie function)[35,36]. Thus, inhibition of Akt in dendritic cells may interfere with different mechanisms of cell survival, and the reduction of dendritic cell viability may affect the immune response by reducing the effectiveness of innate immunity (cytokine production, phagocytosis) and also adaptive immunity (due to impairment in the antigen presentation function and reduction in the production of T and B lymphocyte activating cytokines). On the other hand, inhibition of PI3-Kinase/Akt reduced dendritic cell proliferation (Figure 3), which may be directly related to the activity of cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (such as cyclin D1, p27Kip1) which are direct downstream targets of Akt in macrophages, for example[30]. The introduction of constitutively active forms of Akt in dendritic cells increased their proliferation in vitro and in vivo[37]. Our results suggest that Akt1 plays a leading role in the control of in vitro dendritic cell proliferation, since the use of the Akt1 selective inhibitor (BML-257) had the same impact on the proliferation of BMDCs as compared to the PI3-kinase inhibitor (LY294002). Reduced proliferation of dendritic cells can impair the immune response by reducing the efficacy the innate immune response and, more importantly, of the activation of adaptive immunity. Activation of adaptive immunity by dendritic cells involves primarily the processes of phagocytosis, chemotaxis and antigen processing/presentation.

The Akt1 selective inhibitor (BML-257), the Akt non-selective inhibitor (Triciribine) and the PI3-Kinase inhibitor (LY294002) all decreased the phagocytic activity of BMDCs significantly (Figure 5). Evidence indicates that Akt activity in macrophages phosphorylates/inhibits GSK-3, resulting in accumulation of beta-catenin which is also an Akt-phosphorylated substrate, in particular by Akt1, which increases the transcriptional activity of cytokines (such as interferon) and production of nitric oxide (NO), which are important components of the microbial death process after phagocytosis[38]. Akt2 activity, but not Akt1 or Akt3, is required for extracellular pressure-stimulated phagocytosis in macrophages[39]. Thus, Akt activity may be relevant not only for the phagocytosis, but also for the processes involved in intracellular killing of the phagocytized microorganisms. Since phagocytosis and processing of the phagocytosed microorganisms are influenced by Akt signaling, chemotaxis of dentritic cells may also be affected and compound on a defective activation of adaptive immunity.

There is evidence indicating that the PI3K-Akt axis plays an important role in the migration/chemotaxis of dendritic cells related to hypoxia conditions[40]. There is also evidence of the importance of Akt activity for neutrophil chemotaxis, specifically in the polarization and asymmetric re-organization of the cytoskeleton[41,42]. In neutrophils, Akt2, but not Akt1, is required for chemotaxis[43]. In dendritic cells, expression of the CCR7 receptor is regulated by the transcription factor FOXO1[44], which is a downstream target of Akt. Activation of Akt inhibits FOXO1, which would result in reduced CCR7 expression and, consequently, inhibition of chemotaxis of dendritic cells, which (once activated) need to migrate to the lymph nodes for effective antigen presentation, regulating MHC expression and costimulatory molecules (CD80 and CD86)[45]. Under transient hypoxia conditions, chemotaxis of dendritic cells is partially dependent on PI3K-Akt activity[46]. In this study, even in direct co-cultures that bypasses the need for chemotaxis, inhibition of PI3K, Akt1-3 or Akt1 decreased antigen presenting activity significantly (Figure 6). Strikingly and in contrast with the non-specific Akt inhibitor (Triciribine) and with the PI3-kinase inhibitor (LY294002), chemotaxis of BMDC cells in vitro was not markedly affected by the inhibition of Akt1, suggesting that Akt1 has a role in antigen presentation by dendritic cells that is independent of chemotaxis.

Immature DCs can differentiate into osteoclasts in the presence of RANKL, which is a relevant biological phenotype for chronic inflammatory conditions such as rheumatoid arthritis or periodontitis[47]. Inhibition of PI3-kinase (LY294002) or of Akt1-3 (Triciribine) completely abrogated the RANKL-induced osteoclast differentiation of BMDCs. Inhibition of Akt1 signaling caused a significant decrease in RANKL-induced osteoclast differentiation of

BMDCs (Figure 7), indicating that Akt1 alone plays a major role in the osteoclastic differentiation of BMDCs. Mice lacking Akt1 present diminished osteoclast activity, which was associated with a reduced expression of RANKL[48,49]. The results of the present study indicates that Akt, besides influencing RANKL expression and signaling, has a role in the response of dendritic cells to RANKL, which stresses the relevance of PI3K-Akt axis in osteoclastogenesis.

Collectively, the results indicate that Akt signaling, and Akt1 signaling specifically, has a prominent role in dendritic cell biology. The in vitro experiments using primary bone marrow-derived murine dendritic cells indicate that Akt1 signaling is required for proliferation, phagocytosis, antigen presentation and osteoclastic differentiation of dendritic cells. Inhibiting Akt1 signaling in a transient and controlled manner should be investigated as a possible immunosuppressive therapeutic approach in chronic inflammatory conditions associated with inflammatory bone resorption, such as rheumatoid arthritis and periodontitis.

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5 CONCLUSÕES

- A sinalização via Akt1 em células dendríticas parece ter um papel importante na patogênese da doença periodontal, podendo modificar a intensidade da resposta imune adaptativa assim como sua capacidade na diferenciação osteoclástica, modificando assim o *turnover* fisiológico do osso alveolar

- A sinalização via Akt é necessária para o desenvolvimento das funções biológicas (proliferação, fagocitose, apresentação de antígeno e diferenciação osteoclástica) das células dendríticas.

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APÊNDICES

APÊNDICE A - MATERIAL E MÉTODO (Publicação 2)

Animais

Animais com deleção de Akt1 em células dendríticas foram gerados por meio do sistema Cre- LoxP. Camundongos transgênicos expressando a enzima Cre recombinase sob o controle do promotor do gene CD11c (promotor que é ativo primariamente em células dendríticas) , denominados CD11cCre forma obtidos comercialmente. Estes animais foram cruzados com camundongos nos quais parte do gene AKt1 está flanqueado pela inserção de sequencias de nucleotídeos LoxP, obtidos através do Dr. Morris J. BirnBaum (Perelman School of Medicine, University of Pennsylvania). A prole destes cruzamentos foi genotipada por reação de PCR e os animais de interesse foram: CD11c.Cre^{+}/$Akt1^{L/L}$ (animais com deleção de Akt1 especificamente em células dendríticas) e CD11c.Cre^{-}/$Akt1^{L/L}$ (animais com gene Akt1 flanqueado pelas sequências LoxP, mas SEM deleção do fragmento de DNA intercalado).

Os animais foram aleatoriamente separados em 6 grupos de igual número (n=10) e mantidos em gaiolas plásticas em um ambiente com temperatura controlada (21±1^{o}C), umidade (65-75%) e ciclos de luz (12h claro-12h escuro), e alimentados com água e ração *ad libitum*. Uma vez que a parte in vivo foi realizada na Universidade de Pensilvânia, todas as aprovações regulamentares / éticas foram obtidas dos comitês comptetentes da Instituicao: IACUC (Comite Institucional de Cuidados e Uso Animal) e IRB (Conselho de Revisão Institucional). Após os períodos experimentais, os animais foram eutanasiados por overdose anestésica.

- Animais controle

Os animais não foram submetidos a nenhum tratamento.

- Doença Periodontal Experimental

Nestes animais foi induzida doença periodontal por meio de inoculação oral de $1x10^{9}$ unidades formadoras de colônia (UFC) de *P.gingivalis* (ATCC 33277) e $1x10^{9}$ UFC de *Fusobacterium nucleatum (Fn)* (ATCC 25586) 3 vezes por semana por um período de 2 e 6 semanas; *P. gingivalis* e *Fn* foram cultivados a 37°C em meio anaeróbico Gifu modificado

(GAM) (Nissui, Seiyaku Co.,Ltd., Tokyo, Japão) numa câmara de anaerobiose. Ambas bactérias foram coletadas na fase logarítmica de crescimento e resuspendidas em solução salina tamponada com fosfato (PBS) estéril [52].

A distribuição dos animais, por grupo e procedimentos experimentais, é apresentada no Quadro A1.

Quadro A1

Grupos		Genótipo	Tratamento
Controle	1	CD11c.Cre-/Akt1L/L	Nenhum
	2	CD11c.Cre+/Akt1L/L	Nenhum
Experimental	3	CD11c.Cre-/Akt1L/L	Doença Periodontal 2 semanas
	4	CD11c.Cre+/Akt1L/L	Doença Periodontal 2 semanas
	5	CD11c.Cre-/Akt1L/L	Doença Periodontal 6 semanas
	6	CD11c.Cre+/Akt1L/L	Doença Periodontal 6 semanas

Representação da distribuição dos camundongos por grupo

Fonte: Elaboração própria.

Reabsorção Óssea (μCT / Análise histomorfométrica)

Após a eutanásia, a mandíbula de cada animal foi removida e separada em duas hemi-mandíbulas e cuidadosamente dissecadas. As hemimandíbulas do lado esquerdo foram fixadas em paraformaldeído 4% a 4°C durante 18 horas, lavadas em PBS durante 10 minutos e depois transferidas para etanol 70% até o escaneamento em microtomógrafo computadorizado (μCT). O escaneamento foi executado usando condições padronizadas (kV, mA, filtro de atenuação) com cortes de 18 μm, usando os equipamentos da Universidade da Pensilvânia. A análise da extensão da reabsorção óssea foi realizada após a reconstrução tridimensional e reorientação padronizada das imagens nos três planos espaciais (sagital, frontal e coronal). O conjunto tridimensional de imagens no plano sagital foi binarizado para distinguir os tecidos mineralizados e moles com base na densidade das imagens, utilizando parâmetros também padronizados. A quantidade/extensão relativa dos tecidos mineralizados em cada amostra foi determinada usando uma região de interesse cuboidal padronizado

(ROI) de 750 μm^3 posicionado nas imagens tridimensionais usando marcos anatômicos pré-definidos, na região entre primeiro e segundo molar. A fração do volume desse ROI ocupado por tecidos mineralizados (Bv/Tv, volume ósseo/volume total) foi comparada entre os grupos. Todos estes passos da análise foram realizados com o software OsiriX.

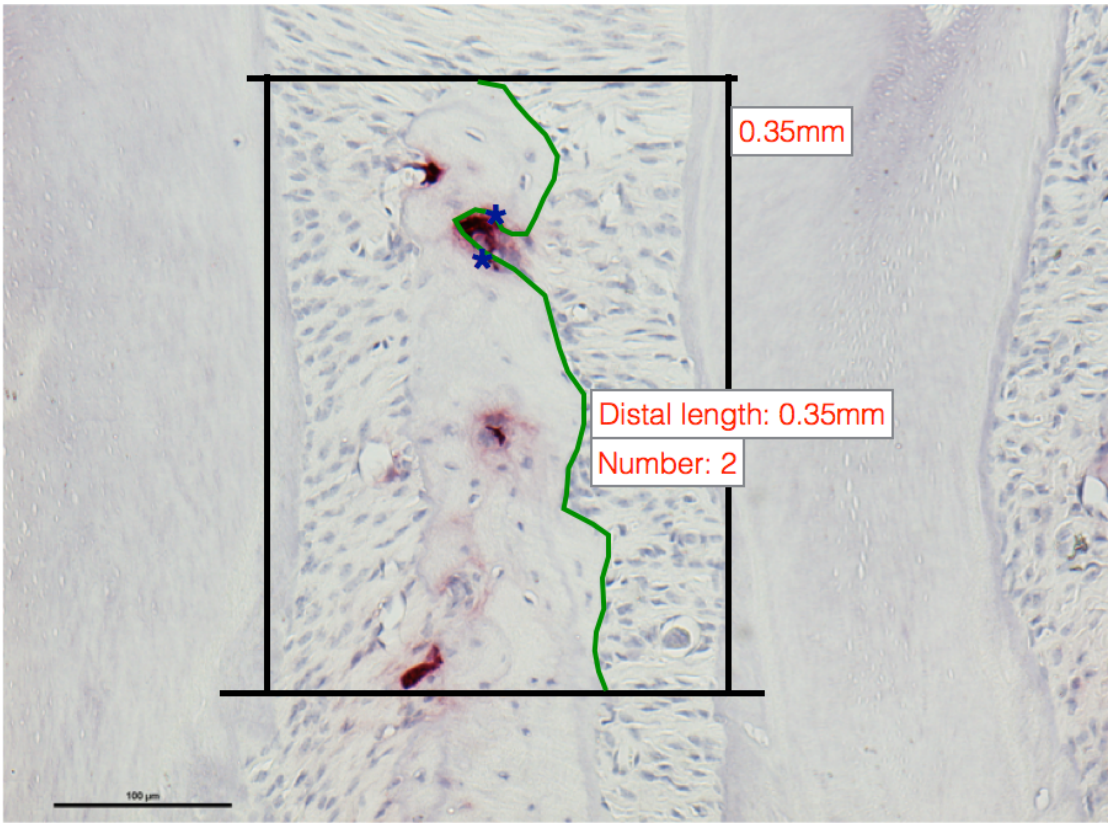
A análise histomorfométrica foi realizada após a digitalização no μCT. As amostras foram lavadas em PBS e desmineralizadas em EDTA (0,5 M, pH8,0) sobre agitação suave e constante à temperatura ambiente durante 6 semanas (com troca da solução de EDTA 3 vezes por semana). Após o processamentos histológico de rotina para a incorporação da parafina, foram obtidas secções em série de 4μm de espessura na direção mésio-distal (anterior-posterior, plano sagital), e montadas em lâminas de vidro sinalizadas. Foram obtidos 60 cortes de cada amostra (abrangendo 300 μm). Foram selecionados três cortes seriados da porção média do primeiro e segundo molar inferior (identificando a porção média pelo diâmetro do canal da raiz distal) de cada mandíbula. As lâminas coradas com H&E foram visualizadas num microscópio óptico com uma ampliação de 10x. A perda óssea foi obtida pela distância entre a distância da junção cemento-esmalte (JCE) até o ponto mais alto do osso interproximal, entre o primeiro e o segundo molar. A perda de inserção foi avaliada pelo distâcincia da junção cemento-esmalte à extensão apical do epitélio, ao longo da superfície radicular da porção distal do primeiro molar e porção mesial do segundo molar, em um aumento de 40X. Ambas as análises foram realizadas por um examinador treinado, sem conhecimento da condição do grupo experimental.

Osteoclastogênese (histoquímica)

Para determinar a influência da sinalização via Akt1 em células dendríticas na osteoclastogênese, utilizamos secções de 4 μm de espessura montadas em lâminas sinalizadas contendo as regiões de primeiro e segundo molar inferior . Para esta análise utilizamos um mínimo de 12 cortes/peça em três regiões diferentes com espaçamento de 80 μm entre elas. Os osteoclastos foram identificados pela marcação positiva da expressão da enzima fosfatase ácida resistente ao tartarato (TRAP) em conjunto com as características morfológicas (tamanho da célula, presença de três ou mais núcleos) e localização (próximo a superfície do tecido ósseo). Brevemente, os cortes histológicos foram desparafinizados em xilol e rehidratados em uma sequência de concentrações decrescentes de etanol. Os cortes foram lavados em PBS e corados com a solução de coloração composta por 0.1M de acetato de sódio tri-hidratado (Sigma S9513), 50nM de tartarato de sódio (Sigma S4797). 5mg de fast red violet lb salt (Sigma F3381), 30mg de naftol AS-MX de fosfato (Sigma N4875), 200ul de

Dimetilformamida (Sigma D4551) e 200ul de 0,1 cloreto de manganês (MnCl2, Sigma M3534) durante 15 minutos, a 37°C no banho maria . Os corte foram contra-corados com hematoxilina por 10 segundos, secados ao ar à temperatura ambiente durante 24 horas e montados com lamínulas. Após 24 horas, imagens digitais foram obtidas em um microscópio óptico com ampliação de 20X, usando configurações constantes para brilho, contraste e exposição. Os osteoclastos foram identificados pela marcação positiva da expressão de fosfatase ácida resistente ao tartarato (TRAP) em conjunto com características morfológicas (tamanho da célula, presença de três ou mais núcleos) e localização (perto da superfície do tecido ósseo); os osteoclastos foram contados a partir da parte distal da crista óssea, entre o primeiro e o segundo molar. A região de interesse estava situada a 0,35 mm da região coronal do osso alveolar (Figura A1). Um examinador treinado e experiente sem conhecimento da condição do grupo experimental realizou a contagem.

Figura A1



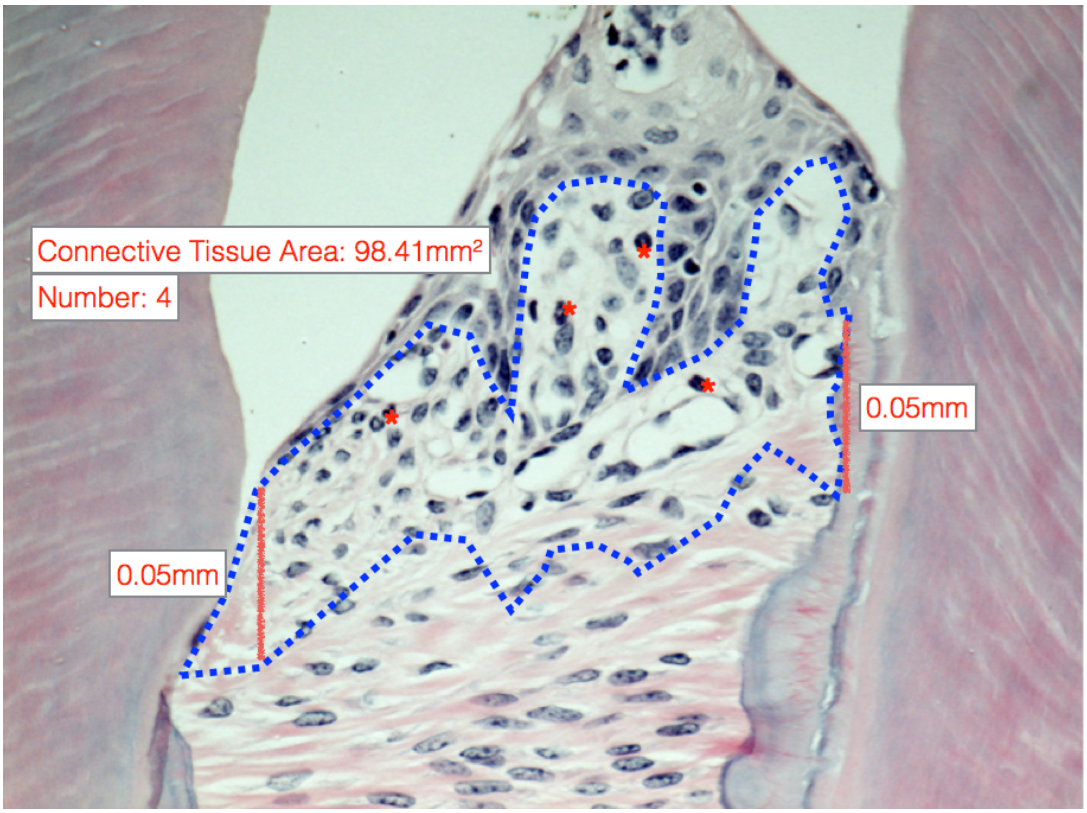
Contagem de osteoclastos. Imagem representativa de como foi delimitada a área para contagem de osteoclastos; Foi trazada uma linea no borde da crista alveolar, entre o primeiro e segundo molar, a partir de essa linea foram delimitados os 0.35mm de osso, uma linea seguindo a superfície de tecido ósseo, na parte distal foi trazada; Os osteoclastos que estavam presentes na superfície do osso foram contados. Aumento de 20X.

Fonte: Autoria própria.

Inflamação (Morfometria)

A avaliação do estado inflamatório foi feita por contagem diferencial de neutrófilos polimorfonucleares (PMNs) situados nos primeiros 0,05 mm de tecido conjuntivo, fora dos vasos sanguíneos. Uma linha seguindo a junção do tecido conjuntivo e epitélio delimitou a área (Figura A2). As laminas coradas com H&E foram visualizadas num microscópio óptico com um ampliação de 60x. As amostras foram analisadas, distribuídas em três grupos de 4 cortes seriados. Entre cada um desses três grupos, houve um espaçamento de 80 µm, permitindo a avaliação de toda a extensão vestíbulo-lingual do primeiro e segundo molar inferior. Um examinador treinado e experiente sem conhecimento da condição do grupo experimental executou o a contagem.

Figura A2



Contagem de PMNs. – Imagem representativa de como foi delimitada a área para contagem dos PMNs situados fora dos vasos sanguíneos; uma linha seguindo a junção do tecido conjuntivo e epitélio delimitou a área, nos primeiros 0,05 mm de tecido conjuntivo foi realizada a contagem de PMNs, fora dos vasos sanguíneos; usando um aumento de 40X.

Fonte: Autoria própria

ELISA

Foi coletado o sangue por punção cardíaca dos camundongos (prévio a eutanásia), e centrifugado a 3000 rpm por 10 minutos a 4^{o}C para a obtenção do soro.

Para a detecção de IgG especificas para *P. gingivalis* e *Fusobacterium nucleatum* (Fn) no soro dos animais, foi realizado o ELISA, utilizando o kit Mouse IgG total Readfy-SET-Go! (88-50400, affymetrix eBioscience) seguindo o protocolo do fabricante, porém com as seguintes modificações: foram utilizadas placas próprias para ELISA da Dynatec, Chantilly e a captura do anticorpo foi feita com 50 µl de *P. gingivalis* ou *Fn* (previamente fixados com formaldeído 1% a 4^{o}C por uma hora com um OD de 0.3) *overnight* a 4 ^{o}C. Após duas lavagens com PBS+0,05% Tween20 a placa coi colocada na estufa a 37 ^{o}C (para secagem) por 2 horas. Depois do bloqueio indicado no protocolo do fabricante, também foi realizado um bloqueio com 2% de soro de rato por uma hora (para evitar ligações inespecíficas/background). Os resultados foram normalizados usando uma curva padrão, utilizandos os padrões fornecidos no próprio kit.

Analise estatística

O objetivo primário da análise dos dados foi comparar os desfechos de interesse considerando a presença (expressão gênica) da via Akt1. Todos os dados obtidos foram submetidos a testes de normalidade (D'Agostino & Pearson normality test) e as análises foram realizadas segundo os métodos adequados a distribuição dos dados, procurando verificar a dissimilaridade (rejeição da hipótese de nulidade de diferenças) nos desfechos de interesse entre os grupos SEM e COM inibição de sinalização via Akt1. O nível de significância adotado foi de 95% ($p<0.05$) para todas as análises. Foi utilizado o software GraphPad 5.0 (GraphPad Software Inc., San Diego, CA, USA).

APÊNDICE B - MATERIAL E MÉTODO (Publicação 3)

Para a parte in vitro foram utilizadas células dendríticas primárias, diferenciadas com GM-CSF a partir de células estromais da medula óssea (BMDC, *bone marrow-derived dendritic cells*) de camundongos C57BL/6, seguindo o protocolo descrito por Lutz et al. [53]. As amostras de medula óssea dos camundongos foram obtidos em colaboração com a Profa. Dra. Alexandra Ivo de Medeiros e estão incluídos no protocolo ético aprovado para o experimento da colaboradora.

Uma vez que o protocolo de diferenciação celular foi otimizado, foi comprovado por meio de citometria que as células obtidas eram realmente células dendríticas näive por meio da expressão dos marcadores característico de células dendríticas (CD11c), e da ausência de expressão de MHC-II por estas células, na ausência de estímulos de ativação/maturação.

Western Blot

BMDC foram plaqueadas numa concentração de $1.5x10^6$ em placas de 60 mm, usando meio RPMI-1640 suplementado com 10% de soro fetal bovino (FBS) inativado por calor, 1 mM de piruvato, 1X aminoácidos não essenciais, 100 U/mL de penicilina e 100 µg/mL de estreptomicina a 37°C em uma atmosfera humidificada e 5% de CO2. Um estoque de *P. gingivalis* (ATCC 33277) foi descongelado e cultivado em placas de Tryptic Soy Agar suplementado com 5% de sangue de carneiro desfibrinado, 0,5 mg/mL hemin e 1 mg/mL de menadiona. A cultura foi mantida em uma câmara anaeróbiose a 37°C em 85% de N2, 5% de CO2 e 10% de H2 durante 3 dias, sendo coletada na fase logarítmica de crescimento e ressuspendida em solução salina tamponada com fosfato (PBS); Para calcular a quantidade de bactérias, ajustamos uma OD de 0,5 (Pg) em um comprimento de onda de 495 nm que foi determinado correlacionar-se com 10^7 CFU/ml. Após confirmar a adesão das BMDCs, as mesmas foram tratadas por 40 minutos com os inibidores bioquímicos LY294002 (Sigma-Aldrich, 10µM) inibidor de PI3-Kinase, Triciribine (Sigma-Aldrich, 1µM) inibidor de Akt1-3 e BML-257 (Cross Biotechnology, 12,5µM) inibidor seletivo para Akt1. DMSO (veículo de diluição dos três inibidores bioquímicos) foi utilizado como controle/veículo. Após tratamento, as células foram estimuladas com *P.gingivalis* numa proporção de 10: 1 (bactérias: célula) durante 20 minutos. No final dos estímulos, os lisados celulares foram preparados com tampão de lise (Cell Lysis Buffer, cat. 9803, Cell Signaling Tech., Inc.) suplementado com inibidores de protease (Complete Protease Inhibitor Cocktail Tablet, livre de EDTA, sc- 29131, Santa Cruz Biotech Inc., EUA) e fosfatase (PhosSTOP, cat.

04906837001, Roche Ltda., Alemanha) para a extração de proteína total. Após a quantificação usando o método de Bradford (Quick Start Bradford protein 1x dye reagent, cat. 500-0205, Bio-Rad Lab. Inc., EUA), 20µg de proteína total foram diluídos em SDS Sample Buffer 4X (NuPAGE, cat. NP0007, Invitrogen) contendo DTT e as proteínas foram desnaturadas por aquecimento a 95°C durante 5 min. A separação eletroforética foi realizada em gel descontínuo de poliacrilamida a 12% (100V de forma constante durante 60 min), seguido de transferência para uma membrana PVDF (300mA de forma constante por 60 minutos). Em seguida, as membranas foram bloqueadas com solução de TBS/Tween contendo 5% de BSA por 2 horas, para evitar ligações não específicas. Durante 18 horas a 4°C sob agitação suave, a membrana foi incubada com o anticorpo primário (Cell Signaling Tech., Cat. 2965S) usando uma diluição de 1:1000 para detectar a fosforilação de Akt no resíduo de treonina (Thr308), seguido de lavagens com TBS/Tween e incubação com anticorpo secundário conjugado com HRP durante 1 hora à temperatura ambiente. A detecção das proteínas alvo foi realizada com um sistema de quimioluminiscência (SuperSignal West Pico, Pierce Biotech., EUA), seguido de visualização em tempo real e documentação em câmara de quimioluminiscência (Chemi-Doc, Bio-Rad, EUA).

Apoptose / viabilidade celular

As BMDCs foram tratadas com os inibidores bioquímicos e estimuladas ou não com agente indutor de apoptose (Camptotecina 8µM, Sigma-Aldrich). A viabilidade celular foi determinada por citometria de fluxo, 8 horas após os estímulos, utilizando os reagentes Annexin V-PE Apoptosis Detection Kit I segundo o protocolo do fabricante (BD Biosciences). As células foram removidas da placa com reagente não enzimático, ressuspendidas e lavadas por 2 vezes em PBS à 4C, contadas em hemocitômetro e ressuspendidas em tampão de ligação na concentração de $1x10^6$ células/mL. Foi adicionado o volume indicado (5µL/amostra) de Anexina V conjugada a PE e 7-AAD, seguindo-se a incubação por 15 minutos ao abrigo da luz. Os dados foram adquiridos no citômetro BD FACSVerse (BD Biosciences), considerando os canais de fluorescência FL2 (PE) e FL3 (7-AAD) e um mínimo de 30.000 eventos/amostra dentro do *gate* criado em *dot-blot Forward x Side scatter* para excluir debris e fragmentos celulares.

Proliferação Celular

Os ensaios de proliferação e viabilidade celular foram realizados ao longo de 72 horas, com contagem de células (totais viáveis, segundo o ensaio de exclusão do corante azul de trypan) nos períodos de 24, 48 e 72 horas. As células foram plaqueadas numa concentração de $3x10^4$ células/poço, em placas de 96 poços, 100µL/poço. Após confirmar a adesão das células, foram acrescentados ao meio de cultura os inibidores bioquímicos LY294002 (Sigma-Aldrich, 10µM) inibidor de PI3-Kinase, Triciribine (Sigma-Aldrich, 1µM) inibidor de Akt1-3, e BML-257 (Santa Cruz Biotechnology, 12.5µM) inibidor seletivo para Akt1. O controle-veículo foi realizado pela adição do maior volume de DMSO utilizado na diluição dos três inibidores. Considerando que o efeito biológico dos inibidores bioquímicos é fugaz, os mesmos foram novamente adicionados às culturas celulares a cada 24 horas, sempre substituindo o meio de cultura para evitar o possível acúmulo de inibidores ou de produtos secretados pelas células. Mitomicina C (Sigma-Aldrich Co., 10µg/mL) foi utilizada como controle negativo. A proliferação celular foi avaliada por contagem direta de células viáveis em hemocitômetro, realizada por examinador treinado e sem conhecimento das condições experimentais. Ao final de cada período experimental (24, 48 e 72 horas), o meio de cultura contendo células em suspensão foi coletado e transferido para um tubo de microcentrífuga. 50µl do reagente enzimático de dissociação (Tryple Express, Gibco/ThermoFisher Scientific) eram acrescentados a cada poço e após 6 minutos de incubação a 25°C eram combinados com o meio de cultura coletado em tubos de microcentrífuga (permitindo a coleta de células aderidas e não aderidas de cada poço). Após lavagem em 1mL de PBS à 4C, as células eram ressuspendidas em 500µL de PBS e duas alíquotas de 100µL diluídas na proporção 1:1 em azul de trypan 0.4% (Gibco/ThermoFisher Scientific) foram contadas em hemocitômetro. Estes experimentos foram realizados em duplicata e repetidos 3 vezes de forma independente.

Quimiotaxia

Nestes ensaios, utilizamos um modelo *transwell* em placas de cultura de 12 poços com membranas de poliéster com poros de 8µm, utilizando CCL-19 (200ng/mL, Peprotech Inc.) como estimulo quimiotático. As BMSCs foram plaqueadas numa concentração de $5x10^4$ sobre a membrana (porção superior da câmara) em 300µL de meio de cultura. Nos poços (porção inferior da câmara) foram acrescentados 600µl de meio de cultura contendo 200ng/mL de CCL-19. Para avaliar a influência da sinalização via PI3-kinase, Akt1-3, Akt1, previamente ao plaqueamento sobre a membrana e imediatamente após a ressuspensão e contagem no

hemocitômetro, as células foram pré-tratadas por 40 minutos com os inibidores bioquímicos ou volume equivalente do diluente (DMSO). Após 18 horas, as células presentes na parte superior das membranas foram removidas gentilmente com *swabs* de algodão estéril, e as membranas foram coradas e montadas em lâminas com agente líquido de montagem contendo DAPI (0.5µg/mL, Dako) para avaliação em microscópio invertido de fluorescência (Evos fl, AMG Micro). As células presentes na porção inferior da membrana (voltada para a câmara inferior d*o transwell*, contendo o estímulos quimiotáctico) foram contadas em 5 campos na magnificação de 400X por um observador treinado e sem conhecimento das condições experimentais. Este experimento foi realizado duas vezes de forma independente e analisado em duplicata.

Atividade Fagocitária

BMDCs foram plaqueadas em uma concentração de $1.5x10^5$ células/poço, e após confirmar que as células estavam aderidas ao substrato plástico de cultura foram tratadas com os inibidores bioquímicos ou com o mesmo volume do veículo DMSO por 40 minutos. Para a avaliação de atividade fagocitária foram utilizadas bactérias vivas (*Porphyromonas gingivalis*, ATCC 33277) marcadas com fluoróforo vermelho (BacLight red bacterial stain, Molecular Probes/ThermoFisher Scientific) por 2 horas. Após este período as células foram lavadas três vezes com PBS a 4°C, fixadas em paraformaldeído 4% (Cytofix, BD Biosciences), permeabilizadas em tampão contendo saponina (Perm/Wash, BD Biosciences) e posteriormente marcadas com Faloidina conjugada a AlexaFluor488 e DAPI. Um examinador previamente treinado e sem conhecimento das condições experimentais realizou a contagem diferencial do total de células e do número de células fagocitantes em 5 campos na magnificação de 200X. A partir destes dados foi calculada a porcentagem de células fagocitantes (índice fagocítico). Este experimento foi realizado de forma independente duas vezes em triplicata.

Apresentação de Antígeno

BMDCs foram marcadas com composto fluorescente (Cytolabeling Orange, cat#ab176737, Abcam) seguindo as indicações do fabricante e plaqueadas numa concentração de $1.5x10^5$ células/poço em 100uL. Após confirmar a adesão das células ao substrato plástico de cultura, e a efetividade da marcação com o composto fluorescente, as células foram tratadas com os inibidores bioquímicos por 40 minutos para depois serem estimuladas com

$1.5x10^6$ *P. gingivalis* (MOI 10:1) por 8 horas. Após 3 lavagens sucessivas das células dendríticas aderidas com PBS, foram acrescentados $1.5x10^5$ esplenócitos obtidos do pâncreas de camundongos C57BL/6. Os esplenócitos ($5x10^5$ células) foram previamente marcados com carboxyfluorescein (CFSE, Sigma-Aldrich) em um volume de 100uL. As co-culturas foram mantidas por 24 horas. Ao final deste período de co-cultura, as células foram lavadas gentilmente 3 vezes com PBS e fixadas em paraformaldeído 4% (Cytofix, BD Biosciences). O DNA genômico/núcleos celulares foram marcados com DAPI (0.5µg/mL, Dako). A avaliação foi feita em microscópio invertido de fluorescência (Evos fl, AMG MIcro) por examinador previamente treinado e sem conhecimento das condições experimentais. Foram contadas: o total de células dendríticas (BMDC) marcadas em vermelho e as BMDC que estavam em contato direto célula:célula com esplenócitos (marcados em verde), em 5 campos aleatórios na magnificação de 200X. Foi calculada a porcentagem de células apresentando o antígeno (i.e., em contato direto com esplenócitos). Este experimento foi realizado três vezes de forma independente.

Osteoclastogênese

BMDCs foram plaqueadas em placa de 96 poços em uma concentração de $5x10^3$ células/poço em 5µl de meio α-MEM suplementado com 10% de soro fetal bovino inativado por calor (FBS) e 100 U/mL de penicilina e estreptomicina no centro de cada poço, para evitar a dissipação das células. Imediatamente depois, as BMDCs foram tratadas durante 40 minutos com inibidores bioquímicos ou veículo, em 50 µl. Após o tratamento com os inibidores, foram adicionados 30 ng/ml de fator de estimulação de colónias de macrófagos (MCSF) e 4 ng/ml de RANKL em 50µl (para um volume final de 100µl). Após 4 dias as células foram fixadas com paraformaldeido 4% e marcadas com TRAP usando o kit Acid Phospatase, Leukocyte (TRAP) (cat # 3871, Sigma-Aldrich) seguindo o protocolo do fabricante. As células TRAP positivas com três ou mais núcleos foram identificadas como osteoclastos. Todos os osteoclastos presentes em cada poço foram contados com a ajuda de um microscópio. A partir destes dados foram feitas as comparações entre os grupos. Este experimento foi realizado em triplicata.

Analise estatística

O objetivo primário da análise dos dados foi comparar os desfechos de interesse considerando o estado de ativação da via Akt1. Todos os dados obtidos foram submetidos a testes de normalidade (D'Agostino & Pearson normality test) e as análises foram realizadas segundo os métodos adequados a distribuição dos dados, procurando verificar a dissimilaridade (rejeição da hipótese de nulidade de diferenças) nos desfechos de interesse entre os grupos SEM e COM inibição de sinalização via Akt1. O nível de significância adotado foi de 95% ($p<0.05$) para todas as análises. Foi utilizado o software GraphPad 5.0 (GraphPad Software Inc., San Diego, CA, USA).

ANEXOS

ANEXO A –Comitê de ética (Publicação 2)

Penn
University of Pennsylvania

Office of Regulatory Affairs
IACUC Protocol Administration

Troy M. Hallman, MS, VMD, Diplomate ACLAM
Director of Animal Welfare, Office of Regulatory Affairs

3624 Market Street, Science Center ♦ Suite 301S ♦ Philadelphia, PA 19104 ♦ Phone: 215-573-2540 ♦ Fax: 215-573-9438

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
(Multiple Project Assurance # A3079-01)

DANA T GRAVES
5113 - Periodontics
DENTAL MEDICINE
240 S. 40TH STREET
PHILA, PA 19104

21-Jan-2015

PRINCIPAL INVESTIGATOR	: DANA T GRAVES
PROTOCOL TITLE	: Lineage specific expression of RANKL in periodontal disease
GRANT TITLE	: Dendritic cells and periodontal disease
SPONSORING AGENCY	: NATIONAL INSTITUTES OF HEALTH
PROTOCOL #	: 804044

Dear DR. GRAVES:

With receipt of the requested revisions for the above protocol your study now stands fully approved as of **20-Jan-2015**. Work may begin at any time. This study will be due for review on or before **20-Jan-2018**. Protocols are only valid for three years from the date of approval. Please use **Ben Reports (https://galaxy.isc-seo.upenn.edu/ws/benreports)** on a routine basis to check the status of your protocols.

If notification of IACUC review is required by the funding source required, please notify our office in writing of the contact person, agency name, address, phone number, fax number, and email as soon as possible.

Please take note of the following information:

Personnel Training: It is the responsibility of the Principal Investigator to ensure that all persons have completed all necessary IACUC and EHRS training prior to participating in the research described in this protocol.

Amendments*: If you wish to change any aspect of this study, such as procedures, sponsor, analgesics, anesthetics, or the investigators, please communicate your requested changes in writing to the Director for Regulatory Affairs. The new procedures cannot be initiated until Committee approval has been given.

Reapproval*: It is the investigator's responsibility to apply for reapproval of ongoing research annually for protocols involving USDA covered species, or more often if required by the funding agency.

*Forms for amendments and re-approval (Form B) are available from the Office of Regulatory Affairs web site [http://www.upenn.edu/regulatoryaffairs].

Completion of Study: Please notify the Director for Regulatory Affairs as soon as the research has been completed.

Thank you for your cooperation with the Committee.

Sincerely,

Troy Hallman, MS, VMD
Director of Animal Welfare, IACUC

ANEXO B - Comitê de ética (Publicação 2)

Penn Animal Welfare

IACUC Protocol Administration
http://www.upenn.edu/animalwelfare

Office of Animal Welfare
Science Center Building
3624 Market Street, Suite 301S
Philadelphia, PA 19104
Phone: 215-898-2615
Fax: 215-746-6308
Email: iacuc@pobox.upenn.edu

UNIVERSITY OF PENNSYLVANIA
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
(Animal Welfare Assurance # A3079-01)

DANA T GRAVES
5113 - Periodontics
DENTAL MEDICINE
240 S. 40TH STREET
PHILA, PA 19104-6030

09-Jan-2018

PRINCIPAL INVESTIGATOR : DANA T GRAVES
PROTOCOL TITLE : Lineage specific expression of RANKL in periodontal disease
PROTOCOL # : 804044
3-YEAR APPROVAL PERIOD : 07-Jan-2018 – 07-Jan-2021

Dear DR. GRAVES:

The above referenced protocol was reviewed and approved by the Institutional Animal Care and Use Committee on **07-Jan-2018**. Protocols are valid for three years from the date of approval. However, please note that protocols with USDA-covered species will require annual continuing reviews. This study will be due for its next review on or before **07-Jan-2021**. Please log into the ARIES electronic protocol system (https://aries.apps.upenn.edu/laProtocol/jsp/fast2.do) on a routine basis to check the status of your protocol, and be cognizant of reminder notifications that will be sent out when new submissions are required.

Please note that the principal investigator should contact ULAR to verify animal housing availability and to coordinate activities for any special animal study needs (including special housing) or equipment requirements if this was not done during the planning phases of the protocol. IACUC protocol approval DOES NOT guarantee the availability of required resources for animal work.

Please take note of the following information:

Personnel Training: It is the responsibility of the Principal Investigator to ensure that all persons have completed all necessary training prior to participating in the research described in this protocol.

Submissions: Please note that all future submissions related to this protocol must be submitted within ARIES. No paper submissions will be accepted.

Amendments: If you wish to change any aspect of this study, such as personnel, sponsors, hazardous materials, drugs, or procedures, please submit an Amendment to the protocol within ARIES. The new changes cannot be initiated until IACUC approval has been given.

Completion of Study: Please notify the Office of Animal Welfare once the research has been completed so the protocol can be terminated. This will prevent you from receiving unnecessary reminder notifications for renewal submissions.

If you have any questions, please contact our office as indicated above. Thank you for your cooperation with the Committee.

Sincerely,

Digitally signed by Gregory R. Reinhard
Date: 2018.01.10 17:37:01 -05'00'

Gregory R. Reinhard, MBA, DVM, DACLAM
Director, Office of Animal Welfare

ANEXO C - –Comitê de ética (Publicação 3)

C E RT I F I C A D O

Certificamos que a proposta intitulada "Avaliação do Perfil Fenotípico e da Capacidade Migratória de Células Dendríticas após Fagocitose de Células Apoptóticas Infectadas ou Não-infectadas", registrada com o Protocolo CEUA/FCF/CAr: 01/2016, sob a responsabilidade de Letícia de Aquino Penteado e Profa. Dra. Alexandra Ivo de Medeiros - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS da Faculdade de Ciências Farmacêuticas do Campus de Araraquara da UNESP em reunião de 15 de abril de 2016.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	Março de 2018
Espécie/linhagem/raça	Camundongo C57BL/6
Nº de animais	24
Peso/Idade	20 g 6 a 14 semanas
Sexo	Fêmea
Origem	Centro Multidisciplinar para Investigação Biológica – CEMIB/UNICAMP

Araraquara, 19 de abril de 2016.

Prof. Dr. CARLOS CESAR CRESTANI
Coordenador da CEUA

ANEXO D - –Artigo publicado (Publicação 1)

Fator do impacto: **3.698**

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Critical Reviews™ in Immunology

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pages 1-13

The Role of Forkhead Box 1 (FOXO1) in the Immune System: Dendritic Cells, T Cells, B Cells, and Hematopoietic Stem Cells

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Dana T. Graves
Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA

RESUMO

Forkhead box-O (FOXO) transcription factors have a fundamental role in the development and differentiation of immune cells. FOXO1 and FOXO3 are FOXO members that are structurally similar and bind to the same conserved consensus DNA sequences to induce transcription. FOXO1 has been studied in detail in the activation of dendritic cells (DCs), where it plays an important role through the regulation of target genes such as ICAM-1, CCR7, and the integrin $\alpha_v\beta_3$. FOXO1 is activated by bacteria challenge in DCs and promotes DC bacterial phagocytosis, migration, homing to lymph nodes, DC stimulation of CD4+ T cells and resting B cells, and antibody production. Deletion of FOXO1 in DCs enhances susceptibility to bacteria-induced periodontal disease. FOXO1 and FOXO3 maintain naive T cell quiescence and survival. FOXO1 and FOXO3 enhance the formation of regulatory T

Araraquara, 26 de março de 2018.

Adriana Alicia Cabrera Ortega