TAIA MARIA BERTO REZENDE

“AGREGADO DE TRIÓXIDO MINERAL (MTA) E RESPOSTA IMUNE: DA IMUNIDADE INATA À REABSORÇÃO ÓSSEA PERIAPICAL.”

FACULDADE DE ODONTOLOGIA
UNIVERSIDADE FEDERAL DE MINAS GERAIS
BELO HORIZONTE
2008

TAIA MARIA BERTO REZENDE

"AGREGADO DE TRIÓXIDO MINERAL (MTA) E RESPOSTA IMUNE: DA IMUNIDADE INATA À REABSORÇÃO ÓSSEA PERIAPICAL."

Tese aprovada em banca de doutorado pelo Colegiado de Pós-graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção de título de doutora em Odontologia.

Área de concentração: Endodontia.

Departamento: Dentística restauradoura.

Orientadores: Leda Quercia Vieira
Tohihisa Kawai
Antônio Paulino R. Sobrinho

BELO HORIZONTE
FACULDADE DE ODONTOLOGIA
UNIVERSIDADE FEDERAL DE MINAS GERAIS
2008

R467a Rezende, Taia Maria Berto
2008 Agregado de trióxido mineral(MTA) e resposta imune: da
T imunidade inata à reabsorção óssea periapical / Taia Maria Berto
Rezende, 2008
147 fls. : il.
Orientadora: Leda Quercia Vieira
Co-orientador: Toshihisa Kawai
Tese(Doutorado)- Universidade Federal de Minas Gerais,
Faculdade de Odontologia
1.Materiais Biocompatíveis – Relação Dose-Resposta -
Imunológica. – Teses
. 2. Reabsorção óssea - Imunologia. – Teses.. I. Vieira, Leda
Quercia . II.Kawai,
Toshihisa. III.Universidade Federal de Minas Gerais, Faculdade
de Odonto-
logia. IV. Título
BLACK D047

UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE DONTOLOGIA
Programa de Pós-Graduação em Odontologia

Tese intitulada **"Agregado de trióxido mineral (MTA) e resposta imune: da imunidade inata à absorção óssea periapical"**, área de concentração Endodontia, apresentada pela candidata **Taia Maria Berto Rezende**, para obtenção do grau de **Doutor em Odontologia**, **APROVADA** pela Comissão Examinadora constituída pelos seguintes professores:

Profa. Dra. Leda Quercia Vieira
Orientadora – ICB-UFMG

Prof. Dr. Toshihisa Kawai
Co-Orientador
Departament of Immunology – FORSYTH INSTITUTE

Prof. Dr. Gilson César Nobre Franco
Universidade de Taubaté – SP

Prof. Dr. Paulo Eduardo Alencar de Souza
PUC-MG

Profa. Dra. Maria Guiomar de Azevedo Bahia
FO-UFMG

Prof. Dr. Mauro Martins Teixeira
ICB- UFMG

Prof. Dr. José Eustáquio da Costa
Coordenador do Colegiado do
Programa de Pós-Graduação em Odontologia

Belo Horizonte, 15 de fevereiro de 2008.

“Não cessaremos de explorar

E ao fim de nossa exploração

Voltaremos ao ponto de partida

Como se não o tivéssemos conhecido”

T. S. Eliot

DEDICATÓRIA

À minha família,

Ao meu marido, ***André****, meu companheiro, incentivador incondicional, que tantas vezes abdicou de minha presença ou de si mesmo. Nunca me esquecerei de seu apoio, presença, carinho, e de tudo o que temos feito em nossos 15 anos de convivência. Com ele recomeçaria tudo novamente e sei que ele também. Amo você, André!*

Aos meus pais, ***Walter e Taia****, meus primeiros mestres. Sei que o trajeto acadêmico é algo muito novo para ambos e nem sempre, o sentido dessa escolha lhes foi claro. Muito obrigada por todo apoio principalmente quando a luz no fim do túnel parecia ausente, tanto para eles quanto para mim. Seu exemplo de luta é incentivo e luz para todos os meus recomeço; com eles tudo faz sentido.*

Às minhas avós, ***Mari-inha*** *e* ***Hildette****, pela jovialidade, inteligência, presença, confiança e apoio constantes à minha procura de conhecimentos e conquistas.*

Aos meus irmãos, ***Juliane, Lectícia, Igor e Isabela,*** *que me incentivam e torcem por mim, de longe ou de perto.*

À minha tia, ***Lourdinha,*** *in memorian, meu anjinho. Nunca me esquecerei do que me ensinou sobre a vida, em tão pouco tempo. Obrigada por olhar por mim.*

A toda a ***minha família Berto Rezende****: pela atenção e por ter me recebido de braços abertos como verdadeira filha.*

A ***minha família Araújo Pinheiro****: por entender minha ausência e incentivar minha busca.*

Por acreditarem sempre em mim, amo vocês!

AGRADECIMENTOS ESPECIAIS

Não poderia ter sorte maior. Deus colocou em minha vida três pessoas competentíssimas, inteligentíssimas, queridas e que serão sempre minha referência. Com elas os pontos que determinam o fim da orientação e o começo da amizade se uniram: o caminho árduo tornou-se muito prazeroso. Entendi que a distância não é empecilho para uma boa orientação.

À professora Doutora ***Leda Quercia Vieira****, pela sua atenção, equilíbrio, paciência, carinho e disponibilidade, em todas as etapas desta empreitada. Seu exemplo de seriedade, serenidade, sua linha de raciocínio e inteligência muito me ajudaram.*

Ao professor Doutor ***Toshihisa Kawai****, pela orientação segura, estímulo constante, que me fizeram crescer muito durante minha estada no Instituto FORSYTH. Seu trabalho árduo e sua busca de conhecimento ensinaram-me que sempre podemos mais. Obrigada por todo carinho, suporte e por me ajudar a superar limites.*

Ao professor Doutor ***Antônio Paulino Ribeiro Sobrinho****, pela orientação sem fim, estímulo constante, dinamismo e bom humor. Sua atuação se fez presente em todas as etapas deste processo. Nunca me esquecerei, entre tantas coisas, do carinho com que você organizou minha ida para os EUA.*

Meus sinceros agradecimentos a todos.

AGRADECIMENTOS

Ao ***curso de Doutorado em Odontologia****, pela oportunidade de crescimento profissional.*

Aos ***laboratórios: Imunologia e Gnotobiologia (UFMG)*** *e* ***Imunologia (Instituto FORSYTH)****, que cederam o material e as instalações para a realização desta pesquisa.*

À ***empresa Odonto-lógika*** *por ter cedido o MTA para este estudo.*

Aos ***professores do doutorado****, pelos ensinamentos transmitidos de maneira primorosa durante todo o curso.*

Ao ***Professor, Doutor Jacques Nicoli****, do departamento de microbiologia (UFMG), pela seriedade e ajuda, sempre.*

Ao ***Professor, Doutor Martin Taubman****, chefe do departamento de Imunologia (Instituto FORSYTH), pela possibilidade de trabalho em tal departamento, pela oportunidade de realizar a disciplina Oral Biology na Faculdade de Odontologia de HARVARD e por todas as sugestões que engrandeceram a realização desta pesquisa.*

Ao ***Professor, Doutor Dan Smith****, do departamento de Imunologia (Instituto FORSYTH), por todas as sugestões e por ter ensinado ao André e a mim o significado do dia de Ação de Graças.*

Ao Sr. ***Ricardo Ferracini*** *e toda a equipe do* ***Centro de Desenvolvimento Tecnológico****, pela eficiente colaboração.*

As ***minhas colegas de Pós-graduação: Soninha, Luciana, Ana Cecília*** *e* ***Letícia****, amigas de risos e conversas, por amenizarem os momentos árduos de trabalho e a distância da família, incentivando minhas viagens para Belo Horizonte.*

Aos meus ***amigos de Brasília****, pela compreensão de minha ausência em tantos momentos importantes.*

Aos alunos de inciação científica, ***Ricardo Reis*** *e* ***Fabiano Cardoso****, por todo o carinho, ajuda, e alegres momentos de convivência.*

Aos meus ***colegas do laboratório de Imunologia e Gnotobiologia****:* ***Denise, Lucas, Marcelo José, Cláudia, Mayra, Juan, Virgínia, Helton,*** *pela parceria nos trabalhos desta pesquisa tanto de perto quanto de longe.*

Aos meus colegas ***do laboratório de Imunologia (Instituto FORSYTH): Cory, Kazu, Tomomi, Greg, Bill e Jean****, pela ajuda e ensino durante toda a fase experimental desenvolvida no instituto FORSYTH.*

Aos ***amigos do Instituto FORSYTH: Flávia, Ricardo e Han****, por todo incentivo e todas as horas de conversa e amizade longe de casa.*

Aos amigos: ***Carla, Cleber, Camile, Raul, Andréia, Brasil, Breno e Gilson*** *pela amizade e companheirismo que fizeram Boston se parecer com o Brasil.*

Às instituições que possibilitaram minha capacitação: ***Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal***

de Nível Superior (CAPES), Universidade Federal de Minas Gerais (UFMG) e Instituto FORSYTH.

A todos que, de alguma forma contribuíram para meu êxito diante de mais um desafio.

*A **Deus** por me oferecer todos os motivos para agradecer.*

Meus singelos e sinceros agradecimentos a cada um.

SUMÁRIO

SUMÁRIO

LISTA

LISTA DE ABREVIATURAS E SIGLAS

1. APC: célula apresentadora de antígeno
2. ATCC: *American Type Collection*
3. Ccl2/MCP-1: proteína quimiotátia de monócito
4. Cc5/RANTES: co-fator de membrane para proteína
5. CD: cluster of differenciation
6. ELISA: Enzime-linked immunoabsorbent assay
7. FDA: *Food and Drug Administration*
8. IL-: interleucina
9. IFN-: interferon
10. Ig: imunoglobulina
11. IRM®: *intermediate restorative material*
12. M1: macrófago do tipo 1
13. M2: macrófago do tipo 2
14. M-CSF: fator estimulador de colônias de monócitos
15. MTA: *Mineral trioxide aggregate*
16. MTT: método de medida da atividade da desidrogenase mitocondrial
17. NK: *natural Killer*
18. NO: óxido nítrico
19. PBS: salina tamponada com fosfato 0,01M pH 7,3
20. PMN: leucócitos polimorfonucleares
21. ROIs: radicais livres de oxigênio
22. RANKL: ligante do receptor ativador do fator nuclear Kappa B
23. SCR: sistema de canais radiculares
24. Super-EBA®: cimento de óxido de zinco e eugenol reforçado
25. Th: célula T-auxiliar (T-helper)
26. TNF: fator de necrose tumoral
27. Treg: célula T regulatória

RESUMO

RESUMO

O agregado de trióxido mineral (MTA), por ser um cimento retro-obturador utilizado em regiões inflamadas e/ou infectadas, deve ser biocompatível e não interferir negativamente na resposta imune periapical. Sendo essa resposta imune um papel protetor, prevenindo a disseminação de infecções, as implicações clínicas de suas alterações precisam ser estudadas. Nesse contexto, nosso objetivo foi observar o papel do MTA na imunidade inata, adaptativa e na reabsorção óssea. A atividade de macrófagos M1 e M2 peritoneais, na presença do MTA, foi objeto de nosso estudo na imunidade inata. Foram analisadas a viabilidade e aderência celular, a fagocitose de *Saccharomyces boulardii*, a produção de espécies reativas de oxigênio (ROI) e de nitrogênio (NO), assim como a atividade da arginase. A produção de IgG em resposta ao *Fusobacterium nucleatum*, a proliferação e produção de citocinas por células T pré-imunes estimuladas por anti-TCR e anti-CD28 e por células T de memória reativas ao *F. nucleatum* e ao *Peptostreptococus anaerobius*, na presença do MTA, foram analisadas no estudo da resposta imune adaptativa. Por último, a influência do MTA na osteoclastogênese mediada por RANKL e a atividade desses osteoclastos também foram pesquisadas. Observou-se que o MTA não afetou nem a atividade antibacteriana, nem a cicatricial dos macrófagos M1 e M2. A produção de IgG ao *F. nucleatum* em MTA foi estimulada em níveis comparáveis aos grupos imunizados com *F. nucleatum* em adjuvante de Freund e adjuvante de hidróxido de alumínio. O MTA estimulou a proliferação das células T pré-imunes estimuladas com anti-TCR e anti-CD 28, elevou a produção das citocinas TNF, RANKL e IFN-γ, além de diminuir a produção de IL-10, por essas células. O MTA reduziu a proliferação das células T de memória antígeno específica. O MTA diminuiu a produção das citocinas IFN-γ e IL-4, pelas células Th1 de memória reativas ao *P. anaerobius* e pelas células

Th2 de memória reativas ao *F. nucleatum*, respectivamente, apesar de não ter afetado a produção das citocinas TNF, RANKL e IL-10. Na reabsorção óssea, o MTA inibiu a osteoclastogênese, e também a atividade dos osteoclastos remanescentes. Concluiu-se que: o MTA não interfere na atividade das células presentes nas fases iniciais da resposta imune; o MTA apresenta alguma atividade nas células presentes nas fases tardias da resposta imune; o MTA inibe os eventos de reabsorção óssea. Considerou-se, portanto, que o MTA é um material que age e interfere, de forma positiva, nos processos de interação material/hospedeiro.

Palavras-chave: MTA, resposta imunológica, reabsorção óssea, resposta periapical.

SUMMARY

SUMMARY

Mineral trioxide aggregate (MTA) is a biocompatible root-end filling material. However MTA's influences on the innate and adaptive immune responses and bone resorption in periapical lesions are still unclear. To investigate the effects of MTA on innate immune response, M1 and M2 macrophages were examined *in vitro* for their viability, adhesion to glass, phagocytosis, production of reactive oxygen and nitrogen species and arginase activity. To assess the effects of MTA on adaptive immune responses, IgG antibody response to *Fusobacterium nucleatum (Fn)* was evaluated *in vivo* and memory T cells (Tm) specific to *Fn* and *Peptostreptococcus anaerobius* (*Pa*) were stimulated *in vitro* with spleen antigen presenting cells and respective antigen in the presence of MTA. MTA's influences on the RANKL-mediated osteoclastogenesis and on the bone resorption activity by mature osteoclasts were tested using RAW264.7 cells and mouse bone marrow cells. MTA did not affect the M1 and M2 activities. MTA increased serum IgG antibody levels against *Fn* ($p<0.05$), indicating an adjuvant activity of MTA. MTA decreased the proliferation of antigen-specific Tm. MTA suppressed IFN-γ or IL-4 ($p<0.05$) production by *Fn*-reactive Th2 or *Pa*-reactive Th1 Tm, respectively, while productions of TNF-α, RANKL and IL-10 from these Tm were not affected by MTA. As to bone resorption, MTA inhibited the osteoclastogenesis and osteoclast activity ($p<0.05$). Conclusion: MTA affected the immune responses as well as bone resorption events in periapical lesions in a manner that benefits host.

Key-words: MTA, immune response, bone resorption, periapical lesion.

1 INTRODUÇÃO

1 INTRODUÇÃO:

A polpa dentária é um tecido conjuntivo que possui abundantes vasos sangüíneos e linfáticos, nervos mielínicos e amielínicos e células mesenquimais indiferenciadas. Semelhante aos outros tecidos conjuntivos do nosso organismo, ela reage à infecção bacteriana ou a outros estímulos (físicos ou químicos) pela resposta inflamatória no órgão pulpar.

A reação imuno-inflamatória que aí se instala procura proteger a polpa contra a agregação microbiana. Porém, essa resposta ao mesmo tempo que apresenta aspectos positivos na eliminação do agente invasor, pode causar danos teciduais. Didaticamente ela pode ser dividida em: imunidade inata e imunidade adquirida.

A imunidade inata provê a primeira linha de defesa contra microrganismos, além de ser uma resposta rápida, imediata à entrada de um dado antígeno e ocorrer de forma semelhante para diferentes agentes agressivos. Já a imunidade adaptativa, também chamada de imunidade específica, apresenta especificidade, diversidade, memória, especialização, autolimitação e tolerância. Essa resposta pode ser dividida em dois subtipos de acordo com os componentes e a forma de eliminação dos microrganismos. A imunidade humoral é mediada por anticorpos especializados produzidos pelos linfócitos B. Seus diferentes tipos podem ativar diferentes mecanismos efetores. É este o principal mecanismo de defesa contra microrganismos extracelulares e toxinas. A imunidade celular é mediada pelos linfócitos T, tanto linfócitos T auxiliares do tipo 1 (Th1) quanto os do tipo 2 (Th2) (Levy, 2006).

Certas características anatômicas do tecido pulpar, porém, tendem a modificar a natureza e o curso da resposta imunológica. O fato de o tecido pulpar confinar-se entre paredes mineralizadas de dentina, inextensíveis, impede a instalação do edema inflamatório. Nesse órgão, a circulação colateral é comprometida, pois, tanto a circulação sangüínea

aferente quanto a eferente penetra e sai do dente, através de forames apicais minúsculos (Shafer W.G. *et al.*, 1987).

O tecido pulpar em condições de normalidade é considerado imunocompetente e tem a capacidade de responder aos estímulos nocivos. Células apresentadoras de antígenos (APC) estão presentes na camada odontoblástica e ao longo dos vasos sanguíneos, além de células semelhantes a macrófagos serem encontradas na porção mais central da polpa (Bergenholtz *et al.*, 1991). Um pequeno número de células T recirculantes é identificado, com a predominância de linfócitos T $CD8^+$, enquanto as células B são raras ou indetectáveis (Hahn *et al.*, 1989). Os plasmócitos estão ausentes (Pulver *et al.*, 1977).

A resposta inicial à exposição pulpar e invasão bacteriana e/ou difusão de seus subprodutos, pelos túbulos dentinários, inclui o influxo de leucócitos polimorfonucleares (PMNs) (Warfvinge *et al.*, 1985) e monócitos (Bergenholtz *et al.*, 1991), caracterizando o infiltrado inflamatório agudo. Com a progressão desse processo, o infiltrado celular torna-se mais intenso, crônico e assume um caráter típico misto. Consiste em células T auxiliares e T citotóxicas. As células B e plasmócitos também estão presentes como elementos específicos, junto com os PMNs, monócitos e células natural killer (NK) como componentes não específicos (Pulver *et al.*, 1977; Hahn *et al.*, 1989). Os níveis de IgG e IgA são elevados (Speer *et al.*, 1977), sugerindo a possibilidade de proteção durante o processo de invasão bacteriana (Hahn e Falkler, Jr., 1992). Geralmente esses mecanismos não são capazes de erradicar a infecção: a destruição tecidual, com a formação de pequenos abscessos e focos necróticos na polpa, ocorrerá com eventual progressão para a necrose pulpar total, na qual o número de linfócitos decresce dramaticamente (Hahn *et al.*, 1989).

Instalada a necrose pulpar, uma rápida colonização bacteriana ocorre no interior do sistema de canais radiculares (SCR). A dinâmica dessa colonização se processa: há uma

microbiota composta por bactérias anaeróbias facultativas, sucedida por bactérias anaeróbias estritas, com predomínio de espécies gram-negativas (Tani-Ishii *et al.*, 1994).

A ligação direta entre o aparecimento, a progressão, a manutenção da resposta inflamatória e a presença bacteriana foi relatada por Kakehashi *et al.* (1965). Nesse estudo constataram-se a formação de necrose pulpar e inflamação periapical após a exposição pulpar de dentes de camundongos convencionais, enquanto observou-se a formação de dentina reparadora em dentes de camundongos isentos de germes, demonstrando a capacidade da polpa em se auto-reparar na ausência de infecção.

Anos mais tarde, Sundquist (1976) confirmou o relacionamento de causa e efeito entre a infecção pulpar e o desenvolvimento de lesão apical. Essa conclusão baseou-se na ausência de bactérias detectáveis em culturas de câmaras pulpares de dentes traumatizados sem lesão apical, enquanto a presença bacteriana foi observada nas culturas dos mesmos dentes com lesão apical (Sundqvist, 1976).

Vários estudos demonstraram que o SCR pode atuar como uma via para a sensibilização do hospedeiro (Kennedy *et al.*, 1957; Barnes e Langeland, 1966). Semelhantemente aos mecanismos descritos no órgão pulpar, as alterações patológicas associadas às alterações perirradiculares são mediadas por reações inflamatórias não específicas e/ou respostas imunes específicas, exceto pela destruição óssea (Torabinejad *et al.*, 1985; Stashenko *et al.*, 1998).

O infiltrado inflamatório das lesões periapicais crônicas caracteriza-se pela presença de linfócitos T e B, PMNs, macrófagos, plasmócitos (Stern *et al.*, 1981; Kawashima *et al.*, 1996), células NK, eosinófilos e mastócitos (Artese *et al.*, 1991; Kawashima *et al.*, 1996). Esse infiltrado representa aproximadamente 50% das células presentes nos granulomas periapicais. Outras células do tecido conjuntivo não inflamatório, aí presentes, incluem os

fibroblastos, endotélio vascular, epitélio proliferativo, osteoblastos e os osteoclastos (Yu e Stashenko, 1987).

Todos os tipos celulares inflamatórios envolvidos tanto na resposta inflamatória específica quanto naquela não-específica infiltram-se na região pulpar e periapical em resposta à infecção. Essas células medeiam inteiramente o espectro da resposta imune, incluindo: ativação dos PMNs e dos macrófagos, hipersensibilidade do tipo retardada, reações citotóxicas, complexo imune e hipersensibilidade mediada por complemento, a resposta anafilática e a produção de citocinas por linfócitos, macrófagos e células do tecido conjuntivo do hospedeiro, metabólitos do ácido aracdônico e cininas (Stashenko *et al.*, 1998).

Uma teoria que explicaria o desenvolvimento dessas alterações periapicais seria a que se segue: com o início da infecção nos tecidos periapicais, começa a resposta imune inata; as APCs fagocitam e processam o antigeno, liberam citocinas e quimiocinas que exacerbarão a tal resposta. Essas citocinas atuarão sobre a reabsorção óssea ao induzir a liberação do RANKL que atuará na diferenciação e atividade dos osteoclastos (Vernal *et al.*, 2006). Durante a apresentação do antígeno pela APC ao linfócito pré-imune, se houver a presença de interleucina (IL)-12, ocorrerá a diferenciação dos linfócitos T no subtipo Th1, que, durante a resposta adaptativa, produzirá as citocinas interferon (IFN)-γ e fator de necrose tumoral (TNF) que exacerbarão a resposta imune na região periapical, contribuindo para o processo de reabsorção óssea. Entretanto, se durante a apresentação de antígeno pela APC ao linfócito pré-imune, houver produção de IL-4, os linfócitos T se diferenciarão no subtipo Th2, produzindo as citocinas IL-4, IL-10 e IL-13, que inibirão o processo de reabsorção óssea. A citocina reguladora IL-10 atuará também regulando o processo de diferenciação das células Th1 (Stashenko *et al.*, 1998).

O tratamento endodôntico em um dente infectado atuará removendo a infecção do SCR e, concomitantemente, promovendo o reparo dos tecidos circunvizinhos. Contudo, em

raras ocasiões, durante esse tratamento, acidentes e complicações podem ocorrer. As perfurações radiculares são a segunda razão mais comum da falha associada ao tratamento endodôntico (Ingle J.I. e Taintor J.F., 1989). Os casos de insucesso, cerca de 9%, culminam no início ou perpetuação de uma alteração periapical (Ingle J.I. e Taintor J.F., 1989). Nesses casos, a necessidade da realização de cirurgia parendodôntica é mandatória e, por muitas vezes, a retro-obturação será necessária. Tanto nas indicações de retro-obturação, quanto nos casos em que se procura selar perfurações radiculares, até recentemente, não existia no mercado um material que preenchesse todos os requisitos para o sucesso do tratamento, como ser radiopaco, ser biocompatível, apresentar bom selamento marginal e não ser reabsorvível.

Em 1993, o agregado de trióxido mineral (MTA), desenvolvido pelo Dr. Mahamoud Torabinejad na Universidade de Loma Linda (CA, EUA), foi descrito pela primeira vez na literatura (Lee *et al.*, 1993). O MTA é um pó constituído de partículas hidrofílicas de silicato tricálcio, aluminato tricálcio, óxido tricálcio, óxido de silicato, cuja presa ocorre na presença de umidade. Suas características dependem do tamanho das partículas, relação água/pó e presença de água (Abedi e Ingle, 1995; Schwartz *et al.*, 1999). Esse produto é fabricado no Brasil pela Odonto-lógika (Londrina, Paraná, Brasil) com os nomes de MTA-Ângelus e MTA Branco. Estudos demonstraram semelhanças entre MTA-Ângelus e o ProRoot, fabricado na Suíça (Abedi e Ingle, 1995; Rezende *et al.*, 2005; De Deus *et al.*, 2005), como também, semelhança entre os cimentos cinza e branco. Na composição desse último não se encontra o elemento ferro (Camilleri *et al.*, 2005). Em função da semelhança de resultados e da não alteração da coloração da câmara pulpar, prefere-se o uso do MTA Branco. Brevemente, a Ângelus disponibilizou no mercado outro MTA de coloração branca, denominado de MTA Bios. O MTA Bios apresenta teor de oxido de alumínio e CaO livre superior ao do MTA Ângelus, e um plastificante no líquido que melhora sua manipulação.

Desde sua primeira descrição por Lee *et al.* (1993), utiliza-se o MTA em aplicações cirúrgicas e não cirúrgicas. Esse material foi inicialmente indicado na clínica endodôntica como material retro-obturador e como selador de perfurações radiculares. Sua utilização clínica, porém, foi tão satisfatória que atualmente ele também é indutor de apexificação e capeador direto (Torabinejad e Chivian, 1999; Adamo *et al.*, 1999; Saidon *et al.*, 2003). Parece ser o material com melhores propriedades para aplicações endodônticas que envolvem reparos radiculares e formação óssea, quando comparado com outros materiais já conhecidos: amálgama, *intermediate restorative material* (IRM®), ionômero de vidro, cimento de óxido de zinco e eugenol reforçado (Super-EBA®). O MTA é ideal para se aplicar junto ao osso, por ser o único que, consistentemente, permite a deposição do cemento, a formação óssea e ainda facilita a regeneração do ligamento periodontal (Schwartz *et al.*, 1999).

Como o MTA geralmente é aplicado sobre feridas cirúrgicas, infectadas ou não, é importante que o material seja biocompatível e não interfira nas respostas dos tecidos pulpares e periapicais inflamados, com os quais o MTA entra em contato. Mas, existem poucos estudos que avaliam esses efeitos. Koh *et al.* (1997) testaram a produção de citocinas por osteoblastos em cultura na presença ou ausência do MTA. Observaram maior produção de IL-1α, IL-1 e IL-6 quando se acrescentava o MTA à cultura. O fator estimulador de colônias de monócitos (M-CSF) não se afetou pelo MTA, presente em altas concentrações. Posteriormente, resultados semelhantes foram obtidos utilizando-se culturas de células MG-63 recuperadas de osteossarcoma humano, na presença ou não do MTA (Koh *et al.*, 1998). Usando as mesmas células MG-63 em cultura, Mitchell *et al.* (1999) observaram a produção de IL-6, grandes quantidades de IL-8 e ausência de IL-1α e IL-11, além de produção de M-CSF, na presença de MTA em comparação com as células na ausência do mesmo. É interessante ressaltar que nesse estudo detectou-se uma pequena quantidade de IL-1α quando grandes quantidades de

MTA foram dispostas na cultura. Abdullah *et al.* (2002) também observaram altos níveis de IL-1β, IL-6, IL-8 e osteocalcina na presença do MTA e de variantes do cimento de Portland.

O efeito da exposição da polpa dental ao MTA, *in vivo,* durante a inflamação aguda (10 dias) e crônica (20 dias) foi descrito por Silva (2005). O MTA reduziu a expressão de ácido ribonucléico mensageiro (mRNA) de co-fator de membrana para proteína (Ccl5/RANTES), de IL-1α e IFN-γ. Não se observou expressão de mRNA de IL-6, IL-4 e proteína quimiotática de monócito (Ccl2/MCP-1). A expressão de mRNA para o TNF-α foi variável. Esses resultados conferem um efeito antiinflamatório ao MTA em função do decréscimo da expressão de citocinas pró-inflamatórias.

Para se avaliar a citotoxicidade de materiais retro-obturadores: amálgama, gallium GF2®, ketac silver® e o MTA, Osorio *et al.* (1998) realizaram culturas de fibroblastos L-929 e as expuseram a esses materiais. As provas de citotoxicidade utilizadas foram: o ensaio MTT e o ensaio de cristal violeta para avaliar o número de células mortas e viáveis. Os resultados demonstraram que o MTA não foi citotóxico; gallium GF2® apresentou leve citotoxicidade; e ketac silver, super-EBA e amálgama demonstraram elevados graus de citotoxicidade.

Haglund *et al.* (2003) observaram que nas culturas de macrófagos e fibroblastos expostas ao MTA, a fresco ou a seco, houve diminuição do crescimento celular. Notou-se, contudo, que, a fresco, o MTA induzia desnaturação de proteínas do meio e causava a morte celular na região adjacente ao material. Contudo, ao redor das culturas mortas, observou-se o crescimento celular normal. Quando exposto a seco, não se verificaram alterações na morfologia celular e não se detectou produção de IL-1β e IL-6. Similarmente, também não houve efeito do MTA na produção de citocinas (TNF-α, IL-12p70 e IL-10) por macrófagos M1 e M2 estimulados com antígenos de *Fusobacterium nucleatum* ou *Peptostreptococcus anaerobius*, com e sem IFN-γ. Como esses diferentes tipos de macrófagos M1 e M2 relacionam-se respectivamente ao combate de infecção e à cicatrização, parece que o MTA

não interfere na resposta imune inata de macrófagos (Rezende *et al.*, 2005). Diante desse resultado, fez-se necessária a complementação dessas informações, analisando-se a atividade fagocítica, antibacteriana e cicatricial desses macrófagos na presença do MTA.

Os linfócitos são extremamente relevantes na resposta que se processa nas alterações perirradiculares, contudo o efeito do MTA sobre essas células é ainda hoje pouco avaliado. Braz *et al.* (2006) e da Silva *et al.* (2006), utilizando o ensaio de gel simples (COMET), observaram que o MTA cinza, o branco e o cimento de portland, em concentrações de 1 a 1000µg/mL, por 1 hora, não danificaram o DNA, de linfócitos humanos do sangue periférico. Como pouco se sabe sobre a interação do MTA com linfócitos, faz-se necessária a avaliação de seus efeitos sobre as respostas de linfócitos Th1, Th2 e linfócitos B.

Nas lesões apicais, a resposta imune que ali se processa induz a destruição óssea, uma peculiaridade dessa patologia em relação às demais respostas imunológicas do organismo. Assim, a perpetuação de tal resposta produz diversos mediadores e culmina na reabsorção óssea, incluindo a produção de RANKL, citocina envolvida tanto na regulação fisiológica ou patológica durante a osteoclastogênese como na ativação osteoclástica (Takahashi *et al.*, 1999).

Pouco se sabe sobre o efeito do MTA no processo de remodelação óssea. A literatura pertinente relata apenas alguns resultados da atividade de osteoblastos na presença desse material, com algumas contradições. Existem trabalhos que relataram elevada produção de citocinas envolvidas no processo de remodelação óssea por osteoblastos na presença do MTA (Koh *et al.*, 1997; Mitchell *et al.*, 1999); no entanto, essas mesmas células não se apresentavam viáveis após 13 dias de cultura (Perez *et al.*, 2003). Surgiu, então, a hipótese de que o sucesso clínico obtido com a utilização do MTA poderia ligar-se não apenas à sua interação com os osteoblastos, mas também com os osteoclastos.

Além desses dados, presentes na literatura, concernentes ao MTA, a possível interferência ou não desse material na modulação da resposta imune também deveria ser estudada uma vez que, analisando-se a composição do MTA, observou-se alta incidência de substâncias contendo o íon cálcio. Sabe-se que este íon e o íon alumínio, são liberados em solução (Tomson *et al.*, 2007). Eles atuam diretamente na remodelação óssea (Boyle *et al.*, 2003; Granchi *et al.*, 2005), e o sal alumínio age como adjuvante (Baylor *et al.*, 2002), podendo potencializar a resposta imune adaptativa local e, como conseqüência, aumentar a atividade anti-bacteriana nas lesões periapicais.

Devido às lacunas no conhecimento da área descrita, esta tese se justifica, baseando-se nas seguintes premissas:

(1) o MTA é um material relativamente novo e indicado em casos nos quais a resposta imune é de extrema importância;

(2) ainda existem questionamentos a respeito dos efeitos biológicos do MTA;

(3) uma vez que é indicado em eventos de reabsorção óssea, é necessário avaliar o efeito do MTA em células pré-osteoclásticas e osteoclastos;

(4) baseando-se na liberação de íons por este material, em cultura, propor uma hipótese sobre o mecanismo de atuação do MTA na osteoclastogênese e ativação dos osteoclastos.

2 OBJETIVO

2 OBJETIVOS:

2.1 Objetivo geral:

O presente trabalho objetiva avaliar o papel do MTA quanto à sua capacidade de atuação na imunidade inata e adaptativa como também na reabsorção óssea.

2.2 Objetivos específicos:

- Avaliar a resposta fagocítica e antibacteriana (aderência, fagocitose e espécies reativas de oxigênio (ROI) e nitrogênio (NO) de macrófagos M1 e M2, *in vitro*, na presença e ausência do MTA;
- Avaliar a atividade de arginase, de macrófagos M1 e M2, *in vitro*, na presença e ausência do MTA;
- Avaliar a indução do anticorpo IgG em resposta ao *F. nucleatum*, *in vivo,* na presença e ausência do MTA;
- Avaliar a proliferação celular e a produção de citocinas (TNF-α, IFN-γ, IL-4, IL-10 e RANKL) por linfócitos T pré-imunes estimulados com anti-TCR e anti-CD28 e por células T de memória ao *F. nucleatum* e ao *P. anaerobius*, *in vitro*, na presença e ausência do MTA;
- Avaliar o efeito do MTA na osteoclastogênese e ativação osteoclástica, *in vitro*;
- Propor uma hipótese sobre o mecanismo do efeito do MTA nas células osteoclásticas.

3 TRABALHOS CIENTÍFICOS

3 TRABALHOS CIENTÍFICOS:

3.1 MTA E A RESPOSTA IMUNE INATA:

TRABALHO 1: "The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages". International Endodontic Journal, 40: 603–611, 2007.

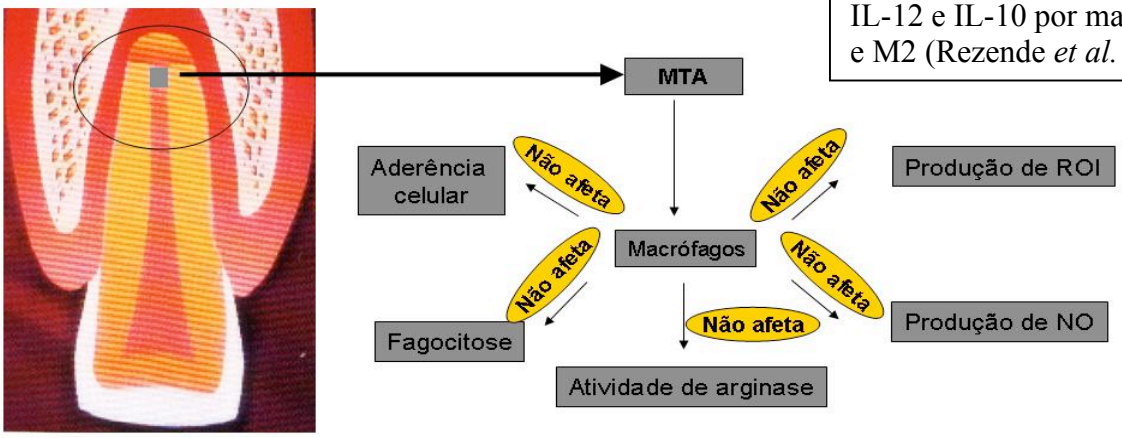
Objetivo: Verificar a influência de co-culturas com o agregado de trióxido mineral (MTA) na fagocitose e produção de espécies reativas de oxigênio (ROI) e nitrogênio (NO) e na atividade de arginase por macrófagos peritoneais M1 e M2.

Metodologia: Viabilidade celular e fagocitose de *Saccharomyces boulardii* foram verificadas na presença do MTA. Macrófagos foram estimulados com zymosan para ensaio de ROI, com *Fusobacterium nucleatum* e *Peptostreptococcus anaerobuius* e IFN-γ para produção de NO e arginase, quando em contato com capilares contendo MTA. Os dados foram analisados pelos testes T, ANOVA, Kruskall-Wallis e Mann-Whitney.

Resultados: Macrófagos M2 apresentaram maior viabilidade celular em tubos de polipropileno, maior habilidade na ingestão de leveduras e menor produção de ROI e maior atividade de arginase quando comparado aos macrófagos M1. Ambos macrófagos, M1 e M2, apresentaram valores similares de aderência celular e produção de NO. A adição de preparações bacterianas aos macrófagos interferiram na produção de NO e de arginase. O MTA não interferiu em nenhum dos parâmetros mensurados.

Conclusão: O MTA não afetou a fagocitose e nem a habilidade dos dois subtipos de macrófagos em eliminar microrganismos.

Resultado prévio: MTA não afeta a produção das citocinas TNF-α, IL-12 e IL-10 por macrófagos M1 e M2 (Rezende *et al.* 2005).



doi:10.1111/j.1365-2591.2007.01255.x

The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages

T. M. B. Rezende[1], L. Q. Vieira[2], F. P. Cardoso[1], R. R. Oliveira[1], S. T. de Oliveira Mendes[1], M. L. R. Jorge[1] & A. P. Ribeiro Sobrinho[1]

[1]Departamento de Dentística Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte; and [2]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Abstract

Rezende TMB, Vieira LQ, Cardoso FP, Oliveira RR, de Oliveira Mendes ST, Jorge MLR, Ribeiro Sobrinho AP. The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages. *International Endodontic Journal*, **40**, 603–611, 2007.

Aim To assess the influence of co-culture with mineral trioxide aggregate (MTA) on phagocytosis and the production of reactive oxygen intermediates (ROI) and nitrogen (NO) species and the arginase activity by M1 and M2 peritoneal macrophages.

Methodology Cellular viability, adherence and phagocytosis of *Saccharomyces boulardii* were assayed in the presence of MTA. Macrophages were stimulated with zymosan for ROI assays and with *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius* and IFN-γ for NO production and arginase activity, when in contact with capillaries containing MTA. Data were analysed by T, ANOVA, Kruskall–Wallis and Mann–Whitney tests.

Results M2 macrophages displayed greater cellular viability in polypropylene tubes, greater ability to ingest yeast and smaller production of ROI and higher arginase activity when compared with M1 macrophages. Both macrophages, M1 and M2, presented similar cell adherence and NO production. The addition of bacterial preparations to macrophages interfered with NO and arginase productions. MTA did not interfere with any of the parameters measured.

Conclusions Phagocytosis and the ability of the two macrophage subtypes to eliminate microbes were not affected by MTA.

Keywords: macrophage, mineral trioxide aggregate, phagocytosis, reactive nitrogen species and arginase activity, reactive oxygen species.

Received 15 August 2006; accepted 12 January 2007

Introduction

Mineral trioxide aggregate (MTA) consists of hydrophilic tri-calcium silicate particles, tri-calcium aluminates, tri-calcium oxide, silicate oxide and other mineral oxides (Lee *et al.* 1993b, Torabinejad *et al.* 1993, Abedi & Ingle 1995, Torabinejad & Chivian 1999). MTA is sold under the brand name ProRoot® (Dentsply Maillefer, Ballaigues, Switzerland) and, in Brazil, MTA-Angelus® (Odonto-lógika, Londrina, Brazil). As MTA is indicated for the use in inflamed or infected areas, it ought to be biocompatible and not affect cell behaviour.

Macrophages predominate amongst the several cells present in the inflamed pulp and periapical tissues (Stern *et al.* 1981, Kawashima *et al.* 1996). Recently, macrophages have been divided into two subtypes: M1 and M2, according to their ability to produce different types of responses (Mills *et al.* 2000, Bastos *et al.* 2002,

Correspondence: Dr Antônio Paulino Ribeiro Sobrinho, Departamento de Odontologia Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil (Tel.: 55-31-34992460; fax: 55-31-34992472; e-mail: sobrinho.bhz@terra.com.br).

Mantovani *et al.* 2002, Mosser 2003, Rezende *et al.* 2005). M1 macrophages are activated in the classical way, in the presence of IL-12, by IFN-γ, to produce nitric oxide (NO) and reactive oxygen intermediates (ROI) in response to microbes (Mosser 2003). In the presence of oxygen, NO generates reactive nitrogen intermediates which will cause damage to cells and hence to infectious agents (Nathan & Shiloh 2000). ROI are intermediate reduction products of oxygen, such as superoxide, hydrogen peroxide, and hydroxyl radical, as well as reactive products of these with halides and amines (Nathan & Shiloh 2000). Together, nitrogen and oxygen intermediates mediate resistance to pathogens (Nathan & Shiloh 2000). On the other hand, M2 macrophages preferably convert arginine into urea and ornithine, which results ultimately in collagen production and cellular proliferation (Mills *et al.* 2000, Bastos *et al.* 2002, Mosser 2003). M2 macrophages have been found to pre-dominate in BALB/c mice and in mice genetically deficient in the p40 chain of IL-12/23 by homologous recombination (IL-12p40$^{-/-}$) (Bastos *et al.* 2002).

During infection, macrophages play a crucial role in eliminating pathogens and in the healing process. Hence, this study endeavors to determine phagocytic activity, production of free radicals and arginase activity in the presence of MTA from two sources (MTA-Angelus® and ProRoot®). M1 macrophages were isolated from C57BL/6 mice, and M2 macrophages from IL-12p40$^{-/-}$ in the C57BL/6 background. Cellular viability, cellular adherence and phagocytosis were assayed, as well as the production of ROI and NO and arginase activity.

Materials and methods

Mice

Male and female 4- to 8-week-old wild-type (wt) C57BL/6 (CEBIO – UFMG, Belo Horizonte, Brazil) and C57BL/6 deficient in the p40 chain for IL-12/IL-23 (IL-12p40$^{-/-}$; Gnotobiology and Immunology Laboratory, UFMG, Belo Horizonte, Brazil) by targeted mutation (Magram *et al.* 1996) were kept in a conventional animal house with barriers, temperature and light control. Food and water were offered *ad libitum*.

Isolation of macrophages

Cells were isolated from the peritoneal cavity of wt and IL-12p40$^{-/-}$ C57BL/6 mice 5 days after injection of 2 mL of 3% thioglycolate medium (Biobrás S.A., Montes Claros, MG, Brazil) in the peritoneum. Cells were resuspended in complete medium: RPMI 1640 without phenol red (Sigma Chemicals Co., St Louis, MO, USA), supplemented with 10% of fetal calf serum (Nutricell, Campinas, SP, Brazil), 0.1% of 0.05 mol L^{-1} β-mercaptoethanol (Sigma Chemicals Co.), 0.2% of penicillin (100 U mL^{-1})/streptomycin (0.1 mg mL^{-1}) and 200 mmol L^{-1} L-glutamine (Oliveira Mendes *et al.* 2003).

Cell cultures

Cells (1×10^5 cells mL^{-1}) were cultured in 1 mL of medium, in 24-well culture plates (Nunclon; Nalge Nunc International, Naperville, IL, USA), for the assessment of viability; 5×10^5 cells mL^{-1} for the phagocytosis assay; 1×10^6 cells mL^{-1} for the verification of cellular adherence, cellular viability, ROI production and arginase activity; and 2×10^6 cells mL^{-1} for NO detection. All incubations were performed in an incubator with humidified atmosphere containing 5% CO_2, at 37 °C.

MTA manipulation

Both MTA brands were prepared in accordance with manufacturers' instructions in sterile conditions. Soon after preparation, MTA was inserted into the tips of previously sectioned sterilized capillary tubes (test group), so that their contact with the cell suspension could be standardized (Oliveira Mendes *et al.* 2003). Empty capillary tubes were used in control cultures. Capillaries were sterilized by exposure to 25 kGray Gamma ray irradiation (CDTN, Belo Horizonte, MG, Brazil).

Cell viability

Cells were cultured for 24 h, in polypropylene tubes. Cellular viability was determined by the trypan blue exclusion assay (Oliveira Mendes *et al.* 2003).

Cell adherence

Polypropylene tubes containing macrophages were incubated for 2 h with capillaries (test and control groups) in an incubator with humidified atmosphere containing 5% CO_2, at 37 °C. Tubes were agitated in a vortex agitator for 5 s, at low speed. Twenty microlitres of the cellular suspension were removed placed into a Newbauer chamber and incubated for 1 h at 37 °C as

above. The percentage of adherent and nonadherent macrophages was then established by counting under an optical microscope (Lee *et al.* 1993a).

Phagocytosis assay

Cells (1×10^6 in 1 mL) were incubated for 2 h in 24-well culture plates (Nunclon, Nalge Nunc International, Miami, FL, USA), onto round glass coverslips (Glasstécnica, São Paulo, SP, Brazil) in an incubator as above. Nonadherent cells were removed by washing with warm complete medium, afterwards 10^7 CFU of *Saccharomyces boulardii* (Floratil; Merck S.A., Rio de Janeiro, RJ, Brazil) and capillaries with or without MTA were added to the medium and plates were incubated for 1 h. Unbound yeast cells were removed by washing with complete medium and the coverslips were covered for 1 min with 1 mL of tannic acid at 1% (Merk), so that the distinction could be made between extracellular and intracellular yeast cells. One drop of fetal calf serum was applied onto each coverslip. The dried coverslips were stained with Panótico Rápido (Laborclin Ltd, Pinhais, PR, Brazil) and glued to microscope glass slides with Entellan (Merk) for observation under optical microscope at 1000× magnification in oil immersion (Giaimis *et al.* 1992). To determine the percentages of macrophages with phagocyted yeast, the percentages of macrophages with adhered yeast, as well as the number of phagocyted yeast/cells, a minimum of 200 cells were counted.

Reactive oxygen intermediates assay

Cells were cultured in polypropylene tubes in an incubator as above. After 24 h, 1×10^6 cells were transferred to a C96 White Maxisorp (Nalgene, Rochester, New York, USA) plate in 100 µL; 10^7 zymosan particles (Sigma Chemical Co.) and 0.05 mmol L^{-1} luminol in 1640 RPMI without phenol red were added to each well. Plates were read every 2 min for 118 min in a luminometer (LumiCount®; Packard Instrument Company Inc., Downers Grove, IL, USA) (Trusk *et al.* 1978). This procedure will measure the luminol-amplified chemiluminescence due to the production of superoxide, hydroxyl radical and oxygen peroxide in response to ingestion of zymosan particles by phagocytes (Trusk *et al.* 1978). Results were expressed as the area under each of the curves obtained in the 118-min period, calculated as follows:

$$I = h[f_2 + f_3 + \cdots + f_{(n-1)}] + \frac{(f_1 + f_n)h}{2}$$

where I is the area underneath the curve, h the time interval, f_1, f_2,…,f_n indicate light units at times 1, 2,…, n (n measurements).

NO production

Cells were cultured with capillaries (test and control groups) in 24-well culture plates (Nunclon) in an incubator (as above) with 10^7 CFU of heat-killed *Fusobacterium nucleatum* (ATCC 10953) or *Peptostreptococcus anaerobius* (ATCC 27337). Some cultures were activated with 10 U mL^{-1} of recombinant IFN-γ (Pharmingen, San Diego, CA, USA). Cells were cultured for 72 h and NO production was assayed as nitrites in the supernatants. Sodium nitrite (0.977–125 µmol L^{-1}) was used as standard (Green *et al.* 1982). Sensibility was 0.5 mmol L^{-1}.

Arginase activity

Cells were cultured with capillaries (test and control groups) in polypropylene tubes in an incubator as above with 10^7 CFU of heat-killed *F. nucleatum* (ATCC 10953) or *P. anaerobius* (ATCC 27337), for 48 h. Arginase activity was measured in cell lysates as described by Corraliza *et al.* (1994). Briefly, cells were lysed with 50 µL of 0.1% Triton X-100 (Sigma Chemicals Co.). After 30 min on a shaker, 50 µL of 10 mmol L^{-1} $MnCl_2$ (Merck), 50 µL of mmol L^{-1} Tris-HCl (Merck) were added, and the enzyme was activated by heating for 10 min at 55 °C. Arginine hydrolysis was conducted by incubating 25 µL of the activated lysate with 25 µL of 0.5 mol L^{-1} L-arginine (Merck; pH 9.7) at 37 °C for 60 min. The reaction was stopped with 400 µL of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). As a degree of arginase activity, the urea concentration was measured at 540 nm after addition of 25 µL of α-isonitrosopropiophenone (Sigma Chemical Co.) dissolved in 100% ethanol, for 45 min at 95 °C. One unit of arginase activity was defined as the amount of enzyme that catalysed the formation of 1 µmol urea min^{-1}. The standard curve was prepared with increasing amounts of urea between 1.625 and 100 µg.

Statistical analysis

Data were analysed using parametric (ANOVA and *t*-test) and nonparametric (Mann–Whitney and Kruskal–Wallis) tests ($P < 0.05$). Analyses were made using the SPSS 8.0 Inc. (Statistical Package for Social Sciences, Chicago, IL, USA) software.

Animal ethical committee

These experiments were authorized by CETEA – UFMG (protocol no. 35/2002).

Results

Cell viability

Cellular viability was greater than 80% in polypropylene tubes (Fig. 1), for both macrophage subtypes. Analyzing macrophages subtypes, M1 macrophages survived better than M2 ($P < 0.05$). MTA did not affect viability when compared with controls in all conditions tested.

Cell adherence

Cell adherence was similar between M1 and M2 macrophages. MTA did not affect the capacity of either macrophage subtype to adhere to glass (Fig. 2).

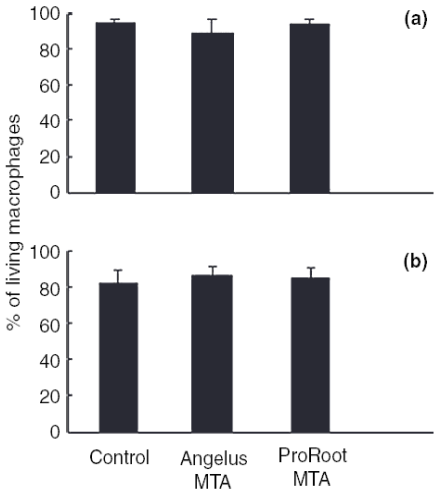


Figure 1 Percentage of living M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice, after incubation in polypropylene tubes with capillaries containing MTA from both commercial sources. Controls were cultured with empty capillaries. Cultures were maintained for 24 h as described in materials and methods. Bars represent the mean of two experiments, lines stand for the standard error of the means.

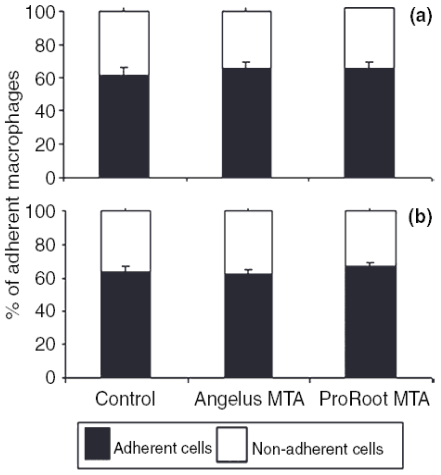


Figure 2 Percentage of adherent M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice, after incubation in culture plates with capillaries containing MTA from both commercial sources. Controls were cultured with empty capillaries. Cultures performed as described in materials and methods. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means.

Phagocytic activity

The percentage of macrophages with adhered yeast to their surface yeast was similar for M1 and M2 subtypes (Fig. 3a,b). However, M2 macrophages presented larger numbers of ingested yeast cells in all conditions examined ($P < 0.05$) (compare Fig. 3c,d). MTA did not affect either parameter. Macrophages in contact with MTA presented bi-refractive structures inside, suggesting that MTA was ingested (Fig. 7).

Production of reactive oxygen intermediates

Analysis of the area under each ROI production's curves showed that in general, M1 macrophages produced more ROI than M2, but when the test and control groups were compared between two macrophages' types there was no statistical differences. Addition of zymosan induced significantly higher levels of ROI production by M1 macrophage ($P < 0.05$). MTA did not affect ROI production in any of the conditions examined (Fig. 4).

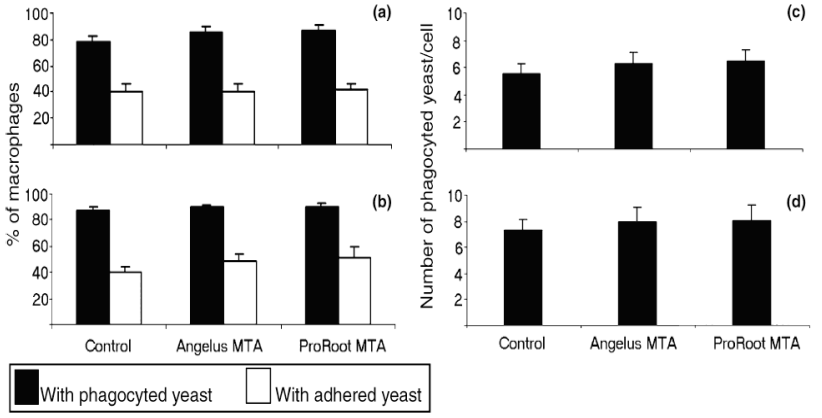


Figure 3 Percentage of M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice displaying phagocyted or adherent yeast cells, after incubation in culture plates with capillaries containing MTA from both commercial sources. Numbers of yeast cells per M1 (c) and M2 (d) macrophages are also shown. Controls were cultured with empty capillaries. Cultures performed as described in materials and methods. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means. All M1 data were statistically different from M2, under the same conditions.

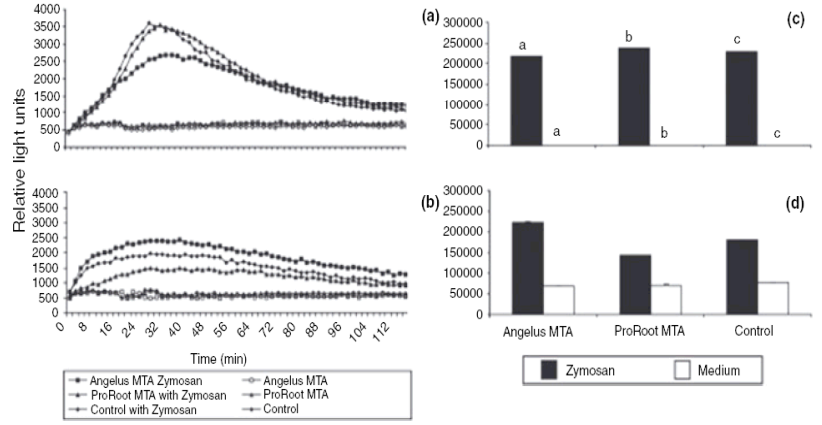


Figure 4 Kinetic of reactive oxygen intermediates (ROI) production by M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice. ROI total production by M1 macrophages (c) and M2 macrophages (d). Cells were cultured with capillaries containing MTA and stimulated with zymosan as described in materials and methods. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means. Different letters indicate statistical difference for ROI production in response to zymosan when compared with macrophages in medium alone, by Mann–Whitney test.

NO production

M1 and M2 macrophages produced similar levels of NO in all conditions examined (Fig. 5). Addition of bacterial preparations to macrophages induced NO production, and activation by IFN-γ increased this production, especially in the cultures stimulated with *F. nucleatum* ($P < 0.05$). MTA had no significant effect on NO production.

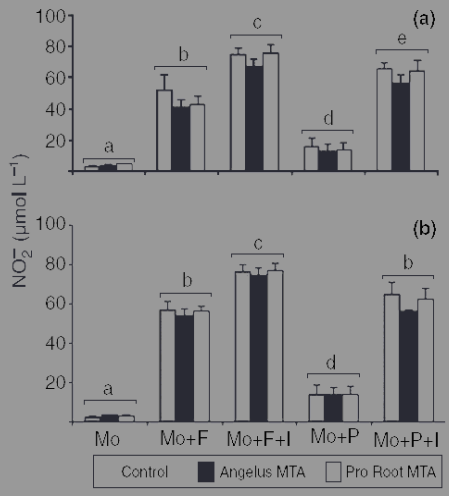


Figure 5 Nitric oxide (NO_2^-) production by M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice. Cells were cultured with capillaries containing MTA and stimulated with *F. nucleatum* (F) or *P. anaerobius* (P), and IFN-gamma (I) as indicated. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means. Different letters indicate statistical difference in the same macrophage subtype (M1 or M2). $P < 0.05$. No differences were found between M1 and M2 macrophages in the same conditions.

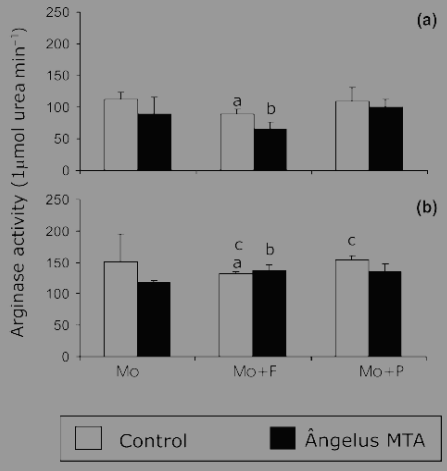


Figure 6 Arginase activity by M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40-/- mice. Cells were cultured with capillaries containing MTA and stimulated with *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P), as indicated. Bars stand for the mean results of three experiments made in duplicates. Lines indicate standard error of the means. a, b indicate $P < 0.05$ for arginase activity when compared two. c indicates $P < 0.05$ when compared to kinds of stimuli in M2 macrophages.

Arginase activity

Mineral trioxide aggregate did not affect the arginase activity of either macrophage subtype (Fig. 6). M2 macrophage presented higher arginase activity than M1 macrophage when it was stimulated with *F. nucleatum*, in MTA and control groups ($P < 0.05$). In M2 macrophages, control group stimulated with *P. anaerobius* presented higher activity than control group stimulated with *F. nucleatum* ($P < 0.05$).

Discussion

Mineral trioxide aggregate is indicated for pulp-capping, pulpotomy, repair of root perforations, fractures and apical barriers in root with incomplete apex formation (Adamo *et al.* 1999, Torabinejad & Chivian 1999, Saidon *et al.* 2003). However, little information is available on MTA interaction with the immune responses to infection, which is relevant since in every

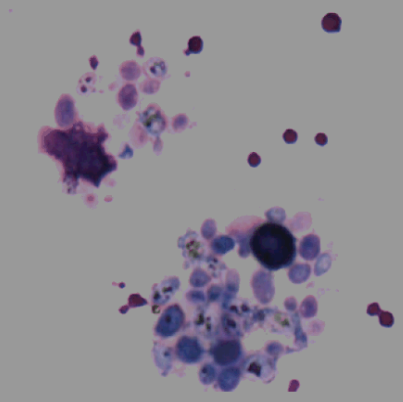


Figure 7 M1 macrophages containing ingested yeast cells (pink, arrows) and numerous adhered yeast cells (purple, stars). Bi-refractive structures are found on and apparently in macrophages, suggesting that MTA might be ingested by macrophages (arrow heads). 1000× under immersion objective.

clinical condition for which this material is indicated there will be contact of MTA with pulpal, periapical, or periodontal tissues.

As soon as the inflammatory process starts, immune-competent cells are attracted to the site, in an attempt to eliminate the aggressors. Of these cells, infiltrating professional phagocytes, neutrophils and macrophages are amongst the first cells to make contact with the foreign bodies, playing therefore the main role in the pathogenesis of the inflammatory process (van Furth *et al.* 1972, Unanue 1978, Stashenko *et al.* 1998, Metzger 2000, Oliveira Mendes *et al.* 2003).

Recent studies propose that two subtypes of macrophages exist, with different response profiles: M1 and M2 (Metzger 2000, Mills *et al.* 2000). IL-12 would drive macrophages to the M1 subtype (Metzger 2000, Mills *et al.* 2000), whilst the absence or low production of this cytokine would lead macrophages to the M2 subtype (Mills *et al.* 2000, Bastos *et al.* 2002). Hence, IL-12p40$^{-/-}$ mice are a good source of M2 macrophages, although this model does not exclude the production of other cytokines that, along with IL-12, induce a Th1 response, such as IL-18 (Joosten *et al.* 2000).

Using both types of macrophages exposed to two different sources of MTA, this study assessed the cellular viability in polypropylene tubes, and showed that M2 macrophages presented lower levels of cellular viability than M1 macrophages. However, differences in cell viability were small amongst culture conditions, and viability was consistently high. Similar values for the test groups (with MTA) and the control groups (without MTA) were observed. In the literature, similar results were reached using fibroblasts (Keiser *et al.* 2000, Saidon *et al.* 2003), osteoblasts (Koh *et al.* 1997, Mitchell *et al.* 1999) and macrophages (Haglund *et al.* 2003, Rezende *et al.* 2005).

Several studies that evaluate biocompatibility of endodontic materials assayed macrophage adherence and spreading (Zhu *et al.* 2000, Oliveira Mendes *et al.* 2003). MTA did not affect either macrophage subtype in their capacity to adhere to glass, which is consistent with results found for osteoblasts and an osteosarcoma cell line exposed to MTA (Koh *et al.* 1997, 1998, Zhu *et al.* 2000).

Mineral trioxide aggregate is applied onto areas that might be inflamed and/or infected, and it is fundamental that it does not interfere with the host's phagocytosis process. In this study, the capability of both macrophage subtypes to ingest *Saccharomyces boulardii* was evaluated in the presence of MTA, and compared with controls. This yeast was selected because of its size, which makes counting easier, and therefore allows for greater data precision. Neither brand of MTA interfered with the percentage of adhered yeast cells to macrophages or with phagocytosis of yeast cells. However, the number of ingested yeast cells was greater in the M2 macrophages. Conversely, M2 macrophages in contact with zinc oxide eugenol based material had their phagocytosis ability inhibited (Oliveira Mendes *et al.* 2003). This phenomenon was also observed by Bastos *et al.* (2002), who found a greater number of *Trypanosoma cruzi* amastigotes in M2 than in M1 macrophages.

Reactive oxygen intermediates and NO are free radicals that are released by phagocytes exposed to microbes (Marton & Kiss 2000). Both macrophages subtypes produced significant amounts of ROI in response to zymosan. Moreover, in accordance to previous report (Mosser 2003), M1 macrophages produced greater level of ROI than M2. Exposure to MTA did not interfere with the peak of ROI production. This is, to our knowledge, the first study in the literature that assayed the effect of MTA on ROI production.

Considering that *F. nucleatum* and the *P. anaerobius* are bacteria usually found in endodontic infections (Sundqvist 1992, Lana *et al.* 2001), they were chosen as stimuli for the production of NO. These heat-killed bacteria were used with or without IFN-γ. There was no difference in NO production between control and MTA and between M1 and M2 macrophages. These results are not supported by the current literature (Mills *et al.* 2000, Bastos *et al.* 2002, Mosser 2003). As we have recently shown that M2 macrophages produce higher levels of IL-10 than M1 (Rezende *et al.* 2005), this production of IL-10 might have been sufficient to suppress ROI production, but could be insufficient to inhibit NO production (Bogdan *et al.* 1991). Alternatively, the higher phagocytic capacity of M2 might have provided a greater stimulation to these cells that to M1 macrophages.

Using the same heat-killed bacteria, arginase activity was measured in cell lysates of M1 and M2 macrophage cultures. It is known that M2 macrophages convert L-arginine to L-ornitine and urea and this reaction results in cell growth and differentiation and collagen production. In this study, MTA did not affect arginase activity. M2 macrophage presented higher arginase activity than M1 macrophages. This result agrees with current literature (Mills *et al.* 2000, Bastos *et al.* 2002, Mosser 2003). The higher levels of IL-10, produced by M2 macrophage previously reported (Rezende *et al.* 2005), as a Th2 cytokine, inhibits the activity of type 2

enzyme nitric-oxide synthase (NOS 2) and induces arginase synthesis (Gordon 2003).

Conclusion

It has recently been reported that MTA does not interfere in the cytokine response by M1 or M2 macrophages (Rezende *et al.* 2005). The present study suggests that MTA did not inhibit phagocytosis, reactive species production and arginase activity, which are protective responses against infections. It is concluded that MTA does not affect effector macrophage activities.

Acknowledgements

Thanks are due to the post-graduate programme of the School of Dentistry of UFMG, the Laboratory of Microbial Ecology and Oral Microbiology Laboratory at UFMG and the Centro de Desenvolvimento Tecnológico Nuclear (CDTN). We are indebted to Antonio Mesquita Vaz for excellent animal care. This study has received financial support from CAPES, CNPq and Fapemig. Dentsply-Maillefer (Ballaigues, Switzerland) and Odonto-lógika (Londrina, PR, Brazil) kindly provided Root® and MTA-Ângelus®.

References

Abedi HR, Ingle JI (1995) Mineral trioxide aggregate: a review of a new cement. *Journal of the California Dental Association* **23**, 36–9.

Adamo HL, Buruiana R, Schertzer L, Boylan RJ (1999) A comparison of MTA, Super-EBA, composite and amalgam as root-end filling materials using a bacterial microleakage model. *International Endodontic Journal* **32**, 197–203.

Bastos KR, Alvarez JM, Marinho CR, Rizzo LV, Lima MR (2002) Macrophages from IL-12p40-deficient mice have a bias toward the M2 activation profile. *Journal of Leukocyte Biology* **71**, 271–8.

Bogdan C, Vodovotz Y, Nathan C (1991) Macrophage deactivation by interleukin 10. *Journal of Experimental Medicine* **174**, 1549–55.

Corraliza IM, Campo ML, Soler G, Modolell M (1994) Determination of arginase activity in macrophages: a micromethod. *Journal of Immunological Methods* **174**, 231–5.

van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bulletin of the World Health Organization* **46**, 845–52.

Giaimis J, Lombard Y, Makaya-Kumba M, Fonteneau P, Poindron P (1992) A new and simple method for studying the binding and ingestion steps in the phagocytosis of yeasts. *Journal of Immunological Methods* **154**, 185–93.

Gordon S (2003) Alternative activation of macrophages. *Nature Review of Immunology* **3**, 23–35.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry* **126**, 131–8.

Haglund R, He J, Jarvis J, Safavi KE, Spangberg LS, Zhu Q, (2003) Effects of root-end filling materials on fibroblasts and macrophages in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **95**, 739–45.

Joosten LA, van De Loo FA, Lubberts E, Helsen MM, Netea MG, van Der Meer JW et al. (2000) An IFN-gamma-independent proinflammatory role of IL-18 in murine streptococcal cell wall arthritis. *Journal of Immunology* **165**, 6553–8.

Kawashima N, Okiji T, Kosaka T, Suda H (1996) Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: a quantitative immunohistochemical study. *Journal of Endodontics* **22**, 311–6.

Keiser K, Johnson CC, Tipton DA (2000) Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. *Journal of Endodontics* **26**, 288–91.

Koh ET, Torabinejad M, Pitt Ford TR, Brady K, McDonald F (1997) Mineral trioxide aggregate stimulates a biological response in human osteoblasts. *Journal of Biomedical Materials Research* **37**, 432–9.

Koh ET, McDonald F, Pitt Ford TR, Torabinejad M (1998) Cellular response to mineral trioxide aggregate. *Journal of Endodontics* **24**, 543–7.

Lana MA, Ribeiro-Sobrinho AP, Stehling R, Garcia GD, Silva BK, Hamdan JS et al. (2001) Microorganisms isolated from root canals presenting necrotic pulp and their drug susceptibility in vitro. *Oral Microbiology and Immunology* **16**, 100–5.

Lee A, Whyte MK, Haslett C (1993a) Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *Journal of Leukocyte Biology* **54**, 283–8.

Lee SJ, Monsef M, Torabinejad M (1993b) Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. *Journal of Endodontics* **19**, 541–4.

Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, Ferrante J et al. (1996) IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* **4**, 471–81.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology* **23**, 549–55.

Marton IJ, Kiss C (2000) Protective and destructive immune reactions in apical periodontitis. *Oral Microbiology and Immunology* **15**, 139–150.

Metzger Z (2000) Macrophages in periapical lesions. *Endodontics Dental Traumatology* **16**, 1–8.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. *Journal of Immunology* **164**, 6166–73.

Mitchell PJ, Pitt Ford TR, Torabinejad M, McDonald F (1999) Osteoblast biocompatibility of mineral trioxide aggregate. *Biomaterials* **20**, 167–73.

Mosser DM (2003) The many faces of macrophage activation. *Journal of Leukocyte Biology* **73**, 209–12.

Nathan C, Shiloh MU (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8841–8.

Oliveira Mendes ST, Ribeiro Sobrinho AP, de Carvalho AT, Souza Cortes MI, Vieira LQ (2003) In vitro evaluation of the cytotoxicity of two root canal sealers on macrophage activity. *Journal of Endodontics* **29**, 95–9.

Rezende TM, Vargas DL, Cardoso FP, Sobrinho AP, Vieira LQ (2005) Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages. *International Endodontic Journal* **38**, 896–903.

Saidon J, He J, Zhu Q, Safavi K, Spangberg LS (2003) Cell and tissue reactions to mineral trioxide aggregate and Portland cement. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **95**, 483–9.

Stashenko P, Teles R, D'Souza R (1998) Periapical inflammatory responses and their modulation. *Critical Reviews in Oral Biology and Medicine* **9**, 498–521.

Stern MH, Dreizen S, Mackler BF, Selbst AG, Levy BM (1981) Quantitative analysis of cellular composition of human periapical granuloma. *Journal of Endodontics* **7**, 117–22.

Sundqvist G (1992) Ecology of the root canal flora. *Journal of Endodontics* **18**, 427–30.

Torabinejad M, Chivian N (1999) Clinical applications of mineral trioxide aggregate. *Journal of Endodontics* **25**, 197–205.

Torabinejad M, Watson TF, Pitt Ford TR (1993) Sealing ability of a mineral trioxide aggregate when used as a root end filling material. *Journal of Endodontics* **19**, 591–5.

Trusk MA, Wilson ME, Dyke KV (1978) The generation of chemiluminescence by phagocytic cells. In: Marlene A. Deluca, ed. *Methods in Enzymology*, London, UK: Academic Press, pp. 462–93.

Unanue ER (1978) The regulation of lymphocyte functions by the macrophage. *Immunology Review* **40**, 227–55.

Zhu Q, Haglund R, Safavi KE, Spangberg LS (2000) Adhesion of human osteoblasts on root-end filling materials. *Journal of Endodontics* **26**, 404–6.

3.2 MTA E A RESPOSTA IMUNE ADAPTATIVA

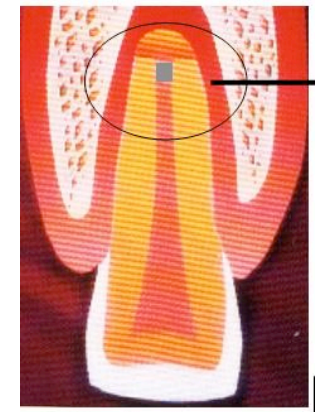
TRABALHO 2: “The influence of Mineral Trioxide Aggregate (MTA) on adaptive immune responses to endodontic pathogens in mice”. Trabalho submetido à publicação no Journal of Endodontics.

Objetivo: Acessar a influência do Agregado de Trióxido Mineral (MTA) nas respostas imunológicas adaptativas.

Métodos: Camundongos BALB/c foram imunizados com *Fusobacterium nucleatum* (*Fn*) mortos pelo calor em MTA ou em adjuvantes e a resposta de IgG sérica ao *Fn* foi mensurada. Tanto células T de memória (Tm) reativas ao *Fn* quanto Tm reativas ao *Peptostreptococcus anaerobius* (*Pa)* foram pre-incubadas, *in vitro* com e sem MTA e re-estimuladas com *Fn* e *Pa*. A proliferação de células T e a produção de citocinas foram verificadas.

Resultados: Comparando com os grupos imunizados apenas com o *Fn*, a resposta a produção de IgG foi aumentada quando os camundongos foram imunizados com *Fn* em MTA em níveis comparáveis a resposta do anticorpo IgG induzida por adjuvante de Freund ou adjuvante de hidróxido de alumino. MTA suprimiu a proliferação de células T de memória Th2 reativas ao *F. nucleatum* e Th1 reativas ao *P. anaerobius,* além de reduzir a expressão das citocinas típicas do subtipo Th1 e Th2, IFN-γ e IL-4, respectivamente; o uso do MTA resulta em pouca ou nenhuma alteração na expressão de outras citocinas (TNF-α, RANKL e IL-10) pelas mesmas células.

Conclusão: O MTA aumentou a resposta imunológica adaptativa humoral e pouco afetou a produção das citocinas pró- e anti-inflamatórias produzidas pelas células Tm.



MTA

Não afeta

Estimula

Efeito do MTA nas células T de memória

Efeito do MTA nas células T de memória

Produção de IgG

Células T naïve TCR/CD28 ativadas

	Capilares com MTA
Proliferação celular	Estimula
IFN-gama	Estimula
TNF-alfa	Estimula
RANKL	Estimula
IL-10	Suprime
IL-4	Sem efeito

Concentração dos nívis de citoinas em baixa escala

Células T de memória reativas ao *F. nucleatum*

	Capilares com MTA
Proliferação celular	Suprrime
IFN-gama	Sem efeito
TNF-alfa	Sem efeito
RANKL	Sem efeito
IL-10	Sem efeito
IL-4	Suprime

Células T de memória reativas ao *P. anaerobius*

	Capilares com MTA
Proliferação celular	Suprime
IFN-gama	Suprime
TNF-alfa	Sem efeito
RANKL	Sem efeito
IL-10	Sem efeito
IL-4	Sem efeito

The influence of Mineral Trioxide Aggregate (MTA) on adaptive immune responses to endodontic pathogens in mice

Authors: Taia Maria Berto Rezende[1], Leda Quercia Vieira[2], Antônio Paulino Ribeiro Sobrinho[3], Ricardo Reis Oliveira[4], Martin A. Taubman[5] and Toshihisa Kawai[6]

[1]Ph.D. student, Departamento de Dentística Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; [2]Associate Professor, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; [3]Associate Professor, Departamento de Dentística Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; [4]Master degree student, Departamento de Dentística Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil;[5] Head of Department of Immunology, The Forsyth Institute, Boston, MA, USA;[6] Associate Member of the Staff, Department of Immunology, The Forsyth Institute, Boston, MA, USA.

Running title: MTA effects on adaptive immune response

Keywords: MTA, IgG antibody, T cells, cytokine, bacteria

Correspondent:

Toshihisa Kawai, DDS, Ph.D., Department of Immunology, The Forsyth Institute

140 The Fenway

Boston, Massachusetts, 02115 USA

Phone: 617-892-8317 – Fax: 617-892-8437

E-mail: tkawai@forsyth.org

Acknowledgments: We thank Dr. Ricardo Teles and Dr. Flávia Teles for their kind gift of the bacteria used in this study and Han Xiaozhe and Jean Eastcott for the flow cytometry experiments. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and NIDCR grants DE 03420, DE 14551 and DE18310.

Abstract

Aim: To assess the influence of mineral trioxide aggregate (MTA) on adaptive immune responses.

Methods: BALB/c mice were immunized with heat-killed *Fusobacterium nucleatum* (*Fn*) in MTA or in other control adjuvants, and serum IgG responses to *Fn* were measured. Either *Fn*- or *Peptostreptococcus anaerobius* (*Pa)*-reactive memory T (Tm) cells were pre-incubated *in vitro* with/without MTA and restimulated with *Fn* or *Pa*. T cell proliferation and cytokine production were assessed.

Results: Compared to groups immunized with control *Fn* immunization alone, IgG-antibody responses were upregulated in mice immunized with *Fn* in MTA at a comparable level of IgG antibody responses induced by Freund's adjuvant or aluminum hydroxide adjuvant. While MTA did not affect the upregulated expression of IL-10, TNF-α or RANKL by Tm cells, it suppressed the proliferation of *Pa*- or *Fn*-Tm cells and inhibited their production of Th1- or Th2-signature cytokines.

Conclusion: MTA upregulated the adaptive humoral immune responses, but had little or no effect on the pro- or anti-inflammatory cytokine production by Tm cells.

Introduction

Bacterial infection at either gum or root sites results in the development of inflammatory lesions of periodontal or periapical tissue, which are accompanied by the natural infiltration of a variety of immune cells (1;2). In general, innate immune cells play an antibacterial role at the acute stage of infection, while adaptive immune cells are committed to eliminating bacteria at the chronic stage of infection. Adaptive immune responses, which include antibody production and cell-mediated immune responses, are considered to be the host protective means against bacterial infection at human periapical inflammatory lesions (3). As the early acute stage of the periapical infectious lesion phases into the chronic stage, bone resorption develops around the root end of a tooth, together with a corresponding increase in the infiltration of adaptive immune cells (4).

The application of mineral trioxide aggregate (MTA) to periapical lesion caused by bacterial infection can only be considered optimally effective if it does not compromise either innate or adaptive immune response. Our group previously demonstrated that MTA does not affect the activity of innate immune macrophages. More specifically, MTA did not show any effects on the antibacterial activities of either M1- or M2-type macrophages, including bacterial phagocytosis, reactive oxygen and nitrogen species production or arginase activity (5). However, the effects, if any, that MTA exerts on adaptive immune cells are still to be elucidated.

To determine the presence or absence of such effects, our experimentation was principally based on the application of aluminum salts, which are known to be a potent adjuvant for immunization with protein antigens. While aluminum hydroxide ($Al(OH)_3$) is most commonly used as an immune adjuvant, some studies showed that aluminum oxide (Al_2O_3) also possesses adjuvant effects (6). Aluminium hydroxide is included as an adjuvant in some vaccines (e.g., Alhydrogel), since it contributes to induction of a good antibody

response. However, it has little capacity to stimulate cellular immune responses, important for protection against many pathogens (7). Very interestingly, MTA is also composed of aluminum oxide, as well as other mineral components, such as lime (CaO) and silica (SiO_2) (8). It has been reported that aluminum in cation form (Al^{3+}) is detected in distilled water incubated with MTA (9). It is therefore conceivable that aluminum in the form of salt or cation is released from MTA under physiological conditions. Based on these lines of evidence, we were initially led to hypothesize that MTA could augment host protective antibody responses, while suppressing tissue destructive cellular immune responses due to its adjuvant effects by the presence of aluminum oxide (Al_2O_3). To test this hypothesis, the present study focused on assessing the influence of MTA on (1) the induction of *in vivo* IgG antibody response to *F. nucleatum*, and (2) the cytokine production pattern of memory T cells, which were primed by two bacteria commonly found in periapical lesions, *F. nucleatum* or *P. anaerobius*.

Materials and Methods

MTA preparation: Ângelus MTA (Odonto-lógika, Londrina, Paraná, Brazil) paste was prepared from the mixture of MTA powder and distilled water in a sterile condition, according to manufacturer's instructions. By the method previously published (10), the MTA paste was then inserted into both ends of sterilized glass capillary tubes (Ø = 1.2 mm; length = 10 mm) so that the contact surface with the cell culture medium could be standardized (area = 2.26 mm^2). Empty capillary tubes without MTA were used as negative controls.

Animals: BALB/c mice (6- to 8-week-old males, n=6/group) were utilized. Animals were kept in a conventional cage and maintained at controlled ambient temperature. Food and

water were offered to animals *ad libitum.* The protocol for this animal experiment was approved by The Forsyth Institute's animal ethics committee.

Antigen preparation: Two types of bacteria, *F. nucleatum* ATCC 10953 and *P. anaerobius* ATCC 27337, were chosen to represent Gram-negative and Gram-positive endodontic pathogens, respectively (11). *F. nucleatum* and *P. anaerobius* were grown in blood agar plate (Becton, Dickinson, Franklin Lake, NJ), harvested during the log growth phase and counted using a spectrophotometer (Thermo Spectronic Genesys, Waltham, MA) (1 OD = $8x10^8$CFU/mL). After re-suspension of *F. nucleatum* and *P. anaerobius* in phosphate buffered saline (PBS), the bacteria were killed at 100°C and used as heat-killed (HK) bacterial antigen, following a previously published method (12).

Immunization with bacterial antigens: (A) Immunization with *F. nucleatum* for examination of IgG antibody response. A total of 4 groups of BALB/c mice (6- to 8-week-old males, n=6/group) were immunized with heat-killed *F. nucleatum* ($3x10^8$ CFU/mouse, s.c. injection) in a mixture of 1) PBS, 2) Freund's adjuvant (Difco Laboratories, Detroit, MI), 3) aluminum hydroxide adjuvant (Alum) (Sigma, St. Louis, MO), or 4) MTA (100mg/mL) every two weeks, for a total of two immunizations (see Fig. 1 B). A third booster immunization was carried out by an injection (s.c.) of heat-killed *F. nucleatum* suspended in PBS alone, two weeks after the second immunization. In particular, for the group receiving Freund's adjuvant, Freund's complete adjuvant and Freund's incomplete adjuvant were used for primary and secondary immunizations, respectively. Otherwise, the same composition of Alum or MTA was used for primary and secondary immunization. Blood was collected on days 0, 14, 28 and 32 and serum obtained. IgG antibody reactions to *F. nucleatum* present in the blood serum specimens were determined by ELISA, as described below (timetable is shown in Fig. 1 B).

(B) Immunization with *F. nucleatum* and *P. anaerobius* for examination of bacterial antigen-specific memory T cell response. In order to develop antigen-specific memory type T cells, two groups of animals were immunized with 1) heat-killed *F. nucleatum* or 2) heat-killed *P. anaerobius*, following the same protocol as described above for the analysis of serum antibody reaction. Two days after booster injection with *F. nucleatum* or *P. anaerobius* suspended in PBS alone, these animals were sacrificed, and mononuclear lymphocytes were isolated from the cervical and auxiliary lymph nodes so that memory T cells specific to *F. nucleatum* or *P. anaerobius* could be primed *in vitro*, as described below.

Measurement of serum IgG antibody responses to bacterial antigens using ELISA: The wells of ELISA plates were coated with heat-killed *F. nucleatum* and heat-killed *P. anaerobius* in 0.2M sodium bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. To optimize the assay system, previous baseline experiments set the concentration of heat-killed *F. nucleatum* and heat-killed *P. anaerobius* at 10^7CFU/mL. The wells of ELISA plates were subjected to blocking with 1% bovine serum albumin (Sigma) and 1% sucrose (Sigma) in PBS supplemented with 0.05% Tween 20 (PBST). Blood serum diluted in PBST was incubated in the wells of ELISA plates for 1 hour at room temperature. Then, each well was reacted with horseradish peroxidase (HRPO)-conjugated anti-mouse IgG (Sigma) for 1 hour at room temperature. *O*-Phenylenediamine dihydrochloride (OPD; Sigma) in 0.1M citrate buffer solution (pH 5.5) supplemented with 2μL/mL of 30% H_2O_2 was applied as a substrate. Colorimetric reaction developed in the wells of ELISA plates was halted by addition of 2N H_2SO_4, and color densities were measured using a plate reader at OD 490 nm (Biokinetics reader EL312e; Bio-Tek Instruments, Winooski, VT). The results were expressed as optical density of the immunoglobulin isotype tested.

Naïve T cell culture: Lymph node cells from BALB/c mice were recovered and passed through a nylon- and glass-wool column to enrich T cells (~90% pure) (13). These T cells (10^6cells in 1mL) were cultured for 3 days in the presence or absence of MTA-filled glass capillaries in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY); 50μM of β-mercaptoethanol; antibiotics, including penicillin, streptomycin and gentamicin (Invitrogen); and L-glutamine in a 24-well plate (Corning, New York, NY). The T cells recovered from pre-incubation in the 24-well plate were restimulated for an additional 3 days in wells of a 96-well plate ($2x10^5$cells/well). The wells had previously been coated with monoclonal antibody (mAb) specific to TCR β-chain (6μg/mL; clone: H57-597) (Pharmingen, San Diego, CA), either with or without anti-CD28 mAb (2μg/mL, clone: 37.51) (Pharmingen). Cytokines produced in the culture supernatant on day 3 and proliferation of T cells in the last 16 hours of a total 4-day culture were verified using ELISA and [^{3}H]-thymidine incorporation assay, respectively.

Memory T cell culture: T line cells specific for *F. nucleatum* and *P. anaerobius* were developed from lymph nodes of animals immunized with heat-killed *F. nucleatum* and heat-killed *P. anaerobius* in Freund's adjuvant following the protocol used for serum IgG antibody induction. T cells were enriched from the mononuclear cell suspension isolated from lymph nodes by passing them through a nylon wool and glass wool column (13). T cells (10^6 cells/mL) were first primed *in vitro* with Mitomycin C (Sigma) (MMC)-treated spleen antigen presenting cells (APC) ($2x10^6$ cells/mL) and *F. nucleatum* or *P. anaerobius* (10^7 CFU/ml) in RPMI 1640 medium supplemented with 10% FBS. After incubation for 1 week, T cells which proliferated in response to each bacterial antigen presentation were separated by gradient centrifugation using Histopaque 1083 (Sigma), and the memory phenotypes were examined using flow cytometry. These *in vitro*-primed memory T cells were restimulated with or

without MTA capillaries in the presence of MMC-treated APC ($2x10^6$ cells/mL) and *F. nucleatum* or *P. anaerobius* (10^7 CFU/mL) in a 24-well plate for an additional 3 days. These MTA-exposed T cells were examined for their reactivity to respective bacterial antigen presentation. Briefly, the T cells were again isolated from APC by gradient centrifugation and stimulated ($2x10^4$ cells/well) with fresh MMC-treated APC ($4x10^5$ cells/well) in the presence or absence of *F. nucleatum* or *P. anaerobius* ($2x10^6$ CFU/well) in a 96-well plate (Corning) for 3 days. Culture supernatant isolated on day 3 was subjected to cytokine measurement using ELISA. The proliferation of T cells was evaluated by their incorporation of [^{3}H] - thymidine (0.5 μCi/well), which was applied during the last 16 hours of a total 4-day culture.

Flow cytometry analysis: In order to evaluate the memory T cell phenotypes, the *in vitro*-primed T cells were incubated with anti-CD4 mAb (clone: YTS191.1, Serotec, Raleigh, NC), anti-CD44 mAb (clone: IM7, Pharmingen), followed by FITC-labeled donkey $F(ab')_2$ anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). Fluorescence data were collected using logarithmic amplification on an EPICS^{TM} Altra flow cytometer (Beckman Coulter, Fullerton, CA).

[^{3}H]-thymidine incorporation assay: The protocol for the measurement of T cell proliferation followed our previously published method (13). Briefly, after collection of the culture supernatant for ELISA on day 3, [^{3}H]-thymidine (0.5 μCi/well) was applied to the T cell culture and incubated overnight (16 hours). Radioactivity incorporated into the lymphocytes was determined using a Tri-Carb liquid scintillation analyzer (model 2100TR; Packard, Meriden, CT).

Cytotoxicity assay: The freshly isolated lymph node T cells or spleen mononuclear cells ($2x10^6$ cells/mL, respectively) were incubated in a 24-well plate in the presence of a capillary filled with or without MTA for 24 hours. Cell death induced during the 24-hour incubation was measured by CytoTox-96™ Non-Radioactive cytotoxicity assay (Promega, Madison, WI). The CytoTox-96 assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis in the same manner as ^{51}Cr is released in a conventional radioactive-based cytotoxicity assay. The control cells incubated in medium alone were all killed after a 24-hour incubation by addition of a Lysis Solution (Promega) and provided a LDH-positive control (100% cell death). The rate of cell death induced by a capillary filled with or without MTA was calculated and expressed as (% cell death) based on the LDH-positive control.

Cytokine ELISA: Cytokine detection was measured in the culture supernatant using commercially available ELISA kits: IFN-γ (Duoset™, R&D Systems, Minneapolis, MN); TNF-α, IL-10, and RANKL (Peprotech, Rocky Hill, NJ) and IL-4 (Pharmingen).

Statistical analysis: All *in vitro* assays were carried out in triplicate. Data were analyzed using parametric Student's *t*-test. The results were considered significant when $p<0.05$.

Results

Effect of MTA on IgG antibody response: Immunization with heat-killed *F. nucleatum* in MTA upregulated IgG antibody to *F. nucleatum* compared to the group immunized with heat-killed *F. nucleatum* in control PBS (Fig. 1). As we expected, immunization with heat-killed *F. nucleatum* in Freund's adjuvant or in aluminum hydroxide

(Alum) adjuvant upregulated the IgG antibody to *F. nucleatum* (Fig. 1). These results suggested that MTA possesses adjuvant effects in the induction of IgG antibody responses against *F. nucleatum.*

Lack of cytotoxicity to lymphocytes by MTA: In order to evaluate the possible cytotoxic effects of MTA on the adaptive immune cells, 1) mononuclear cells isolated from spleen, which contain about 35% T cells and 60 % B cells, and 2) naïve T cells isolated from lymph nodes of BALB/c mice were incubated with or without MTA. The level of cell toxicity measured at 24 hours after MTA exposure is shown as % cell death in comparison to the positive control of 100% cell death that was induced by lysis of all cells in a well after 24 hour incubation in medium alone (Fig. 2 A, naïve T cells; B, spleen mononuclear cells). B cells are, by their own nature, more prone to die by apoptosis in the regular *in vitro* culture condition. Therefore, as expected, spleen cells which contain not only naïve T cells, but also B cells (Fig. 2 B), showed a higher percentage of cell death than the group of naïve T cells (Fig. 2 A). Most importantly, the presence of MTA did not affect cell death of lymphocytes induced in the *in vitro* culture, indicating that MTA is not cytotoxic to lymphocytes.

Effect of MTA on memory T cells: The memory T cells reactive to *F. nucleatum* or *P. anaerobius* from immunized BALB/c mice were tested for specificity by proliferation and cytokine expression in response to specific bacterial antigen. *F. nucleatum*-reactive memory T cells did not show proliferation to *P. anaerobius*-antigen presentation, and *P. anaerobius*-reactive memory T cells proliferation did not respond to *F. nucleatum*-antigen presentation (not shown). Also, flow cytometry results indicated that both *F. nucleatum*- and *P. anaerobius*-reactive memory T cells express CD44 (c.a. 90% of total of each T cells) and CD4 (c.a. 60% of total of each T cells)(data not shown). Both *F. nucleatum*- and *P.*

anaerobius-reactive BALB/c memory T cells showed increased proliferation (Fig. 2C and D). Expression of cytokines in response to antigen (Fig. 3A and F, IFN-γ; B and G, TNF-α; E and J, IL-10) was also examined in memory cells. *F. nucleatum*-reactive memory T cells presented high IL-4 and low IFN-γ expression, whereas *P. anaerobius*-reactive memory T cells produced high IFN-γ and low IL-4 levels. Both memory T cell subsets showed elevated RANKL expression when they were incubated with APC, irrespective of the presence of bacteria (Fig. 3C and H). Having established these basal proliferation patterns and cytokine expression profiles by *F. nucleatum*- and *P. anaerobius*-reactive memory T cells, the influence of MTA could then be evaluated for the basal responses of these memory type T cells to their specific-antigen presentation.

The exposure of BALB/c memory T cells to MTA suppressed the antigen-specific proliferation of both *F. nucleatum*- and *P. anaerobius*-reactive memory T cells (Fig. 2C and D). MTA exposure also inhibited IL-4 production by *F. nucleatum*-reactive memory T cells (Fig. 3D) and inhibited IFN-γ production by *P. anaerobius*-reactive BALB/c memory T cells (Fig. 3F). However, exposure to MTA did not affect the expression of RANKL, TNF-α and IL-10 by antigen-stimulated BALB/c memory T cells (Fig. 3B, C and E, *F. nucleatum*-reactive memory T cells; G, H and J, *P. anaerobius*-reactive memory T cells). These results therefore indicate that MTA exerts most of its influence on the T cell growth factors (IFN-γ and L-4), while the expression of the inflammatory cytokine, TNF-α, the bone destructive cytokine, RANKL, and the anti-inflammatory cytokine, IL-10, all remained unaffected by MTA. This lack of influence of MTA on the expression of RANKL, TNF-α and IL-10 by these two different types of memory T cells indicates that MTA may have no effects on the inflammation mediated by memory T cells in the context of periapical lesions.

Effect of MTA on the Naïve T cell reactions to TCR/CD28 activation: In addition to memory type T cells, which appear to be the predominant T cells in periapical lesions (4), naïve T cells may also be involved in the process of cell-mediated immune responses to pathogens present in the root canal system. Therefore, the present study also examined the influence of MTA on the activity of naïve T cells, including the effects of MTA exposure on the proliferation of naïve T cells in response to TCR/CD28 activation, as well as their cytokine expression pattern. First, in terms of proliferation, naïve T cells stimulated with TCR/CD28 showed an incorporation of [^{3}H]-thymidine level which was comparable to that demonstrated by the proliferation of Th1- and Th2-type memory T cells (not shown). However, in contrast to the memory type T cells, where MTA suppressed the antigen-specific proliferation, the pre-exposure of MTA slightly increased TCR/CD28-mediated proliferation of naïve T cells compared to the control naïve T cells cultured without MTA (not shown). Second, while naïve T cells and Th1- or Th2-type memory T cells were applied in equal numbers to the respective proliferation assay, naïve T cells stimulated with TCR/CD28 showed significantly lower expression of all examined cytokines, including IFN-γ, IL-4, IL-10, TNF-α and RANKL, than the Th1- or Th2-type memory T cells activated with their specific antigens (Fig. 3K, L, M, N and O). Although pre-exposure of MTA slightly increased IFN-γ, TNF-α and RANKL expression by TCR/CD28-activated naïve T cells (Fig. 3, K, L and M; see the inset figures with magnified scale), the expression level of these three cytokines was still negligibly lower than that expressed by the two types of activated memory T cells.

Discussion

Periapical lesions are characterized by inflammation in the connective tissues accompanied by bone destruction around the infected root-end of teeth (4;14). The cellular

infiltration of the periapical lesion has been characterized by a variety of cell types, mainly represented by macrophages and T and B lymphocytes (15;16). MTA is applied to the end of an infected tooth root canal where it is directly in contact with host immune cells infiltrating the inflammatory lesions of periapical connective tissues. Therefore, it is plausible that immune responses at the periapical lesions could be influenced by the presence of this endodontic material. The effects of MTA on cytokine expression by host immune cells in response to endodontic pathogens had not previously been addressed until we reported the influence of MTA on cytokine production by bacteria-stimulated mouse macrophages (17). Based on this study, it was observed that MTA did not affect TNF-α, IL-12 and IL-10 cytokine production by M1 or M2 macrophages under the conditions used in the present study, i.e., *F. nucleatum* and *P. anaerobius* stimulation (17). Since the influence of MTA on innate immune scavenger cells, or macrophages, had been previously addressed, it was intriguing to evaluate its potential effects on adaptive immune responses, including T cell and B cell responses. Accordingly, the present study demonstrated that MTA appears to only minimally affect memory T cell activity to specific bacterial antigen presentation. However, we originally hypothesized that MTA might increase adaptive immune response because one of its components, aluminum oxide, is reported to possess adjuvant effects. In terms of B cell-mediated humoral responses, this hypothesis was confirmed by IgG antibody responses to *F. nucleatum* which were upregulated by the presence of MTA.

Aluminum salt has been used as a vaccine adjuvant to induce antibody responses to bacteria such as *Corynebacterium diphtheriae*, *Clostridium tetani*, *Pertussis*, *Haemophilus influenzae* type b, *Pneumococcus conjugates*, and *HeP. anaerobiustitis* A and B (18). *F. nucleatum* immunization in the presence of MTA resulted in upregulation of IgG antibody responses to the bacterium to an extent comparable to *F. nucleatum* immunization in PBS. Since IgG-positive cells represent 70% of the immunoglobulin-producing cells in periapical

granulomas and radicular cysts (19), it is conceivable that locally produced IgG antibody in association with the use of MTA could, in fact, contribute to antibacterial activity in periapical lesions. In other words, to the extent that sustained IgG antibody response repels bacterial infection and thus retains the host antimicrobial responses at the periapical lesions, the overall MTA effect on IgG antibody response, as indicated above, should benefit the host.

In terms of the influence of MTA on naïve T cells, the present study demonstrated that MTA increased the production of RANKL, IFN-γ and TNF-α by TCR/CD28-activated naïve T cells. However, such increased amount of cytokine production by TCR/CD28-activated naive T cells in the presence of MTA was negligibly lower than the same cytokines produced by memory T cells, whereas both naïve and memory T cells showed comparable levels of proliferation. It is plausible that both inflammatory and anti-inflammatory cytokines derived from activated memory T cells would more prominently affect periapical lesions than those same cytokines derived from naïve T cells, supported by the report that majority of T cells in the periapical lesion is memory type T cells (4).

We addressed whether MTA could alter the expression level of pro- and anti-inflammatory cytokines by either *F. nucleatum*- or *P. anaerobius*-reactive memory T cells. Significantly, MTA suppressed the proliferation of these two different types of memory T cells along with the diminished expression of T cell growth cytokine, IL-4 or IFN-γ, for *F. nucleatum*- or *P. anaerobius*-reactive memory T cells, respectively. Even though both types of memory T cells expressed IL-10 and RANKL (TNF-α only for *P. anaerobius*-reactive memory T cells), it is significant that MTA, under the conditions tested, was not observed to alter the expression of these anti-inflammatory and bone destructive cytokines. It is noteworthy that IL-$10^{-/-}$ mice had significantly greater infection-stimulated bone resorption *in vivo* compared with wild-type mice, which indicates that this cytokine may be engaged in the down-modulation of this process (20). The fact that MTA did not interfere with the

production of anti-inflammatory and bone destructive cytokines from memory T cells contradicted to our original hypothesis. Since MTA did not alter the RANKL expression from memory T cells, it is intriguing to test if MTA can influence the osteoclastogenesis induced by such RANKL produced from memory T cells in the context of periapical lesion. This question of MTA's effects on osteoclastogenesis will be addressed in our future study.

In summary, the results of the present study have demonstrated that 1) MTA upregulated the IgG antibody responses, 2) MTA suppressed proliferation of *F. nucleatum*-reactive and *P. anaerobius*-reactive memory T cells and reduced their expression of T cell growth cytokines, IL-4 and IFN-γ, respectively, and 3) MTA use results in little or no alteration of the other cytokine expression (TNF-α, RANKL and IL-10) by *F. nucleatum*-reactive and *P. anaerobius*-reactive memory T cells. These results indicated that MTA's influence on adaptive immune response still favors to the host in the context of periapical lesion caused by bacterial infection.

Reference List

1. Stern, M. H., Dreizen, S., Mackler, B. F., Selbst, A. G., and Levy, B. M. Quantitative Analysis of Cellular Composition of Human Periapical Granuloma. J.Endod. 1981;7(3):117-22.

2. Yu, S. M. and Stashenko, P. Identification of Inflammatory Cells in Developing Rat Periapical Lesions. J.Endod. 1987;13(11):535-40.

3. Tani, N., Osada, T., Watanabe, Y., and Umemoto, T. Comparative Immunohistochemical Identification and Relative Distribution of Immunocompetent Cells in Sections of Frozen or Formalin-Fixed Tissue From Human Periapical Inflammatory Lesions. Endod.Dent.Traumatol. 1992;8(4):163-9.

4. Stashenko, P., Teles, R., and D'Souza, R. Periapical Inflammatory Responses and Their Modulation. Crit Rev.Oral Biol.Med. 1998;9(4):498-521.

5. Rezende, T. M., Vieira, L. Q., Cardoso, F. P., Oliveira, R. R., Oliveira Mendes, S. T., Jorge, M. L., and Ribeiro Sobrinho, A. P. The Effect of Mineral Trioxide Aggregate on Phagocytic Activity and Production of Reactive Oxygen, Nitrogen Species and Arginase Activity by M1 and M2 Macrophages. Int.Endod.J. 2007;40(8):603-11.

6. Naim, J. O., van Oss, C. J., Wu, W., Giese, R. F., and Nickerson, P. A. Mechanisms of Adjuvancy: I--Metal Oxides As Adjuvants. Vaccine 1997;15(11):1183-93.

7. Petrovsky, N. and Aguilar, J. C. Vaccine Adjuvants: Current State and Future Trends. Immunol.Cell Biol. 2004;82(5):488-96.

8. Asgary, S., Parirokh, M., Eghbal, M. J., and Brink, F. Chemical Differences Between White and Gray Mineral Trioxide Aggregate. J.Endod. 2005;31(2):101-3.

9. Tomson, P. L., Grover, L. M., Lumley, P. J., Sloan, A. J., Smith, A. J., and Cooper, P. R. Dissolution of Bio-Active Dentine Matrix Components by Mineral Trioxide Aggregate. J.Dent. 2007;35(8):636-42.

10. Oliveira Mendes, S. T., Ribeiro Sobrinho, A. P., de Carvalho, A. T., Souza Cortes, M. I., and Vieira, L. Q. In Vitro Evaluation of the Cytotoxicity of Two Root Canal Sealers on Macrophage Activity. J.Endod. 2003;29(2):95-9.

11. Lana, M. A., Ribeiro-Sobrinho, A. P., Stehling, R., Garcia, G. D., Silva, B. K., Hamdan, J. S., Nicoli, J. R., Carvalho, M. A., and Farias, Lde M. Microorganisms Isolated From Root Canals Presenting Necrotic Pulp and Their Drug Susceptibility in Vitro. Oral Microbiol.Immunol. 2001;16(2):100-5.

12. Ribeiro Sobrinho, A. P., Melo Maltos, S. M., Farias, L. M., de Carvalho, M. A., Nicoli, J. R., de Uzeda, M., and Vieira, L. Q. Cytokine Production in Response to Endodontic Infection in Germ-Free Mice. Oral Microbiol.Immunol. 2002;17(6):344-53.

13. Kawai, T., Eisen-Lev, R., Seki, M., Eastcott, J. W., Wilson, M. E., and Taubman, M. A. Requirement of B7 Costimulation for Th1-Mediated Inflammatory Bone Resorption in Experimental Periodontal Disease. J.Immunol. 15-2-2000;164(4):2102-9.

14. Wang, C. Y. and Stashenko, P. Kinetics of Bone-Resorbing Activity in Developing Periapical Lesions. J.Dent.Res. 1991;70(10):1362-6.

15. Kawashima, N., Okiji, T., Kosaka, T., and Suda, H. Kinetics of Macrophages and Lymphoid Cells During the Development of Experimentally Induced Periapical Lesions in Rat Molars: a Quantitative Immunohistochemical Study. J.Endod. 1996;22(6):311-6.

16. Stashenko, P. and Yu, S. M. T Helper and T Suppressor Cell Reversal During the Development of Induced Rat Periapical Lesions. J.Dent.Res. 1989;68(5):830-4.

17. Rezende, T. M. B., Vargas, D. L., Cardoso, F. P., Sobrinho, A. P. R., and Vieira, L. Q. Effect of Mineral Trioxide Aggregate on Cytokine Production by Peritoneal Macrophages. International Endodontics Journal 2005;38:896-903.

18. Baylor, N. W., Egan, W., and Richman, P. Aluminum Salts in Vaccines--US Perspective. Vaccine 31-5-2002;20 Suppl 3:S18-S23.

19. Pulver, W. H., Taubman, M. A., and Smith, D. J. Immune Components in Human Dental Periapical Lesions. Arch.Oral Biol. 1978;23(6):435-43.

20. Sasaki, H., Hou, L., Belani, A., Wang, C. Y., Uchiyama, T., Muller, R., and Stashenko, P. IL-10, but Not IL-4, Suppresses Infection-Stimulated Bone Resorption in Vivo. J.Immunol. 1-10-2000;165(7):3626-30.

Figure Legends:

Figure 1: Influence of MTA on IgG antibody response to endodontic pathogen immunization (A). BALB/c mice received immunizations with heat killed-*F. nucleatum* in PBS (Fn+PBS), heat killed-*F. nucleatum* in Freund's adjuvant (Fn+Freund's), heat killed-*F. nucleatum* in aluminum hydroxide adjuvant (Fn+Alum) or heat killed-*F. nucleatum* in MTA (Fn+MTA). MTA injection alone without bacteria was used as control. The schedule for immunization is indicated in the diagram inserted in the figure (B). Column and bar indicate the mean and SD of relative IgG titer value from six different mice, respectively. The methods to measure IgG antibody are described in the materials and methods section.

* indicates that the value of the column is statistical different than the value of heat killed-*F. nucleatum* in PBS group, in the same day, by *t*-test ($p<0.01$).

# indicates that the value of the column is statistically higher than the same group in day 0, by *t*-test ($p<0.05$).

Figure 2: Effects of MTA on isolated lymphocytes incubated *in vitro*. Lack of cytotoxicity to lymphocytes by MTA (A and B): Freshly isolated lymph node T cells (A) or spleen mononuclear cells (B) ($2x10^6$ cells/mL, respectively) were incubated in a 24-well plate in the presence capillary filled with or without MTA for 24 hours. Cell death induced during the 24-hour incubation was measure by CytoTox-96^{TM} Non-Radioactive cytotoxicity assay. # indicates no statistical differences between the groups with and without MTA. **Effects of MTA on proliferation of bacteria-specific memory T cells (C and D):** *F. nucleatum*-reactive memory T cells (C) and *P. anaerobius*-reactive memory T cells (D) were established from BALB/c mice. These bacterial-reactive memory T cells were pre-incubated with or without MTA in the presence of respective bacterial antigen and APC for 3 days. After pre-

incubation, the T cells were re-stimulated with respective bacterial antigen and APC for 4 days. Proliferation of bacterium-specific memory T cells was measured. Column and bar indicate the mean and SD of triplicates, respectively, of one representative experiment. Abbreviations: *Fn*, *F. nucleatum; Pa, P. anaerobius*. Column and bar indicate the mean and SD of triplicates, respectively. This figure shows one representative result out of a total of 3 experiments. * indicates that the mean number of the histogram column is statistically higher than control empty capillaries by *t*-test ($p<0.05$). • indicates the statistical differences between the two columns connected with brackets by *t*-test ($p<0.05$).

Figure 3: Effects of MTA on cytokine production by bacteria-specific memory T cells and naïve T cells. *F. nucleatum*-reactive memory T cells and *P. anaerobius*-reactive memory T cells were established from BALB/c mice. These bacterial-reactive memory T cells were pre-incubated with or without MTA in the presence of respective bacterial antigen and APC for 3 days. After pre-incubation, the T cells were re-stimulated with respective bacterial antigen and APC for 4 days (A-E, *F. nucleatum*-reactive memory T cells; F-J, *P. anaerobius*-reactive memory T cells). Otherwise, naïve T cells isolated from lymph nodes of BALB/c mice (K-O) were pre-incubated with or without MTA for 3 days. After pre-incubation, T cells were activated with immobilized anti-TCR MAb and anti-CD28 MAb for 4 days. Production of cytokines, including IFN-γ, TNF-α, RANKL, IL-4 and IL-10 by TCR/CD28-activated T cells, were measured in the culture supernatant. Column and bar indicate the mean and SD of triplicates, respectively. As to the figures for cytokine expression profile of naïve T cells (K-O), the inserted figures show the magnified y-axis scale for the detected amount of cytokines, while the y-axis of external figures keep the same scale as the one used for memory T cells. In other words, the insert and external figures for naïve T cells show the same data in different

scales. Abbreviations: *Fn*, *F. nucleatum*; *Pa, P. anaerobius*. Column and bar indicate the mean and SD of triplicates, respectively. This figure shows one representative result out of a total of 3 experiments. * indicates that the mean number of the histogram column is statistically higher than control empty capillaries by *t*-test ($p<0.05$). • indicates the statistical differences between the two columns connected with brackets by *t*-test ($p<0.05$).

Figure 1

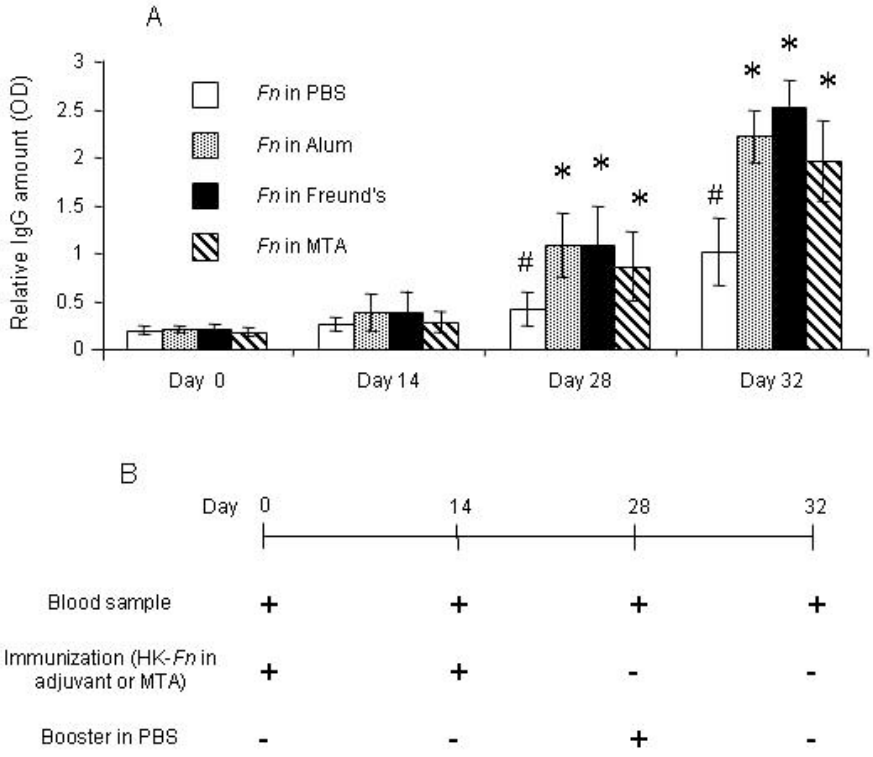

A
Relative IgG amount (OD)
3
2.5
2
1.5
1
0.5
0
Fn in PBS
Fn in Alum
Fn in Freund's
Fn in MTA
#
*
Day 0
Day 14
Day 28
Day 32
B
Day 0 14 28 32
Blood sample + + + +
Immunization (HK-Fn in adjuvant or MTA) + + - -
Booster in PBS - - + -

Figure 2

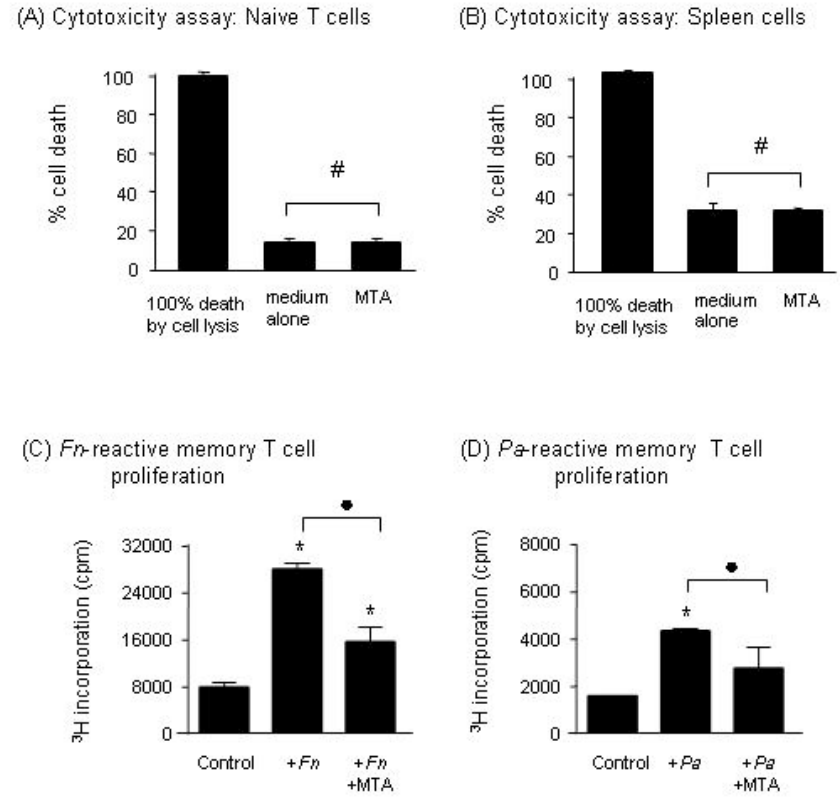
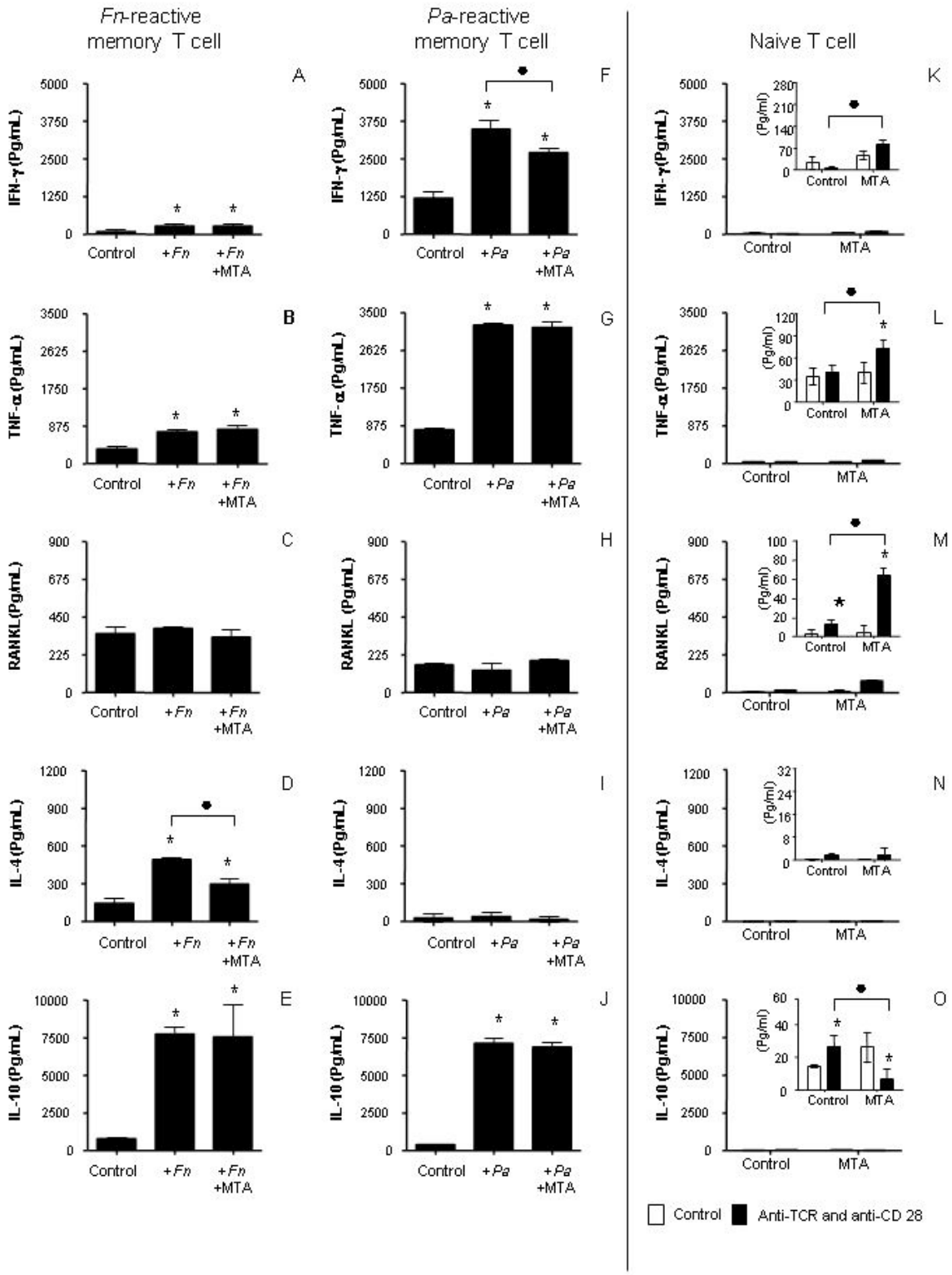

(A) Cytotoxicity assay: Naive T cells
% cell death
100
80
60
40
20
0
#
100% death by cell lysis
medium alone
MTA
(B) Cytotoxicity assay: Spleen cells
% cell death
100
80
60
40
20
0
#
100% death by cell lysis
medium alone
MTA
(C) Fn-reactive memory T cell proliferation
3H incorporation (cpm)
32000
24000
16000
8000
0
*
*
*
Control
+Fn
+Fn +MTA
(D) Pa-reactive memory T cell proliferation
3H incorporation (cpm)
8000
6000
4000
2000
0
*
*
Control
+Pa
+Pa +MTA

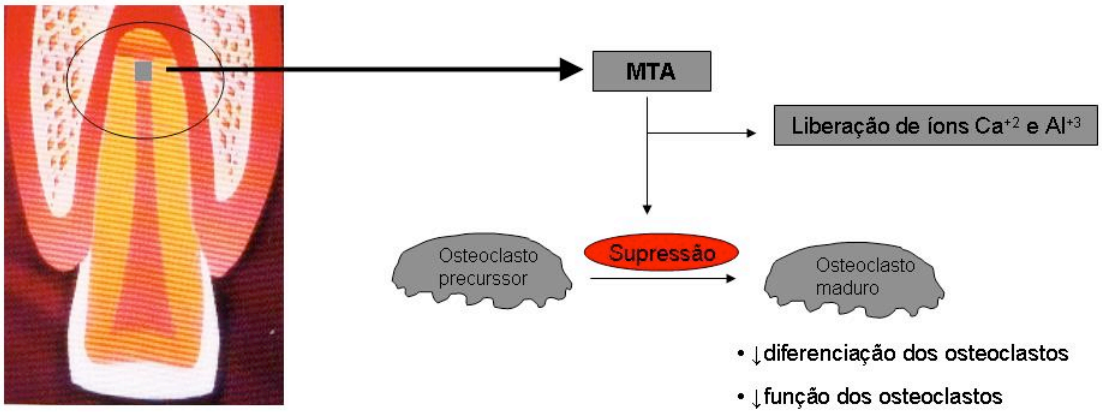
Figure 3


Fn-reactive memory T cell
Pa-reactive memory T cell
Naive T cell
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
IFN-γ(Pg/mL)
TNF-α(Pg/mL)
RANKL(Pg/mL)
IL-4(Pg/mL)
IL-10(Pg/mL)
(Pg/ml)
Control
+Fn
+Fn +MTA
+Pa
+Pa +MTA
MTA
Control
Anti-TCR and anti-CD 28

3.3 MTA E A REABSORÇÃO ÓSSEA

TRABALHO 3: "Influence of Mineral Trioxide Aggregate (MTA) on RANKL-mediated osteoclastogenesis and osteoclast activation". Trabalho em preparação.

O agregado de trióxido mineral (MTA) é utilizado como material retroobturador em ápices radiculares infectados, onde a reabsorção óssea inflamatória é causada pela osteoclastogênese mediada pelo ligante do receptor ativador do NF-κ B (RANKL). O objetivo deste estudo foi verificar os efeitos do MTA na reabsorção óssea dependente de osteoclastos mediada por RANKL, incluindo a diferenciação dos precursores em osteoclastos maduros e a atividade destes osteoclastos. Dois tipos de precursores osteoclásticos, linhagem de células RAW264.7 (RAW) e células da medula óssea (com M-CSF adicionado), foram estimuladas com ou sem RANKL recombinante (rRANKL) na presença ou ausência de MTA por 6 a 8 dias. A influência do MTA nestes dois precursores osteoclásticos foi medida por: número de células multinucleadas positivas para fosfatase ácida resistente a tartarato (TRAP) (RAW e células da medula), atividade de TRAP (RAW), expressão do gene da Catepsina K (RAW) e formação de áreas de reabsorção (RAW). Em ambas as células, o número de osteoclastos TRAP-positivos induzidos por rRANKL foi significativamente inibido pela presença do MTA quando comparado ao controle ($p<0,05$), juntamente com a atividade da enzima TRAP ($p<0,05$) e da expressão gênica da Catepsina K ($p<0,05$). Em contraste com o controle, a área de reabsorção dentinária foi significativamente menor nas culturas de osteoclastos maduros incubados com o MTA ($p<0,05$). Baseado nos indicadores acima, o MTA suprimiu significativamente a osteoclastogênese mediada por RANKL e a atividade dos osteoclastos, parecendo ser capaz de suprimir eventos de reabsorção óssea em lesões periapicais.


MTA
Liberação de íons Ca^{+2} e Al^{+3}
Osteoclasto precurssor
Supressão
Osteoclasto maduro
• ↓diferenciação dos osteoclastos
• ↓função dos osteoclastos

Influence of Mineral Trioxide Aggregate (MTA) on RANKL-mediated osteoclastogenesis and osteoclast activation.

Authors: Rezende TMB[1,3], Vieira LQ[2], Ribeiro Sobrinho AP[1], Taubman MA[3] and Kawai T[3]

[1]Departamento de Dentística Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; [2]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; [3]Department of Immunology, The Forsyth Institute, Boston, MA, USA.

Running title: MTA effects on osteoclasts cells

Keywords: MTA, RANKL, Cathepsin K, TRAP activity, pit formation.

Correspondent:

Toshihisa Kawai, DDS, Ph.D., Department of Immunology, The Forsyth Institute

140 The Fenway

Boston, Massachusetts, 02115 USA

Phone: 617-892-8317

E-mail: tkawai@forsyth.org

Abstract

MTA is used as root-end filling material for bacterially infected root apex where inflammatory bone resorption is caused by receptor activator NF-κ B ligand (RANKL)-mediated osteoclastogenesis. The aim of this study is to assess the effect(s) of MTA on *in vitro* RANKL-mediated osteoclast dependent bone resorption events, including differentiation to mature osteoclasts from their precursors and osteoclast activity. Two types of osteoclast precursors, RAW264.7 (RAW) cell line or bone marrow cells (with M-CSF added), were stimulated with or without recombinant (r) RANKL with or without MTA for 6 to 8 days. Influence of MTA on these types of osteoclast precursors were measured by number of differentiated tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (RAW & bone marrow cells), TRAP enzyme activity (RAW), Cathepsin K gene expression (RAW) and resorptive pit formation (RAW) by mature osteoclasts. In bone marrow and RAW cells, the number of TRAP-positive mature osteoclast cells induced by rRANKL was significantly inhibited by the presence of MTA compared to control rRANKL stimulation without MTA ($p<0.05$), along with the reduction of TRAP enzyme activity ($p<0.05$) and the low expression of Cathepsin K gene ($p<0.05$). In contrast to control mature osteoclasts incubated, the resorption area on dentin was significantly decreased for mature osteoclasts incubated with MTA ($p<0.05$). Measured by the indicators above, MTA significantly suppressed RANKL-mediated osteoclastogenesis, osteoclast activity and, therefore, appears able to suppress bone resorptive events in periapical lesions.

Introduction

Periapical lesions are destructive inflammatory pathologies and immune response against microorganisms that affect the periapical periodontum. They are characterized by periradicular periodontal ligament and bone destruction as a consequence of bacterial infection of the dental pulp (Stashenko *et al.*, 1998). Diverse inflammatory mediators - interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, tumor necrosis factor (TNF)-alpha, granulocyte-macrophage colony stimulating factor (GM-CSF), nitric oxide (NO), interferon (IFN)-gamma, prostaglandins, and metalloproteinases - have been associated with periradicular lesions (Stashenko *et al.*, 1998; Kawashima and Stashenko, 1999).

Receptor Activator of Nuclear factor Kappa B (RANK), a hematopoietic surface receptor, has been identified as a key regulator of bone (Lacey *et al.*, 1998) and calcium metabolism. RANK ligand (RANKL) is a protein produced by osteoblasts, cells of bone stroma and by activated T lymphocytes (Vernal *et al.*, 2006) and it is classified into the superfamily of TNF and TNF receptor. RANKL is a cytokine involved in both the physiological and pathological regulation of osteoclastogenesis and osteoclast activation (Takahashi *et al.*, 1999). The activation of RANK by its ligand leads to the expression of osteoclast-specific genes during differentiation, the activation of resorption by mature osteoclasts, and their survival and participation in new rounds of bone degradation at neighboring sites (Zhang *et al.*, 2001).

Mineral Trioxide Aggregate (MTA) is a root-end filling material developed by Mahamoud Torabinejad (Torabinejad *et al.*, 1993) as a powder composed by tricalcium silicate, bismuth oxide, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, and calcium sulfate dehydrate. MTA was first developed in a gray color but recently, the white-colored formula that lacks the tetracalcium aluminoferrite has became available (Camilleri *et al.*, 2005). Bios MTA present only little differences comparing with Ângelus

MTA. It presents a higher percentage of aluminum oxide and free CaO and it contains a plastification in the liquid. As a root-end filling material, MTA is used for bacterially infected root apex where inflammatory bone resorption is caused by RANKL-mediated osteoclastogenesis (Vernal *et al.*, 2006). In our previous work, we observed that MTA did not affect the RANKL production by *Fusobacterium nucleatum* (*Fn)* and *Peptostreptococcus anaerobius (Pa*)-reactive memory T cells from BALB/c mice (unpublished data).

Despite the good clinical results, including mineralized tissue formation which results in periapical lesions repair, obtained with the MTA use (Camilleri and Pitt Ford, 2006), little is known about how this process happens. One hypothesis is that some released elements from MTA on the periapical area can stimulate the osteoblast activity while inhibit the osteoclast activity. Atomic spectroscopy analysis of distillated water incubated with MTA, per 72 hours, reveals that the highest ions released were represented by aluminum and calcium in this solution (Tomson *et al.*, 2007). The aim of this study was to assess the *in vitro* effect(s) of MTA on the osteoclasts, analyzing the osteoclastogenesis and the osteoclast function in the presence of MTA and to propose the Ca^{+2} and Al^{+3} mechanism for these effects.

Materials and methods

MTA preparation: White Ângelus MTA and Bios MTA (Odonto-lógika, Londrina, Paraná, Brazil) was prepared in accordance with manufacturers' instructions in sterile conditions. After preparation, MTA was inserted into the tips of previously sectioned and sterilized capillary tubes (test group), so that the contact surface with the cell suspension could be standardized (area = 0.50mm^2 and 0.01mm^2 for the cultures realized in 24-well plates and 96-well plates respectively). Empty capillary tubes were used in control cultures (Rezende *et al.*, 2005).

Animals: Male and female BALB/c were used. Animals were kept in a conventional cage and maintained at controlled ambient temperature. Food and water were offered to animals *ad libitum.* The protocol for this animal experiment was approved by The Forsyth Institute's animal ethics committee.

RAW cells culture: Non-confluent culture of RAW264.7 osteoclast precursor (RAW) cell line were harvested and cultured with minimum essential medium eagle alpha modification (alpha-MEM) (Sigma, St. Louis, MO) supplemented with 2.2G/L of Na bicarbonate (Sigma), 15% of fetal calf serum (Altana, Lawrenceville, GA), 1% of penicillin/streptomycin (1000U/mL) (Invitrogen, Grand Island, NY), 1% of MEM amino acids solution (Invitrogen), 1% of L-glutamine (Invitrogen) and 0.1% of gentamycin (Invitrogen). Cells ($2x10^4$cells) were cultured in a 24-well plate (Corning, New York, NY) with capillaries with or without MTA, per 24 hours for cell viability assay. To observe osteoclast differentiation, 100ng/mL of recombinant (r) RANKL (Peprotech, Rock Hill, NJ) was added in this culture and every 3 days, half of medium and rRANKL (Peprotech) were changed and the culture was stopped on days 5, 6 and 8. The RNA extractions were harvested after 48h of cell incubation. To verify the effect of aluminum and calcium released by MTA in the osteoclastogenesis, RAW cells were incubated with/without 40µg/mL of Aluminum oxide, 99.99% (Sigma) and Calcium oxide Reagent Plus, 99.99% (Sigma), with/without rRANKL (Peprotech) per 7 days.

Bone marrow cells culture: Bone marrow cells were recovered by flushing Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 0.0001% of gentamycin (Invitrogen) and 0.002% fungizone amphotericin B (Invitrogen) in the marrow space of femur and tibia of BALB/c mice. Culture was washed twice with DMEM (Invitrogen) and separated from blood cells and lymphocytes by histopac 1083 (Sigma). Culture was washed twice in alpha-MEM medium (Sigma) supplemented as above. Cells

($2.5x10^6$cells/mL) were cultured with 100ng/mL of rRANKL (Peprotech), 10ng/mL of rM-CSF (Peprotech) and capillaries with or without MTA, per 6 days. Half of medium was changed on day 3 by replacing half new medium and new rM-CSF (Peprotech) and rRANKL (Peprotech).

MTT assay: Half of medium was removed from the culture and it was incubated with MTT solution (Sigma) in incubator, per 4 hours. 0.04N HCl in isopropanol was added in the culture and the formazan crystals were dissolved by pipetting. The culture was transferred to a 96-well plate (Corning) and color was read in ELISA reader (Biokinetics reader, Bio-tek instruments, Winooski, Vermont, USA) at 575nM. The results were expressed in optical density after reduced the blank's optical density.

Tartrate-resistant acidic phosphatase (TRAP) stain: Cells were fixed with 5% formalin-saline, per 20 minutes. Then, cells were washed with PBS, 4 times and incubated with Trap 150mM tartrate (pH 5.5), per 2 hours, at room temperature. Acid phosphatase substrate was applied in the culture and it was incubated per 2 hours, to develop red color. Methyl green was used to counterstain cell nuclei. Differentiated osteoclasts were identified as tartrate-resistant acid phosphatase (TRAP) positive cells with three or more nuclei. Each well of 24-well plate was divided in four quadrants and differentiated osteoclasts cells of one of these quadrants were counted while for the cultures realized in the 96-well plate the number of these cells were counted in the totality of the well (Kawai *et al.*, 2000).

TRAP activity: Twenty five microlitres of lysed cells and a standard curve of TRAP (Sigma) enzyme were incubated with 225µL TRAP buffer (150mM tartrate buffer and 2mM $MgCl_2$) and 1mg/mL of phosphatase substrate (Sigma), per 90 minutes. To stop the reaction 50µL of 2N NaOH was added to the plate and the optical density was observed in the ELISA reader (Biokinetics reader, Bio-tek instruments) (405nm).

Reverse transcriptase-polymerase chain reaction (RT-PCR): Total RNA was extracted from RAW cells after 48 hours of incubation with or without rRANKL (Peprotech) and MTA using RNA-Bee (Tel-Test, Friends Wood, Texas). One micrograms of cDNA was reversed transcribed using SuperScript^{TM} II RT (Invitrogen), 0.1M DTT (Invitrogen) and 25mM dNTP (TaKaRa Bio Inc., Otsu, Shiga, Japan). cDNA was amplified using the TaKaRa Ex Taq System (TaKaRa Bio Inc.). Sequences of specific primer sets were as follows: β actin (house keeping gene): sense 5'-GACGGGGTCACCCACACTGT-3', anti-sense 5'-AGGAGCAATGATCTTGATCTTC-3'; Cathepsin K: sense 5'-CTGAAGATGCTTTCCCATATGTGGG-3', anti-sense 5'-GCAGGCGTTGTTCTTATTCCGAGC-3'. An optimized protocol of thermal cycling was used: 94°C for 2min, followed by 35 cycles of 98°C for 10s, 50°C for 30s, and 72°C for 60s, and final extension at 72°C for 7min. PCR products were separated in 1.5% agarose gels and stained with ethidium bromide (Sigma). The results were expressed as a ratio between the optical densitometry of Cathepsin K gene expression signal/β-actin gene expression signal, using the NIH image software analyser.

Pit Formation assay: RAW cells were cultured as described above with 100ng/mL of rRANKL (Peprotech) in a plate coated with type I collagen rat tail (BD Biosciences Bedford, MA) and 0.02N acetic acid (Fisher, Trenton, NJ), until the osteoclasts cells were differentiated. Cells were harvested with tripsin-EDTA (Invitrogen) and the cellular concentration adjusted to $4x10^5$cells/well. The cells were applied onto dentin slices (Immunodiagnostic systems, Boldon, UK), with 100ng/mL of rRANKL (Peprotech) and capillaries with or without MTA. After osteoclast differentiation, dentin slices were stained with toluidine blue 1% (Fisher). Four pictures were taken from each group and resorpted pit area was counted in these pictures using the NIH image software analyser at a 100-fold magnification.

Statistical analysis: The experiments were repeated 3 times. Data were analyzed using parametric Student's *t*-test, of one representative experiment. The results were considered significant when $p<0.05$.

Results

Effect of MTA on osteoclast differentiation induced by rRANKL in RAW and bone marrow cells: First, the effect of MTA on the osteoclast precursor cell line RAW264.7 viability was observed. MTA did not affect RAW264.7 cells viability, by MTT assay, when compared to the control group (Figure 1A). Same results of RAW cell's viability were observed when 2 MTA-filled capillaries were used (data not shown). A model of osteoclastogenesis induced by rRANKL was used to verify the effect of MTA on the osteoclast differentiation. The addition of rRANKL into the RAW264.7 (Figure 1B and photos J-O) and BALB/c bone marrow (Figure 1C and photos D-I) cells culture medium increased the number of osteoclast differentiated cells ($p<0.05$). MTA did suppress the number of TRAP-positive mature osteoclast cells induced by rRANKL, in both RAW264.7 cells (Control: 302 cells±33.17, Ângelus MTA: 213.17 cells±34.52*, Bios MTA: 175.17 cells±20.35*, *different from control by Student's *t*-test, $p<0.05$) and bone marrow cells (Control: 219.33 cells±11.02, Ângelus MTA: 168 cells±30.61*, Bios MTA: 183 cells±15.28*, *different from control by Student's *t*-test, $p<0.05$). Same results were, also observed, when these cells were cultured with 2 MTA-filled capillaries (data not shown). The suppression of TRAP-positive stain cells in RAW264.7 cells culture by MTA was observed since day 5 to day 8 (Figure 1P). In all incubation time points, cultures with MTA presented lower number of osteoclast cells. On day 8, Bios MTA significantly decreased the number of TRAP positive cells when it was compared with Ângelus MTA (Ângelus MTA: 53 cells±1.41, Bios MTA: 27.33 cells±3.06*, *different by Student's *t*-test, $p<0.05$). Despite this MTA effect on the

osteoclastogenesis, when the number of nuclei/osteoclast cell was observed, no statistical differences were found between the cultures with or without MTA in all incubation time points (Figure 1Q). These data indicated that after the multinucleated cells were recruited by the action of RANKL and have adhered to the bone in a resorption area, both MTAs could inhibit the cyto-differentiation process to the mature osteoclasts.

Effect of MTA on the osteoclast function: Three parameters were analyzed: the TRAP activity, the Cathepsin K gene expression and the resorptive pit area, using the same model of osteoclastogenesis induced by rRANKL, in RAW264.7 osteoclast precursor cell line. After the osteoclast differentiation, RANKL stimulates osteoclast activation, which includes the activation of TRAP and Cathepsin K enzymes for subsequent bone resorption. The addition of rRANKL into the cell culture medium increased the TRAP activity ($p<0.05$) and the Cathepsin K expression ($p<0.05$). MTA did suppress the TRAP activity (Control: 1.26µg/mL±0.07, Ângelus MTA: 0.38µg/mL±0.11*, Bios MTA: 0.16µg/mL±0.03*, *different from control by Student's *t*-test, $p<0.05$) (Figure 2A) induced by rRANKL. RAW264.7 cells, stimulated with rRANKL in the presence of Bios MTA, presented lower TRAP activity than the same culture with Ângelus MTA ($p<0.05$). The Cathepsin K gene expression was adjusted by the house keeping gene β-actin. MTA suppressed the Cathepsin K gene expression in groups with or without rRANKL. In the samples incubated with rRANKL, MTA suppress the Cathepsin K gene expression to 9% of the control group (Figure 2B and 2C). As a consequence of the osteoclats function, the third parameter analyzed was the resorptive pit area by mature osteoclasts. Again, the addition of the rRANKL to the cell culture increased the resorptive pit area ($p<0.05$). The addition of both MTAs on the mature osteoclast culture, previously differentiated by rRANKL stimuli on RAW264.7 cell culture, reduced the resorptive pit area comparing with the same culture in the absence of MTA (rRANKL+Control: 0.266cm^2±0.07, rRANKL+Ângelus MTA: 0.115cm^2±0.05*,

rRANKL+Bios MTA: 0.035cm^2±0.01*, *different from rRANKL+control by Student's *t*-test, p<0.05) (Figure 2D-H). Not only the total area of resorptive pit area was smaller in groups with MTA, but also the size of each pit area was smaller in this group (Figure 2E-H). Comparing both MTAs, Bios MTA presented the lowest resorpted pit area (p<0.05), similar to the control group without rRANKL. These data indicated that MTA had an effect on the osteoclast activity and probably presented some protective action in periapical lesions through resorptive events and that Bios MTA presented better results than Ângelus MTA.

Effect of calcium oxide and aluminum oxide on the rRANKL mediated osteoclastogenese: MTA releases aluminum and calcium ions in solution and the effect of these two ions in the RAW264.7 osteoclast precursor cell line stimulated with rRANKL was analyzed. Using the same model described above, both ions reduced the RANKL-mediated osteoclastogenesis (Fig. 3A). Aluminum oxide was the major suppressor of osteoclast cells differentiation (Control: 236±12.49 cells; Calcium oxide: 101.67±7.23 cells*; Aluminum oxide: 80.33±4.73 cells*, *different from control by Student's *t*-test, p<0.05). This result indicates that calcium and aluminum ions released by aluminum oxide and calcium oxide suppress the RANKL mediated osteoclastogenesis (Figure 3B). The release of these ions, by MTA, can be one of the protective mechanisms of this material on the periapical resorptive events, what has permitted the successful use of MTA in apical lesions.

Discussion

Receptor activator of nuclear factor κB-ligand is a bone-resorptive cytokine and an essential molecule in all phases of the osteoclast's life span. It has been catalogued as a key regulator of the physiological and pathological control of bone metabolism (Takahashi *et al.*, 1999). RANKL has been associated with diverse pathologies characterized by bone destruction, such as rheumatoid arthritis, osteoporosis, Paget's disease, bone tumors, facial

osteolytic lesions and periodontitis (Taubman and Kawai, 2001). It has been proposed as the common final pathway of the bone-resorptive and regulatory cytokines, in osteoclast differentiation and activation and in bone resorption. RANK and RANKL-deficient mice exhibit severe osteopetrosis, characterized radiographically by opacity in long bones, vertebral bodies, and ribs and by significantly increased total and trabecular bone density (Dougall *et al.*, 1999).

As a destructive inflammatory pathology, periapical lesion involves bone loss and the RANKL gene is highly over-expressed in granuloma lesions (Vernal *et al.*, 2006). In this case, the use of MTA as a root-end filling material must be indicated. In a previous unpublished data, we observed that MTA did not affect the RANKL expression by *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius* reactive memory T cells. At this point it was not known if this result was or not a benefit point for bone resorptive events, so the understanding of MTA effect on the osteoclast life became an extremely important fact.

The bone resorption starts with the recruitment of the multinucleated polykaryons cells by the CSF-1 and RANKL action, its adherence to the bone and cell-differentiation into the mature osteoclasts. The osteoclast activity is initiated by RANKL stimulation and this one induces the secretion of protons and lytic enzymes into the sealed resorption vacuole formed between the basal surface of the osteoclast and the bone surface. Acidification of this compartment by secretion of protons leads to the activation of TRAP and cathepsin K, the two main enzymes responsible for the degradation of bone mineral and collagen matrices leading to the bone resorption (Boyle *et al.*, 2003). It was observed that MTA affected both phases of osteoclast's life span: the osteoclast differentiation and osteoclast function.

MTA did not affect the viability of RAW264.7 osteoclast precursor cell line and this biocompatibility was described for others different cell types (Holland *et al.*, 2002; Bonson *et al.*, 2004; Rezende *et al.*, 2005; De Deus *et al.*, 2005; Braz *et al.*, 2006; Rezende *et*

al., 2007). In spite of the biocompatibility of this material, MTA decreased the number of osteoclast differentiated cells by osteoclast precursor cell line and by BALB/c bone marrow cells (with M-CSF) stimulated with rRANKL but it did not affect the number of nuclei/osteoclast cells. The reduction of the number of differentiated osteoclast cells by the presence of MTA was observed from day 5 to day 8. On day 8, BIOS MTA presented better results than Ângelus MTA.

Analyzing the osteoclast function, MTA suppressed the TRAP enzyme activity and the Cathepsin K gene expression. The cathepsin K is an osteoclastic proteolytic enzyme that cleaves the helical and telopeptide regions of collagen at an acid optimum pH (Zaidi *et al.*, 2001). The Cathepsin K is expressed in and secreted by osteoclasts near the ruffled border with its expression being promoted by RANKL (Zaidi *et al.*, 2001). It is a promising target for interrupt the osteoclast function once; Cathepsin K knockout mice display marked osteopetrosis (Gelb *et al.*, 1996) and its overexpression results in an enhanced bone resorption (Kiviranta *et al.*, 2001).

The other osteoclastic functional parameter suppressed by MTA was the TRAP enzyme. This enzyme is involved in the degradation of bone constituents by osteoclasts, what was demonstrated by the incubation of osteoclasts with anti-TRAP antibodies which results in reduction of bone resorption (Zaidi *et al.*, 1989; Moonga *et al.*, 1990). TRAP is a purple acid phosphatase that resists inhibition by tartrate. It is expressed at high levels in osteoclasts and it is used as a cytochemical marker for osteoclasts and their precursors (Hayman *et al.*, 2001). TRAP-deficient mice display osteopetrotic bone phenotype with resorptive defective osteoclasts (Hollberg *et al.*, 2002). TRAP also plays an important role in intracellular vesicular transport. It is primarily found in multinucleated osteoclasts and mononuclear osteoclast precursors. The TRAP activation is not affected by the absence of Cathepsin K, as it was observed in the osteoclasts isolated from Cathepsin K knockout mice (Perez-Amodio *et*

al., 2006). To finish the end process of bone response in the presence of MTA, the resorptive pit area was observed by mature osteoclasts. As a consequence of the down-regulation of the osteoclast activity, the resorpted pit area by mature osteoclasts was smaller in the presence of MTA.

Trying to understand the mechanism in which MTA acts in the down-modulation of the osteoclast differentiation and function, we observed its mineral composition and its release of ions in extraction solution, specially calcium (405.23mg/L) and aluminum (25.42mg/L) ions (Duarte *et al.*, 2003; Tomson *et al.*, 2007). It is known that high extra cellular calcium concentration modulates the osteoclast formation and/or function (Zaidi *et al.*, 1999) and that osteoclastic bone resorption is directly regulated by calcitonin hormone, and locally, by ionized calcium (Ca^{2+}) generated as a result of osteoclastic bone resorption. This fact was confirmed when freshly isolated rat osteoclasts were settled onto bone slices and incubated in a high Ca^{2+} concentration (5–20 mM), which resulted in a dramatic and concentration-dependent reduction in their bone-resorpting activity (Zaidi *et al.*, 1993). Parallel to this, the elevation of the extra cellular calcium concentration causes a dramatic reduction of cell size, accompanied by a marked diminution of tartrate resistant acid phosphatase in a dependent manner within an hour of exposure to high Ca^{2+} and the abolition of bone resorption (Datta *et al.*, 1989). Interestingly, resorpting osteoclasts produced lower quantities of acid phosphatase than those plated on plastic, suggesting that locally produced Ca^{2+} may inhibit enzyme secretion (Moonga *et al.*, 1990). In all these experiments, Ca^{2+} was added extracellularly after osteoclasts were plated onto devitalized bone. Recently, the addition of 10% of $CaCl_2$ in the MTA composition has been related. This addition improved the sealing ability (Bortoluzzi *et al.*, 2006), increased the initial pH, required lower amounts of water and increased the calcium released (Antunes *et al.*, 2006), what can be benefit for the suppression of the bone resorptive events.

In the other hand, aluminum oxide, present in the MTA composition, is a common adjuvant used in vaccines (Naim *et al.*, 1997) and it is also used as a ceramic-on-ceramic hip prosthesis used in orthopedic surgery for total hip replacement, in patients affected by osteoarthritis of the hip (Granchi *et al.*, 2004; Granchi *et al.*, 2005). In this kind of surgery, 60-70% of hip arthroplasties ultimately fail due to the bone loss around the implant due to bone resorption and ultimately aseptic loosening of the prothesis. Granchi *et al*. (2005) observed that supernatant of human osteoblasts with aluminum oxide in a mononuclear cell culture showed a decrease in c-*fos* expression together with an unchanged expression of c–*src*, suggesting that the passage of macrophages into osteoclast lineage is deviated. In this study, the authors observed that prosthesis that did not have aluminum oxide in its constitution produced higher levels of RANKL (Granchi *et al.*, 2005).

We proposed that the down-regulation of osteoclast differentiation and function, by MTA, is caused by the release of these ions in the solution, what was confirmed by the down-regulation of the osteoclast differentiation in the presence of the calcium oxide and aluminum oxide alone in the cell culture medium, stimulated by rRANKL. It is known that the release of calcium in MTA extract solutions is much higher than the same amount of aluminum (Tomson *et al.*, 2007) but, we observed that the down-regulation of the osteoclast differentiation was much more effective when the aluminum oxide was added in the solution than when the calcium oxide was added. An additional support to our hypothesis is the fact that BIOS MTA presents 6% aluminum oxide in its composition, while Ângelus MTA presents only 4% and BIOS MTA also present high percentage of free CaO than Ângelus MTA. This fact can justify the high down-regulation of the TRAP enzyme and the smaller resorptive pit area in cultures with BIOS MTA comparing with Ângelus MTA. The addition of high quantities of aluminum oxide in the MTA composition can be a benefit point for treatments that involves bone resorption area.

Little is known about the effect of MTA on the bone turn-over. Only some aspects of osteoblast activity were evaluated in the presence of MTA but with some contradictory results. Koh *et al.* (1997) observed that osteoblasts-like cells (MG-63) incubated with MTA produced high levels of IL-1α, IL-1β and IL-6, after 6-144h, than the same cells incubated with polymethylmethacrylate. In the other hand, Mitchell *et al.* (1999), using the same cells did not observe the IL-1α production and did observe the IL-6 and IL-8 production in the presence of MTA. This result indicated that MTA promotes healing through stimulation of bone turn-over. Pelliccioni *et al.* (2004) observed that another osteoblast-like cell (Saos-2) attached to the rough surface of MTA and spread onto the rough surface. Moreover, the cells on MTA were viable, grew, and released some collagen even at 72h, while cell metabolism and growth was dramatically reduced onto super EBA and amalgam surfaces. But another question about the effect of MTA on osteoblast activity was risen by Perez *et al*. (2003). They observed that whilst primary osteoblast (MG-63 cells) initially bound to white MTA, they did not survive on the surface by the end of 13 days. Analyzing these MTA contradictory effects on the osteoblast activity and considering the MTA effect on the osteoclast activity, maybe the good clinical results observed with the use of MTA on the resorpted area was not only based on the high modulation of the osteoblast activity but also the down modulation of the osteoclast activity. Then, the healing process promoted by MTA in the periapical bone loss area involves not only the mineralized tissue formation that results in periapical lesions repair (Camilleri and Pitt Ford, 2006), but also the suppression of the osteoclast differentiation and osteoclast function.

In summary, the results of the present study have demonstrated that 1) MTA down-regulated the osteoclast differentiation, 2) MTA down-regulated the osteoclast function (TRAP activity, Cathepsin K gene expression and resorpted pit area), and 3) Calcium and aluminum ions are the active components for the MTA effect on the osteoclast. The use of

MTA is recommended in bone loss area, once it promotes the formation of mineralized tissue and down-regulate the osteoclast differentiation and function.

Acknowledgments

Odonto-lógika (Londrina, PR, Brazil) kindly provided MTA-Ângelus. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

Reference

Antunes,BE, Juarez,BN, Antonio Hungaro,DM, Oliveira Demarchi,AC, and Monteiro,BC (2006). The use of a setting accelerator and its effect on pH and calcium ion release of mineral trioxide aggregate and white Portland cement. *J Endod* 32(12):1194-1197.

Bonson,S, Jeansonne,BG, and Lallier,TE (2004). Root-end filling materials alter fibroblast differentiation. *J Dent Res* 83(5):408-413.

Bortoluzzi,EA, Broon,NJ, Bramante,CM, Garcia,RB, de Moraes,IG, and Bernardineli,N (2006). Sealing ability of MTA and radiopaque Portland cement with or without calcium chloride for root-end filling. *J Endod* 32(9):897-900.

Boyle,WJ, Simonet,WS, and Lacey,DL (2003). Osteoclast differentiation and activation. *Nature* 423(6937):337-342.

Braz,MG, Camargo,EA, Salvadori,DM, Marques,ME, and Ribeiro,DA (2006). Evaluation of genetic damage in human peripheral lymphocytes exposed to mineral trioxide aggregate and Portland cements. *J Oral Rehabil* 33(3):234-239.

Camilleri,J, Montesin,FE, Brady,K, Sweeney,R, Curtis,RV, and Ford,TR (2005). The constitution of mineral trioxide aggregate. *Dent Mater* 21(4):297-303.

Camilleri,J and Pitt Ford,TR (2006). Mineral trioxide aggregate: a review of the constituents and biological properties of the material. *Int Endod J* 39(10):747-754.

Datta,HK, MacIntyre,I, and Zaidi,M (1989). The effect of extracellular calcium elevation on morphology and function of isolated rat osteoclasts. *Biosci Rep* 9(6):747-751.

De Deus,G, Ximenes,R, Gurgel-Filho,ED, Plotkowski,MC, and Coutinho-Filho,T (2005). Cytotoxicity of MTA and Portland cement on human ECV 304 endothelial cells. *Int Endod J* 38(9):604-609.

Dougall,WC, Glaccum,M, Charrier,K, Rohrbach,K, Brasel,K, De Smedt,T *et al.* (1999). RANK is essential for osteoclast and lymph node development. *Genes Dev* 13(18):2412-2424.

Duarte,MA, Demarchi,AC, Yamashita,JC, Kuga,MC, and Fraga,SC (2003). pH and calcium ion release of 2 root-end filling materials. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 95(3):345-347.

Gelb,BD, Shi,GP, Chapman,HA, and Desnick,RJ (1996). Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* 273(5279):1236-1238.

Granchi,D, Amato,I, Battistelli,L, Ciapetti,G, Pagani,S, Avnet,S *et al.* (2005). Molecular basis of osteoclastogenesis induced by osteoblasts exposed to wear particles. *Biomaterials* 26(15):2371-2379.

Granchi,D, Ciapetti,G, Amato,I, Pagani,S, Cenni,E, Savarino,L *et al.* (2004). The influence of alumina and ultra-high molecular weight polyethylene particles on osteoblast-osteoclast cooperation. *Biomaterials* 25(18):4037-4045.

Hayman,AR, Macary,P, Lehner,PJ, and Cox,TM (2001). Tartrate-resistant acid phosphatase (Acp 5): identification in diverse human tissues and dendritic cells. *J Histochem Cytochem* 49(6):675-684.

Holland,R, Souza,V, Nery,MJ, Faraco,J, I, Bernabe,PF, Otoboni Filho,JA *et al.* (2002). Reaction of rat connective tissue to implanted dentin tubes filled with a white mineral trioxide aggregate. *Braz Dent J* 13(1):23-26.

Hollberg,K, Hultenby,K, Hayman,A, Cox,T, and Andersson,G (2002). Osteoclasts from mice deficient in tartrate-resistant acid phosphatase have altered ruffled borders and disturbed intracellular vesicular transport. *Exp Cell Res* 279(2):227-238.

Kawai,T, Eisen-Lev,R, Seki,M, Eastcott,JW, Wilson,ME, and Taubman,MA (2000). Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *J Immunol* 164(4):2102-2109.

Kawashima,N and Stashenko,P (1999). Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. *Arch Oral Biol* 44(1):55-66.

Kiviranta,R, Morko,J, Uusitalo,H, Aro,HT, Vuorio,E, and Rantakokko,J (2001). Accelerated turnover of metaphyseal trabecular bone in mice overexpressing cathepsin K. *J Bone Miner Res* 16(8):1444-1452.

Koh,ET, Torabinejad,M, Pitt Ford,TR, Brady,K, and McDonald,F (1997). Mineral trioxide aggregate stimulates a biological response in human osteoblasts. *J Biomed Mater Res* 37(3):432-439.

Lacey,DL, Timms,E, Tan,HL, Kelley,MJ, Dunstan,CR, Burgess,T *et al.* (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93(2):165-176.

Mitchell,PJ, Pitt Ford,TR, Torabinejad,M, and McDonald,F (1999). Osteoblast biocompatibility of mineral trioxide aggregate. *Biomaterials* 20(2):167-173.

Moonga,BS, Moss,DW, Patchell,A, and Zaidi,M (1990). Intracellular regulation of enzyme secretion from rat osteoclasts and evidence for a functional role in bone resorption. *J Physiol* 429:29-45.

Naim,JO, van Oss,CJ, Wu,W, Giese,RF, and Nickerson,PA (1997). Mechanisms of adjuvancy: I--Metal oxides as adjuvants. *Vaccine* 15(11):1183-1193.

Pelliccioni,GA, Ciapetti,G, Cenni,E, Granchi,D, Nanni,M, Pagani,S *et al.* (2004). Evaluation of osteoblast-like cell response to Proroot MTA (mineral trioxide aggregate) cement. *J Mater Sci Mater Med* 15(2):167-173.

Perez,AL, Spears,R, Gutmann,JL, and Opperman,LA (2003). Osteoblasts and MG-63 osteosarcoma cells behave differently when in contact with ProRoot MTA and White MTA. *Int Endod J* 36(8):564-570.

Perez-Amodio,S, Jansen,DC, Schoenmaker,T, Vogels,IM, Reinheckel,T, Hayman,AR *et al.* (2006). Calvarial osteoclasts express a higher level of tartrate-resistant acid phosphatase than long bone osteoclasts and activation does not depend on cathepsin K or L activity. *Calcif Tissue Int* 79(4):245-254.

Rezende,TM, Vieira,LQ, Cardoso,FP, Oliveira,RR, Oliveira Mendes,ST, Jorge,ML *et al.* (2007). The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages. *Int Endod J* 40(8):603-611.

Rezende,TMB, Vargas,DL, Cardoso,FP, Sobrinho,APR, and Vieira,LQ (2005). Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages. *International Endodontics Journal* 38:896-903.

Stashenko,P, Teles,R, and D'Souza,R (1998). Periapical inflammatory responses and their modulation. *Crit Rev Oral Biol Med* 9(4):498-521.

Takahashi,N, Udagawa,N, and Suda,T (1999). A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. *Biochem Biophys Res Commun* 256(3):449-455.

Taubman,MA and Kawai,T (2001). Involvement of T-lymphocytes in periodontal disease and in direct and indirect induction of bone resorption. *Crit Rev Oral Biol Med* 12(2):125-135.

Tomson,PL, Grover,LM, Lumley,PJ, Sloan,AJ, Smith,AJ, and Cooper,PR (2007). Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. *J Dent*.

Torabinejad,M, Watson,TF, and Pitt Ford,TR (1993). Sealing ability of a mineral trioxide aggregate when used as a root end filling material. *J Endod* 19(12):591-595.

Vernal,R, Dezerega,A, Dutzan,N, Chaparro,A, Leon,R, Chandia,S *et al.* (2006). RANKL in human periapical granuloma: possible involvement in periapical bone destruction. *Oral Dis* 12(3):283-289.

Zaidi,M, Adebanjo,OA, Moonga,BS, Sun,L, and Huang,CL (1999). Emerging insights into the role of calcium ions in osteoclast regulation. *J Bone Miner Res* 14(5):669-674.

Zaidi,M, Alam,AS, Shankar,VS, Bax,BE, Bax,CM, Moonga,BS *et al.* (1993). Cellular biology of bone resorption. *Biol Rev Camb Philos Soc* 68(2):197-264.

Zaidi,M, Moonga,B, Moss,DW, and MacIntyre,I (1989). Inhibition of osteoclastic acid phosphatase abolishes bone resorption. *Biochem Biophys Res Commun* 159(1):68-71.

Zaidi,M, Troen,B, Moonga,BS, and Abe,E (2001). Cathepsin K, osteoclastic resorption, and osteoporosis therapy. *J Bone Miner Res* 16(10):1747-1749.

Zhang,YH, Heulsmann,A, Tondravi,MM, Mukherjee,A, and Abu-Amer,Y (2001). Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J Biol Chem* 276(1):563-568.

Legends

Figure 1: MTA effects on the osteoclast differentiation. MTT viability assay with RAW cells (A), number of Trap positive stained cells induced from RAW264.7 cells (B) and bone marrow cells (C) by stimulation with 100ng/mL of rRANKL and 100ng/mL of rRANKL associated with 10ng/mL of rM-CSF, respectively, in contact with or without MTA filled capillaries. Columns and bars indicate the mean and SD of triplicates, respectively of one representative experiment. * indicates, statistical differences ($p<0.05$), by *t*-test, when compared to the group only with medium. ● indicates $p<0.05$, by *t*-test. Representative photos of trap positive stain cells induced by induced by RAW cells (D-I) and bone marrow cells (J-O). Scale bar: 100μm. Kinetic of osteoclast differentiation from day 5 to day 8 in culture induced by RAW cells stimulated with 100ng/mL of rRANKL (P). * indicates, statistical differences ($p<0.05$), by *t*-test, when compared with Ângelus MTA and Bios MTA. ● indicates $p<0.05$, by *t*-test, when compared with Bios MTA. Number of nuclei/5 trap positive cells (Q) in RAW cell cultured with 100ng/mL of rRANKL. No statistical difference was found between triplicates of all groups.

Figure 2: MTA effects on the osteoclast function. Trap activity (A) induced from RAW264.7 cells by stimulation or not with 100ng/mL of rRANKL, in contact with or without MTA filled capillaries, per 6 days. Columns and bars indicate the mean and SD of triplicates, respectively of one representative experiment. * indicates, statistical differences ($p<0.05$), by *t*-test, when compared to the group only with medium. ● indicates $p<0.05$, by *t*-test. β-actin (house keeping gene) and cathepsin K gene expression (B) by RAW264.7 cells by stimulation or not with 100ng/mL of rRANKL, in contact with or without MTA, per 48 hours. Columns indicate the ratio between cathepsin K gene expression/β-actin gene expressions (C). Resorpted pit

area (D) induced from mature osteoclasts stimulated with 100ng/mL of rRANKL, in contact with or without MTA filled capillaries. Columns and bars indicate the mean and SD of triplicates, respectively of one representative experiment. * indicates, statistical differences ($p<0.05$), by *t*-test, when compared to the control without rRANKL. ● indicates $p<0.05$, by *t*-test. Representative photos of pit formation induced (E-H) by mature osteoclasts. Scale bar: 100μm.

Figure 3: Effect of calcium oxide and aluminum oxide on the rRANKL mediated osteoclastogenese. Number of Trap positive stained cells induced from RAW264.7 cells (A) by stimulation with 100ng/mL of rRANKL, in contact with or without MTA filled capillaries. * indicates, statistical differences ($p<0.05$), by *t*-test, when compared to the RAW cells without rRANKL. ● indicates $p<0.05$, by *t*-test. Representative scheme of how the release of Calcium and Aluminum ions, by MTA, can affect the differentiation and activity of mature osteoclasts (B).

Figure 1

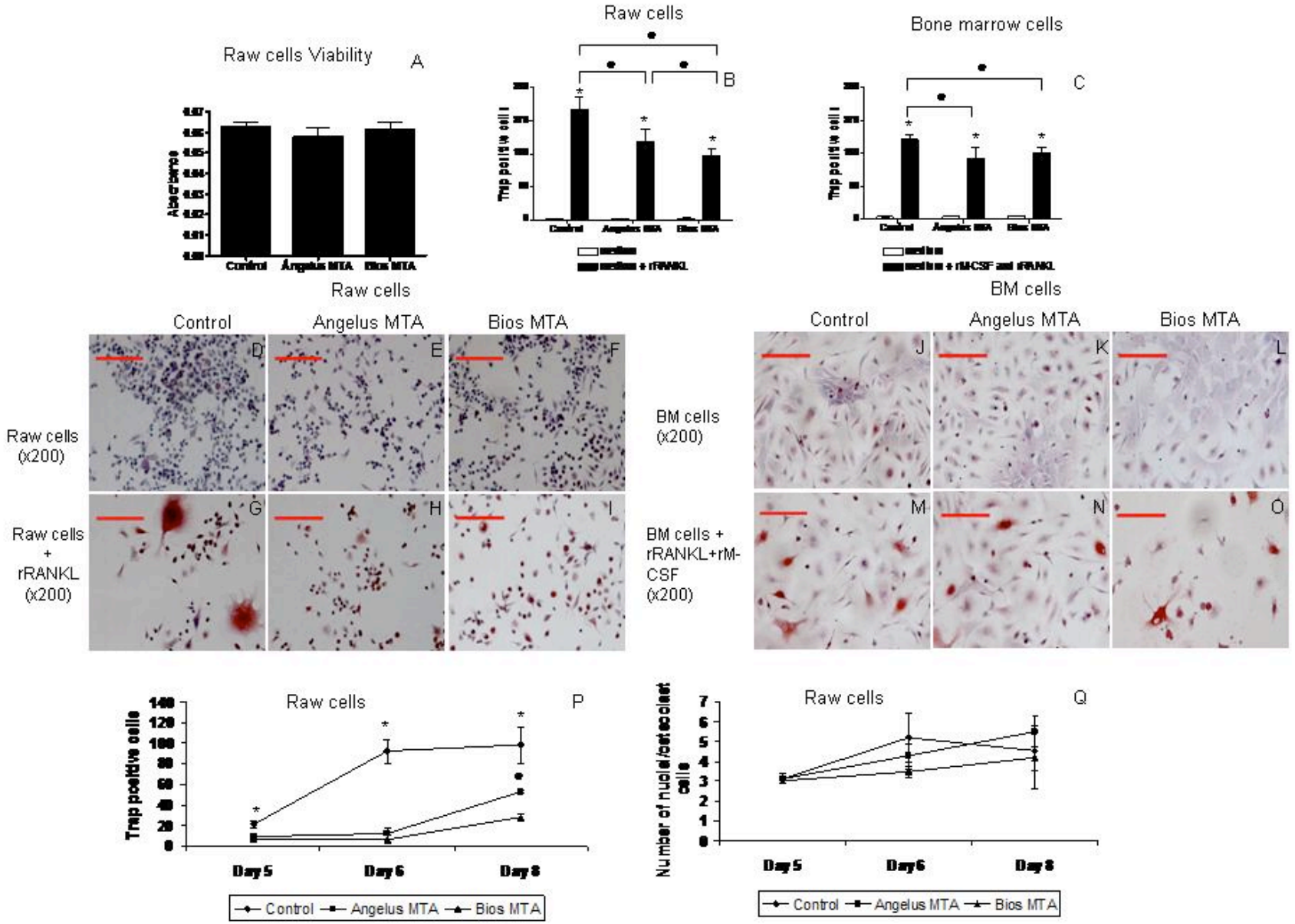

Raw cells Viability
A
Raw cells
B
Bone marrow cells
C
Raw cells
BM cells
Control
Angelus MTA
Bios MTA
Raw cells (x200)
Raw cells + rRANKL (x200)
BM cells (x200)
BM cells + rRANKL+rM-CSF (x200)
D
E
F
G
H
I
J
K
L
M
N
O
P
Raw cells
Trap positive cells
Day 5
Day 6
Day 8
Control
Angelus MTA
Bios MTA
Q
Raw cells
Number of nuclei/osteoclast cells

Figure 2

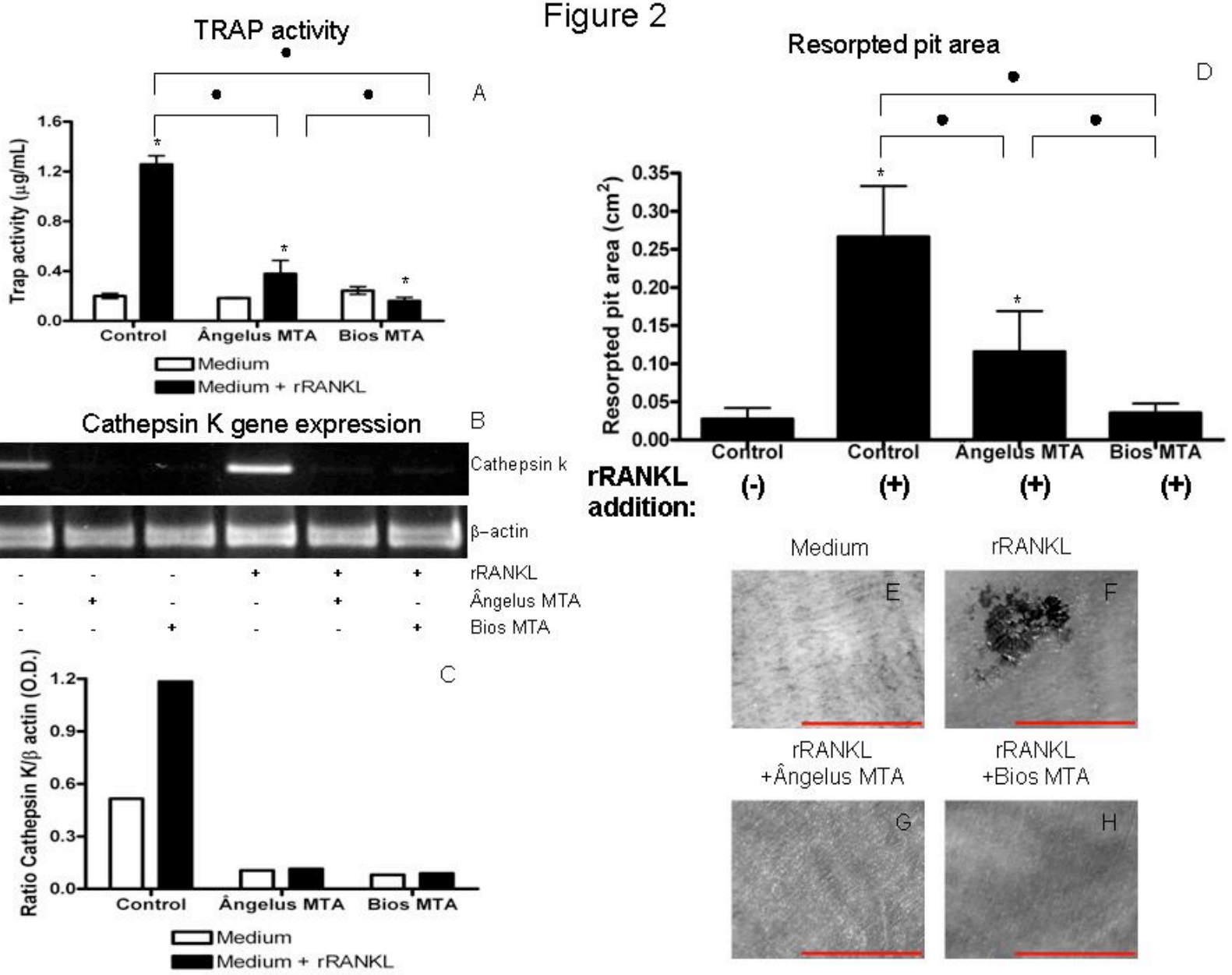
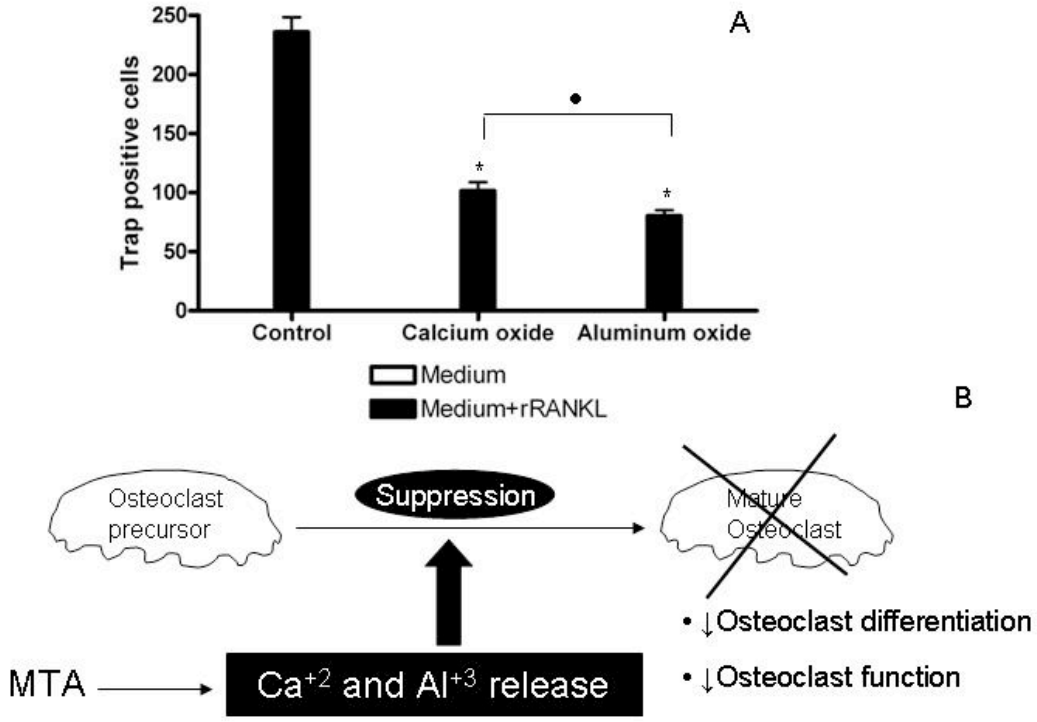

TRAP activity
A
Trap activity (µg/mL)
1.6
1.2
0.8
0.4
0.0
Control
Ângelus MTA
Bios MTA
Medium
Medium + rRANKL
Cathepsin K gene expression
B
Cathepsin k
β–actin
rRANKL
Ângelus MTA
Bios MTA
C
Ratio Cathepsin K/β actin (O.D.)
1.2
0.9
0.6
0.3
0.0
Control
Ângelus MTA
Bios MTA
Medium
Medium + rRANKL
Resorpted pit area
D
Resorpted pit area (cm²)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
Control
Ângelus MTA
Bios MTA
rRANKL addition:
(-)
(+)
(+)
(+)
Medium
rRANKL
E
F
rRANKL +Ângelus MTA
rRANKL +Bios MTA
G
H

Figure 3


A
Trap positive cells
250
200
150
100
50
0
Control
Calcium oxide
Aluminum oxide
Medium
Medium+rRANKL
B
Osteoclast precursor
Suppression
Mature Osteoclast
• ↓Osteoclast differentiation
• ↓Osteoclast function
MTA
Ca+2 and Al+3 release

4 DISCUSSÃO

4 DISCUSSÃO:

O MTA trouxe uma nova perspectiva para a endodontia. Esse material, inicialmente indicado no tratamento de perfurações radiculares e como material retro-obturador, atualmente também é utilizado em aplicações não cirúrgicas (Torabinejad e Chivian, 1999; Adamo *et al.*, 1999; Saidon *et al.*, 2003). Apresenta-se como um pó que deve ser incorporado à água destilada estéril; é composto, basicamente, de óxidos minerais, íons cálcio e fosfato, que também são componentes dos tecidos dentais. A composição do pó, como se vê, confere-lhe biocompatibilidade (Fischer *et al.*, 1998; Adamo *et al.*, 1999).

Como é um material relativamente novo, tem sido alvo de várias pesquisas, *in vivo* e *in vitro*. Elas demonstram que o MTA apresenta excelente biocompatibilidade, não provocando inflamação tecidual significativa no local de sua aplicação. O MTA permite que os reparos se processem, induzindo a deposição de tecido dentinário, cementário e ósseo (Torabinejad *et al.*, 1995a; Torabinejad *et al.*, 1995b; Holland *et al.*, 1999; Holland *et al.*, 2001). No Brasil, esse produto está disponível no mercado em duas formulações: nas cores cinza e branca. Este último pode ser adquirido de duas formas: MTA Branco e MTA Bios (Odonto-lógika).

Apesar dos resultados favoráveis relatados na literatura e do sucesso clínico obtido com a utilização desse material, conhece-se pouco a cerca de sua interação com os mecanismos de resposta do hospedeiro (Torabinejad *et al.*, 1995a; Torabinejad *et al.*, 1995b; Koh *et al.*, 1997; Koh *et al.*, 1998; Osorio *et al.*, 1998; Mitchell *et al.*, 1999; Zhu *et al.*, 2000; Keiser *et al.*, 2000; De Deus *et al.*, 2005; da Silva *et al.*, 2006; Braz *et al.*, 2006). O que se sabe, atualmente, observando-se os resultados dos estudos já citados, é que o MTA não interfere em alguns aspectos da resposta imune, porém interfere em outros, podendo

potencializar respostas, incluindo a cicatrização tecidual. Esse fato, ainda pouco explorado, é de extrema importância, pois, em todas as condições clínicas em que esse material é indicado, ocorre seu contato direto com o tecido pulpar, periapical ou periodontal inflamado, em diferentes fases da resposta imune.

Nosso grupo iniciou as pesquisas com o MTA avaliando a resposta de macrófagos à presença do MTA. Os macrófagos exercem um papel central na resposta imune inata, apresentando como principais funções a fagocitose de partículas estranhas e a produção de citocinas (Abbas A.K. *et al.*, 2005). Essas células podem ser didaticamente divididas em M1 e M2 por apresentarem diferentes funções dentro do contexto da resposta imunológica: atividade antimicrobiana e cicatricial, respectivamente. Na primeira etapa do trabalho, verificou-se a produção das citocinas TNF-α, IL-12p70 e IL-10 por macrófagos M1 e M2, na presença ou ausência do MTA (Anexo 1: "Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages"). Nesse estudo, *in vitro*, as culturas foram estimuladas com antígenos de bactérias altamente prevalentes nas lesões apicais (Lana *et al.*, 2001; Brito *et al.*, 2007), *F. nucleatum* e *P. anaerobius*, com e sem IFN-γ. Observou-se que os macrófagos M1 e M2 produziram as citocinas testadas pela estimulação com os antígenos bacterianos com e sem IFN-γ e que esta produção não foi alterada na presença do MTA. Outro resultado observado foi a polarização M1 e M2 caracterizada pelo perfil das citocinas produzidas: os macrófagos M2 uma produção de IL-10 superior à dos macrófagos M1 (Rezende *et al.*, 2005).

De posse desses dados, passamos a analisar as demais funções dos macrófagos M1 e M2 na presença do MTA (TRABALHO 1: "The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages".). Analisaram-se a aderência, a fagocitose de *S. boulardii*, a produção de ROIs e NO, além da atividade de arginase, que também se utiliza para definir a

polarização M1 e M2. A L-arginina pode ser usada por dois diferentes tipos de enzimas - a arginase e a óxido nítrico sintase - que competem entre si por esse substrato (Bronte e Zanovello, 2005). No caso da utilização da L-arginina pela óxido nítrico sintase, o produto final gerado será o NO, que apresenta atividade antimicrobiana (Mantovani *et al.*, 2002; Mosser, 2003). Quando a L-arginina é empregada para a produção de arginase, ocorrerá a formação de uréia e da L-ornitina, gerando poliaminas e prolinas que, como produto final, induzem a proliferação celular e a produção de colágeno, respectivamente, envolvidos nos processos cicatriciais (Mantovani *et al.*, 2002; Mosser, 2003). Neste estudo, os macrófagos M1 e M2 demonstraram sua polarização em virtude da elevada produção de ROI pelos macrófagos M1 e da grande atividade da arginase pelos macrófagos M2. O MTA não interferiu nas atividades macrofágicas analisadas, o que demonstrou a manutenção da resposta macrofágica inicial na presença do MTA, favorecendo, assim, sua indicação clínica.

Com esses resultados animadores, passamos a analisar o efeito do referido material na resposta imunológica adaptativa (TRABALHO 2: "The influence of Mineral Trioxide Aggregate (MTA) on adaptive immune responses to endodontic pathogens in mice"). Como os linfócitos T e B são os principais tipos celulares presentes nessa fase (Abbas A.K. *et al.*, 2005), observou-se a produção de IgG *F. nucleatum*-específica e a proliferação e produção de citocinas por linfócitos Th pré-imunes e Th de memória. Comprovou-se que comparando com os grupos imunizados apenas com o *Fn*, a resposta a produção de IgG foi aumentada quando os camundongos foram imunizados com *Fn* em MTA em níveis comparáveis a resposta do anticorpo IgG induzida por adjuvante de Freund ou adjuvante de hidróxido de alumino. Demonstrando que o MTA estimulou a produção de IgG ao *F. nucleatum*. Com relação às células Th, observou-se que o MTA aumentou a proliferação das células T pré-imunes. Essas células produziram baixos níveis de citocinas do estímulo policlonal. Na presença de MTA, porém, houve um aumento da produção das citocinas

inflamatórias (TNF-α e IFN-γ) e da citocina ligada à reabsorção óssea (RANKL). Nas alterações perirradiculares predomina um infiltrado crônico (Stashenko *et al.*, 1998). Procurou-se, então, analisar os mesmos parâmetros já descritos para as células T de memória reativas ao *F. nucleatum* e ao *P. anaerobius*. Curiosamente, observou-se que as células T de memória reativas ao *F. nucleatum* apresentaram perfil Th2 (IFN-γ<IL-4) enquanto as células T de memória, reativas ao *P. anaerobius,* mostraram perfil Th1 (IFN-γ>IL-4). Ao contrário do que se comprovou para as células T pré-imunes, apesar do MTA ter decrescido a proliferação das células T de memória, ele não afetou a produção das citocinas TNF-α, RANKL e IL-10; apresentou efeito apenas sobre as citocinas típicas dos subtipos Th1 (IFN-γ) e Th2 (IL-4). Observamos que o MTA pouco afetou os parâmetros celulares avaliados, sugerindo que suas ações são favoráveis à resposta imunológica adaptativa, influenciando positivamente ainda mais sua indicação clínica.

No transcurso da instalação de uma alteração perirradicular, a reabsorção óssea apresenta-se como um fator peculiar dessa patologia, sendo altamente influenciada pelo padrão de citocinas produzidas. Diante deste fato, o passo seguinte deste estudo foi avaliar os efeitos do MTA na osteoclastogênese e na atividade dos osteoclastos, em um modelo de reabsorção óssea mediada por RANKL (TRABALHO 3: "Influence of Mineral Trioxide Aggregate (MTA) on RANKL-mediated osteoclastogenesis and osteoclast activation"). Como se observou anteriormente, a presença do óxido de alumínio na composição do MTA mereceu destaque, bem como a alta quantidade de componentes ligados ao íon cálcio. Verificou-se a liberação de grandes quantidades desses íons quando se adicionou o MTA às culturas (Tomson *et al.*, 2007). Sabendo-se que tais íons participam dos eventos de reabsorção óssea (Zaidi *et al.*, 1993; Zaidi *et al.*, 1999; Granchi *et al.*, 2004; Granchi *et al.*, 2005), tornou-se relevante observar essa relação. Em virtude do breve lançamento do novo MTA Branco produzido pela Odonto-lógika, o MTA Bios, no transcurso desta pesquisa, o mesmo foi

também inserido neste estudo. Comprovou-se que o MTA afetou não só a osteoclastogênese como também a atividade dos osteoclastos remanescentes. Nesse aspecto o MTA Bios apresentou melhores resultados em comparação com o MTA Branco. A partir daí, observou-se que o mecanismo de atuação do MTA nos eventos de reabsorção óssea está realmente relacionado à liberação desses íons: diminuição da osteoclastogênese na presença exclusiva dos íons e maior percentual dos mesmos na composição do MTA Bios. Levantou-se, então, uma nova hipótese: o sucesso clínico, quando da utilização do MTA, advém não apenas de sua atuação sobre osteoblastos (Koh *et al.*, 1997; Mitchell *et al.*, 1999; Granchi *et al.*, 2004; Pelliccioni *et al.*, 2004; Granchi *et al.*, 2005), mas também da supressão dos eventos que levam à reabsorção óssea. Os resultados deste trabalho são muito positivos para a utilização do MTA em eventos de reabsorção óssea, e nas lesões apicais, e lhe confere um real poder cicatricial nessas patologias.

A busca por respostas aos vários questionamentos relativos à biocompatibilidade de materiais endodônticos objetivam, em última análise, fundamentar a utilização de materiais com ações terapêuticas eficazes. Nesse aspecto, torna-se importante observar a interação hospedeiro/material endodôntico, após sua utilização nos SCRs, para se avaliar se ocorreu o almejado sucesso clínico. Na década passada, o enfoque sobre novos materiais biomédicos tomou um novo rumo. O antigo conceito de material "passivo", aquele que deveria apresentar propriedades ideais, deu lugar ao conceito de materiais que ativamente interagem e se integram ao ambiente biológico no qual o mesmo é utilizado (Anderson, 2006). Assim, dentro desse novo contexto de biocompatibilidade, o MTA pode ser considerado hoje, como o mais completo material a ser usado nas indicações de selamento de perfuração e de retro-obturação. Apesar de sua comprovada eficácia, o MTA vem sofrendo modificações desde sua fabricação inicial. Com os resultados obtidos nesta tese pode-se sugerir que alguns aspectos de sua composição deveriam ser reavaliados, como por exemplo: o aumento no percentual dos

elementos que atuam sobre os processos de reabsorção óssea. Sabendo-se que as respostas imunes inatas e adquiridas parecem ter um papel protetor, prevenindo a disseminação de infecções endodônticas (Teles *et al.*, 1997), as implicações clínicas da exacerbação ou da redução dessas respostas em infecções de origem endodôntica precisam ser mais bem explicadas em estudos epidemiológicos. Nesse contexto, a pesquisa relacionada à interação hospedeiro/material endodôntico pode ser a porta para o desenvolvimento de materiais mais biocompatíveis com atuação positiva sobre a resposta imune, acelerando assim os processos de cicatrização e aumentando seu percentual de sucesso dentro da especialidade endodôntica. Acreditamos, portanto, que nossa nova meta deve ser a busca da compreensão dos mecanismos de interação do MTA com seu ambiente clínico, *in vivo*.

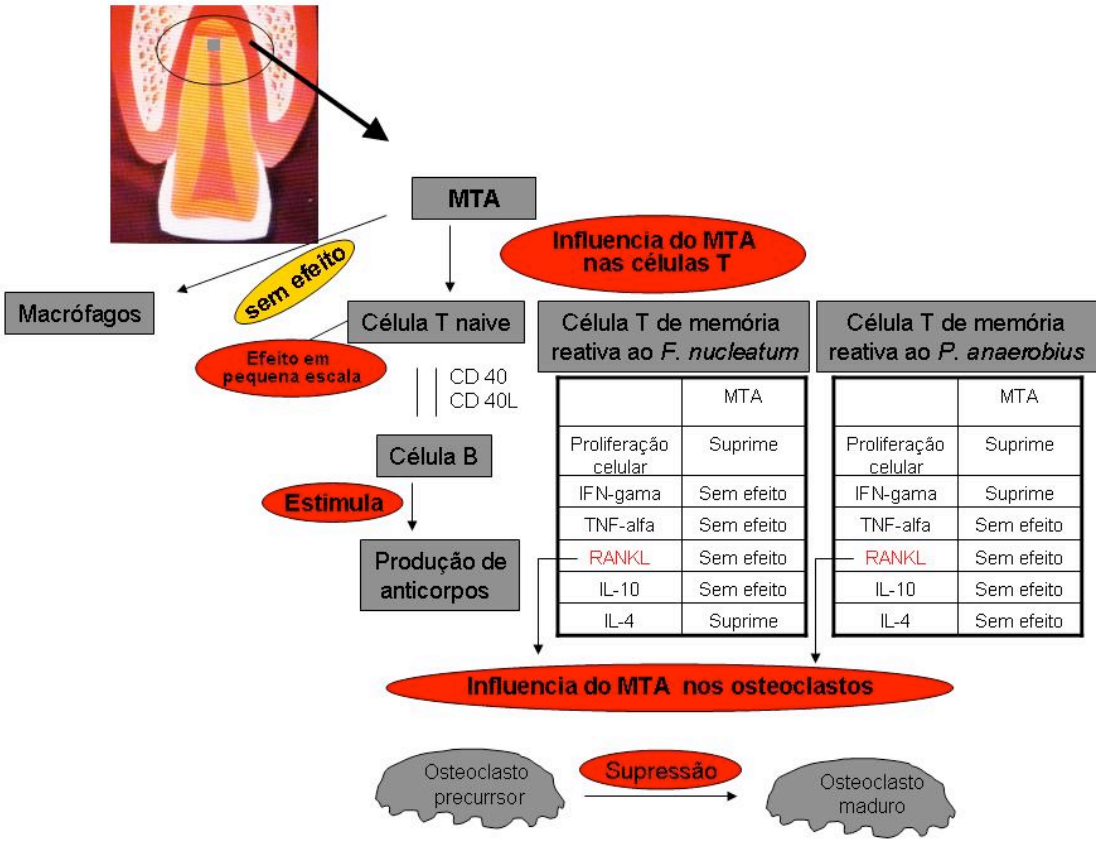
5 CONCLUSÃO

5 CONCLUSÃO:

Pôde-se observar que o MTA:

- Na resposta imune inata:
  - Não interferiu na viabilidade e aderência celular de macrófagos M1 e M2;
  - Não interferiu na fagocitose da *S. boulardii* por macrófagos M1 e M2;
  - Não interferiu na produção de ROI e NO, nem na atividade da arginase, quando os macrófagos M1 e M2 foram ou não estimulados.
- Na resposta imune adaptativa:
  - Estimulou a produção de IgG ao *F. nucleatum*;
  - Estimulou a proliferação de células T pré-imunes;
  - Estimulou a produção de citocinas inflamatórias e de reabsorção óssea;
  - Inibiu a proliferação de células T de memória reativas ao *F. nucleatum* e ao *P. anaerobius*;
  - Não interferiu na produção das citocinas TNF-α, RANKL e IL-10 pelas células T de memória reativas ao *F. nucleatum* e ao *P. anaerobius;*
  - Inibiu a produção das citocinas IFN-γ e IL-4, nos modelos Th1 e Th2, respectivamente.
- Na reabsorção óssea:
  - Inibiu a osteoclastogênese;

- Inibiu a atividade dos osteoclastos;
- Apresentou maior inibição da osteoclastogênese pelo MTA Bios que pelo MTA Branco;
- A liberação dos íons alumínio e cálcio apresentam-se como elementos ativos no processo de inibição óssea.



	MTA
Proliferação celular	Suprime
IFN-gama	Sem efeito
TNF-alfa	Sem efeito
RANKL	Sem efeito
IL-10	Sem efeito
IL-4	Suprime

	MTA
Proliferação celular	Suprime
IFN-gama	Suprime
TNF-alfa	Sem efeito
RANKL	Sem efeito
IL-10	Sem efeito
IL-4	Sem efeito

Concluiu-se que: o MTA não interfere na atividade das células presentes nas fases iniciais da resposta imune; o MTA apresenta alguma atividade nas células presentes nas fases tardias da resposta imune; o MTA inibe os eventos de reabsorção óssea.

REFERÊNCIAS BIBLIOGRÁFICAS

REFERÊNCIAS BIBLIOGRÁFICAS

1. Abbas A.K., Lichtman A.H., Pober J.S. (2005). Cellular and molecular immunology. 5 ed.

2. Abdullah D, Ford TR, Papaioannou S, Nicholson J, McDonald F . An evaluation of accelerated Portland cement as a restorative material. **Biomaterials**, v. 23, n. 19, p.4001-4010, 2002.

3. Abedi HR, Ingle JI . Mineral trioxide aggregate: a review of a new cement. **J Calif Dent Assoc**, v. 23, n. 12, p.36-39, 1995.

4. Adamo HL, Buruiana R, Schertzer L, Boylan RJ . A comparison of MTA, Super-EBA, composite and amalgam as root-end filling materials using a bacterial microleakage model. **Int Endod J**, v. 32, n. 3, p.197-203, 1999.

5. Anderson JM . The future of biomedical materials. **J Mater Sci Mater Med**, v. 17, n. 11, p.1025-1028, 2006.

6. Artese L, Piattelli A, Quaranta M, Colasante A, Musani P . Immunoreactivity for interleukin 1-beta and tumor necrosis factor-alpha and ultrastructural features of monocytes/macrophages in periapical granulomas. **J Endod**, v. 17, n. 10, p.483-487, 1991.

7. Barnes GW, Langeland K . Antibody formation in primates following introduction of antigens into the root canal. **J Dent Res**, v. 45, n. 4, p.1111-1114, 1966.

8. Baylor NW, Egan W, Richman P . Aluminum salts in vaccines--US perspective. **Vaccine**, v. 20 Suppl 3, p.S18-S23, 2002.

9. Bergenholtz G, Nagaoka S, Jontell M . Class II antigen expressing cells in experimentally induced pulpitis. **Int Endod J**, v. 24, n. 1, p.8-14, 1991.

10. Boyle WJ, Simonet WS, Lacey DL . Osteoclast differentiation and activation. **Nature**, v. 423, n. 6937, p.337-342, 2003.

11. Braz MG, Camargo EA, Salvadori DM, Marques ME, Ribeiro DA . Evaluation of genetic damage in human peripheral lymphocytes exposed to mineral trioxide aggregate and Portland cements. **J Oral Rehabil**, v. 33, n. 3, p.234-239, 2006.

12. Brito LC, Teles FR, Teles RP, Franca EC, Ribeiro-Sobrinho AP, Haffajee AD *et al.* Use of multiple-displacement amplification and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections. **J Clin Microbiol**, v. 45, n. 9, p.3039-3049, 2007.

13. Bronte V, Zanovello P . Regulation of immune responses by L-arginine metabolism. **Nat Rev Immunol**, v. 5, n. 8, p.641-654, 2005.

14. Camilleri J, Montesin FE, Brady K, Sweeney R, Curtis RV, Ford TR . The constitution of mineral trioxide aggregate. **Dent Mater**, v. 21, n. 4, p.297-303, 2005.

15. da Silva GN, Braz MG, de Camargo EA, Salvadori DM, Ribeiro DA . Genotoxicity in primary human peripheral lymphocytes after exposure to regular and white mineral trioxide aggregate. **Oral Surg Oral Med Oral Pathol Oral Radiol Endod**, v. 102, n. 5, p.e50-e54, 2006.

16. De Deus G, Ximenes R, Gurgel-Filho ED, Plotkowski MC, Coutinho-Filho T . Cytotoxicity of MTA and Portland cement on human ECV 304 endothelial cells. **Int Endod J**, v. 38, n. 9, p.604-609, 2005.

17. Fischer EJ, Arens DE, Miller CH . Bacterial leakage of mineral trioxide aggregate as compared with zinc-free amalgam, intermediate restorative material, and Super-EBA as a root-end filling material. **J Endod**, v. 24, n. 3, p.176-179, 1998.

18. Granchi D, Amato I, Battistelli L, Ciapetti G, Pagani S, Avnet S *et al.* Molecular basis of osteoclastogenesis induced by osteoblasts exposed to wear particles. **Biomaterials**, v. 26, n. 15, p.2371-2379, 2005.

19. Granchi D, Ciapetti G, Amato I, Pagani S, Cenni E, Savarino L *et al.* The influence of alumina and ultra-high molecular weight polyethylene particles on osteoblast-osteoclast cooperation. **Biomaterials**, v. 25, n. 18, p.4037-4045, 2004.

20. Haglund R, He J, Jarvis J, Safavi KE, Spangberg LS, Zhu Q . Effects of root-end filling materials on fibroblasts and macrophages in vitro. **Oral Surg Oral Med Oral Pathol Oral Radiol Endod**, v. 95, n. 6, p.739-745, 2003.

21. Hahn CL, Falkler WA, Jr. Antibodies in normal and diseased pulps reactive with microorganisms isolated from deep caries. **J Endod**, v. 18, n. 1, p.28-31, 1992.

22. Hahn CL, Falkler WA, Jr., Siegel MA . A study of T and B cells in pulpal pathosis. **J Endod**, v. 15, n. 1, p.20-26, 1989.

23. Holland R, de S, V, Murata SS, Nery MJ, Bernabe PF, Otoboni Filho JA *et al.* Healing process of dog dental pulp after pulpotomy and pulp covering with mineral trioxide aggregate or Portland cement. **Braz Dent J**, v. 12, n. 2, p.109-113, 2001.

24. Holland R, de S, V, Nery MJ, Otoboni Filho JA, Bernabe PF, Dezan JE . Reaction of rat connective tissue to implanted dentin tubes filled with mineral trioxide aggregate or calcium hydroxide. **J Endod**, v. 25, n. 3, p.161-166, 1999.

25. Ingle J.I., Taintor J.F. Endodontia. Rio de Janeiro: Guanabara S.A. 1989.

26. Kakehahsi S, Stanley HR, Fritzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. **Oral Surg Oral Med Oral Pathol**, v. 20, p.340-349, 1965.

27. Kawashima N, Okiji T, Kosaka T, Suda H . Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: a quantitative immunohistochemical study. **J Endod**, v. 22, n. 6, p.311-316, 1996.

28. Keiser K, Johnson CC, Tipton DA . Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. **J Endod**, v. 26, n. 5, p.288-291, 2000.

29. Kennedy DR, Hamilton TR, Syverton JT. Effects on monkeys of introduction of hemolytic streptococci into root canals. **J Dent Res**, v. 36, n. 4, p.496-506, 1957.

30. Koh ET, McDonald F, Pitt Ford TR, Torabinejad M . Cellular response to Mineral Trioxide Aggregate. **J Endod**, v. 24, n. 8, p.543-547, 1998.

31. Koh ET, Torabinejad M, Pitt Ford TR, Brady K, McDonald F . Mineral trioxide aggregate stimulates a biological response in human osteoblasts. **J Biomed Mater Res**, v. 37, n. 3, p.432-439, 1997.

32. Lana MA, Ribeiro-Sobrinho AP, Stehling R, Garcia GD, Silva BK, Hamdan JS *et al.* Microorganisms isolated from root canals presenting necrotic pulp and their drug susceptibility in vitro. **Oral Microbiol Immunol**, v. 16, n. 2, p.100-105, 2001.

33. Lee SJ, Monsef M, Torabinejad M . Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. **J Endod**, v. 19, n. 11, p.541-544, 1993.

34. Levy JA . HIV pathogenesis: knowledge gained after two decades of research. **Adv Dent Res**, v. 19, n. 1, p.10-16, 2006.

35. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A . Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. **Trends Immunol**, v. 23, n. 11, p.549-555, 2002.

36. Mitchell PJ, Pitt Ford TR, Torabinejad M, McDonald F . Osteoblast biocompatibility of mineral trioxide aggregate. **Biomaterials**, v. 20, n. 2, p.167-173, 1999.

37. Mosser DM . The many faces of macrophage activation. **J Leukoc Biol**, v. 73, n. 2, p.209-212, 2003.

38. Naim JO, van Oss CJ, Wu W, Giese RF, Nickerson PA . Mechanisms of adjuvancy: I--Metal oxides as adjuvants. **Vaccine**, v. 15, n. 11, p.1183-1193, 1997.

39. Osorio RM, Hefti A, Vertucci FJ, Shawley AL . Cytotoxicity of endodontic materials. **J Endod**, v. 24, n. 2, p.91-96, 1998.

40. Pelliccioni GA, Ciapetti G, Cenni E, Granchi D, Nanni M, Pagani S *et al.* Evaluation of osteoblast-like cell response to Proroot MTA (mineral trioxide aggregate) cement. **J Mater Sci Mater Med**, v. 15, n. 2, p.167-173, 2004.

41. Perez AL, Spears R, Gutmann JL, Opperman LA . Osteoblasts and MG-63 osteosarcoma cells behave differently when in contact with ProRoot MTA and White MTA. **Int Endod J**, v. 36, n. 8, p.564-570, 2003.

42. Pulver WH, Taubman MA, Smith DJ . Immune components in normal and inflamed human dental pulp. **Arch Oral Biol**, v. 22, n. 2, p.103-111, 1977.

43. Rezende TMB, Vargas DL, Cardoso FP, Sobrinho APR, Vieira LQ . Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages. **International Endodontics Journal**, v. 38, p.896-903, 2005.

44. Saidon J, He J, Zhu Q, Safavi K, Spangberg LS . Cell and tissue reactions to mineral trioxide aggregate and Portland cement. **Oral Surg Oral Med Oral Pathol Oral Radiol Endod**, v. 95, n. 4, p.483-489, 2003.

45. Schwartz RS, Mauger M, Clement DJ, Walker WA, III . Mineral trioxide aggregate: a new material for endodontics. **J Am Dent Assoc**, v. 130, n. 7, p.967-975, 1999.

46. Shafer W.G., Hine M.K., Levy B.M. (1987). Doenças da polpa e dos tecidos periapicais. In: Tratado de Patologia Bucal. Editora Guanabara Koogan S.A., editor. Rio de Janeiro: pp. 443-471.

47. Silva MJB (2005). Avaliação imunológica e histológica do tecido pulpar e periodontal expostos ao MTA. Departamento de Bioquímica e Imunologia. Universidade Federal de Minas Gerais.

48. Speer ML, Madonia JV, Heuer MA . Quantitative evaluation of the immunocompetence of the dental pulp. **J Endod**, v. 3, n. 11, p.418-423, 1977.

49. Stashenko P, Teles R, D'Souza R . Periapical inflammatory responses and their modulation. **Crit Rev Oral Biol Med**, v. 9, n. 4, p.498-521, 1998.

50. Stern MH, Dreizen S, Mackler BF, Selbst AG, Levy BM . Quantitative analysis of cellular composition of human periapical granuloma. **J Endod**, v. 7, n. 3, p.117-122, 1981.

51. Sundqvist G (1976). Bacteriological studies of necrotic pulps. Ümea: University Odontol.

52. Takahashi N, Udagawa N, Suda T . A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. **Biochem Biophys Res Commun**, v. 256, n. 3, p.449-455, 1999.

53. Tani-Ishii N, Wang CY, Tanner A, Stashenko P . Changes in root canal microbiota during the development of rat periapical lesions. **Oral Microbiol Immunol**, v. 9, n. 3, p.129-135, 1994.

54. Teles R, Wang CY, Stashenko P . Increased susceptibility of RAG-2 SCID mice to dissemination of endodontic infections. **Infect Immun**, v. 65, n. 9, p.3781-3787, 1997.

55. Tomson PL, Grover LM, Lumley PJ, Sloan AJ, Smith AJ, Cooper PR . Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. **J Dent,** v. 35, n. 8, p. 636-642, 2007.

56. Torabinejad M, Chivian N . Clinical applications of mineral trioxide aggregate. **J Endod**, v. 25, n. 3, p.197-205, 1999.

57. Torabinejad M, Eby WC, Naidorf IJ . Inflammatory and immunological aspects of the pathogenesis of human periapical lesions. **J Endod**, v. 11, n. 11, p.479-488, 1985.

58. Torabinejad M, Hong CU, Lee SJ, Monsef M, Pitt Ford TR . Investigation of mineral trioxide aggregate for root-end filling in dogs. **J Endod**, v. 21, n. 12, p.603-608, 1995a.

59. Torabinejad M, Hong CU, Pitt Ford TR, Kaiyawasam SP . Tissue reaction to implanted super-EBA and mineral trioxide aggregate in the mandible of guinea pigs: a preliminary report. **J Endod**, v. 21, n. 11, p.569-571, 1995b.

60. Torabinejad M, Watson TF, Pitt Ford TR . Sealing ability of a mineral trioxide aggregate when used as a root end filling material. **J Endod**, v. 19, n. 12, p.591-595, 1993.

61. Vernal R, Dezerega A, Dutzan N, Chaparro A, Leon R, Chandia S *et al.* RANKL in human periapical granuloma: possible involvement in periapical bone destruction. **Oral Dis**, v. 12, n. 3, p.283-289, 2006.

62. Warfvinge J, Dahlen G, Bergenholtz G . Dental pulp response to bacterial cell wall material. **J Dent Res**, v. 64, n. 8, p.1046-1050, 1985.

63. Yu SM, Stashenko P . Identification of inflammatory cells in developing rat periapical lesions. **J Endod**, v. 13, n. 11, p.535-540, 1987.

64. Zaidi M, Adebanjo OA, Moonga BS, Sun L, Huang CL . Emerging insights into the role of calcium ions in osteoclast regulation. **J Bone Miner Res**, v. 14, n. 5, p.669-674, 1999.

65. Zaidi M, Alam AS, Shankar VS, Bax BE, Bax CM, Moonga BS *et al.* Cellular biology of bone resorption. **Biol Rev Camb Philos Soc**, v. 68, n. 2, p.197-264, 1993.

66. Zhu Q, Haglund R, Safavi KE, Spangberg LS . Adhesion of human osteoblasts on root-end filling materials. **J Endod**, v. 26, n. 7, p.404-406, 2000.

ANEXOS

ANEXOS

ANEXO 1: “Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages”.

Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages

T. M. B. Rezende[1], D. L. Vargas[1], F. P. Cardoso[1], A. P. R. Sobrinho[1] & L. Q. Vieira[2]

[1]Departamento de Dentística Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais; and [2]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Abstract

Rezende TMB, Vargas DL, Cardoso FP, Sobrinho APR, Vieira LQ. Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages. *International Endodontic Journal*, **38**, 896–903, 2005.

Aim To test the effect of two commercial brands of grey mineral trioxide aggregate (ProRoot® and MTA-Ângelus®) on cytokine production by M1 and M2 inflammatory macrophages.

Methodology M1 (from C57BL/6 mice) and M2 peritoneal inflammatory macrophages (from C57BL/6 IL12p40$^{-/-}$ mice) were obtained and cultured *in vitro* in the presence of MTA. The cellular viability and the production of tumour necrosis factor-α, interleukin (IL)-12 and IL-10 in response to stimulation with interferon-γ and *Fusobacterium nucleatum* or *Peptostreptococcus anaerobius* were evaluated. Data were analysed by Mann–Whitney, Kruskal–Wallis and ANOVA tests.

Results The cements did not interfere with cellular viability or with cytokine production by either type of macrophage. However, M2 macrophages produced higher levels of IL-10 when stimulated with *F. nucleatum* than M1 macrophages ($P < 0.05$).

Conclusions The brands of MTA evaluated did not interfere in the cytokine response by M1 or M2 macrophages to the two bacteria tested. However, a difference in cytokine production between the two types of macrophages was found.

Keywords: biocompatibility, cytokines, innate immunity, macrophages, mineral trioxide aggregate.

Received 9 March 2005; accepted 15 August 2005

Introduction

Since its first description (Lee *et al.* 1993), mineral trioxide aggregate (MTA) has been used in a variety of surgical and nonsurgical endodontic applications. Research has demonstrated that MTA has better properties in terms of root repair and bone formation, when compared with other commonly used materials such as intermediate restorative material (IRM®; Dentsply, Milford, DE, USA), glass ionomer and reinforced zinc oxide–eugenol cement (Super-EBA®; Harry J. Bosworth Company, Skokie, IL, USA) (Torabinejad *et al.* 1995a, 1998). As MTA is usually applied on infected or sterile surgical wounds, it is important that it is biocompatible and does not interfere with the immune response of the patient.

Inflamed periradicular and pulp tissues, upon which MTA is generally applied, present a variety of immunocompetent cells. Macrophages are the predominant cells (Stern *et al.* 1981, Kawashima *et al.* 1996) at the site. These cells play a central role in the pathogenesis of inflammation (Van Furth *et al.* 1972, Unanue 1978) and cytokine production is one among their several actions.

Recent studies have revealed that there may be three types of macrophages, based on their ability to produce different responses (Mosser 2003). As defined by Mills *et al.* (2000), M1 macrophages (obtained from C57BL/6 mice) will utilize arginine to produce nitric oxide (NO), while M2 macrophages (obtained from BALB/c mice) will produce low amounts of NO and high amounts of TGF-β. In addition, M1 macrophages are

Correspondence: Dra Leda Quercia Vieira, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Caixa Postal 486, 30.161-970, Belo Horizonte, MG, Brasil (Tel:. 55 31 34992656; e-mail: lqvieira@icb.ufmg.br).

high producers of oxygen reactive species (Mills *et al.* 2000, Mosser 2003), while there is evidence that M2 macrophages do not produce interleukin (IL)-12 but release high amounts of IL-10 (Gerber & Mosser 2001). A third type, defined as the alternatively activated macrophage, converts arginine into ornithine by action of arginase. Ornithine is the precursor of polyamines that will mediate collagen synthesis and cellular proliferation. Hence, M1 macrophages (obtained from C57BL/6 mice) would be high producers of IL-12 and low producers of IL-10, in accordance with their pro-inflammatory role, while M2 macrophages (obtained from BALB/c mice) would produce lower levels of IL-12 and higher levels of IL-10 and TGF-β, which would render them an anti-inflammatory role (Mills *et al.* 2000, Gerber & Mosser 2001, Mosser 2003). Recently, Bastos *et al.* (2002) showed that mice deficient in the p40 chain for IL-12/IL-23 by targeted mutation have predominantly macrophages of the M2 type, like the BALB/c mice. This model is elegant, as it provides macrophages of the same genetic background (in this case the C57BL/6) but with different phenotypes (M1 or M2).

As MTA has only recently been used as an endodontic material, there are few studies evaluating its effects on the host defense mechanisms (Koh *et al.* 1997, 1998, Mitchell *et al.* 1999). This study aimed to analyze the action of two commercial brands of MTA (MTA-Ângelus®, Odonto-lógika, Londrina, PN, Brazil; ProRoot®, Dentsply Maillefer, Ballaigues, Switzerland) on the activity of M1 and M2 murine macrophages, assessing cellular viability and cytokine production.

Materials and methods

Preparation of MTA

Two different commercial brands of grey MTA were used: ProRoot® (Dentsply Maillefer, Ballaigues, Switzerland) and MTA-Ângelus® (Odonto-lógika, Londrina, PR, Brazil). The cements were prepared according to the manufacturer's specifications, and their manipulation was carried out in a laminar flow hood under aseptic conditions. Cements were inserted into the ends of capillary tubes (Mícron, Trianon Ind. e Com. Ltda, São Paulo, SP, Brazil), previously cut and sterilized (capillary diameter: 3.52 mm^2), according to the technique described previously (Oliveira Mendes *et al.* 2003). Capillaries containing cements were stored in six well culture plates (Nunclon; Nalge Nunc International, Naperville, IL, USA) for final setting. Subsequently, the plate was sterilized using 25 kGray of gamma ray irradiation (Centro de Desenvolvimento Tecnológico/CDTN, Belo Horizonte, MG, Brazil).

Mice

Males and females of 4–8 weeks of two mouse strains were used: C57BL/6 (CEBIO – UFMG, Belo Horizonte, Brazil) and C57BL/6 deficient in the p40 chain for IL-12/IL-23 by targeted mutation (Magram *et al.* 1996). Matrices for IL-12p40$^{-/-}$ were obtained from Dr Luiz Vicente Rizzo (University of São Paulo, SP, Brazil), mice were bred at the Gnotobiology Laboratory, UFMG, Belo Horizonte, Brazil. Experimental animals were kept in a conventional animal house with barriers, temperature and light control. Food and water were offered *ad libitum*. The research protocol was authorized by the committee of ethics and research on animals (CETEA – UFMG, protocol number 35/2002).

Macrophages

Inflammatory macrophages were obtained from the peritoneal cavity, 5 days after injection of 2 mL of 3% thioglicolate medium, containing 1% sterile agar (Biobrás S.A., Montes Claros, MG, Brazil). The animals were then sacrificed and 10 mL of sterile RPMI 1640 medium, without phenol red (Sigma Chemical Co., St Louis, MO, USA), was injected into the peritoneal cavity. The largest possible content was aspirated, and the cells were centrifuged at 350 *g* for 10 min, at 4 °C. Supernatant medium was discarded and cells were resuspended in RPMI 1640 medium without phenol red, supplemented with 10% fetal bovine serum (Nutricell; Campinas, SP, Brazil), 0.05 mmol L^{-1} β-mercaptoethanol (Sigma Chemical Co.), 0.2% gentamicin and 200 mmol L^{-1} L-glutamine. The cells were counted in a Newbauer chamber, and the final concentration was adjusted for each experiment.

Viability

To evaluate the cellular viability in the presence of MTA, the capillaries containing MTA and empty controls were added onto the cellular suspension (1×10^5 cells mL^{-1}) present in 24-well plates (Nunclon). Cells were incubated for 24, 48 or 72 h. The viability analysis was accomplished with an inverted microscope, following the addition of 100 μL of 0.5% tryptan blue solution (Sigma Chemical Co.) in PBS (0.15 M NaCl and 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4).

Live and dead cells were counted, amounting to at least 300 cells (Oliveira Mendes *et al.* 2003).

Bacterial preparations

To induce the production of tumour necrosis factor (TNF), IL-12 and IL-10, *Fusobacterium nucleatum* (ATCC 10953) and *Peptostreptococcus anaerobius* (ATCC 27337) were prepared, and used in a proportion of 10 UFC for each macrophage in culture (Ribeiro Sobrinho *et al.* 2002). These preparations were used to stimulate cell cultures.

Cell cultures and cytokine assays

Fusobacterium nucleatum and *P. anaerobius* (10^7 UFC mL^{-1}) preparations were added to the 24-well plates (Nunclon), containing 1 mL of peritoneal cell suspension (2×10^6 cells mL^{-1}), in the presence or absence of 10 U mL^{-1} of recombinant murine interferon (IFN)-γ (Pharmingem, San Diego, CA, USA), in addition to the MTA-containing or empty capillaries. Plates were incubated for 24 h for TNF and IL-12 assays, and for 72 h for IL-10. Supernatants were harvested, and cytokine readings were carried out (Duo Set Elisa kits for TNF, IL-12p70 and IL-10; Development System, R & D Systems, Mineapolis, MN, USA). Sensitivity for the assays were as follows: TNF: 31.2 pg mL^{-1}; IL12p70: 23.4 pg mL^{-1}; IL-10: 15.6 pg mL^{-1}.

Statistical analysis

Results were compared by ANOVA, Mann–Whitney and Kruskal–Wallis tests as specified for each experiment. The analyses were carried out with SPSS 8.0 Inc. software (Statistical Package for Social Sciences, Chicago, Ill, USA).

Results

Cell viability in the presence of MTA

The results of cellular viability are shown in Fig. 1. The mean cellular viability was >97% in all the M1 and M2 macrophage cultures, regardless of exposure to MTA. There was no difference in the percentages of live cells between the two cements. However, the proportion of M2 macrophage live cells exposed for 24 h to MTA-Ângelus was statistically higher than the M1 macrophages ($P < 0.05$). The incubation time did not influence the percentage of live M1 macrophages. M2 macrophages were less viable after 72 h of culture ($P < 0.05$). However, as viability was >97% in all cultures, cytokine production in these cultures was investigated.

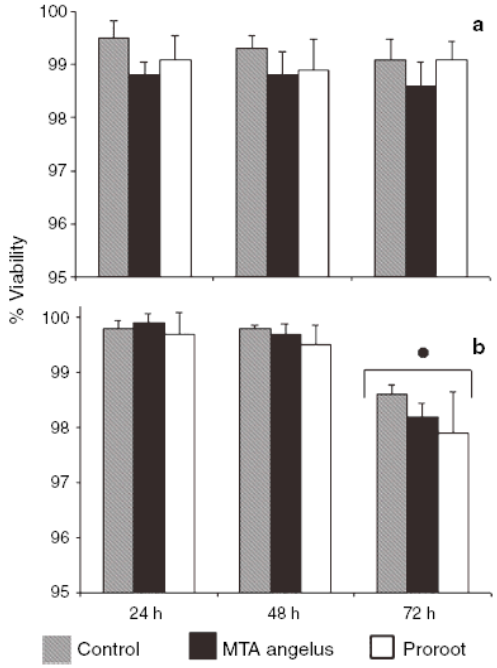


Figure 1 Percentage of live M1 (a) and M2 (b) macrophages 12, 24 and 48 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the means. • indicates $P < 0.05$ when compared with 24 and 48 h by Kruskal–Wallis tests.

TNF-α

Inflammatory M1 and M2 macrophages produced TNF-α in culture in the absence of bacterial stimuli (Fig. 2). TNF-α production by M1 or M2 was not influenced by the presence of either brand of MTA. M1 macrophages produced TNF-α in response to all stimuli. However, because of variability among experiments and the consequent high standard deviations, statistical differences were found only among the control cultures and the cultures stimulated with *P. anaerobius* and IFN-γ. M2 macrophages stimulated with IFN-γ and either *F. nucleatum* or *P. anaerobius* produced higher levels of TNF-α than nonstimulated cultures.

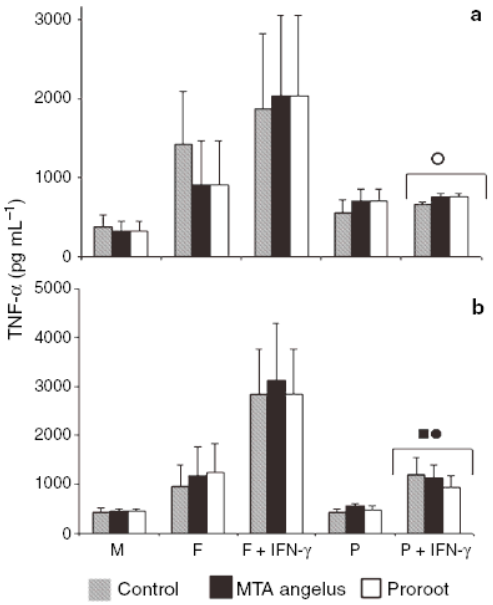


Figure 2 Production of tumour necrosis factor-α by M1 (a) and M2 (b) macrophage cultures 24 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Cells were cultured in the medium alone (M) and *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon (IFN)-γ (I) was added where indicated at 10 units mL^{-1} (1 μg = 8430 units). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the means. ○ indicates $P < 0.05$ by Kruskal–Wallis test when compared with macrophages in medium alone. ● and ■ indicates $P < 0.05$ when compared with bacterial preparations without IFN-γ.

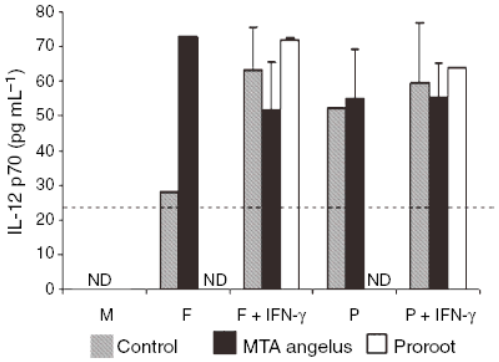


Figure 3 Production of interleukin (IL)-12p70 by M1 macrophage cultures 24 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Cells were cultured in the medium alone (M) and *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon-γ (I) was added where indicated at 10 units mL^{-1} (1 μg = 8430 units). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the mean values; - - - indicates sensitivity (23.4 pg mL^{-1}) of the assay; ND, not detected.

IL-12p70

Production of IL-12p70 was undetected in several conditions, and, when detected, was just above the detection limit of the assay. Neither brand of MTA influenced the production of this cytokine by M1 macrophages. Consistent IL-12 production was found when cells were stimulated with both bacterial preparations and IFN-γ (Fig. 3).

IL-10

Once again, neither brand of MTA influenced the production of cytokine by M1 or M2 macrophages (Fig. 4). Neither type of macrophage produced IL-10 in the absence of bacterial stimuli. M1 macrophages produced IL-10 in response to all stimuli, and addition of IFN-γ did not influence the levels of IL-10 produced. *Fusobacterium nucleatum* induced significantly higher levels of IL-10 production by M2 macrophages than *P. anaerobius*. IFN-γ inhibited the production of IL-10 stimulated by *F. nucleatum*. Moreover, *F. nucleatum*-stimulated M2 macrophages produced higher levels of IL-10 than *F. nucleatum*-stimulated M1 macrophages.

Discussion

Since its introduction as an endodontic cement (Lee *et al.* 1993), MTA has been studied both *in vivo* (Holland *et al.* 2002, Saidon *et al.* 2003) and *in vitro* (Koh *et al.* 1998, Osorio *et al.* 1998, Mitchell *et al.* 1999, Zhu *et al.* 2000, Abdullah *et al.* 2002, Haglund *et al.* 2003). These studies have demonstrated that MTA has excellent biocompatibility, causing no significant tissue inflammation at the site of its application. It allows repair, inducing the deposition of dentinal (Tziafas *et al.* 2002), cemental (Torabinejad *et al.* 1995b, Holland *et al.* 2001) and bony tissues (Torabinejad *et al.* 1995b, 1998).

It is known that during the pulp inflammatory process, an array of immunocompetent cells is attracted to the site in an attempt to eliminate the aggressor

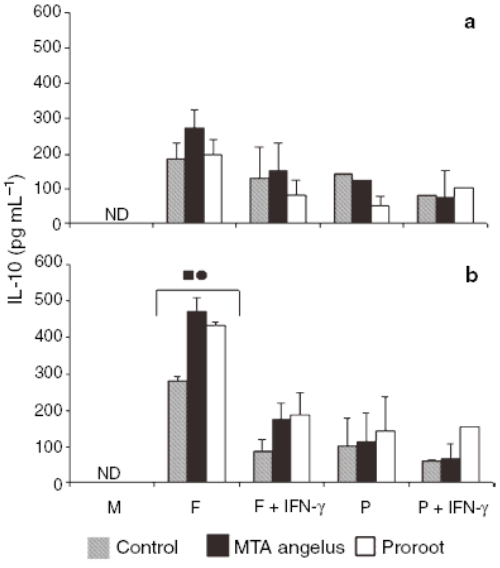


Figure 4 Production of interleukin-10 by M1 (a) and M2 (b) macrophage cultures 72 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Cells were cultured in the medium alone (M) and *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon-γ (I) was added where indicated at 10 units mL^{-1} (1 μg = 8430 units). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the mean value. ■ indicates $P < 0.05$ for M2 macrophages stimulated with *F. nucleatum* compared with all other conditions by ANOVA followed by Tukey's multiple comparisons. ● indicates $P < 0.05$ when M1 and M2 macrophages stimulated with *F. nucleatum* were compared by ANOVA test followed by Tukey's multiple comparisons.

stimulus. Macrophages are among the first cells to come in contact with foreign bodies and play the main role in the pathogenesis of the inflammatory process (Van Furth *et al.* 1972, Unanue 1978). Some authors (Yu & Stashenko 1987) believe that lymphocytes are the most prevalent cells in the periapical inflammatory infiltrate whereas others believe that macrophages are most prevalent (Stern *et al.* 1981, Kawashima *et al.* 1996). Macrophages produce several cytokines that promote the initiation, perpetuation, directing and the inhibition of the immunological response process (Stashenko *et al.* 1998, Metzger 2000).

In this study, macrophages were exposed to manipulated MTA *in vitro*, using a previously described system (Oliveira Mendes *et al.* 2003) that allows a controlled exposure of cell cultures to the cement. In these conditions, viability was >97% at all periods examined, and was similar in the presence of the two commercial brands; however, it was lower for the M1 macrophages in contact with MTA-Ângelus, for 24 h. This difference was not observed in the subsequent periods. The high cellular viability in the presence of MTA coincide with that for fibroblasts (Mitchell *et al.* 1999, Keiser *et al.* 2000, Saidon *et al.* 2003) and osteoblasts (Koh *et al.* 1997, Mitchell *et al.* 1999). Only one study was found in the literature on the viability of macrophages exposed to MTA (Haglund *et al.* 2003). In that paper, the authors observed a normal morphology of macrophages in contact with dry MTA. MTA also allowed increased viability when compared with the other materials evaluated. However, lower cell numbers were found in cultures exposed to MTA when compared with nonexposed controls. A very large area of each material was exposed to cells in culture, and, perhaps, this was the reason for the higher mortality in MTA-exposed cultures when compared with controls, in that study.

The decrease in viability of the M2 macrophages in culture is intriguing. No data are available in the literature as to differences in viability of M1 and M2 macrophages in culture. It is interesting that a cell type that is supposedly not producing IFN-γ (Mosser 2003) and lower levels of NO (Mills *et al.* 2000, Bastos *et al.* 2002) than M1 dies more easily. However, the decrease in viability was small. In the present study, the 72-h cultures were used to detect IL-10. Thus, it is possible that slightly higher values of this cytokine could be detected in M2 cultures, if the cells were completely viable.

Periapical inflammation is not only the result of the direct action of microorganisms, but also of pro-inflammatory mediators, like the cytokines (Stashenko *et al.* 1998). These mediators act on the innate immunity, antigen presentation, differentiation within the bone marrow, cell recruiting and activation, and adhesion molecule expression (Borish & Steinke 2003). The present study has investigated the production of TNF-α, IL-12 and IL-10 by macrophages exposed *in vitro* to MTA, as these cytokines are involved in the onset of the inflammatory process (TNF-α), the interconnection of the innate and adaptive immune responses (IL-12), and the regulation or the inflammatory process (IL-10). In addition, TNF-α is an important mediator in the activation of macrophages is osteoclastic activation factor. IL-12 and IL-10 are macrophage polarization markers. Addition of bacterial

antigens and IFN-γ to the cell cultures was an attempt to reproduce the clinical conditions, where the presence of anaerobe bacteria (Sundqvist 1992), as well as the presence of other cytokines (Stashenko *et al.* 1998) is a frequent finding. IFN-γ acts by stimulating antigen presentation and cytokine production by macrophages and dendritic cells, in addition to activating the effector functions of the macrophages. These functions include adherence, phagocytosis, secretion, respiratory explosion and the production of nitric oxide (Borish & Steinke 2003).

Tumour necrosis factor-α is mainly produced by mononuclear phagocytes with the purpose of stimulating neutrophil and monocyte recruitment to the infection site, and activating these cells to eradicate microorganisms. In apical lesions, TNF-α, IL-1-α, IL-1-β, TNF-β, IL-6 and IL-11 act on bone resorption, and they collectively constitute the osteoclastic activation factor (Stashenko 1990). In the present study, large amounts of TNF-α were observed following incubation for 24 h with bacterial preparations and with these preparations and IFN-γ. The production of TNF-α by macrophages was also observed when in contact with the capillaries with and without the MTA, which is not surprising given that macrophages were harvested from the peritoneal cavity of mice after an inflammatory stimulus. Hence, MTA did not affect the capacity of macrophages to produce TNF-α on response to triggering stimuli.

Interleukin-12 induces the production of IFN-γ by natural killer cells and T cells, a fact that adds to the activation of phagocytic cells, in addition to favouring the differentiation of T-helper cells into the Th1 subset. In addition to these actions, it works as a link between the innate and the adaptive antigen-specific responses (Trinchieri & Scott 1995). We observed that the production of IL-12 increased both in presence of gram-positive and gram-negative bacteria, and that the addition of IFN-γ seemed to lead to an even greater IL-12 production (however, no statistical significance was found when cultures with and without addition of IFN-γ were compared). These data are in accordance with those reported in the literature (Ma *et al.* 1996, Hayes *et al.* 1998). Again, MTA did not affect the capacity of macrophages to produce IL-12.

Interleukin-10 induces tissue homeostasis, leading to the inhibition of pro-inflammatory cytokine production by activated T cells and macrophages (Kawashima *et al.* 1996, Stashenko *et al.* 1998, Gerber & Mosser 2001, Borish & Steinke 2003). Both *F. nucleatum* and *P. anaerobius* triggered IL-10 production. *Fusobacterium nucleatum* stimulated M2 macrophages to produce higher levels of IL-10 than M1 macrophages. This is in accordance with the literature, which shows that M2 macrophages are high producers of IL-10 (Mosser 2003). However, *P. anaerobius* did not stimulate M1 and M2 differently. For some time it had been believed that gram negative bacteria would be better inducers of IL-10 than gram positive bacteria (Hessle *et al.* 2000, Cross *et al.* 2004). Here, this observation was confirmed: the gram negative bacterium (*F. nucleatum*) stimulated more IL-10 than the gram positive (*P. anaerobius*). None of the studies in which the gram positive/gram negative, IL-12/IL-10 dichotomies were addressed used mouse-derived M1 or M2 macrophages. In one study human monocytes were used (Hessle *et al.* 2000), in the other, a macrophage cell line (Cross *et al.* 2004). Hence, our results show that for M2 macrophages a gram negative bacterium induces more IL-10 than a gram positive bacterium. The same was not found for the low IL-10 producing M1 macrophages. Also of note is the fact that IFN-γ down-modulated production of IL-10 only by *F. nucleatum*-stimulated M2 macrophages. In short, it was found that the two different types of macrophages have only differed as to the production of IL-10 (besides the obvious lack of IL-12 production by the population of M2 used here), which is in agreement with previous reports (Mosser 2003).

It has been shown that MTA did not affect the production of the cytokines, either alone, or in conjunction with gram positive or gram negative bacteria and IFN-γ. This appears to be the first report in which the effect of MTA on the production of polarizing cytokines by M1 and M2 macrophages has been determined. One other report on the effect of MTA on cytokines that mediate bone resorption has shown that this cement does not, by itself, induce the production of these cytokines (Haglund *et al.* 2003). However, it should be borne in mind that the compatibility of MTA demonstrated here and elsewhere (Koh *et al.* 1997, Haglund *et al.* 2003, Saidon *et al.* 2003) has all the limitations of *in vitro* work. Additional work should be carried out to assess the effects of MTA *in vivo*, as cytokine synthesis *in vivo* is a very complex event, and its expression and effects are ruled by many factors, including other cells and mediators (Mitchell *et al.* 1999).

Conclusion

Mineral trioxide aggregate had excellent results concerning macrophage viability, and noninterference

with the production and secretion of the cytokines TNF, IL-12 and IL-10, by M1 and M2 macrophages.

Acknowledgements

We are indebted to Prof. Jacques Robert Nicoli for the advice and use of the laboratory, and to Antonio Mesquita Vaz for excellent animal care. This study has received financial support from CAPES, CNPq and Fapemig CBS 6509. Tulsa-Dental (Ballaigues, Switzerland) and Odonto-lógika (Londrina, PR, Brazil) kindly provided ProRoot® and MTA-Ângelus®.

References

Abdullah D, Pitt Ford TR, Papaioannou S, Nicholson J, McDonald F (2002) An evaluation of accelerated Portland cement as a restorative material. *Biomaterials* **23**, 4001–10.

Bastos KR, Alvarez JM, Marinho CR, Rizzo LV, Lima MR (2002) Macrophages from IL-12p40-deficient mice have a bias toward the M2 activation profile. *Journal of Leukocyte Biology* **71**, 271–8.

Borish LC, Steinke JW (2003) 2. Cytokines and chemokines. *Journal of Allergy and Clinical Immunology* **111**, S460–75.

Cross ML, Ganner A, Teilab D, Fray LM (2004) Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. *FEMS Immunology and Medical Microbiology* **42**, 173–80.

Gerber JS, Mosser DM (2001) Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *The Journal of Immunology* **166**, 6861–8.

Haglund R, He J, Jarvis J, Safavi KE, Spangberg LS, Zhu Q (2003) Effects of root-end filling materials on fibroblasts and macrophages in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **95**, 739–45.

Hayes MP, Murphy FJ, Burd PR (1998) Interferon-gamma-dependent inducible expression of the human interleukin-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in Epstein-Barr virus-transformed lymphoblastoid cells. *Blood* **91**, 4645–51.

Hessle C, Andersson B, Wold AE (2000) Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. *Infection and Immunity* **68**, 3581–6.

Holland R, de Souza V, Nery MJ et al. (2001) Reaction of rat connective tissue to implanted dentin tube filled with mineral trioxide aggregate, Portland cement or calcium hydroxide. *Brazilian Dental Journal* **12**, 3–8.

Holland R, Souza V, Nery MJ et al. (2002) Reaction of rat connective tissue to implanted dentin tubes filled with a white mineral trioxide aggregate. *Brazilian Dental Journal* **13**, 23–6.

Kawashima N, Okiji T, Kosaka T, Suda H (1996) Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: a quantitative immunohistochemical study. *Journal of Endodontics* **22**, 311–6.

Keiser K, Johnson CC, Tipton DA (2000) Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. *Journal of Endodontics* **26**, 288–91.

Koh ET, Torabinejad M, Pitt Ford TR, Brady K, McDonald F (1997) Mineral trioxide aggregate stimulates a biological response in human osteoblasts. *Journal of Biomedical Materials Research* **37**, 432–9.

Koh ET, McDonald F, Pitt Ford TR, Torabinejad M (1998) Cellular response to Mineral Trioxide Aggregate. *Journal of Endodontics* **24**, 543–7.

Lee SJ, Monsef M, Torabinejad M (1993) Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. *Journal of Endodontics* **19**, 541–4.

Ma X, Chow JM, Gri G et al. (1996) The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *The Journal of Experimental Medicine* **183**, 147–57.

Magram J, Connaughton SE, Warrier RR et al. (1996) IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* **4**, 471–81.

Metzger Z (2000) Macrophages in periapical lesions. *Endodontics and Dental Traumatology* **16**, 1–8.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. *The Journal of Immunology* **164**, 6166–73.

Mitchell PJ, Pitt Ford TR, Torabinejad M, McDonald F (1999) Osteoblast biocompatibility of mineral trioxide aggregate. *Biomaterials* **20**, 167–73.

Mosser DM (2003) The many faces of macrophage activation. *Journal of Leukocyte Biology* **73**, 209–12.

Oliveira Mendes ST, Ribeiro Sobrinho AP, de Carvalho AT, Souza Cortes MI, Vieira LQ (2003) In vitro evaluation of the cytotoxicity of two root canal sealers on macrophage activity. *Journal of Endodontics* **29**, 95–9.

Osorio RM, Hefti A, Vertucci FJ, Shawley AL (1998) Cytotoxicity of endodontic materials. *Journal of Endodontics* **24**, 91–6.

Ribeiro Sobrinho AP, Melo Maltos SM, Farias LM et al. (2002) Cytokine production in response to endodontic infection in germ-free mice. *Oral Microbiology and Immunology* **17**, 344–53.

Saidon J, He J, Zhu Q, Safavi K, Spangberg LS (2003) Cell and tissue reactions to mineral trioxide aggregate and Portland cement. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **95**, 483–89.

Stashenko P (1990) Role of immune cytokines in the pathogenesis of periapical lesions. *Endodontics and Dental Traumatology* **6**, 89–96.

Stashenko P, Teles R, D'Souza R (1998) Periapical inflammatory responses and their modulation. *Critical Reviews in Oral Biology and Medicine* **9**, 498–521.

Stern MH, Dreizen S, Mackler BF, Selbst AG, Levy BM (1981) Quantitative analysis of cellular composition of human periapical granuloma. *Journal of Endodontics* **7**, 117–22.

Sundqvist G (1992) Ecology of the root canal flora. *Journal of Endodontics* **18**, 427–30.

Torabinejad M, Hong CU, Lee SJ, Monsef M, Pitt Ford TR (1995a) Investigation of mineral trioxide aggregate for root-end filling in dogs. *Journal of Endodontics* **21**, 603–8.

Torabinejad M, Rastegar AF, Kettering JD, Pitt Ford TR (1995b) Bacterial leakage of mineral trioxide aggregate as a root-end filling material. *Journal of Endodontics* **21**, 109–12.

Torabinejad M, Pitt Ford TR, Abedi HR, Kariyawasam SP, Tang HM (1998) Tissue reaction to implanted root-end filling materials in the tibia and mandible of guinea pigs. *Journal of Endodontics* **24**, 468–71.

Trinchieri G, Scott P (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Research in Immunology* **146**, 423–31.

Tziafas D, Pantelidou O, Alvanou A, Belibasakis G, Papadimitriou S (2002) The dentinogenic effect of mineral trioxide aggregate (MTA) in short-term capping experiments. *International Endodontic Journal* **35**, 245–54.

Unanue ER (1978) The regulation of lymphocyte functions by the macrophage. *Immunology Reviews* **40**, 227–55.

Van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bulletin of the World Health Organization* **46**, 845–52.

Yu SM, Stashenko P (1987) Identification of inflammatory cells in developing rat periapical lesions. *Journal of Endodontics* **13**, 535–40.

Zhu Q, Haglund R, Safavi KE, Spangberg LS (2000) Adhesion of human osteoblasts on root-end filling materials. *Journal of Endodontics* **26**, 404–6.

ANEXO 2: Aprovações do Comitê de Ética em Pesquisa Animal (CETEA-UFMG).

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL (CETEA)

CERTIFICADO

Certificamos que o protocolo nº 35/2002 relativo ao projeto intitulado "Agregado mineral trióxido (MTA): efeitos sobre a atividade de macrófagos peritoneais" que tem como responsáveis Antônio Paulino Sobrinho, Leda Quércia Vieira e Maria Ilma de Souza Cortês, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** (CETEA/UFMG), tendo sido aprovado em reunião de **06.11.2002.**

Este certificado expira-se em 06.11.2007

CERTIFICATE

We certify that the protocol nº 35/2002 related to the project entitled "Trioxide mineral aggregate (MTA): effects on the activity of peritoneal macrophages", under the supervision of Antônio Paulino Sobrinho, Leda Quércia Vieira and Maria Ilma de Souza Cortês, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the **Ethics Committee in Animal Experimentation** (CETEA/UFMG) and was approved in **06.11.2002.**

This certificate expires in 06.11.2007

Belo Horizonte, 06/11/2002

Dra. CLEUZA MARIA F. REZENDE
Presidente do CETEA/UFMG
Universidade Federal de Minas Gerais
Reitoria – Campus Pampulha
Avenida Antônio Carlos 6627
CEP: 31270-901 - Belo Horizonte MG
Brasil

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL (CETEA)

CERTIFICADO

Certificamos que o protocolo nº **126/04** relativo ao projeto intitulado "***Efeito do MTA (Agregado de Trióxido Mineral) sobre a produção de citocinas durante a apresentação de antígenos e após a diferenciação dos linfócitos T***", que tem como responsáveis Leda Quercia Vieira e Antônio Paulino Ribeiro Sobrinho, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** (CETEA/UFMG), tendo sido aprovado em reunião de **15.12.2004.**

Este certificado expira-se em **15.12.2009**

CERTIFICATE

We certify that the protocol nº **126/04** related to the project entitled "***Effect of MTA on the production of cytokines during the antigen presentation and after differentiation of T lymphocytes***", under the supervision Leda Quercia Vieira and Antônio Paulino Ribeiro Sobrinho, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** (CETEA/UFMG) and was approved in **15.12.2004.**

This certificate expires in **15.12.2009.**

Belo Horizonte, 15 de dezembro de 2004

Dra. CLEUZA MARIA F. REZENDE
PRESIDENTE DO CETEA/UFMG

Universidade Federal de Minas Gerais -Reitoria – Campus Pampulha
Avenida Antônio Carlos 6627-7 Andar CEP: 31270-901 - Belo Horizonte MG-Brasil
Telefone: (31) 3499-4592 – Fax: (31) 3499-4027 – Home page: www. ufmg.br/prpq/cetea

ANEXO 3: Trabalho adicional publicado durante o período de doutoramento.

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03318.x

Diminished forkhead box P3/CD25 double-positive T regulatory cells are associated with the increased nuclear factor-kB ligand (RANKL+) T cells in bone resorption lesion of periodontal disease

C. W. O. Ernst,* J. E. Lee,*‡ T. Nakanishi,* N. Y. Karimbux,‡ T. M. B. Rezende,* P. Stashenko,† M. Seki,*§ M. A. Taubman* and T. Kawai*

*Department of Immunology and †Department of Cytokine Biology, The Forsyth Institute, Boston, MA, USA, ‡Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, MA, USA, and §Research and Development Division, Mitsubishi Pharma Corporation, Tokyo, Japan

Accepted for publication 14 December 2006
Correspondence: Toshihisa Kawai DDS, PhD, Department of Immunology, The Forsyth Institute, 140 The Fenway, Boston, MA, 02115 USA. E-mail: tkawai@forsyth.org

Summary

Periodontal disease involves multi-bacterial infections accompanied by inflammatory bone resorption lesions. The abundant T and B lymphocyte infiltrates are the major sources of the osteoclast differentiation factor, receptor activator for nuclear factor-kB ligand (RANKL) which, in turn, contributes to the development of bone resorption in periodontal disease. In the present study, we found that the concentrations of RANKL and regulatory T cell (T_{reg})-associated cytokine, interleukin (IL)-10, in the periodontal tissue homogenates were correlated negatively, whereas RANKL and proinflammatory cytokine, IL-1β, showed positive correlation. Also, according to the fluorescent-immunohistochemistry, the frequency of forkhead box P3 (FoxP3)/CD25 double-positive cells was diminished strikingly in the bone resorption lesion of periodontal disease compared to healthy gingival tissue, while CD25 or FoxP3 single positive cells were still observed in lesions where abundant RANKL+ lymphocytes were present. Very importantly, few or no expressions of FoxP3 by the RANKL+ lymphocytes were observed in the diseased periodontal tissues. Finally, IL-10 suppressed both soluble RANKL (sRANKL) and membrane RANKL (mRANKL) expression by peripheral blood mononuclear cells (PBMC) activated *in vitro* in a bacterial antigen-specific manner. Taken together, these results suggested that FoxP3/CD25 double-positive T_{reg} cells may play a role in the down-regulation of RANKL expression by activated lymphocytes in periodontal diseased tissues. This leads to the conclusion that the phenomenon of diminished CD25+FoxP3+ T_{reg} cells appears to be associated with the increased RANKL+ T cells in the bone resorption lesion of periodontal disease.

Keywords: FoxP3, periodontal/oral immunology, RANKL, T cells, T_{reg} cells

Introduction

Many research reports have demonstrated conclusively that regulatory T cells (T_{reg} cells) suppress pathogenic adaptive immune responses, including those involved in autoimmune diseases, infectious diseases and organ transplantation [1]. Although the presence of T_{reg} cells in periodontal diseased tissues has been demonstrated by the reverse transcription–polymerase chain reaction (RT–PCR) for forkhead box P3 (FoxP3), interleukin (IL-10) and transforming growth factor (TGF)-β [2], the role of T_{reg} cells in the context of bone resorption lesion of periodontal disease remains unclear. For example, involvement of lymphocytes in bone loss processes was demonstrated only recently in both *in vitro* [3] and *in vivo* models for rheumatoid arthritis [4] and periodontal disease [5,6]. This finding resulted from the discovery of osteoclast differentiation factor, receptor activator for nuclear factor-kB ligand (RANKL) [7], which is expressed not only in bone marrow stromal cells and osteoblasts but also in activated lymphocytes [8]. However, the linkage between T_{reg} cells and RANKL production by lymphocytes in the context of bone resorption lesion of periodontal disease remains unknown.

An active periodontal lesion is characterized by the prominent infiltration of B cells [9,10] and T cells [11,12]. Specifically, the occupancy of 50–60% of such cellular infiltrates by plasma cells makes periodontal disease very distinct from other chronic infectious diseases [13,14]. This is demonstrated by our recent discovery that the osteoclast differentiation factor, RANKL, is expressed distinctively by activated

T cells and B cells in gingival tissues with periodontal disease, but not by these lymphocytes in healthy gingival tissues [15]. These RANKL$^+$ lymphocytes isolated from patients' gingival tissues were functionally sufficiently potent to induce *in vitro* osteoclastogenesis in an RANKL-dependent manner [15]. Supporting this finding, we also demonstrated that *Actinobacillus actinomycetemcomitans* (*Aa*) Omp29-specific T helper 1 (Th1)-type T cells or *Aa*-reactive B cells can trigger periodontal bone resorption in rat models [5,6,16]. Because it has never been reported that any bacteria *per se* invade and resorb bone independently of osteoclast activation, RANKL expression by T cells and B cells is considered to be a major stimulus of osteoclast precursor cells.

To gain an insight into inflammatory bone resorption mechanisms involving RANKL-expressing activated lymphocytes, a question is raised as to whether FoxP3$^+$ T_{reg} cells are associated with suppression of such lymphocyte expression of RANKL in the context of periodontal bone resorption lesions. It is reported that CD4$^+$ T cells with regulatory phenotypes, CD25 or CTLA4, along with the FoxP3, TGF-β and IL-10 mRNA, are present in diseased gingival tissues [2]. Another study has also demonstrated that a majority of CD4$^+$ T clone cells (almost 100%), which are those established from T cells infiltrating periodontal diseased tissues, are positive for FoxP3 and IL-10 mRNA [17]. The latter results support the presence of FoxP3$^+$CD25$^+$ T cells in gingival tissue. It is conceivable that the protein expression level of FoxP3 in the T cells infiltrating in periodontal tissues may not be proportional to the FoxP3 mRNA. This result, however, contradicts studies of rheumatoid arthritis in which the prevalence of CD25$^+$CD4$^+$ T cells in the total CD4$^+$ T cells of patients' peripheral blood and sites of inflammation was shown to be less than 10%, respectively [18]. Therefore, in the present study, we focus on investigating the expression pattern of FoxP3 protein by the T cells infiltrating in periodontal diseased tissue. Our step-by-step experimental methodology is described below.

Materials and methods

Human gingival tissue samples

For the *in situ* analysis of FoxP3 expression, healthy and diseased gingival tissue samples were collected in laboratories of the Department of Periodontology at the Harvard School of Dental Medicine (HSDM, Boston, MA). Healthy gingival tissue was characterized by the lack of bleeding on probing (gingival pocket depth ≤ 3 mm; n = 4, 1 male and 3 females, ages 25–51 y). Inflamed gingival tissues were collected from patients with periodontal disease at surgery for non-responsive sites after basic periodontal therapy. These samples were characterized by the presence of radiological bone resorption features and bleeding on probing (gingival pocket depth > 5 mm; n = 4, 2 males and 2 females, ages 36–55 y).

Tissue samples were also collected in the Department of Periodontology at HSDM in order to examine the cytokine expression in the gingival tissue homogenates. In this case, healthy gingival tissues were sampled, after cosmetic or preventive surgery, for non-responsive sites (gingival pocket depth ≤ 3 mm, age 27–41 y old, n = 3, one female and 2 males). Inflamed gingival tissues from patients with periodontal disease were sampled at surgery for periodontal therapy as described above (gingival pocket depth > 3 mm, average 5.7 ± 1.3 mm SD, age 32–58 y old, n = 11, 8 females and 3 males). The Institutional Review Board of HSDM approved the collection of gingival tissues after periodontal surgery, and informed consent was obtained from each subject prior to inclusion in this study.

Double-color confocal microscopy

The presence of FoxP3 expression in the human gingival tissues was determined using double-color confocal microscopy (DMRXE/TCS/SP-2 laser scan confocal microscope, Leica, Wetzler, Germany), following the previously published protocol [15]. FITC-conjugated anti-human FoxP3 antibody (clone 259D, BioLegend, San Diego, CA) and Alexa Fluor 647-conjugated anti-human CD25 antibody (clone BC96, BioLegend), with appropriate control antibodies, were reacted with frozen sections of healthy (n = 4) and diseased (n = 4) human gingival tissues. Staining pattern of FoxP3 in the healthy gingival tissues, as well as periodontal diseased lesions with RANKL$^+$ lymphocytes, was evaluated by staining with FITC-conjugated anti-human FoxP3 antibody and with osteoprotegerin-Fc-biotin (OPG-Fc-biotin), followed by TexasRed-avidin [15]. OPG-Fc-biotin was prepared by reacting sulfo-NHS-biotin (Pierce, Rockford, IL) to OPG-Fc (gift from Dr. Dunston, Amgen), following the previously published procedure [15]. The number of FoxP3, CD25 and RANKL positive cells in the microscopic field (×1000) were counted, following the method published previously [15].

Measurement of sRANKL, IL-10 and IL-1β in gingival tissue homogenates using ELISA

Gingival tissue homogenates were prepared following the previously published method [15]. The concentrations of IL-1β and IL-10 in the homogenates were measured using DuoSet ELISA kit (R&D Systems, Minneapolis, MN). Detection of soluble RANKL (sRANKL) was carried out using ELISA kit (PeproTech, Rocky Hill, NJ).

In vitro stimulation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from healthy subjects (n = 2), and one patient with periodontal disease, by a gradient centrifugation using Histopaque™ (Sigma, St. Louis, MO) [15]. The resulting

single-cell suspension of PBMC was stimulated in the RPMI 1640 medium supplemented with 10% FBS and antibiotics in a 96-well culture plate (10^5/well) for 4 days. The PBMC were stimulated with *Aa* Y4 (10^7/ml) in the presence or absence of anti-HLA-DR MAb (clone G46–6, BD PharMingen, San Diego, CA), control isotype matched MAb (PF18 [19]), or recombinant IL-10 (50 ng/ml). On Day 3, culture supernatant was harvested and subjected to human sRANKL ELISA (PeproTech). ^{3}H thymidine (0.5 uCi) was added to the culture during the last 16 hours of a total incubation of 4 days.

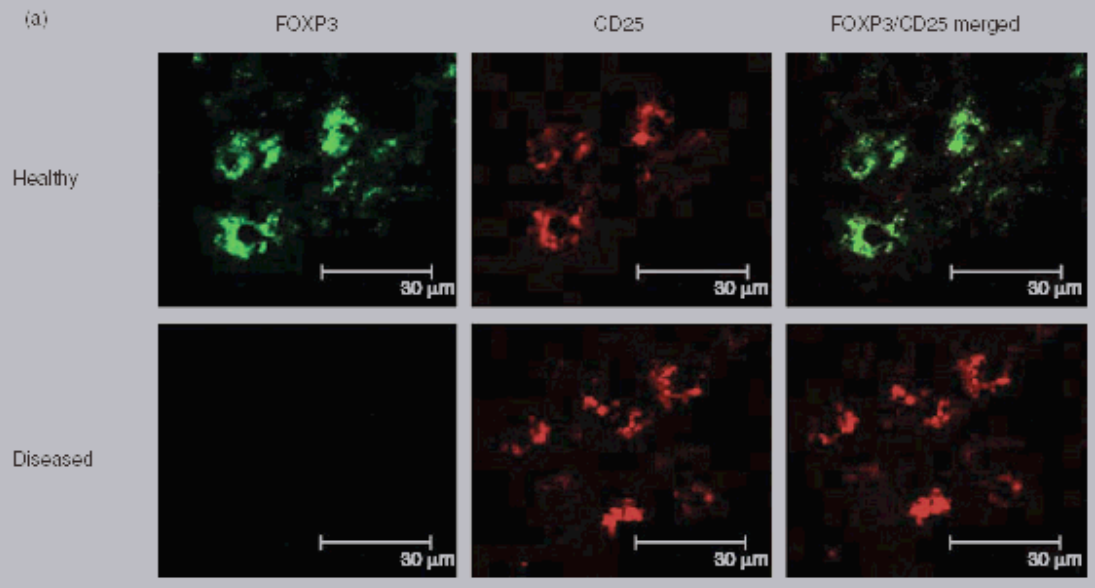
For the confocal microscopy analysis, PBMC harvested on Day 3 were fixed on a glass slide using Cytospin (Shandon Elliott, Surrey, England) and stained with FITC-conjugated-anti-human FoxP3 antibody, Alexa Fluor 647-conjugated-anti-human CD25 antibody, Alexa Fluor 647-conjugated-anti-human CD4 and OPG-Fc-biotin/TexasRed-conjugated-avidin.

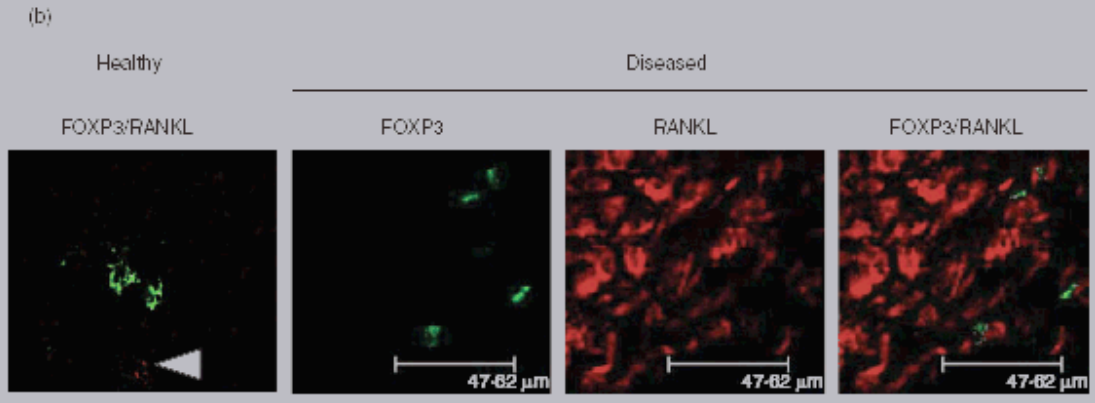
Results

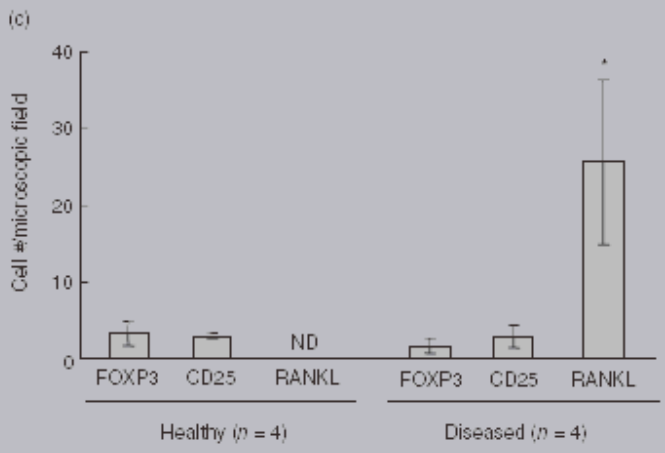
First, the expression pattern of FoxP3 protein in the gingival tissues with periodontal disease, as well as healthy gingival tissues, was determined by fluorescein isothiocyanate (FITC)-conjugated anti-FoxP3 antibody using double-colour confocal microscopy (Fig. 1a). In the healthy gingival tissues, FoxP3/CD25 double-positive lymphocytes were found in the connective tissues beneath the sulcular epithelium (Fig. 1a, healthy). Very strikingly, however, while the diseased tissue showed the presence of either CD25 or FoxP3 single-positive cells (Fig. 1a,b), the CD25$^+$ lymphocytes did not show a positive staining pattern for FoxP3. In the gingival tissues with periodontal disease, a few FoxP3$^+$ cells were detected within the dense infiltration of RANKL$^+$ lymphocytes (Fig. 1b). Nevertheless, the FoxP3$^+$ cells in the diseased tissues displayed very little or no staining pattern for membrane RANKL (mRNAKL), considerably lessening the likelihood that FoxP3$^+$ cells contribute to local bone resorption. The relative number of cells counted for FoxP3, CD25 and mRANKL in a microscopic field is shown in Fig. 1c (× 1000). Although the number of mRANKL$^+$ lymphocytes was increased significantly in the diseased gingival tissues compared to healthy tissues, there was no significant difference in the number of CD25$^+$ cells or FoxP3$^+$ cells between diseased and healthy tissues. The percentage of FoxP3$^+$ cells, based on the number of mRANKL$^+$ lymphocytes in the diseased tissues, was 4·7 ± 5·6% (average ± s.d., $n = 4$), indicating that a very low rate of FoxP3$^+$ lymphocytes is present in the diseased tissues. Very importantly, the percentage of CD25/FoxP3 double-positive cells in the total CD25$^+$ cells was high (about 77%) in healthy gingival tissue, while no CD25/FoxP3 double-positive cells were detected in diseased gingival tissues (Fig. 1d).

Secondly, we employed enzyme-linked immunosorbent assay (ELISA) (Fig. 2) to examine cytokines present in the gingival homogenates, including sRANKL, IL-1β and IL-10, to determine whether the T_{reg}-associated cytokine, IL-10, is expressed in periodontal diseased tissue in relation to sRANKL. The concentrations of IL-10 and sRANKL in the gingival tissues showed negative correlation (Fig. 2b), whereas a positive correlation was detected between IL-1β and sRANKL (Fig. 2a). Interestingly, there were no significant differences in IL-10 concentrations between healthy and diseased tissues (healthy, 13·1 ± 3·6* pg/mg tissue; diseased, 11·2 ± 4·9* pg/ml tissue, *average ± s.d.). Therefore, based on reports that CD4$^+$ T cells with regulatory phenotype CD25 produce IL-10 mRNA in diseased gingival tissues [2], the FoxP3$^+$ cells found in healthy and diseased gingival tissues (Fig. 1) appeared to be at least one of the cellular sources of IL-10 in gingival tissues.

Next, in order to interpret the phenomenon of negative correlation found between IL-10 and sRANKL in the above-described human gingival tissue homogenates (Fig. 2), healthy human peripheral blood mononuclear cells (PBMC) were stimulated with the periodontal bacterium *Aa* in the presence or absence of IL-10. Our mouse and rat models of periodontal disease demonstrate the relevance of antigen-specific T cell activation in the context of RANKL-mediated periodontal bone loss. Therefore, an antigen-specific T cell activation assay was employed. Proliferative response of PBMC upon *Aa* stimulation was determined using a [^{3}H]-thymidine incorporation assay (Fig. 3a). *Aa*-mediated PBMC activation was inhibited by anti-HLA-DR antibody, indicating that *Aa*-mediated PBMC proliferation is derived from antigen-dependent T cell proliferation via *Aa*-antigen presentation from human leucocyte antigen D-related (HLA-DR$^+$) professional antigen-presenting cells (APC). The healthy subject also possessed positive serum IgG reaction to *Aa* as determined by ELISA (not shown), which provides evidence that this healthy subject possesses both T and B cell-mediated adaptive immune responses to *Aa* in an antigen-dependent manner. The stimulation of PBMC with *Aa* also up-regulated sRANKL expression by PBMC (Fig. 3b). Very importantly, the addition of IL-10 suppressed both proliferation and production of sRANKL by *Aa*-activated PBMC, indicating that a negative correlation between IL-10 and sRANKL in gingival tissue homogenates (Fig. 2a) may result from the suppression function of IL-10, as it acts to reduce sRANKL production by activated T cells. It is noteworthy that PBMC stimulation with immobilized anti-CD3 monoclonal antibody (MoAb) (UCTH-1) and anti-CD28 MoAb (clone CD28·2) also showed results similar to those of *Aa* stimulation (not shown) [15]. Furthermore, PBMC isolated from a patient with periodontal disease, who possessed elevated serum IgG response to *Porphyromonas intermedia* (*Pi*), also showed a soluble RANKL expression pattern similar to that of the healthy subject, as this patient's PBMC was stimulated with *Pi* in the presence or absence of IL-10 (not shown). To summarize, these results all demonstrated that IL-10 can suppress RANKL expression induced in T cells in an antigen-dependent manner, indicating that IL-10 present in the gingival tissues may be engaged in the


(a)
FOXP3
CD25
FOXP3/CD25 merged
Healthy
Diseased
30 μm


(b)
Healthy
Diseased
FOXP3/RANKL
FOXP3
RANKL
FOXP3/RANKL
47·62 μm


(c)
Cell #/microscopic field
40
30
20
10
0
ND
FOXP3
CD25
RANKL
FOXP3
CD25
RANKL
Healthy (n = 4)
Diseased (n = 4)

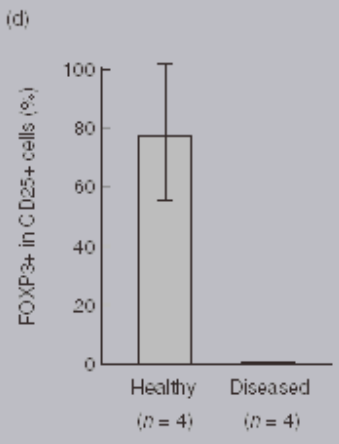

(d)
FOXP3+ in CD25+ cells (%)
100
80
60
40
20
0
Healthy
(n = 4)
Diseased
(n = 4)

Fig. 1. Forkhead box P3 (FoxP3) expression in CD25-positive cells diminished in periodontal diseased gingival tissue where abundant nuclear factor-kB ligand (RANKL+) lymphocytes infiltrate. Healthy gingival tissues (gingival pocket depth < 3 mm; $n = 4$, one male and three females, ages 25–51 years) and inflamed gingival tissues (gingival pocket depth > 5 mm; $n = 4$, two males and two females, ages 36–55 years) were analyzed for the expression of FoxP3, CD25 and RANKL on the infiltrating lymphocytes in the tissues. (a) The presence of FoxP3 expression in the human gingival tissues was determined using double-colour confocal microscopy. Fluorescein isothiocyanate (FITC)-conjugated anti-human FoxP3 antibody (green) and Alexa Fluor 647-conjugated anti-human CD25 antibody (red) were reacted with frozen sections of human gingival tissues of healthy ($n = 4$) and diseased ($n = 4$) gingival tissues. Representative staining patterns of healthy and diseased gingival tissues are shown. (b) Staining pattern of FoxP3 in the healthy gingival tissues and periodontal diseased lesions with RANKL+ lymphocytes was evaluated by staining with FITC-conjugated anti-human FoxP3 antibody (green) and with osteoprotegerin (OPG)-Fc-biotin, followed by TexasRed–avidin (red). The stained tissues were also analysed by the confocal microscope. In the healthy gingival tissue sample, an arrow indicates RANKL-positive cells. (c) The number of FoxP3, CD25 and RANKL-positive cells in the microscopic field (× 1000) were counted. Data from healthy and diseased gingival tissues ($n = 4$ and $n = 4$, respectively) are expressed as average cell number ± s.d. per microscopic field (n.d., not detectable). *Significantly higher than the number of FoxP3 or CD25-positive cells in either healthy or diseased tissues by Student's t-test ($P < 0{\cdot}05$). (d) The number of FoxP3/CD25 double-positive cells and total CD25 single-positive cells were counted in each microscopic field. The percentage of FoxP3-positive cells in CD25+ cells is calculated and shown as an average percentage ± s.d.

suppression of T cell activation and production of RANKL by the activated T cells.

Finally, the influence of IL-10 on RANKL expression by PBMC in relation to FoxP3 expression was examined using PBMC isolated from the same healthy subject shown in Fig. 3. After 3 days of *in vitro* stimulation with *Aa*, the PBMC were fixed onto a glass slide and expressions of CD25, FoxP3 and mRANKL were determined using double-colour confocal microscopy. The stimulation with *Aa*, irrespective of the addition of IL-10, increased the number of $CD25^+$ cells and $FoxP3^+$ cells, respectively, whereas the number of CD25/FoxP3 double-positive cells remained similar to control non-stimulated PBMC (Fig. 4a,d,g, indicated by arrows). It is noteworthy that two levels of FoxP3 expression, which we term 'bright' and 'dim', were observed and then differentiated as follows: the CD25/FoxP3 double-positive cells appeared to be bright in FoxP3 expression level ($FoxP3^{bright}$), while the $CD25^-/FoxP3^+$ cells appeared to be dim in FoxP3 expression level ($FoxP3^{dim}$).

Aa stimulation also up-regulated mRANKL expression by PBMC (Fig. 4e) compared to control (Fig. 4b). The data enumerated for CD25, FoxP3 and mRANKL expression by *in vitro*-stimulated PBMC are shown in Fig. 4l,m. In contrast to the stable number of $FoxP3^{bright}/CD25^+$ cells, regardless of stimulations, the increase in cell numbers for the other three types of cells, $FoxP3^{dim}/CD25^+$, $FoxP3^{dim}/CD25^-$ and $FoxP3^-/CD25^+$, was significant upon stimulation with *Aa* (Fig. 4m). Of particular importance, the increased number of $FoxP3^{dim}/CD25^+$ and $FoxP3^-/CD25^+$ cells seemed to be due to the transient inflammatory stress caused by mitogenic components present in *Aa*. This finding is confirmed by the observation that these same expression patterns were no longer found in the *Aa*-activated PBMC on day 7 (Fig. 4j,k). This phenomenon is supported by the study of Gorska *et al.*, who

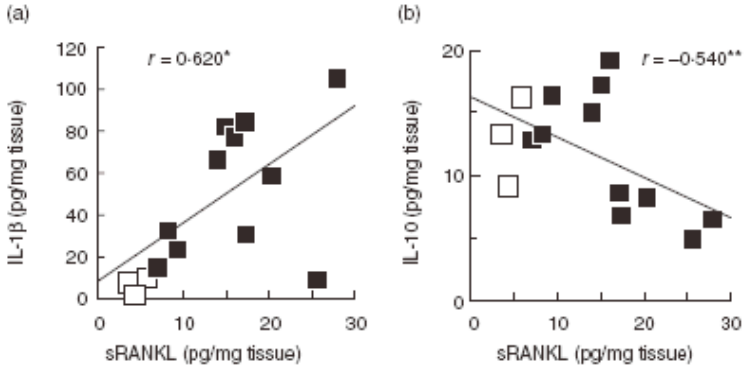


Fig. 2. Measurement of soluble nuclear factor-kB ligand (sRANKL), interleukin (IL-10) and IL-1β in gingival tissue homogenates. Gingival tissue homogenates were prepared from inflamed gingival tissues from patients with periodontal disease (gingival pocket depth > 3 mm, average 5·7 ± 1·3 s.d. mm, age 32–58 years, $n = 11$, eight females and three males) and healthy gingival tissues (gingival pocket depth ≤ 3 mm, age 27–41 years, $n = 3$, one female and 2 males), following the procedure described in the Materials and Methods. Concentrations of IL-1β, IL-10 and sRANKL in the gingival tissue homogenates were measured using the DuoSet enzyme-linked immunosorbent assay (ELISA) kit. Open or closed symbols indicate the data from healthy or diseased gingival tissue homogenates, respectively. (a) Concentrations of IL-1β and sRANKL and (b) concentrations of IL-10 and sRANKL are shown in scatter diagrams. *Positive correlation between two parameters ($n = 14$, $P < 0{\cdot}05$); **negative correlation between two parameters ($n = 14$, $P < 0{\cdot}05$).

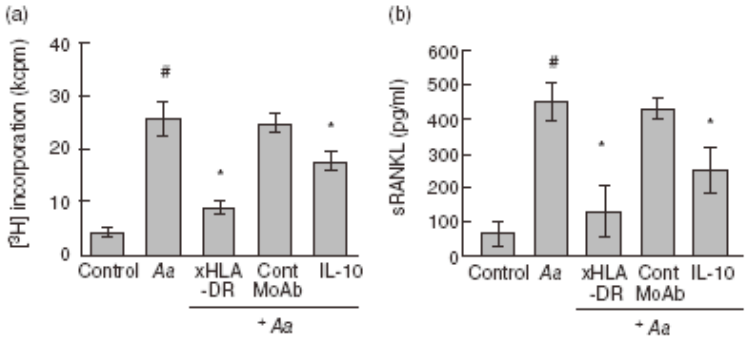


Fig. 3. IL-10 suppressed *in vitro* soluble nuclear factor – KB ligand (sRANKL) expression by activated peripheral blood mononuclear cells (PBMC). *In vitro* stimulation of peripheral blood mononuclear cells (PBMC) induced predominant nuclear factor-kB ligand (RANKL) expression in forkhead box P3 (FoxP3)-negative cells, but little or no expression in FoxP3-positive cells. PBMC isolated from a healthy subject were stimulated with *Aa* Y4 (10^7/ml) in the presence or absence of anti-human leucocyte antigen D-related (HLA-DR) monoclonal antibody (MoAb) (clone G46-6), control isotype-matched MoAb (PF18), or recombinant interleukin (IL)-10 (50 ng/ml). On day 3, culture supernatant was harvested and subjected to human sRANKL enzyme-linked immunosorbent assay (ELISA). [^{3}H]-thymidine (0·5 μCi) was added to the culture during the last 16 h of a total incubation of 4 days. The data from a healthy subject were expressed as average ± s.d. of (a) [^{3}H]-thymidine incorporation (kcpm = 1000 c.p.m) or (b) secreted sRANKL concentration (pg/ml), respectively. PBMC isolated from another healthy subject and a patient with periodontal disease showed similar expression patterns of sRANKL in response to bacterial stimulation in the presence of IL-10 (not shown). *Significantly lower than *Aa* stimulation alone (#) by Student's *t*-test ($P < 0·05$).

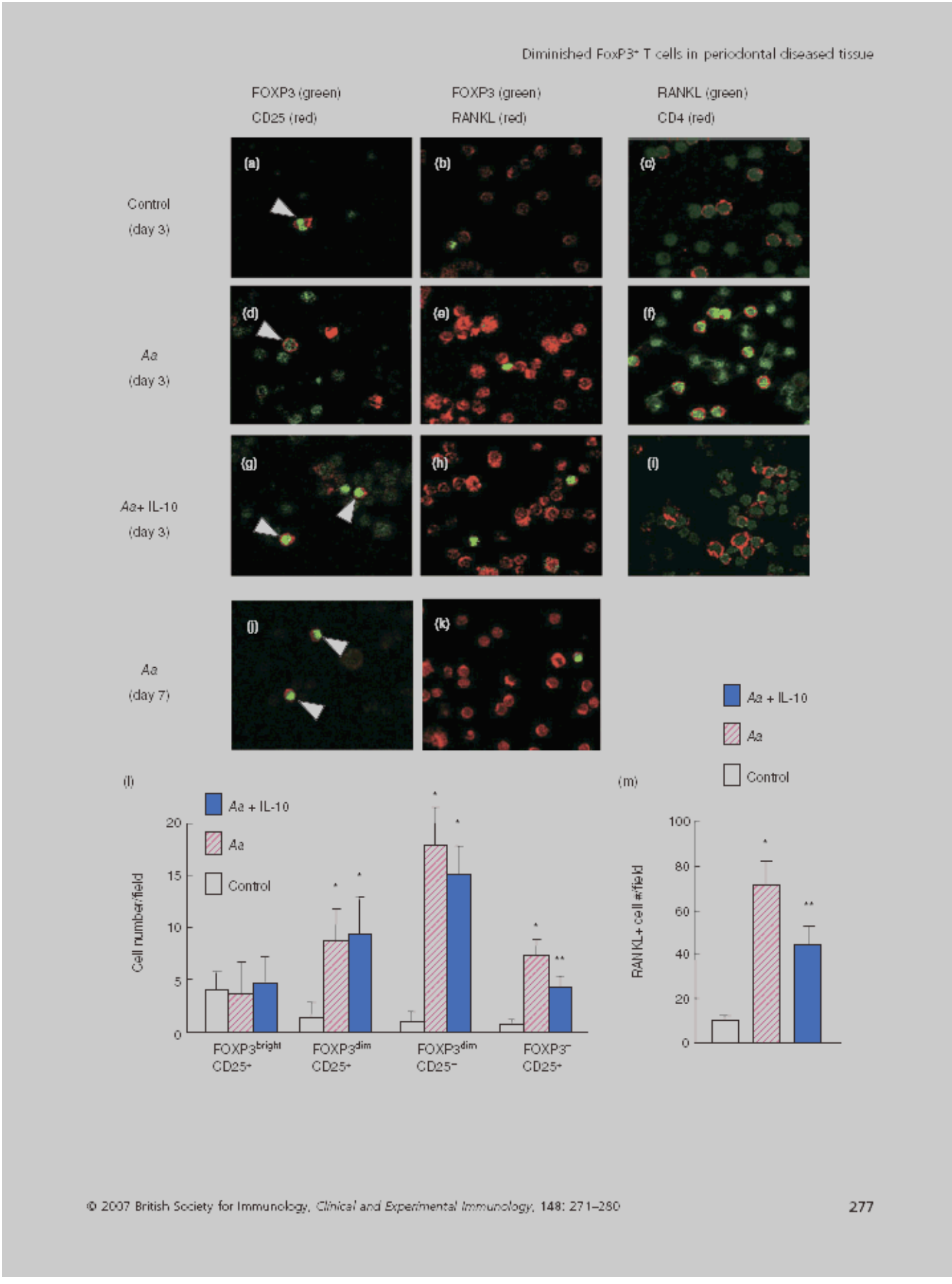
reported that the frequency of CD25$^+$ cells in the PBMC decreases significantly after proper periodontal surgery [20], which eliminates the generic inflammation in periodontal tissues.

The FoxP3^{bright} cells demonstrated little or no mRANKL expression, whereas FoxP3^{dim} cells expressed mRANKL to a relatively strong degree (Fig. 4b,e,h). Although FoxP3^{dim} cells disappeared on day 7 in *Aa*-stimulated PBMC, FoxP3^{bright} cells, as well as mRANKL$^+$ cells, remained at the same levels of frequency and intensity as determined on day 3 (Fig. 4j,k). However, the addition of IL-10 suppressed such *Aa*-mediated mRANKL expression by PBMC on day 3 (Fig. 4h,i,m) and on day 7 (not shown). The increased expression of mRANKL was observed in the CD4$^+$ T cells (Fig. 4f). These results indicate that, upon activation of PBMC with *Aa*, FoxP3^{dim} expression could be induced transiently in T cells. mRANKL expression was expressed prominently in FoxP3 negative cells and in FoxP3^{dim} cells, but not in FoxP3^{bright} cells, which were most probably represented by the presence of CD25/FoxP3 double-positive cells. It must be emphasized that CD25/FoxP3 double-positive cells are considered to function as T_{reg} cells [1]. As such, the production of T_{reg}-associated cytokine IL-10 seems to suppress RANKL expression by T cells, which indicates that T_{reg} cells can down-regulate RANKL expression by activated T cells, as long as T_{reg} cells produce IL-10 in the periodontal tissues, as reported previously [17].

Discussion

Overall, the present study demonstrated the following novel findings about T_{reg} cells in the context of periodontal disease:

Fig. 4. *In vitro* stimulation of peripheral blood mononuclear cells (PBMC)-induced predominant nuclear factor-kB ligand (RANKL) expression in forkhead box P3 (FoxP3)-negative cells compared to FoxP3 positive cells. PBMC isolated from healthy subjects were stimulated with formalin-fixed *Actinobacillus actinomycetemcomitans* (*Aa*) Y4 (10^7/ml) in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in the presence or absence of human recombinant interleukin (IL)-10. Three days after incubation, the cells were fixed and stained with fluorescein isothiocyanate (FITC)-conjugated anti-human FoxP3 antibody, Alexa Fluor 647-conjugated anti-human CD25 antibody, Alexa Fluor 647-conjugated anti-human CD4 and osteoprotegerin (OPG)-Fc-biotin/TexasRed-conjugated avidin. The staining pattern for each molecule was analysed using the double-colour confocal microscope. Representative staining patterns of merged images between green and red colours are shown at ×640 magnification. The arrows in (a), (d) and (g) indicate the FoxP3/CD25 double-positive cells. All three samples, including two healthy subjects and one patient with periodontal disease, showed similar expression patterns of CD25, FoxP3 and RANKL after stimulation with *Aa* in the presence or absence of IL-10. (a–i) PBMC of a healthy subject stimulated for 3 days; (j, k) PBMC of a healthy subject stimulated with *Aa* for 7 days. The numbers of cells positive for respective molecules were counted in a microscopic field containing a total of 100 PBMC cells (l, FoxP3 and/or CD25; mM, RANKL). *Significantly higher than non-stimulation control by Student's *t*-test ($P < 0·05$); **significantly lower than *Aa* stimulation alone by Student's *t*-test ($P < 0·05$).


Diminished FoxP3+ T cells in periodontal diseased tissue
FOXP3 (green)
CD25 (red)
FOXP3 (green)
RANKL (red)
RANKL (green)
CD4 (red)
Control
(day 3)
Aa
(day 3)
Aa+ IL-10
(day 3)
Aa
(day 7)
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Aa + IL-10
Aa
Control
Cell number/field
20
15
10
5
0
FOXP3bright CD25+
FOXP3dim CD25+
FOXP3dim CD25−
FOXP3− CD25+
(m)
Aa + IL-10
Aa
Control
RANKL+ cell #/field
100
80
60
40
20
0

1 FoxP3/CD25 double-positive cells are present in healthy gingival tissues, whereas FoxP3 or CD25 single-positive cells, but not FoxP3/CD25 double-positive cells, are found in diseased gingival tissues.
2 The percentage of FoxP3$^+$ cells is as low as 5% within the otherwise massive infiltration of RANKL$^+$ lymphocytes found in the diseased gingival tissues.
3 A negative correlation between concentrations of sRANKL and IL-10 in the gingival tissue homogenates has been demonstrated.
4 In the peripheral blood lymphocytes stimulated with bacteria (*Aa*) in an antigen-dependent fashion, mRANKL expression is expressed prominently in FoxP3 negative cells and in FoxP3^{dim} cells, but not in FoxP3^{bright} cells, which most probably represents the presence of CD25/FoxP3 double-positive cells.
5 T_{reg}-associated cytokine IL-10 can suppress RANKL expression by PBMC stimulated in an antigen-specific manner.

Several examples of these finding can be found in the literature. Recurrent aphthous ulcerations (RAU) is a chronic inflammatory disease with evidence of inappropriate immune response towards microorganisms found in the oral cavity of patients with RAU [21–23]. Peripheral blood of these patients is reported to show a decreased number of CD4$^+$CD25^{+high} T_{reg} cells along with the predominance of type 1 cytokine production by cultured PBMC [24]. Another example is found in systemic lupus erythematosus, where a diminished number of T_{reg} cells is also implicated [25]. These lines of evidence suggest that a diminished T_{reg} cell count may be attributed to 'out-of-control' immune responses in certain diseases.

The current study found that an amount equal to only 5% of FoxP3$^+$ cells is present in the RANKL$^+$ lymphocytes infiltrating diseased gingival tissues. This finding, however, contradicts the report by Ito *et al.* [17], where they demonstrate that a majority of CD4 T clone cells (nearly 100%) established from T cells infiltrating periodontal diseased tissues is positive for FoxP3 and IL-10 mRNA. None the less, it is reported that almost all human CD25$^-$CD4$^+$ T cells express FoxP3 upon activation, peaking at 72 h, whereas in mice only CD25$^+$CD4$^+$ T_{reg} cells, but not CD25$^-$CD4$^+$ T cells, express FoxP3, irrespective of activation [26,27]. Furthermore, other studies have demonstrated that most human CD4$^+$ clone cells are FoxP3-positive [28]. The FoxP3^{dim} cells found in *Aa*-stimulated PBMC, which were also RANKL$^+$ on day 3 (Fig. 4e,h), may represent those FoxP3$^+$ cells that can be induced in an activation-dependent manner. It is significant that all FoxP3^{dim} expression in *Aa*-stimulated PBMC diminished dramatically, whereas RANKL expression remained positive in those cells (Fig. 4k). Therefore it is conceivable that, in the study by Ito *et al.*, T cell stimulation with CD3 and IL-2 may have induced FoxP3 mRNA in an activation-dependent manner, which then resulted in an extraordinarily high percentage of FoxP3$^+$ T cell clones. In terms of the fluorescent-immunohistochemistry results for the gingival tissue sections shown in Fig. 1, the FoxP3-positive cells found in the diseased tissue appeared to be FoxP3^{dim} cells, which are activated in a transitory manner under inflammatory conditions, whereas the FoxP3/CD25 double-positive cells found in healthy gingival tissues appeared to be FoxP3^{bright} cells (Fig. 1b).

It is also reported that systemic administration of IL-10 can suppress periodontal bone resorption induced by adoptive transfer of RANKL expressing human T cells into non-obese diabetic (NOD)/SCID-mice [29]. In this SCID mouse model, although it is unclear if IL-10 reduces the amount of RANKL expression from the adoptively transferred human T cells, systemic IL-10 administration clearly suppressed the frequency of Th1-type human T cells [interferon (IFN)-γ producers] in the NOD/SCID-mice. We also previously demonstrated in the mouse periodontal disease model with *P. gingivalis* infection that periodontal bone loss is increased in the IL-10 gene knockout mice compared to the wild-type mice [30]. Findings in the present study have demonstrated (1) the negative correlation between IL-10 and sRANKL concentrations in the human gingival tissues and (2) the suppression function of IL-10 on RANKL expression and proliferation by activated PBMC cells. These findings are therefore consistent with the results of the two mouse models noted above, which show the suppression role of IL-10 in periodontal bone loss induced by RANKL production by activated T cells.

In addition to the suppression role of IL-10, it is noteworthy that TGF-β also plays an important role in the immune suppression function of T_{reg} cells. As TGF-β mRNA appears to be expressed in the T_{reg} cells present in the gingival tissue [2], the possible engagement of TGF-β in RANKL-mediated periodontal disease is yet to be elucidated. In particular, TGF-β is reported to increase osteoprotegerin (OPG) expression by bone marrow stromal cells [31]. Therefore, it is very plausible that T_{reg} cells play a suppressive regulatory role in the inflammatory bone resorption lesion of periodontal disease, while elaboration of such a hypothesis in both *in vitro* and *in vivo* studies is still required to elucidate the relevance of T_{reg} cells in the context of periodontal bone resorption.

Kopitar *et al.* [32] found that commensal oral bacteria antigens prepared from *Bacteroides fragilis, Streptococcus mitis* and *Propionibacterium* acnes prime human dendritic APC to induce Th1, Th2 and T_{reg} differentiation, respectively, indicating that antigenic properties of bacteria can modulate the APC instruction mechanism on T_{reg} as well as adaptive T cells (Th1 and Th2) [32]. Therefore, the composition of bacterial flora in the periodontal crevice may affect the induction of T_{reg} cells in the periodontal tissue. None the less, the present study demonstrated clearly that FoxP3/CD25 double-positive cells diminish in the bone resorption lesion

of periodontal disease where abundant RANKL+ lymphocytes infiltrate. It is speculated that chemoattractant factors that recruit FoxP3/CD25 double-positive T_{reg} cells are down-regulated, or antagonized, in the diseased gingival tissue and that this results in the decrease of FoxP3/CD25 double-positive T_{reg} cells in the bone resorption lesion of periodontal disease.

In addition to T_{reg}-associated IL-10 cytokine production, it is remarkable that IL-10 is also produced by Th2-type cells. Although it is considered a controversial finding in the literature, both Th1 and Th2 cell types are found in periodontally diseased tissues [33,34]. Interestingly, our preliminary *in vitro* studies demonstrated that another Th2 cytokine, IL-4, can up-regulate the sRANKL production by CD3/CD28-stimulated naive T cells isolated from both humans and mice (unpublished data). Therefore, it is plausible that IL-4 produced by Th2 type T cells may antagonize the suppression of RANKL-expression by IL-10. Consequently, the cellular source and expression pattern of IL-10 and IL-4 in periodontal lesions may play a key role in the regulation of RANKL production by the T effector cells.

In summary, the present study demonstrated that diminished FoxP3/CD25 double-positive T_{reg} cells, in the context of periodontal diseased tissue, is associated with pathogenic RANKL expression by activated lymphocytes. Possible production of immune suppressive cytokine IL-10 by T_{reg} cells also appeared to play a pivotal role in the regulation of such RANKL expression.

Acknowledgements

We thank Dr Colin Dunstan for OPG-Fc (Amgen Inc, Thousand Oak, CA, USA) and Dr Takashi Yanaoka and Mr Tadahiko Kounoike for their support of this study. This work was supported by grants DE-03420, DE-09018 and DE-14551 from the National Institute of Dental and Craniofacial Research.

References

1 Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005; 6:345–52.
2 Nakajima T, Ueki-Maruyama K, Oda T *et al.* Regulatory T-cells infiltrate periodontal disease tissues. J Dent Res 2005; 84:639–43.
3 Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation *in vitro*. Biochem Biophys Res Commun 1999; 265:144–50.
4 Kong YY, Feige U, Sarosi I *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999; 402:304–9.
5 Valverde P, Kawai T, Taubman MA. Selective blockade of voltage-gated potassium channels reduces inflammatory bone resorption in experimental periodontal disease. J Bone Miner Res 2004; 19:155–64.
6 Kawai T, Eisen-Lev R, Seki M, Eastcott JW, Wilson ME, Taubman MA. Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. J Immunol 2000; 164:2102–9.
7 Lacey DL, Timms E, Tan HL *et al.* Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93:165–76.
8 Takayanagi H, Sato K, Takaoka A, Taniguchi T. Interplay between interferon and other cytokine systems in bone metabolism. Immunol Rev 2005; 208:181–93.
9 Mackler BF, Frostad KB, Robertson PB, Levy BM. Immunoglobulin bearing lymphocytes and plasma cells in human periodontal disease. J Periodontal Res 1977; 12:37–45.
10 Seymour GJ, Greenspan JS. The phenotypic characterization of lymphocyte subpopulations in established human periodontal disease. J Periodont Res 1979; 14:39–46.
11 Taubman MA, Stoufi ED, Ebersole JL, Smith DJ. Phenotypic studies of cells from periodontal disease tissues. J Periodont Res 1984; 19:587–90.
12 Okada H, Kida T, Yamagami H. Identification and distribution of immunocompetent cells in inflamed gingiva of human chronic periodontitis. Infect Immun 1983; 41:365–74.
13 Malberg K, Molle A, Streuer D, Gangler P. Determination of lymphocyte populations and subpopulations extracted from chronically inflamed human periodontal tissues. J Clin Periodontol 1992; 19:155–8.
14 Seymour GJ, Powell RN, Davies WI. The immunopathogenesis of progressive chronic inflammatory periodontal disease. J Oral Pathol 1979; 8:249–65.
15 Kawai T, Matsuyama T, Hosokawa Y *et al.* B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am J Pathol* 2006; 169:987–98.
16 Han X, Kawai T, Eastcott JW, Taubman MA. Bacterial-responsive B lymphocytes induce periodontal bone resorption. J Immunol 2006; 176:625–31.
17 Ito H, Honda T, Domon H *et al.* Gene expression analysis of the CD4+ T-cell clones derived from gingival tissues of periodontitis patients. Oral Microbiol Immunol 2005; 20:382–6.
18 Dejaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. Immunology 2006; 117:289–300.
19 Kawai T, Ito H, Sakado N, Okada H. A novel approach for detecting an immunodominant antigen of *Porphyromonas gingivalis* in diagnosis of adult periodontitis. Clin Diagn Lab Immun 1998; 5:11–17.
20 Gorska R, Laskus-Perendyk A, Gregorek H, Kowalski J. The influence of surgical treatment of periodontal disease on selected lymphocyte subpopulations important for cellular and humoral immune responses. J Periodontol 2005; 76:1304–10.
21 Hasan A, Childerstone A, Pervin K *et al.* Recognition of a unique peptide epitope of the mycobacterial and human heat shock protein 65–60 antigen by T cells of patients with recurrent oral ulcers. Clin Exp Immunol 1995; 99:392–7.
22 Hasan A, Shinnick T, Mizushima Y, van der Zee R, Lehner T. Defining a T-cell epitope within HSP 65 in recurrent aphthous stomatitis. Clin Exp Immunol 2002; 128:318–25.
23 Sun A, Chia JS, Chiang CP. Increased proliferative response of peripheral blood mononuclear cells and T cells to *Streptococcus mutans* and glucosyltransferase D antigens in the exacerbation stage of recurrent aphthous ulcerations. J Formos Med Assoc 2002; 101:560–6.

24 Lewkowicz N, Lewkowicz P, Banasik M, Kurnatowska A, Tchorzewski H. Predominance of type 1 cytokines and decreased number of CD4(+) CD25(+high) T regulatory cells in peripheral blood of patients with recurrent aphthous ulcerations. Immunol Lett 2005; 99:57–62.

25 Miyara M, Amoura Z, Parizot C *et al.* Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 2005; 175:8392–400.

26 Walker MR, Kasprowicz DJ, Gersuk VH *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. J Clin Invest 2003; 112:1437–43.

27 Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006; 24:209–26.

28 Morgan ME, van Bilsen JH, Bakker AM *et al.* Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum Immunol 2005; 66:13–20.

29 Zhang X, Teng YT. Interleukin-10 inhibits gram-negative-microbe-specific human receptor activator of NF-kappaB ligand-positive CD4+-Th1-cell-associated alveolar bone loss *in vivo*. Infect Immun 2006; 74:4927–31.

30 Sasaki H, Okamatsu Y, Kawai T, Kent R, Taubman M, Stashenko P. The interleukin-10 knockout mouse is highly susceptible to *Porphyromonas gingivalis*-induced alveolar bone loss. J Periodont Res 2004; 39:432–41.

31 Takai H, Kanematsu M, Yano K *et al.* Transforming growth factor-beta stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. J Biol Chem 1998; 273:27091–6.

32 Kopitar AN, Ihan Hren N, Ihan A. Commensal oral bacteria antigens prime human dendritic cells to induce Th1, Th2 or Treg differentiation. Oral Microbiol Immunol 2006; 21:1–5.

33 Salvi GE, Brown CE, Fujihashi K *et al.* Inflammatory mediators of the terminal dentition in adult and early onset periodontitis. J Periodont Res 1998; 33:212–25.

34 Takeichi O, Haber J, Kawai T, Smith DJ, Moro I, Taubman MA. Cytokine profile of T lymphocytes from gingival tissues with pathological pocketing. J Dent Res 2000; 79:1548–55.