Giovanna Ribeiro Souto

Relação entre Citocinas, Quimiocinas e Maturação das Células Dendríticas, em Indivíduos Fumantes e Não-Fumantes, com Periodontite Crônica

Faculdade de Odontologia

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Tese apresentada ao Colegiado do Programa de Pós-Graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do grau de Doutor em Odontologia – área de concentração em Patologia Bucal.

Orientador: Prof. Dr. Ricardo Alves Mesquita

Co-Orientador: Prof. Dr. Fernando de Oliveira Costa

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Relação entre Citocinas, Quimiocinas e Maturação das Células Dendríticas em Indivíduos com Periodontite Crônica Fumantes e Não-Fumantes

GIOVANNA RIBEIRO SOUTO

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ODONTOLOGIA, como requisito para obtenção do grau de Doutor em ODONTOLOGIA, área de concentração PATOLOGIA BUCAL.

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ATA DA DEFESA DE TESE DA ALUNA
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Realizou-se, no dia 21 de fevereiro de 2014, às 14:00 horas, sala 3403, Faculdade de Odontologia UFMG, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada:

Relação entre Citocinas, Quimiocinas e Maturação das Células Dendríticas em Indivíduos com Periodontite Crônica Fumantes e Não- Fumantes, apresentada por GIOVANNA RIBEIRO SOUTO, número de registro 2010750130, graduada no curso de ODONTOLOGIA, como requisito parcial para a obtenção do grau de Doutor em ODONTOLOGIA, à seguinte Comissão Examinadora: Prof(a). Ricardo Alves de Mesquita - Orientador (UFMG), Prof(a). Telma Campos Medeiros Lorentz (Universidade Federal de Minas Gerais), Prof(a). Tarcilia Aparecida da Silva (UFMG),Prof(a). Rodrigo Villamarim Soares (PUC-MG), Prof(a). Fernando de Oliveira Costa (FOUFMG),Prof(a). Ana Carolina Uchoa Vasconcelos (UFPel).

A Comissão considerou a tese:
(X) Aprovada
( ) Reprovada

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Belo Horizonte, 21 de fevereiro de 2014.

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e minhas irmãs Lívia e Cecília,

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“As pessoas mais felizes não têm as melhores coisas.
Elas sabem fazer o melhor das oportunidades
que aparecem em seus caminhos” – Clarice Lispector

RESUMO

A periodontite crônica (PC) é a principal forma de doença periodontal destrutiva e resulta da interação entre bactérias e resposta inflamatória do hospedeiro, podendo ser afetada por fatores ambientais como o fumo. O objetivo do estudo foi avaliar a expressão de citocinas e quimiocinas, assim como a densidade de células dendríticas (CDs) imaturas e maduras, e densidade do infiltrado inflamatório no tecido gengival de indivíduos não-fumantes (NF) e fumantes (F), diagnosticados com periodontite crônica. O estudo foi aprovado pelo COEP-UFMG (423/11) e foram recrutados 24 indivíduos NF e 21 F. Seis amostras de mucosa normal (MN) foram usadas como controle de quimiocinas e citocinas. Foi realizado exame periodontal para avaliação de profundidade de sondagem (PS), nível de inserção clínica (NIC) e sangramento à sondagem (SS). Indivíduos que apresentavam NIC ≥3 mm na proximal em ≥2 dentes não adjacentes foram diagnosticados com PC. Imunoistoquímica foi feita para avaliar a densidade (células/mm^2) de CDs imaturas CD1a+ e Fator XIIIa+, e CDs maduras CD83+. A densidade do infiltrado inflamatório em cortes corados por hematoxilina e eosina também foi avaliada. As quimiocinas CCL2, CCL3, CCL5, CCL19, CCL20 e CXCL8 foram medidas usando o ensaio ELISA e as citocinas IL-2, IL-10, IL-4, IL-6, IFN-ɣ, TNF-α, IL-17A foram medidas usando o método CBA, nas amostras de tecido gengival. Os indivíduos foram questionados em relação ao tempo do hábito de fumar (HF/anos) e o número de cigarros fumados por dia (C/dia). A densidade de CDs maduras CD83+, níveis de CCL3 e CXCL8 diminuíram nos fumantes. Os níveis de CCL5 estavam aumentados nos fumantes. Negativas correlações entre o número de C/dia com níveis de IL-17A e o número de dentes, e entre o tempo do HF/anos e níveis de CCL19 foram observadas. As expressões de IL-6, CCL2 e CCL20 foram positivamente correlacionadas com o número de CDs imaturas CD1a+. Os níveis de IL-2, TNF-α, IFN-ɣ, IL-10, IL-17A, CCL3, CCL5, CCL19 e CXCL8 estavam aumentados no tecido com PC quando comparados com MN. A porcentagem de sítios com NIC≥3

mm foi positivamente correlacionada com densidade do infiltrado inflamatório, níveis de expressão de CCL3 e CXCL8, e negativamente correlacionada com densidade de CDs imaturas CD1a+ e níveis de IL-2. Pode-se concluir que o hábito de fumar afeta células e mediadores inflamatórios da resposta imune de indivíduos diagnosticados com PC. Além disso, o aumento das CDs imaturas pode estar associado com maiores níveis de IL-6, CCL2 e CCL20, embora somente os níveis de IL-2, TNF-α, IFN-ɣ, IL-10, IL-17A, CCL3, CCL5, CCL19 e CXCL8 apresentaram aumentados nos tecidos gengivais com PC. Maior densidade de infiltrado inflamatório, níveis de CCL3 e CXCL8, e menor densidade de CDs imaturas CD1a+ e níveis de IL-2 parecem estar associados com maior gravidade da doença.

Palavras-chave: Periodontite crônica, quimiocinas, citocinas, células dendríticas, fumo

ABSTRACT

Relation between cytokines, chemokines, and maturation of dendritic cells, in smokers and non-smokers, diagnosed with chronic periodontitis

Chronic periodontitis (CP) is the most frequent form of destructive periodontal disease, and results from the interaction between bacterial biofilm and the host inflammatory response. Environmental factor as smoking is essential in determining to individual susceptibility. The present study aimed to evaluate chemokine and cytokine expressions, densities of immature and mature dendritic cells (DCs), and inflammatory infiltrate density in the gingival tissue of non-smokers (NS) and smokers (S) diagnosed with CP. The effect of smoking in chemokines, cytokines and immature and mature DCs were also evaluated. The study was approved by COEP-UFMG (423/11), and it was selected 24 NS and 21 S. Six samples of normal mucosa (NM) were used to control of chemokine and cytokine levels. It was performed periodontal examination to determine probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP). Individuals presented proximal CAL ≥3 mm in ≥2 non-adjacent teeth were diagnosed with CP. Immunohistochemistry was performed to evaluate the density (cell/mm^2) of Factor XIIIa+ and CD1a+ immature DCs, and CD83+ mature DCs. Inflammatory infiltrate density was measured using hematoxylin and eosin stained sections. Chemokines (CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8) were measured using ELISA assay, while cytokines (IL-2, IL-10, IL-4, IL-6, IFN-ɣ, TNF-α, and IL-17A) were measured using CBA method, in samples of gingival tissue. The individuals were asked about the time of their smoking habit in years (SH/years) and how many cigarettes they smoked per day (C/day). CD83+ mature DCs, CCL3 and CXCL8 levels decreased in smoker group. While CCL5 levels were increased in smokers. Negative correlations between C/day and IL-17A levels and number of teeth, and between SH/years and CCL19 were observed. IL-6, CCL2, CCL20 levels were

positively correlated with CD1a+ immature DCs densities. IL-2, TNF-α, IFN-ɣ, IL-10, IL-17A, CCL3, CCL5, CCL19, and CXCL8 levels were increased in gingival tissue with CP when compared with NM. The percentage of sites with CAL≥3 mm were positively correlated with inflammatory infiltrate density, CCL3 and CXCL8 levels, and negatively correlated with CD1a+ immature DCs and IL-2 levels. In conclusion, it was observed that the smoking affect cells and inflammatory mediators of the immune response, and this can contribute for increase the susceptibility at CP in smokers. In addition, the increase of CD1a+ immature DCs was associated with higher levels of IL-6, CCL2, and CCL20. However, only IL-2, TNF-α, IFN-ɣ, IL-10, IL-17A, CCL3, CCL5, CCL19, and CXCL8 levels presented increased in gingival tissues with CP. Higher density of inflammatory infiltrate, CCL3 and CXCL8 levels, as well as lower density of CD1a+ immature DCs, and IL-2 levels can result in a more severe degree of human CP.

Key-words: Chronic periodontitis, chemokines, cytokines, dendritic cells, smoking

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LISTA DE SIGLAS E ABREVIATURAS

CDs – células dendríticas

Th – células T helper

Treg – células T regulatórias

IL – interleucina

IFN - interferon

CL – células de Langerhans

TNF – tumor necrosis factors

DPOC – doença pulmonar obstrutiva crônica

ELISA - Enzyme-linked Immunosorbent Assay

CBA - Cytometric Bead Array

C/dia – cigarros fumados por dia

HF/anos – hábito de fumar em anos

COEP-UFMG – Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais

PS – profundidade de sondagem

NIC – nível de inserção clínica

SS – sangramento à sondagem

F – fumantes

NF – não fumantes

HE – hematoxilina e eosina

DAB – diaminobenzidina

EB – epitélio bucal

ES – epitélio do sulco

LP – lâmina própria

SUMÁRIO

1 – Introdução

A periodontite crônica é a forma mais frequente de doença periodontal destrutiva, caracterizada por um processo inflamatório dos tecidos de suporte dos dentes que resulta da complexa interação entre bactérias patogênicas e resposta imuno-inflamatória do indivíduo (Armitage, 2004; Tonetti & Claffey, 2005; Akpinar et al., 2012). Sabe-se que a resposta do hospedeiro tem um papel essencial na patogênese da periodontite crônica. Contudo, é bem conhecido que fatores de risco ambientais como o fumo podem modificar a patogênese da doença (Akpinar et al., 2012; Genco & Borgnakke, 2013).

Dessa forma, a manifestação clínica da periodontite crônica depende da natureza da resposta, que é determinada pela susceptibilidade inata do paciente (Cullinan et al., 2003). Indivíduos podem responder de forma variável à presença da placa, em relação a outros, mas a forma como alguns fatores podem contribuir para a susceptibilidade do paciente são ainda pouco entendidos (Ohlrich et al,. 2009). Assim, nas últimas décadas, numerosos estudos tentam entender os mecanismos envolvidos na iniciação e progressão da periodontite (Genco & Borgnakke, 2013). Porém, ainda não está bem esclarecido como patógenos causam diretamente os sinais e sintomas clínicos da doença, e como fatores genéticos e fatores de risco ambientais influenciam o sistema imune e a resposta inflamatória (Akpinar et al., 2012; Genco & Borgnakke, 2013).

De maneira geral, sabe-se que os epitélios sulcular e juncional permitem a passagem seletiva de antígenos e células, além de produzirem moléculas de defesa. Uma vez quebrada a barreira epitelial, a resposta imune adaptativa se inicia. Assim, células dendríticas (CDs) da imunidade inata, funcionam como uma ponte entre a resposta imune inata e adaptativa, capturando antígenos bacterianos e iniciando a resposta imune (Banchereau & Steinman, 1998). Após captura de antígenos, as CDs tornam-se maduras, expressam moléculas co-estimulatórias e produzem distintos

padrões de citocinas, que determinam a migração seletiva de subtipos de células T CD4 auxiliares (Cutler & Jotwani, 2004; Tew et al., 2012).

Células T CD4 podem ser subdivididas em subtipos Th1, Th2, Th17 e Treg de acordo com a padrão de expressão de citocinas (Murphy & Reiner, 2002; Sallusto & Lanzavecchia, 2009). Citocinas Th1 protetoras (como Interleucina (IL)-2 e interferon (INF)-ɣ) estão envolvidos na resposta imune celular (Jankovic et al., 2001; Lappin et al., 2001; Gemmell et al., 2007). Citocinas Th2 (como IL-4 e IL-10) estão associadas com imunidade humoral e propriedades anti-inflamatórias (Jankovic et al., 2001; Lappin et al., 2001; Murphy & Reiner, 2002; Gemmell et al., 2007). Resposta Th17 (IL-17A) foi identificada como células que recrutam neutrófilos e macrófagos para participar e amplificar a reação inflamatória (Laurence & O'Shea, 2007; Allam et al., 2011; Zhao et al., 2011). Quais citocinas regulam os mecanismos estimulatórios e supressores da resposta Treg ainda são incertas. Mas sabe-se que a resposta Treg modula a resposta imune contra antígenos externos e próprios (Murphy & Reiner, 2002). No contexto da patogênese da doença periodontal, o papel das CDs e citocinas relacionadas com perfil de células T auxiliares necessita ser mais bem esclarecido. Além disso, ainda não é bem entendido como o efeito do hábito de fumar sobre CDs e citocinas pode contribuir para a susceptibilidade da periodontite crônica.

Nesse mesmo contexto, estudos também sugerem que CDs imaturas aumentam continuamente no epitélio da gengiva saudável para gengivite e para periodontite crônica (Seguier et al., 2000; Jotwani & Cutler, 2003; Cury et al., 2008). Estas CDs imaturas, após a captura de antígenos, tornam-se maduras, vão para os linfonodos onde apresentam antígenos para os linfócitos T, e estes migram para o local da inflamação (Banchereau et al., 2000). Estudos mostram que a migração de CDs e linfócitos T pode ocorrer em resposta a sinais quimiotáticos (McWilliam et al., 1994; Xu et al., 1996; Cutler & Jotwani, 2004). Em estudos *In vitro*, CDs imaturas e maduras respondem a um grande espectro de quimiocinas como CCL2/MCP-1, um ligante para o receptor CCR2 presente nas CDs imaturas (Barker et al., 1991; Merad

et al., 2002), CCL3/MIP-1α ligante para receptores CCR1 e CCR5 (Rossi & Zlotnik, 2000), CCL5/RANTES interage com os receptores CCR3 e CCR5 (Siveke & Hamann, 1998; Graves et al., 1999), CCL20/MIP-3α que corresponde ao receptor CCR6 presente em CDs imaturas, além das quimiocinas CCL19/MIP-3β e CCL21 que ambas respondem ao receptor CCR7 de CDs maduras (Dieu et al., 1998).

Sendo assim, o papel das quimiocinas no recrutamento de CDs imaturas e maduras, bem como o efeito do estágio de maturação das CDs nos níveis de citocinas na patogênese da periodontite crônica ainda é pouco entendido. Além disso, ainda necessita ser esclarecido qual o efeito do hábito de fumar nas quimiocinas, citocinas e CDs imaturas e maduras do tecido gengival de indivíduos com periodontite crônica.

2– Revisão de literatura

2.1 – Classificação e patogênese da periodontite crônica

Em 1999, o Workshop Internacional para Classificação da Doença periodontal apresentou um sistema de classificação para doença periodontal onde foram listadas mais de 40 diferentes doenças gengivais. Basicamente, estas doenças foram classificadas em doença gengival não-induzida por placa – como estomatite herpética primária, manifestação gengival de líquen plano, reação alérgica em gengiva, dentre outras – e doença gengival induzida por placa – onde estão incluídas a gengivite por placa e gengivite ulcerativa necrozante (Armitage, 1999). Em algumas das lesões gengivais induzidas por placa, a perda de inserção e a destruição do osso alveolar podem ocorrer. Neste caso, essas são classificadas em sete categorias de doença periodontal destrutiva: periodontite crônica, periodontite como uma manifestação de doença sistêmica, periodontite agressiva localizada e generalizada, periodontite ulcerativa necrozante, abscesso periodontal e lesão de endopério. Assim, a gengivite crônica é uma doença periodontal associada à placa que, em alguns casos, pode evoluir para uma doença periodontal destrutiva como a periodontite crônica (Armitage, 2004).

No diagnóstico da periodontite crônica, algumas características clínicas são observadas: é uma lesão mais prevalente em adultos, a quantidade de destruição é consistente com a presença de fatores locais, cálculo subgengival é um achado comum e está associada a uma variedade de padrões microbiológicos. Além disso, a progressão da doença pode variar de leve à moderada, mas apresentando períodos de progressão rápida. A doença ainda pode ser modificada por doenças sistêmicas como diabetes ou outros fatores externos como fumo e o estresse (Armitage, 2004).

No diagnóstico da doença, critérios de sondagem clínica periodontal também são considerados. Em 2005, o encontro do Workshop Europeu de Periodontia propôs dois critérios de definição de periodontite crônica. Um para definição da doença,

quando ocorre perda de inserção proximal ≥3 mm em ≥2 dentes não-adjacentes. Outro critério para definir a extensão e severidade, naqueles casos em que ocorre perda de inserção clínica ≥5mm em mais de 30% dos dentes presentes (Tonetti & Claffey 2005).

A periodontite crônica é a forma mais comum de doença periodontal destrutiva e uma das principais causas de perda dentária, além de ser um importante fator modificador da saúde sistêmica dos pacientes (Tonetti & Claffey 2005). O principal fator etiológico da doença é a colonização bacteriana na superfície dos dentes, nas regiões de sulco gengival. Além disso, fatores da resposta imunológica individual, associados a fatores do meio ambiente, interagem com a infecção bacteriana levando ao desenvolvimento da doença (Van Dyke, 2007).

Dessa forma, é bem estabelecido que bactérias da placa dentária sejam a causa da inflamação periodontal (Seymour & Taylor 2004). Três espécies de bactérias foram consideradas como mais importantes patógenos periodontais: Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans e Tannerella forsythia pelo Workshop Mundial de Periodontia Clínica de 1996. Posteriormente foram descritas três espécies que formam um complexo associado a formas avançadas de periodontite crônica: P. gingivalis, Treponema denticola e T. forsythia (Socransky et al., 1998). No entanto, sabe-se que a presença apenas dessas bactérias não é suficiente para desenvolvimento da doença (Cullinan et al., 2003). Outros fatores são também de grande importância e podem determinar o controle das respostas imune inata e adaptativa, dentre eles fatores genéticos e fatores ambientais como o fumo e estresse (Seymour, 1991; Seymour & Taylor 2004).

A periodontite crônica é, portanto, uma resposta inflamatória dos tecidos gengivais, no qual a natureza da resposta imunológica determina a susceptibilidade do indivíduo (Van Dyke, 2007). Assim, apenas a presença de periodontopatógenos não é suficiente para desenvolvimento da doença (Graves, 2008). É a resposta imune persistente do hospedeiro contra estes patógenos que resultará na destruição dos tecidos periodontais (Graves, 2008; Liu et al., 2010).

A resposta imunológica inicia com o reconhecimento de patógenos pelas células apresentadoras de antígenos como as CDs (Cutler & Jotwani, 2004). As CDs após se tornarem maduras produzem diferentes padrões de citocinas e quimiocinas (Murphy & Reiner, 2002; Kalinski & Moser, 2005; Bluestone et al., 2009). Esses mediadores induzem a expressão de moléculas de adesão, o aumento da permeabilidade vascular e a quimiotaxia de neutrófilos para o sulco gengival (Ford et al., 2010). Dessa forma, é bem estabelecido que a progressão da periodontite crônica seja determinada pelo complexo balanço de citocinas induzido pela presença de uma combinação de patógenos periodontais (Van Dyke, 2007; Genco & Borgnakke, 2013).

2.2 – Células dendríticas

As CDs são células apresentadoras de antígenos, derivadas da medula óssea, que funcionam como sentinelas do sistema imune (Caux et al., 1996). São células da imunidade inata e funcionam como uma ponte entre a resposta imune inata e adaptativa (Banchereau & Steinman, 1998). Estão presentes em todos os órgãos e são descritos com diferentes fenótipos no sangue periférico, pele e órgãos linfoides (Caux et al., 1996; Dubois et al., 1999). Através do sangue, as CDs precursoras migram da medula óssea para quase todos os tecidos, onde geralmente se tornam CDs imaturas residentes (McWilliam et al., 1994; Robert et al., 1999). As células de Langerhans (CLs) são exemplos de células dendríticas imaturas, presentes na epiderme/epitélio, caracterizadas por apresentarem alta capacidade de capturar antígenos, mas baixa capacidade de apresentá-los, devido ao seu fenótipo imaturo (Caux et al., 1996). Durante os processos patológicos, as CLs capturam antígenos e rapidamente deixam o epitélio. Elas migram através da derme, atravessam o endotélio dos vasos linfáticos e vão para os linfonodos. Para que ocorra essa migração, as CLs sofrem mudanças funcionais e fenotípicas (Delgado et al., 1998). As principais delas são a perda da capacidade de capturar antígenos, aumento da expressão de outras moléculas e capacidade de apresentar antígenos aos linfócitos T, tornando-se células apresentadoras de antígenos. Nos linfonodos, elas migram até as áreas ricas em

células T onde apresentam antígenos para as células T naive. Após apresentarem os antígenos, a maioria sofre apoptose (Steinman, 1991; Banchereau & Steinman, 1998; Sallusto & Lanzavecchia, 1999).

As CDs mieloides parecem se diferenciar de progenitores hematopoiéticos CD34+ na presença de GM-CSF+TNF (tumor necrosis factors)-alfa em duas CDs fenotipicamente distintas: CD14-CD1a+ e CD14+CD1a-, que correspondem respectivamente a CDs epiteliais/epidérmicas e CDs dérmicas/intersticiais. Precursores CD1a+ típicos são caracterizados pela expressão de CD1a, grânulos de Birbeck, antígeno Lag e E-Caderina. Enquanto CD14+ se diferenciam em CDs com características de CDs dérmicas com a expressão de Fator XIIIa, CD68, CD9 e CD2 (Caux et al., 1996). Dessa forma, é observado que CDs epiteliais imaturas ou CL são caracterizadas pela expressão seletiva de CD1a (Caux et al., 1996) e expressam vários receptores de capturar antígenos (Valladeau et al., 2000). As CDs intersticiais imaturas ou da derme são encontradas no interstício da maioria dos órgãos e tecidos. São caracterizadas pela expressão de CD68 e Fator XIIIa, além da ausência de marcadores de CLs, como CD1a (Caux et al., 1996).

Estudos *in vitro* utilizam incorporação de partículas fagocíticas a fim de induzirem efeitos sobre as CDs. Assim, estas CDs foram estimuladas, tornarem-se maduras e expressaram moléculas co-estimuladoras de células T como CD80, CD86, HLA-DR e CD83, enquanto o marcador de célula dendrítica imatura CD1a tornou-se ausente (Randolph et al., 1998). Portanto, CD83 é um importante marcador expressado durante o processo de maturação das CDs imaturas em resposta a estímulos inflamatórios (Lechmann et al., 2002; Lechmann et al., 2002).

O recrutamento de CDs precursoras da circulação para os tecidos ocorre em resposta à produção de quimiocinas no local da inflamação (McWilliam et al., 1994; McWilliam et al., 1996). CDs imaturas respondem *in vitro* a diversas quimiocinas como CCL3/MIP-1alfa, CCL4/MIP-1beta, CCL5/RANTES, CCL7/MCP-3 (Delgado et al., 1998), CCL20/MIP-3alfa, CCL19/MIP-3Beta (Dieu et al., 1998), que emitem sinais para

diversos receptores como CCR1 (Delgado et al., 1998; Sallusto et al., 1998), CCR2 (Sallusto et al., 1998), CCR5 (Delgado et al., 1998; Sallusto et al., 1998), CCR6 (Dieu et al., 1998) e CCR7 (Dieu et al., 1998; Sallusto et al., 1998). A determinação dos níveis de maturação das CDs, sua origem e secreção de sinais regulatórios de quimiocinas é um pré-requisito para entender a regulação da migração das CDs *in vivo* (McWilliam et al., 1994; McWilliam et al., 1996).

2.3 – Células dendríticas e periodontite crônica

Estudos de CDs nos tecidos gengivais tem mostrado um aumento dessas células com o aumento da inflamação gengival em associação com placa bacteriana (Newcomb et al., 1982; DiFranco et al., 1985; Hitzig et al., 1989; Jotwani et al., 2001; Dereka et al., 2004; Cury et al., 2008; Anjana et al., 2012) e diminuição após terapia periodontal não-cirúrgicas (Dereka et al., 2004). Contudo, avaliando células inflamatórias na gengivite e periodontite, Séguier et. al. (2000) encontraram aumento no número de linfócitos, enquanto as CDs estavam significativamente diminuídas, quando comparadas com tecidos gengivais saudáveis. Por outro lado, Gemmel et.al. (2002) não encontraram diferenças em relação às CDs entre tecido gengival inflamado e controles. Variações nos resultados observados podem ser explicadas por diferenças nas metodologias utilizadas (Seguier et al., 2000), bem como efeitos secundários de outras substâncias (Nurmenniemi et al., 1999; Souto et al., 2011). Redução significativa das CDs foi encontrada no tecido gengival de indivíduos utilizando medicação imunossupressora, quando comparado com tecido controle (Nurmenniemi et al., 1999). Além disso, o hábito de fumar demonstrou estar relacionado com a diminuição da CLs na gengivite crônica (Souto et al., 2011).

Foi sugerido que a infecção por *Porphyromonas gingivalis* está associada com ativação de CDs imaturas em CD maduras na periodontite crônica (Cutler et al., 1999). Também foi observado por Jotwani et al. (2001) que o aumento da densidade de CDs em periodontite crônica foi induzido pela *Porphyromonas gingivalis* acompanhado da ativação e maturação de CDs, com liberação de citocinas e estimulação de células T.

Na gengiva saudável, gengivite e periodontite crônicas foi demonstrada a presença de numerosas CDs CD1a imaturas e CDs CD83 maduras na lâmina própria e no epitélio (Jotwani et al., 2001), além de CDs dérmicas na lâmina própria (Jotwani & Cutler, 2003; Dereka et al., 2004). Entretanto, Gemmell et.al. (2002) não observaram células CD83 positivas no epitélio gengival, ao contrário das células CD1a positivas, enquanto ambas foram notadas na lâmina própria.

2.4 – Células dendríticas e perfil Th1, Th2 e Th17 na patogênese da periodontite crônica

A resposta imune do hospedeiro inicia quando patógenos são reconhecidos por células apresentadoras de antígenos, como as CDs (Cutler & Jotwani, 2004). Após se tornarem CDs maduras elas produzem diferentes padrões de citocinas que determinarão a polarização e ativação de linfócitos antígeno-específicos (Banchereau & Steinman, 1998). A polarização da resposta imune é determinada por diferentes tipos de citocinas que estão envolvidas na migração seletiva de subtipos de linfócitos T auxiliares (Cutler & Jotwani, 2004; Tew et al., 2012).

Células T CD4 se dividem em subtipos Th1, Th2 e Th17 de acordo com o perfil de citocinas produzido (Murphy & Reiner, 2002; Sallusto & Lanzavecchia, 2009). Como regra geral, a resposta imune mediada por células T polariza para um fenótipo Th1 e resulta em uma resposta caracteristicamente celular e pró-inflamatória (Jankovic et al., 2001). Enquanto células Th2 são associadas com imunidade humoral e propriedades anti-inflamatórias (Jankovic et al., 2001; Murphy & Reiner, 2002). O perfil Th17 foi identificado em 2005 e está associado com recrutamento de neutrófilos e macrófagos, amplificando a resposta inflamatória (Laurence & O'Shea, 2007).

No passado, foi sugerida a hipótese de que as células Th1 estavam associadas com lesões periodontais estáveis, enquanto Th2 com lesões progressivas (Gemmell & Seymour, 1994; Manhart et al., 1994). No entanto, outros estudos mostraram um predomínio da resposta Th1 e reduzida resposta Th2 em tecidos periodontais doentes (Sigusch et al., 1998; Takeichi et al., 2000). Recentemente, tem sido sugeridas que

ambas as respostas Th1 e Th2 estejam envolvidas na doença periodontal tanto em humanos quanto nos modelos em ratos (Berglundh et al., 2002; Gemmell et al., 2007).

Anteriormente ao conhecimento do perfil Th17, a patogênese da doença periodontal era classificada de acordo com o paradigma Th1/Th2, onde citocinas e células Th1 pró-inflamatórias eram expressas na periodontite na fase inicial (Garlet et al., 2005) ou na de inicio precoce (Bartova et al., 2000), ao passo que citocinas e células Th2 anti-inflamatórias eram produzidas na fase tardia (Tokoro et al., 1997; Garlet et al., 2005). No entanto, esse modelo não explica adequadamente alguns achados em relação à imunidade de células T (Gor et al., 2003; Zhao et al., 2011). Assim, o perfil de resposta imune na doença periodontal pode ser profundamente influenciado por outras citocinas pró-inflamatórias como IL-17 (Cardoso et al., 2009).

Tem sido bem demonstrado que IL-17 tem um importante papel na patogênese da artrite reumatoide associada com destruição óssea (Sato et al., 2006) No entanto, é sugerido ainda que IL-17 seja um importante regulador da resposta do hospedeiro associada com tráfico de neutrófilos (Linden & Adachi, 2002). Similar à artrite reumatoide, a destruição óssea na doença periodontal também é resultado da resposta imune (Yu et al., 2007).

Marcadores dos subtipos Th1, Th2 e Th17 tem sido descritos nos tecidos periodontais inflamados e a produção, por células apresentadoras de antígenos, de mediadores característicos de todos os perfis de resposta T podem ser estimulados por bactérias bucais (Gemmell et al., 2002; Garlet et al., 2003; Cardoso et al., 2009; Zhao et al., 2011).

2.5 - Células dendríticas e Quimiocinas

Quimiocinas ou citocinas quimiotáticas são proteínas secretadas de 8 a 10kDa que regulam a migração e ativação/diferenciação não somente de leucócitos, como as CDs, e também de células do estroma (Schall & Bacon, 1994). Com a descoberta de numerosas quimiocinas, moléculas semelhantes foram registradas com nomes diferentes. Assim, o Simpósio de Quimiocinas de Keystone introduziu uma nova

nomenclatura que distingue as quimiocinas pela posição das cisteínas (Zlotnik & Yoshie, 2000). Dessa forma, duas subfamílias de quimiocinas são distinguidas de acordo com a posição das primeiras duas cisteínas em C-X-C, quando separadas por um aminoácido, ou C-C, quando não separadas (Baggiolini et al., 1997). Essa diferença estrutural distingue propriedades dessas moléculas. A maioria das quimiocinas C-X-C atraem neutrófilos, mas não monócitos, enquanto a maioria das C-C parecem atrair apenas monócitos (Schall & Bacon, 1994).

Quimiocinas agem via receptores de domínio transmembrana ligados a proteína G que estão na superfície de células inflamatórias alvo (Schall & Bacon, 1994). Receptores CCR ligam-se as quimiocinas do grupo CC-, enquanto receptores CXCR ligam-se as quimiocinas CXC- (Dieu-Nosjean et al., 1999). No entanto, uma única quimiocina CC- ou CXC- pode se ligar a mais de um receptor (tabela 1). Por exemplo, nos neutrófilos são expressos dois receptores para IL-8, CXCR1 e CXCR2 (Baggiolini et al., 1997).

Tabela 1 – Relação de receptores, suas quimiocinas ligantes e antiga nomenclatura.

Receptor	Ligante
CCR1	CCL3/MIP-1alfa, CCL5/RANTES, CCL8/MCP-2, CCL7/MCP-3, CCL12/MIP-5
CCR2	CCL2/MCP-1, CCL8/MCP-2, CCL7/MCP-3, CCL13/MCP-4
CCR3	CCL11/Eotaxin, CCL7/MCP-3, CCL13/MCP-4, CCL5/RANTES
CCR4	CCL17/TARC, CCL22/MDC
CCR5	CCL3/MIP-1alfa, CCL4/MIP-1beta, CCL5/RANTES,
CCR6	CCL20/MIP-3alfa
CCR7	CCL19/MIP-3beta
CXCR1	CXCL-8/IL-8

As CDs imaturas são caracterizadas pela eficiência na captura e processamento de antígenos e migram em resposta às quimiocinas CCL20/MIP-3alfa, CCL5/RANTES e CCL3/MIP-1alfa (Sozzani et al., 1995; Xu et al., 1996; Sozzani et al., 1997). As quimiocinas CCL2/MCP-1 e CCL8/MCP-2 também foram identificados como quimiotáticos para as células CD1a+ (Xu et al., 1996). No entanto, CDs maduras perdem a capacidade de internalizar antígenos e de responderem a essas quimiocinas, mas respondem à CCL19 /MIP-3beta (Dieu et al., 1998). Além disso, sinais como LPS, TNF-alfa ou sinais de células T como CD40L induzem a perda de resposta a CCL20/MIP-3alfa, CCL3/MIP-1alfa e CCL5/RANTES e adquirem resposta a CCL19/MIP-3beta. Estes sinais induzem a maturação das CDs que perdem rapidamente a capacidade de capturarem antígenos (Sallusto & Lanzavecchia, 1994; Dieu et al., 1998).

O Receptor CCR5, alvo das quimiocinas CCL3/MIP-1alfa, CCL4/MIP-1beta e CCL5/RANTES, apresenta-se com expressão diminuída nas CDs maduras tornando-as não responsivas a essas quimiocinas. Mas apresenta-se altamente expresso nas CDs imaturas (Delgado et al., 1998).

2.6 Quimiocinas CCL2, CCL3, CCL5, CCL19, CCL20 e CXCL8

2.6.1 CCL2

A quimiocina CCL2, também conhecida como MCP-1 (proteína quimiotática de monócito-1), e que apresenta-se como ligante do receptor CCR2, é produzida por queratinócitos em resposta a presença de INF-gama, em sítios inflamados (Barker et al., 1991). Estudos *in vitro* demostram que a quimiocina CCL2 é capaz de produzir quimiotaxia tanto para monócitos, como as CDs, quanto para linfócitos T em resposta a presença de antígenos inflamatórios (Barker et al., 1991; Carr et al., 1994; Xu et al., 1996). Lu et al. (1998), demonstraram que apesar de estudos *in vitro* mostrarem que outras quimiocinas são capazes de ligarem aos receptores CCR2, somente a quimiocina CCL2 é responsável por estimular a infiltração de células mononucleares, em modelos inflamatórios *in vivo* .

A expressão de CCL2 tem sido observada em pacientes apresentando várias doenças associadas a inflamações crônicas como artrite reumatóide (Villiger et al., 1992), arterosclerose (Nelken, et al., 1991), tumores (Graves et al., 1992) e fibrose pulmonar idiopática (Antoniades, et al., 1992). Níveis aumentados dessa quimiocina no fluido crevicular gengival também foram observados com a progressão da doença periodontal, e sua diminuição após tratamento da doença (Pradeep et al., 2009b). Pradeep et al. (2009a) ainda observaram que o aumento de CCL2 estava correlacionado com o aumento de IL-18, no fluido crevicular gengival de pacientes com doença periodontal, comparado com indivíduos saudáveis. Além disso, CCL2 demonstrou estar aumentado em tecido gengival inflamado (Kabashima et al., 2002) e na doença periodontal agressiva (Emingil et al., 2004).

Em contraste, Gemmel et al (2001) demonstraram através de estudo imunoistoquímico em amostras gengivais de indivíduos tanto com periodontite crônica como saudáveis/gengivite, pouca expressão de CCL2/MCP-1 por leucócitos teciduais. Sugerindo que essa quimiocina possa ter importância na migração de monócitos/macrófagos apenas nos estágios iniciais de inflamação gengival.

2.6.2 CCL3

A proteína inflamatória de macrófagos-1 (MIP-1) foi assim denominada devido as suas propriedades inflamatórias. Posteriormente, estudos bioquímicos caracterizam duas proteínas distintas: MIP-1alfa e MIP-1beta (Sherry et al., 1988). Com introdução da nova nomenclatura, MIP-1alfa foi denominada CCL3 e MIP-1beta denominada CCL4 (Zlotnik & Yoshie, 2000).

Dois receptores têm sido identificados para CCL3: receptores CCR1 e CCR5 (Kaufmann et al., 2001). Linfócitos T, monócitos (Su et al., 1996), eosinófilos (Sabroe et al., 1999) e CDs (Sallusto et al., 1999) expressam CCR1 em sua superfície. Os níveis de CCR1 e CCR5 altamente presentes nas CDs imaturas são estimulados a diminuírem durante a maturação dessas células, e ao mesmo tempo observa-se aumento da expressão de CCR7 (Sallusto et al., 1999). O aumento da produção de

CCL3 está relacionado ao aumento da expressão de moléculas de adesão por leucócitos e células do endotélio, mostrando ter um importante papel no mecanismo de recrutamento de leucócitos na iniciação ou durante a resposta inflamatória (Lukacs et al., 1994).

A quimiocina CCL3 tem sido relacionada com recrutamento de leucócitos em várias doenças como artrite reumatóide (Patel et al., 2001), asma (Holgate et al., 1997) e bronquite crônica (Capelli et al., 1999). Observa-se ainda aumento da expressão dessa quimiocina em tecido gengival inflamado, quando comparado com tecido saudável (Kabashima et al., 2002). De acordo com Garlet et al. (2003), CCL3 apresenta-se aumentada tanto na periodontite agressiva como na periodontite crônica, em relação ao grupo controle.

Gemmel et al (2001) demonstraram através de estudo imunoistoquímico em amostras gengivais de indivíduos saudáveis/gengivite ou com periodontite crônica uma maior expressão de CCL3/MIP-1alfa quando comparado com IP-10, CCL2/MCP-1 e CCL5/RANTES. Além disso, foi observado que as três quimiocinas diminuem com o aumento da inflamação, enquanto a quimiocina CCL3 aumenta. Níveis salivares elevados de CCL3 também foram associados com severidade da periodontite crônica e diminuíram após terapia periodontal (Sexton et al., 2011). Além disso, significantes correlações foram observadas entre os níveis de CCL3 e os parâmetros clínicos periodontais, podendo sugerir uma relação com perda óssea periodontal (Al-Sabbagh et al., 2012).

Ainda com relação à perda óssea, CCL3 também tem sido associada com reabsorção óssea em processos inflamatórios, agindo diretamente na diferenciação de osteoclastos (Watanabe et al., 2004). Além disso, os receptores CCR1 (Yu et al., 2004; Vallet et al., 2007) e CCR5 (Vallet et al., 2007), alvos da quimiocina CCL3, são associados a precursores de osteoclastos ou células capazes de levar a sua diferenciação ou ativação, atuando nos processos de reabsorção óssea.

2.6.3 CCL5

A quimiocina CCL5, também conhecida como RANTES (secretada e expressa por células T normais e reguladas), mostrou ser capaz de interagir com os receptores CCR1 (Proudfoot, et al. 2001), CCR3 (Daugherty et al., 1996) e CCR5 (Proudfoot et al., 2001). No entanto, o receptor CCR5 mostrou ser altamente expressado em linfócitos Th1, enquanto CCR3 foi detectado somente em linfócitos Th2 (Loetscher et al., 1998). Observa-se ainda que CCL5 está relacionada com estimulação de eosinófilos (Rot et al., 1992), monócitos (Gamonal et al., 2001), basófilos (Bischoff et al., 1993) e quimiotaxia para células T (Schall et al., 1990).

Estudos avaliando tecido gengival mostraram aumento da expressão de CCL5 associado ao aumento da inflamação gengival de indivíduos com periodontite crônica (Gemmell et al., 2001; Kabashima et al., 2002), além de aumento da expressão na periodontite crônica e agressiva quando estes indivíduos foram comparados com indivíduos controle (Garlet et al., 2003). Avaliações do fluido gengival crevicular também mostraram aumento da expressão de CCL5 em indivíduos com periodontite agressiva, quando comparados com indivíduos saudáveis. Além disso, a expressão de CCL5 foi positivamente correlacionada com medidas de profundidade de sondagem e perda de inserção (Emingil et al., 2004). A quimiocina CCL5 e seu receptor CCR5 foram detectados no tecido gengival de pacientes com doença periodontal. Neste caso também foi observado diminuição da expressão de CCL5 no fluido gengival após terapia periodontal (Gamonal et al., 2001).

2.6.4 CCL19

A resposta a determinadas quimiocinas parece ser uma importante via de caracterização da maturação das CDs. Assim, CDs passam a não responderem a determinadas quimiocinas e se tornam sensíveis a uma única quimiocina, a CCL19/MIP-3beta (Dieu et al., 1998). CCL19, também conhecida como proteína inflamatória de macrófagos-3beta (MIP-3beta), é uma quimiocina produzida pelas CDs

maduras, e também capaz de atuar nos receptores CCR7 presentes nestas CDs maduras (Sallusto et al., 1998; Charbonnier et al.,1999).

Após serem estimuladas por produtos bacterianos como LPS ou TNF-alfa, CDs sofrem maturação e passam a expressar altos níveis dos receptores CCR4, CXCR4 e principalmente CCR7. Dessa forma, tornam-se responsivas a CCL19/MIP-3beta, quimiocina altamente seletiva para esse último receptor (Sallusto et al., 1998). Além disso, tem sido observado que a produção de CCL19/MIP-3beta pelas CDs é importante no tráfico de células T no timo e de células T e B para os órgãos linfoides secundários (Kim et al.,1998). Foi demonstrado o acúmulo de CCR7/CCL19/MIP-3beta em CDs maduras presentes nas áreas ricas em células T de tecidos linfoides (Dieu et al., 1998).

Assim, CCR7 pode ser expresso por algumas CDs imaturas, também chamadas de semimaduras devido ao fenótipo intermediário, e apresenta dois ligantes CCL19 e CCL21. O ligante CCL19 é expresso por CDs maduras, enquanto o ligante CCL21 expresso por células do endotélio linfático aferente (Ohl et al., 2004; Johnson & Jackson, 2010). Contudo, CDs CCR7+ e linfócitos CCR7+ são capazes de migrar em resposta a ambos os ligantes CCL19 e CCL21 (Britschgi et al., 2010).

2.6.5 CCL20

CCL20, também conhecida como proteína inflamatória de macrófagos-3alfa (MIP-3alfa) (Zlotnik & Yoshie, 2000), está associada com a migração de monócitos, alguns subtipos de linfócitos, eosinófilos e mastócitos (Graves et al., 1999), bem como pelas CDs imaturas derivadas de células CD34+ (Dieu et al., 1998). Estudos demonstraram que a quimiocina CCL20/MIP-3alfa é constitutivamente expressa por queratinócitos das camadas epidérmicas da pele e está associada com migração de CLs CD1a+ e CL precursoras, por expressarem altos níveis de CCR6 (Charbonnier et al., 1999). Fatores de células T como CD40L, IL-17 e INF-gama induzem a expressão de fortes níveis de CCL20/MIP-3alfa pelas células epiteliais, sugerindo que sobre

condições inflamatórias a produção de CCL20/MIP-3alfa pode ter um papel importante no recrutamento de CLs na pele (Caux et al., 2000).

Hosokawa et al. (2005) demonstraram que fibroblastos gengivais humanos são induzidos a produzir CCL20 na presença de IL1-beta, TNF-alfa e LPS bacteriano. No entanto, INF-gama reduziu drasticamente a produção da quimiocina CCL20 por essas células. Também foi observada a expressão de CCR6 pelos fibroblastos gengivais humanos e que o seu ligante, CCL20, é capaz de induzir a expressão de fator de crescimento endotelial vascular pelos fibroblastos. Assim, CCL20 produzida pelos fibroblastos gengivais humanos pode estar envolvido na infiltração de células inflamatórias, promovendo a progressão da doença periodontal (Hosokawa et al., 2005).

2.6.6 CXCL8

CXCL8/IL-8 é a principal quimiocina envolvida no recrutamento de neutrófilos para os sítios inflamatórios (Baggiolini et al., 1989). Sua secreção está associada a vários tipos celulares como macrófagos, células endoteliais e epiteliais (Baggiolini et al., 1989; Wolff et al., 1998). Muitos receptores de superfície são capazes de se ligarem a CXCL8/IL-8, mas os tipos mais frequentemente estudados são CXCR1 e CXCR2, presentes nos neutrófilos (Rossi & Zlotnik, 2000; Zlotnik & Yoshie, 2000).

Os neutrófilos representam a primeira linha de defesa do hospedeiro contra infecção microbiana (Baggiolini et al., 1989; Baggiolini & Clark-Lewis, 1992; Fredriksson, 2012). Além disso, tem importante papel na manutenção da saúde periodontal (Mathur et al., 1996). Defeitos em sua função podem estar associados a várias formas de doença periodontal, bem como sua hiperatividade é relacionada à destruição periodontal (Waddington et al., 2000). Fredriksson et al. (2012), demonstraram que indivíduos com periodontite crônica apresentam uma subpopulação de neutrófilos que é mais responsivos a CXCL8/IL-8 do que indivíduos controle.

Níveis aumentados de CXCL-8/IL-8 no fluido crevicular gengival tem sido detectados em tecidos periodontais inflamados e relacionados ao aumento da

severidade da doença (Tsai et al., 1995). No entanto, Zhang et al. (2002) não observaram aumento da expressão de CXCL-8/IL-8 e níveis de neutrófilos do fluido crevicular gengival de indivíduos após 3 dias sem higienização, sugerindo que a migração de neutrófilos pelo acúmulo de placa ocorre mais tardiamente e após presença de sinais clínicos de inflamação.

2.7 Citocinas, quimiocinas e células dendríticas em periodontite crônica de fumantes

Evidências de estudos transversais e caso-controle em várias populações demonstram que fumantes têm três vezes mais probabilidades de desenvolver periodontite do que não fumantes (Johnson & Hill, 2004). O hábito de fumar está associado a indivíduos com periodontite de maior gravidade e que apresentam menor resposta às terapias periodontais (Grossi et al., 1997). Além disso, alterações microbiológicas também são observadas no periodonto de fumantes (Kamma et al., 1999; Johnson & Hill, 2004).

Indivíduos fumantes apresentam índices de placa, médias de profundidade de sondagem e perda óssea periodontal significativamente maiores do que não fumantes, enquanto os índices de sangramento do sulco gengival são menores (Haffajee & Socransky, 2001). Além disso, fumantes também apresentam maiores perdas dentárias devido à periodontite crônica (Rudzinski & Banach, 2011). Adicionalmente, observa-se além da profundidade de sondagem e nível de inserção clínica alterados, menor contagem de células vermelhas do sangue e hemoglobina nos fumantes em comparação com não fumantes (Jain et al., 2012).

Um estudo avaliando o tratamento periodontal não cirúrgico observou melhora dos parâmetros clínicos em ambos fumantes e não fumantes com periodontite crônica, entretanto diminuições significativas nos níveis de antioxidantes foram observadas apenas no fluido crevicular gengival de indivíduos não fumantes (Akpinar et al., 2012). Assim, fatores ambientais como o hábito de fumar modificam a resposta do organismo à presença de placa bacteriana levando a uma maior progressão da doença periodontal (Johnson & Hill 2004).

Os primeiros estudos avaliando a relação do fumo com CDs foram feitos no tecido pulmonar. Foi demonstrado que o hábito de fumar é capaz de afetar os mecanismos de defesa respiratórios inibindo, dentre outros fatores, o número de CDs do pulmão (Robbins et al., 2004). Estudos em tecido pulmonar também demostram que a expressão de quimiocinas e citocinas são afetadas pelos componentes do cigarro (Demedts et al., 2007; Mortaz et al., 2009; Bauer et al., 2010). Bracke et al. (2006) observaram aumentos dos níveis de CCL20 no tecido pulmonar de ratos expostos aos componentes do cigarro. Em humanos, essas alterações foram confirmadas em um estudo que verificou aumento do número de CDs e dos níveis de CCL20 no tecido pulmonar de fumantes com doença pulmonar obstrutiva crônica (DPOC) quando comparado com não fumantes e fumantes sem DPOC. Além disso, observou-se que o número de CDs aumentava com a severidade da DPOC (Demedts et al., 2007). O tecido pulmonar de ratos expostos ao fumo também apresentou aumentada expressão de CCL3, CXCL2 (Mortaz et al., 2009), CCL2 e CCL7 (Bauer et al., 2010). Também foi observado que componentes do cigarro são capazes de afetar a produção de citocinas pelos macrófagos alveolares, inibindo a produção de TNF-α, MIP-1α, IL-12, enquanto IL-10 apresentava-se aumentada (Therriault et al., 2003).

Além disso, também foram avaliados os efeitos do cigarro em citocinas e CDs em tecido sinovial inflamado de pacientes apresentando artrite reumatoide. Verificou-se que a expressão do gene IL17A estava diminuída na sinóvia de fumantes. Contudo, diferenças na expressão de IL-6, IL17F, IL-22 e IL-23 não foram confirmadas (Kazantseva et al., 2012).

A diminuição de inflamação clínica no tecido periodontal é resultado de alterações da resposta imunológica em fumantes (Kumar & Faizuddin, 2011; Srinivas et al., 2012). Assim, estudos *in vitro* e *in vivo* tentam demonstrar os efeitos dos componentes do cigarro na quimiotaxia de células inflamatórias e produção de citocinas (Eichel & Shahrik, 1969; Bridges et al., 1977).

Recentemente, Yanagita et al. (2012a) demonstraram *in vitro* que CDs diferenciadas na presença de nicotina (CDni) e estimuladas por LPS induziram a diferenciação de células T CD4 naive em células Th2, enquanto as CDs não diferenciadas na presença de nicotina induziram uma resposta imune Th1. Uma significante redução na expressão de CD1a também foi observado em CDni. Além disso, outro estudo com cultura de células sanguíneas de indivíduos com periodontite crônica estimuladas por LPS de *Neisseria meningitidis* demonstrou uma relação significativamente menor no balanço de citocinas Th1/Th2 nos fumantes, sugerindo uma pronunciada resposta Th2 nesse grupo (de Heens et al., 2009). Adicionalmente, CDs do sangue periférico humano foram estimuladas com LPS de *Porphyromonas gingivalis* na presença de nicotina e observou-se menor proliferação de células T e expressão de antígeno HLA-DR, além de menor produção de IFN-ɣ, IL-10, IL-12, TNF-α e RANTES/CCL5 em comparação com CDs não estimuladas. Contudo, aumento significativo MCP-1 e MDC foram observados (Yanagita et al., 2012b).

Em indivíduos com periodontite crônica, poucos estudos avaliaram o efeito do fumo na produção de citocinas e quimiocinas pelas CDs, e variações nas metodologias usadas dificultam comparação entre os mesmos estudos (de Heens et al., 2009a; de Heens et al., 2009b; Tymkiw et al., 2011; Goutoudi et al., 2012; Yanagita et al., 2012a; Yanagita et al., 2012b). Dessa forma, amostras de sangue de pacientes com periodontite crônica demonstraram que o fumo estimula uma pronunciada proliferação de subpopulações de células tipo Th2 (de Heens et al., 2009). Enquanto, estudo avaliando o fluido crevicular gengival mostrou que o fumo diminuiu significativamente a quantidade de citocinas pró-inflamatórias (IL-1β, IL-6 e IL-12p40), quimiocinas (IL-8, MCP-1/CCL2, MIP-1 e RANTES/CCL5) e reguladores de células T e NK (IL-7 e IL-15), tanto nos sítios com periodontite crônica quanto nos controles (Tymkiw et al., 2011). IL-8 e IL-6 também foram avaliadas no fluido crevicular gengival de indivíduos com periodontite crônica. A média dessas citocinas foi significativamente maior em sítios doentes quando comparado com sítios não-doentes. No entanto, em

sítios não doentes, IL-8 foi maior em não fumantes comparado com fumantes, mas aumentou em fumantes após terapia periodontal. Enquanto em sítios doentes, fumantes apresentaram aumento apenas após terapia periodontal (Goutoudi et al., 2012).

3 - Objetivos

3.1 Objetivo Geral

O objetivo geral deste estudo foi avaliar a expressão de citocinas e quimiocinas, assim como a densidade de CDs imaturas e maduras, e densidade do infiltrado inflamatório no tecido gengival de indivíduos fumantes e não-fumantes, diagnosticados com periodontite crônica.

3.2 Objetivos Específicos:

- Comparar a expressão de citocinas, quimiocinas, densidade de CDs imaturas e maduras, densidade do infiltrado inflamatório, parâmetros clínicos periodontais e número de dentes entre os indivíduos fumantes e não-fumantes.
- Correlacionar a expressão de citocinas, quimiocinas, densidade de CDs imaturas e maduras, densidade do infiltrado inflamatório, parâmetros clínicos periodontais e número de dentes com o número de cigarros fumados por dia (C/dia) e tempo do hábito de fumar em anos (HF/anos).
- Correlacionar os diferentes perfis de citocinas pró-inflamatórias (IL-6 e TNF-α), Th1 (IL-2 e IFN-ɣ), Th2 (IL-4 e IL-10) e Th17 (IL-17A) com os parâmetros clínicos periodontais e número de dentes.
- Correlacionar os níveis de quimiocinas CCL2, CCL3, CCL5, CCL19, CCL20 e CXCL8 com os parâmetros clínicos periodontais e número de dentes.
- Analisar a densidade das células de dendríticas imaturas CD1a+ e Fator XIIIa+, e CDs maduras CD83+, presentes no epitélio do sulco, epitélio bucal e lâmina própria das amostras e correlacionar com os perfis de citocinas (pró-inflamatórias, Th1, Th2 e Th17) os níveis de quimiocinas (CCL2, CCL3, CCL5, CCL19, CCL20 e CXCL8), os parâmetros clínicos periodontais e número de dentes.

- Correlacionar a densidade do infiltrado inflamatório das amostras com a densidade das células de dendríticas imaturas CD1a+ e Fator XIIIa+, e CDs maduras CD83+, os perfis de citocinas (pró-inflamatórias, Th1, Th2 e Th17) os níveis de quimiocinas (CCL2, CCL3, CCL5, CCL19, CCL20 e CXCL8), os parâmetros clínicos periodontais e número de dentes.

4 - Hipóteses

Sugere-se uma diminuição na densidade do infiltrado inflamatório, células dendríticas imaturas e maduras na periodontite crônica em indivíduos fumantes quando comparados com indivíduos não-fumantes. Sugere-se uma diminuição nos níveis de expressão de citocinas e quimiocinas na periodontite crônica em indivíduos fumantes quando comparados com indivíduos não-fumantes. Sugere-se um aumento de citocinas, quimiocinas e células dendríticas na periodontite crônica, e que alguns desses mediadores e tipos de células dendríticas avaliados podem estar associados com estágios mais avançados da doença.

5 – Metodologia

5.1 População de estudo e critérios de inclusão e exclusão

O estudo foi submetido e aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais (COEP-UFMG) sob o número 423/11 (Anexo1). Foram recrutados 45 pacientes que assinaram o Termo de Consentimento Livre e Esclarecido (Anexo 2) para participarem do estudo. Todos os pacientes foram submetidos a exame periodontal da boca completa avaliando a profundidade de sondagem (PS), nível de inserção clínica (NIC) e sangramento à sondagem (SS) (Anexo 3). Indivíduos que apresentavam NIC≥3mm na proximal em ≥2 dentes não adjacentes foram diagnosticados com periodontite crônica. Indivíduos apresentando NIC≥ 5mm em ≥ 30% dos dentes foram diagnosticados com periodontite crônica avançada, enquanto os demais diagnosticados com periodontite crônica moderada (Tonetti & Claffey, 2005).

Para caracterização do hábito de fumar os indivíduos foram classificados de acordo com os critérios de Tomar e Asma (Tomar & Asma, 2000; Demoor et al., 2009) em fumantes (F) e não-fumantes (NF). São considerados F aqueles que já fumaram ≥100 cigarros durante a vida, e NF aqueles que não fumaram ≥100 cigarros (Tomar & Asma, 2000). Os indivíduos foram questionados sobre o número de cigarros fumados por dia (C/dia) e o tempo do hábito de fumar em anos (HF/anos) (Anexo 3). As amostras gengivais foram obtidas durante o acerto de tecido mole de extrações dentárias realizadas por razões protéticas, endodônticas ou periodontais. Os indivíduos do estudo apresentavam periodontite crônica, mas ainda não haviam começado o tratamento de raspagem e alisamento radicular. Os pacientes avaliados neste estudo não relataram doenças sistêmicas, anormalidades imunológicas ou faziam uso de medicamentos anti-inflamatórios e antibióticos.

Seis amostras foram coletadas da mucosa que recobria terceiros molares totalmente inclusos e que foram indicados para exodontia. Os indivíduos não

apresentavam periodontite crônica e as amostras foram utilizadas para controle dos níveis de citocinas e quimiocinas.

5.2 – Processamento da amostra e análise do infiltrado inflamatório

Dois fragmentos gengivais foram removidos. Um fragmento foi pesado e armazenado em solução de PBS (0,4mM NaCl, 10mM NaPO4) contendo inibidores de proteases (0,1mM PMSF, 0,1mM benzethonium clorídrico, 10mM EDTA e 0,01 mg/mL aprotinina A, pH 7,4) e Tween 20 (0,05%) na proporção de 1 ml de solução para 100 mg de tecido e armazenado a -80ºC. Para a extração das proteínas, as amostras foram em seguida homogenizadas empregando-se macerador e a seguir centrifugadas a 10.000 r.p.m por 10 min a 4ºC. O sobrenadante obtido foi utilizado para análise das citocinas através do método de citometria de fluxo (CBA - Cytometric Bead Array), e análise das quimiocinas pelo teste ELISA *sandwich.*

O segundo fragmento foi fixado em solução de formol tamponado a 10% e enviado para o Serviço de Patologia Bucomaxilofacial da Faculdade de Odontologia da UFMG para inclusão do tecido em parafina e confecção de uma lâmina de hematoxilina e eosina (HE) e confecção das lâminas utilizadas na imunoistoquímica. A avaliação quantitativa do infiltrado inflamatório foi realizada por um único observador (GRS). As células inflamatórias do infiltrado foram contadas nos cortes coradas em HE, em toda a lâmina. Para isso, os campos foram fotografados através do sistema de captura de imagens da Zeiss, utilizando o microscópio Axio Scoup A1 (Zeiss, Göttingen, Germany) acoplado a uma câmera fotográfica, em um aumento de 400x. As imagens digitalizadas foram então utilizadas para contagem das células inflamatórias através do programa Image Tool 3.0 (University of Texas Health Science Center, San Antonio, Texas, USA). Foram obtidas as médias de densidade de células por mm^2 para cada amostra. Em seguida, os dados foram agrupados em percentis e as amostras foram dicotomizadas pela mediana e classificadas de acordo com o grau de inflamação: infiltrado inflamatório discreto e infiltrado inflamatório acentuado.

5.3 – Métodos de citometria de fluxo (CBA - Cytometric Bead Array)

As citocinas (IL-6, TNF-α, IL-2, IFN-ɣ, IL-4, IL-10 e IL-17A) foram simultaneamente medidas pelo método CBA, nas amostras de tecido gengival. O CBA é um método de captura de proteínas, contendo esferas de tamanho e fluorescência conhecidos, podendo assim capturar múltiplas proteínas simultaneamente. Cada esfera está associada a um único anticorpo específico para cada citocina analisada. Assim, as esferas conjugadas a fluorocromos emitem fluorescência. A concentração de cada citocina é obtida pela intensidade de fluorescência emitida. O Kit Th1/Th2/Th17 humano (BD Biosciences, San Jose, CA, USA) foi aplicado seguindo as instruções do fabricante. O limite de detecção para cada citocinas é 0.1 pg/ml (IL-2), 0.03 pg/ml (IL-4), 1.4 pg/ml (IL-6), 0.5 pg/ml (IFN-ɣ), 0.9 pg/ml (TNF-α), 0.8 pg/ml (IL-17A), 16.8 pg/ml (IL-10). A aquisição das medidas obtidas foi feita com o citrômetro de fluxo FACSCanto II (BD Biosciences, San Jose, CA, USA). A sensitividade e desempenho do instrumento foram conferidos pelo Cytometer Setup & Tracking beads (BD Biosciences, San Jose, CA, USA) antes da aquisição dos dados. Os resultados quantitativos foram gerados pelo software FCAP Array v1.0.1 (Soft Flow Inc., Pecs, Hungary).

5.4 - ELISA sandwich (Enzyme-Linked Immunosorbent Assay)

Os níveis de quimiocinas (CCL2, CCL3, CCL5, CCL19, CCL20, CXCL8) foram obtidos através do método de ELISA *sandwich, n*as amostras de tecido gengival. O ensaio ELISA *sandwich* foi realizado com kits disponíveis comercialmente (R&D Systems, Minneapolis, MN, USA) seguindo-se as recomendações do fabricante. Nesse ensaio, um anticorpo de captura é ligado a uma fase sólida na base dos poços de uma placa de 96 poços. O antígeno, então, adicionado, permite a formação de um complexo pela ligação com o anticorpo, removendo-se, com a lavagem, os antígenos que não se ligarem. Um segundo anticorpo, de detecção, é adicionado e se liga ao antígeno, formando o “sanduíche”. É adicionada solução contendo estreptoavidina-peroxidase e, posteriormente, o seu substrato, o-fenilenodiamina (OPD) e H_2O_2. A

reação é paralizada com o uso de ácido sulfúrico 1M. A diferença na densidade óptica do produto de oxidação do OPD é detectada por colorimetria em um leitor de ELISA no comprimento de onda de 492nm. Os resultados foram expressos em picogramas de citocinas por 100mg de tecido, com base em uma curva padrão.

5.5 - Imunoistoquímica

As reações de imunoistoquímica foram feitas usando o método indireto. As CDs imaturas foram marcadas pelos anticorpos anti-CD1a e anti-Factor XIIIa. O anti-CD1a é considerado marcador de células de Langerhans, também caracterizadas pela expressão de grânulos de Birbeck, enquanto o anti-Factor XIlla é descrito como marcador de CDs dérmicas (Caux et al.,1996; Caux et al., 1997). Para análise das CDs maduras utilizou-se o anti-CD83 (Dereka et al., 2004).

Inicialmente foram obtidos, a partir de blocos de parafina, cortes histológicos de 3µm de espessura, sendo estes estendidos sobre lâminas previamente gelatinizadas e colocados em estufa a 55 ºC durante 24 horas. Os cortes foram desparafinizados em banhos de xilol: um de 24 horas e outro de 15 minutos à temperatura ambiente. Em seguida, os cortes foram hidratados em banhos decrescentes de etanol (100%, 90% e 70%) por 5 minutos cada.

Para a recuperação antigênica, as lâminas foram imersas em soluções apresentadas na tabela 2 e incubadas em panela de pressão (Manttra Inc, Índia) por 12 minutos, à 98^{o}C para anti-CD1a and anti-CD83, e em banho-maria, por 30 minutos à 98^{o}C para anti-Fator XIIIa. Após a recuperação, essas lâminas foram mantidas por 20 minutos em temperatura ambiente para resfriar, sendo lavadas em cinco banhos de água destilada.

Tabela 2- Anticorpos primários utilizados, diluição, temperatura e tempo de incubação, agente de recuperação antigênica.

Anticorpo (Clone)	*Diluição*	*Temperatura de incubação/ tempo*	*Agente de recuperação / pH*
CD1a (MTB1)[1]	1:20	Ambiente/ 1 hora	Tampão 3 em 1/ 9,0[4]
Fator XIIIa(AC-1A1)[2]	1:500	Ambiente/1 hora	Ácido citric / 6,0[5]
CD83 (1H4b)[3]	1:100	Ambiente/ 1 hora	Tampão 3 em 1/ 9,0[4]

[1](BioSB, Santa Bárbara, CA, EUA); [2](BioSB, Santa Barbara, CA, USA); [3](Abcam, Cambridge, UK); [4]Spring bioscience, Pleasanton, CA, USA; [5]10 mM ácido cítrico (Lab Synth, Diadema, Brazil, A2270).

A fim de inibir a peroxidase endógena, os cortes foram incubados em dois banhos de peróxido de hidrogênio a 10 volumes por 15 minutos cada, em seguida, lavados em cinco banhos de água destilada. Seguiu-se então a incubação em três banhos em solução tampão com 2,42 g de TRIS hidroximetil amino metano PA (Nuclear, São Paulo, Brasil, código 318768) e 8,5 g de cloreto de sódio PA (Vetec Química Fina, Rio de Janeiro, Brasil, código 106) em 1000 mL de água destilada (TRIS-HCL), pH 7,4 por cinco minutos. Os cortes foram então incubados com os anticorpos primários monoclonais cujas diluições, temperaturas e tempo de incubação encontram-se dispostos na tabela 2. Após a incubação, os cortes foram submetidos a três banhos de solução tampão TRIS-HCL, pH 7,4. Em seguida, foi feita a incubação com o anticorpo secundário e complexo terciário usando para o anticorpo CD1a o sistema Advance HRP (Dako, Carpinteria, CA, EUA), para o CD83 o sistema Reveal (Spring bioscience, Pleasanton, CA, USA) e para o Fator XIIIa o Kit LSAB+ Peroxidase (DAB, Sigma-Aldrich, St. Louis, MO) à temperatura ambiente em câmara úmida por 30 minutos e lavagem em três banhos em solução tampão TRIS-HCL, pH 7,4. A revelação da reação foi executada com sistema de substrato cromógeno constituído de uma gota de DAB-cromógena (3,3' diaminobenzidina em solução) em 1ml de substrato tamponado (Dako, Carpinteria, CA, EUA) por 2 minutos. Após a lavagem

com 5 banhos de água destilada, a contra-coloração foi feita com solução de hematoxilina de Mayer por 40 segundos. Os cortes foram então desidratados em soluções de etanol em concentrações crescentes: 70%, 90% e 100% por cinco minutos e mais duas vezes a 100% por dez minutos, diafanizadas em um banho de cinco e outro de dez minutos em xilol. As lâminas serão montadas com lamínulas de vidro e Permount (Fisher Scientific, Fair Lawn, EUA).

5.6 Análise da imuno-expressão e contagem das células

A análise da imuno-expressão foi feita em toda a extensão do epitélio bucal (EB), epitélio do sulco (ES), lâmina própria (LP) e foram obtidas as densidades/mm^2. Para análise da densidade de células positivas/mm^2 as amostras foram fotografadas utilizando o sistema de captura de imagens da Zeiss, utilizando o microscópio Axio Scoup A1 (Zeiss, Göttingen, Germany) acoplado a uma câmera fotográfica, em um aumento de 400x. As imagens digitalizadas foram então utilizadas para contagem e medida da área obtida através do Sofware AxioVision 4.2 (Microsoft, Carl Zeiss Vision GmbH, Gottingen, Germany). A avaliação quantitativa da imunomarcação foi realizada por um único observador (GRS). De cada imagem foi obtido o número de células positivas em relação à área em mm^2.

5.7 Análise estatística

Os dados foram submetidos à análise estatística utilizando o software SPSS (versão 17.0, Chicago, IL, USA). Foram realizadas avaliações de normalidade usando o teste Shapiro-Wilks. Em amostras com distribuição normal foram aplicados os testes teste-t de Student e correlações de Pearson. Significância estatística foi alcançada quando os valores de P foram <0,05. Em amostras com distribuição não-normal foram aplicados os testes Kruskal–Wallis, e após correção de Bonferroni, significância estatística foi alcançada quando os valores de P foram <0,016. Nas amostras não-normais também foram aplicados os testes Mann-Whitney U e correlações de Spearman. As correlações foram graduadas de acordo com a correlação de Cohen (1988) leve (<0.30), moderado (0.30 a 0.50) e forte (>0.50).

Para o cálculo amostral foram considerados erros tipo I e II. Assim, foram assumidos intervalos de 95% e poder do teste de 80%. Os parâmetros dos valores de níveis de citocinas e contagem de células foram obtidos dos estudo de Behfarnia et al., (2010) e Souto et al. (2011), respectivamente. De acordo com tais estudos, o número de pacientes é consistente com a amostra do presente estudo. Para contagem de células, foi feito correlação intraclasse e os valores da correlação foram significantes ($P<0.05$)

6 - Resultados e Discussão

6.1 – Artigo 1 - Aceito para publicação na revista “Plos One”

TITLE: Pro-inflammatory, Th1, Th2, Th17 cytokines and dendritic cells: a cross-sectional study in chronic periodontitis

RUNNING TITLE: Cytokines and dendritic cells

KEYWORDS: **Cytokines, Cellular immunology, Chronic periodontitis, innate immunology**

Abstract

There are a limited number of studies correlating the different stages of dendritic cells (DC) maturation with cytokines in individuals presented chronic periodontitis (CP). The aim of the study was to evaluate the correlation among the expression of IL-2, IL-10, IL-4, IL-6, IFN-ɣ, TNF-α, and IL-17A with the presence of DC and mild-moderate or advanced CP. Gingival samples were obtained from 24 individuals with CP and six samples of normal mucosa (NM) overlapping third molar for controls of the levels of cytokines. Periodontal examination was performed. Immunohistochemical staining was carried out, revealing CD1a+ immature, Fator XIIIa+ immature, and CD83+ mature DCs. The inflammatory infiltrate was counted, and the cytokines were measured by flow cytometry. Densities of DCs and inflammatory infiltrate, cytokines, subtypes of CP, and clinical periodontal parameters were correlated and compared. IL-6 expression was correlated positively with the increased numbers of CD1a+ immature DCs. Levels of IL-2, TNF-α, IFN-ɣ, IL-10, and IL-17A were increased when compared with NM. The percentage of sites with clinical attachment level (CAL)>3 were positively correlated with densities of inflammatory infiltrate, and negatively correlated with densities of immature DCs. IL-6 can contribute to the increase of the immature DCs in the CP. Higher levels of IL-2, TNF-α, IFN-ɣ, IL-10, and IL-17A cytokines were observed in CP. Higher densities of inflammatory infiltrate, as well as lower densities of immature DCs can result in a more severe degree of human CP.

Introduction

Chronic periodontitis (CP) [1] is the most frequent form of periodontitis [2,3]. The bacterial biofilm is required, but not sufficient, for disease initiation. Major periodontal tissue destruction results from persistent host inflammatory immune reaction in response to bacteria [4-6]. The host inflammatory immune reaction begins when the recognition of the bacterial pathogens occurs by means of antigen-presenting cells, such as dendritic cells (DCs) [7].

Myeloid DCs, also known as conventional DCs, present a strong capability of capturing antigens, which enables them to stimulate T cells [8]. In this context, in CP, DC activation occurs after coming into contact with lipopolysaccharide (LPS) [9,10] or immune complexes [11] produced by periodontal pathogens [12,13]. After activation, these DCs become mature, express co-stimulatory molecules, and produce distinct cytokine patterns, such a INF-ɣ and IL-17, which will determine the selective migration of CD4 T-helper subsets and the subsequent production of characteristic cytokines [7,11].

CD4 T-cells can be subdivided into Th1, Th2, Th17, and Treg subsets on the basis of their pattern of cytokine production [14,15]. Protective Th1-related cytokines (Interleukin (IL)-2 and interferon (IFN)-ɣ) are involved in cellular immune responses [16-20]. Th2-related cytokines (IL-4 and IL-10) are associated with humoral immunity and anti-inflammatory properties [16-20]. Th2-related cytokinees (IL-4 and IL-10) are associated with humoral immunity and anti-inflammatory properties. However, these arise in a later period of periodontitis and are involved in the chronic progression of the disease[14,17-19]. Th17 was identified as cells that recruited neutrophils and macrophages to participate and amplify the inflammatory reaction [4,13,21].

The role of T-helper cells and DCs in the pathogenesis of CP has not yet been fully clarified. There are a limited number of studies correlating the different stages of DC maturation with pro-inflammatory (IL-6 and tumor necrosis factor (TNF)-α), Th1 (IL-

2 and interferon (IFN)-ɣ), Th2 (IL-4 and IL-10), and Th17 (IL-17A) cytokines in individuals with different stages of CP. In this light, the present study aimed to investigate the correlation among the expression of pro-inflammatory, Th1, Th2, and Th17 cytokines with the presence of DCs in gingival samples of mild-moderate and advanced CP.

Material and Methods

Patients and periodontal samples

This study was approved by the Committee of Ethics in Research from Universidade Federal de Minas Gerais (UFMG), Brazil (423/11). Thirty patients provided written informed consent to participate in this study. All individuals received a full-mouth periodontal examination in which probing depth (PD), clinical attachment level (CAL), bleeding on probing, and oral hygiene index were determined [2,22]. Individuals presenting four or more teeth with one or more sites containing PD≥4 and CAL≥3 within the same site were diagnosed as CP. Individuals presenting four or more teeth with one or more sites containing CAL≥5 were diagnosed as advanced CP; otherwise, participants were diagnosed as mild-moderate CP [2]. The patients evaluated in this study reported no presence of systemic diseases or immunologic abnormalities. Individuals with smoking habit were excluded [23]. All subjects included in the study had untreated CP at the moment of the sample collection. After diagnostic of mild-moderate or advanced CP, two samples of gingival tissues were collected from each individual during extractions for prosthetic or endodontic reasons in tooth with CP and CAL≥5. The first sample was stored in a buffer (0.4 mM NaCl, 10 mM NaPO4, pH 7.4) containing inhibitors of proteases (0.1 mM PMSF – phenylmethylsulfonyl fluoride – 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4, at a ratio of 1 ml of solution per 100 mg of tissue to perform the cytometric bead array (CBA). The second sample was set in 10% buffered formalin, histologically processed, sectioned, and stained with hematoxylin and eosin or

subjected to immunohistochemistry. Due to losses during processing of samples, 24 samples were examined by means of Cytometric Bead Array (CBA), and 22 samples by means of hematoxylin and eosin and immunohistochemistry.

Six samples were collected from the mucosa overlapping the third molar that had been recommended for extraction. These samples were used to control the cytokine levels.

Detection of tissue cytokines

Multiple gingival tissue cytokines (IL-2, IL-10, IL-4, IL-6, IFN-ɣ, TNF-α, and IL-17A) were simultaneously measured by flow cytometry by means of CBA. The human Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA, USA) was applied following manufacturer instructions. The limit of detection of each cytokine is 0.1 pg/ml (IL-2), 0.03 pg/ml (IL-4), 1.4 pg/ml (IL-6), 0.5 pg/ml (IFN-ɣ), 0.9 pg/ml (TNF-α), 0.8 pg/ml (IL-17A), 16.8 pg/ml (IL-10). Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument was checked for sensitivity and overall performance using Cytometer Setup & Tracking beads (BD Biosciences, San Jose, CA, USA) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary).

Inflammatory assessment

The inflammatory infiltrate in gingival tissue was measured using hematoxylin and eosin stained sections (Figure 1A). These sections were digitized using a microscope (Axio Scoup A1, Zeiss, Göttingen, Germany) at 400x magnification and interfaced to a computer. Cell counts were taken during the sectioning by a blinded examiner (GRS). The mean of inflammatory infiltrate was determined by counting the number of inflammatory cells on the lamina propria (LP) in consecutive fields of all gingival samples (field area: 0.04652 mm^2) using the Image Tool software (version 3.0, University of Texas Health Science Center, San Antonio, TX). The mean number of cells per unit area (cell number per square millimeters) was obtained. Inflammatory infiltrate density was correlated with periodontal indexes, densities of DCs, and

cytokine levels. Furthermore, data were dichotomized by medians: mild and intense inflammatory infiltrate [23]. The periodontal indexes, densities of DCs, and cytokine levels were compared between mild and intense inflammatory infiltrate groups.

Immunohistochemistry

An immunohistochemical reaction was performed using the streptavidin-biotin standard protocol. Anti-CD1a and anti-Factor XIIIa are considered markers for immature DCs. Anti-CD1a is typical epidermal Langerhans cells (LC) characterized by the expression of Birbeck granules, while Factor XIIIa is described as dermal dendritic cells [24-26] Anti-CD83 is considered a marker for mature DCs [27]. Serial sections of 3µm from paraffin-embedded blocks were deparaffinized and dehydrated. Antigen retrieval was carried out using the Dewaxing & Antigen Retrieval Buffer 4 (pH 9.0, Spring bioscience, Pleasanton, CA, USA) for 12 minutes at 98^{o}C for anti-CD1a and anti-CD83. Antigen retrieval was carried out using a 10-mM citrate buffer (Laboratory Synth, Diadema, SP, Brazil), pH =6.0, for 20 minutes at 98^{o}C for anti-Factor XIIIa. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Primary antibodies were incubated at room temperature for 1 hour. The following monoclonal antibodies were used: anti-CD1a (clone MTB1; BioSB, Santa Barbara, CA, USA), at a dilution of 1:20; anti-Factor XIIIa (clone AC-1A1; BioSB, Santa Barbara, CA, USA), at a dilution of 1:500; and anti-CD83 (clone 1H4b; Abcam, Cambridge, UK), at a dilution of 1:10. Detection was performed using the Advance HRP system (Dako, Carpinteria, CA, EUA) for CD1a, the LSAB system (Dako, Carpinteria, CA, EUA) for Fator XIIIa, and the Reveal system (Spring bioscience, Pleasanton, CA, USA) for CD83. Counter-staining was performed using 3.3'-diaminobenzidine tetrahydrochloride chromogen (DAB, Sigma-Aldrich, St. Louis, MO) and Mayer hematoxylin.

Immunoexpression analysis and cell counts

Densities of immunolabeled cells (cell number per square millimeters) were calculated for CD1a, Factor XIIIa, and CD83. Positive cell counts were restricted to immunolabeled cells that exhibited well-defined nuclei. The slices were digitized with a

microscope (Axio Scoup A1, Zeiss, Göttingen, Germany) at 400x magnification and interfaced to a computer. Cell counts were performed throughout the sections by a blinded examiner (GRS). Areas were delineated using a mouse and measured using the software AxioVision (version 4.8, Zeiss).

Statistical analyses

SPSS statistic software (SPSS Inc., version 17.0, Chicago, IL, USA) was used for statistical analyses. Normal distribution was tested using the Shapiro-Wilks procedure. In samples with a normal distribution, the Student t test was applied and P values were <0.05. Kruskal–Wallis and Mann-Whitney U tests were used to analyze the samples with abnormal distributions. After Bonferroni correction, the statistical significance was achieved when P values were <0.016. For correlation tests, all samples demonstrated abnormal distribution, and Spearman correlation was applied; the α level was set to 0.05. The correlation was graded according to the Cohen classification as weak (<0.30), moderate (0.30 to 0.50), or strong (>0.50) [28].

Sample calculation considered both type I and II errors. For this, we assumed a 95% confidence interval, 80% power of test, and parameters of values of levels of IL17A obtained in study of periodontal disease of Behfarnia et al. [29].

Results

Clinical date

The studied population presented a mean age of 44.50±12.19 years (12 males, mean age: 45.70; and 12 females, mean age: 43.30). Periodontal clinical parameters of the samples are presented in Table 1. In accordance with periodontal parameters for cytokine analysis and DC evaluation, individuals were classified as presenting either mild-moderate CP (n=10) or advanced CP (n=14 for cytokines; n=12 for DCs).

Inflammatory infiltrate cells, dendritic cells and CP

All regions of the gingival tissue were evaluated for each antibody. Factor XIIIa+ immature DCs could be observed only in the LP (Figure 2A and 2D); CD1a+ immature

DCs could only be observed in the oral epithelium (OE), sulcular epithelium (SE), and LP (Figure. 2B, 2E and 2F); while CD83+ mature DCs could be observed in the OE and LP regions (Figure 2C, 2G, 2H). CD1a+ immature DCs showed cytoplasmic processes in the OE and SE (Figure 2E and 2F, respectively), CD1a+ immature DCs in the LP (Figure 2F), Factor XIIIa+ immature DCs in the LP (Figure 2D), and CD83+ mature DCs in the OE and LP (Figure 2G and 2H, respectively), all of which presented a rounded or oval shape. The density of inflammatory cells and DCs was compared between mild-moderate CP and advanced CP (Figure 1B, 1C, 1D, 1E, 1F, 1G, and 1H). Despite the lack of statistical significance, the group of individuals with advanced CP presented slightly less inflammatory infiltrate cells and DCs than did individuals with mild-moderate CP. A statistically significant difference could be observed for the CD1a+ immature DCs of the LP (Figure 1F).

Regarding possible altered phenotypes between the female and male genders, when comparing the density of inflammatory infiltrate cells and the density of immature and mature DCs, no significant differences between genders could be observed.

Intensity of inflammatory infiltrate and DC densities

The present study evaluated whether or not the density of inflammatory infiltrate cells influenced the quantity of DCs. Eleven samples were grouped in mild inflammatory infiltrate and eleven in intense inflammatory infiltrate. The density of Factor XIIIa+ immature DCs was higher in individuals presenting an intense, as compared to mild, inflammatory infiltrate density ($P<0.05$) (table 2). For this reason, correlations between inflammatory infiltrate cells and DCs also were performed. In this regard, there was a positive correlation ($P<0.05$) between the density of Factor XIIIa+ immature DCs and inflammatory infiltrate cells. Furthermore, positive correlations ($P<0.05$) could be observed between the density of CD1a+ immature DCs in the OE and CD1a+ immature DCs in the LP with inflammatory infiltrate cells. By contrast, negative correlations ($P<0.05$) were observed between the density of CD83+ mature DCs in the OE and inflammatory infiltrate cells.

Pro-inflammatory, Th1, Th2, and Th17 cytokine profiles correlated with CP

Pro-inflammatory (IL-6 and TNF-α), Th1 (IL-2 and INF-ɣ), Th2 (IL-4 and IL-10), and Th17 (IL-17A) cytokine profiles were compared among the groups with mild-moderate CP (n=10), advanced CP (n=14), and NM (n=6) (Figure 3). IL-2, INF-ɣ, IL-10, and IL-17A levels were higher (P<0.016) in the individuals presenting mild-moderate and advanced CP, when compared to NM (Figure 3C, 3D, 3F and 3G, respectively). By contrast, TNF-α levels were higher (p<0.016) in the individuals presenting advanced CP, as compared to NM (Figure 3A). In addition, cytokines correlations was performed and presented in the table 3. Pro-inflammatory, Th1, Th2, and Th17 profiles of cytokines were correlated among themselves. Then, the samples were grouped according to the classification of CP and cytokines were correlated (table 3). No differences between females and males could be observed when comparing cytokines.

Correlations among cytokine levels, dendritic cells, inflammatory infiltrate cells, and clinical periodontal parameters of CP

Positive correlation (P<0.05) could be observed between IL-6 and the density of CD1a+ immature DCs, as well as the density of inflammatory infiltrate cells, in both the SE and LP. A positive correlation (P<0.05) could also be observed between IL-6 and the percentage of sites with PD>4. By contrast, a negative correlation (P<0.05) could be identified between IL-2 and the percentage of sites with CAL>3.

Positive correlations (p<0.05) could be observed between the percentage of sites with PD>4 and the density of inflammatory infiltrate cells, CD1a+ immature DCs in the SE, and Factor XIIIa+ immature DCs. Moreover, a positive correlation (p<0.05) could also be identified between the percentage of sites with CAL>3 and the density of inflammatory infiltrate cells. However, negative correlations (p<0.05) could be observed between the number of teeth with CAL>3 and CD1a+ immature DCs in the SE, as well as between the number of teeth with CAL>5 and CD1a+ immature DCs in the SE and LP.

Discussion

DCs are the most potent antigen-presenting cells (APCs), whose function is to initiate an adaptive immune response. Activation and maturation of DCs are induced by infectious agents and inflammatory products [7,30], as well as by host cytokines [31, 32]. Cytokines affect DC differentiation and are important in controlling states of perpetual inflammation, as can be seen in CP, when DCs are continuously exposed to antigens [33]. Prior reports have shown that, in regions such as the lymph nodes, spleen, and liver, where DCs are continually exposed to potential antigens, IL-6 avoids a state of perpetual inflammation and protects central immune organs from overstimulation [34,35]. In this regard, *in vitro* studies have shown that IL-6 plays a major role in maintaining immature DCs and in blocking DC maturation. IL-6-mediated negative feedback may well contribute to the down-regulation of the immune response initiated by pathogens or in persistent infections [36]. In fact, in the present study, the IL-6 expression in gingival tissues was positively correlated with the increased number of immature DCs in patients presenting CP. It is therefore possible to conclude that IL-6 may well contribute to the inhibition of DC maturation in human CP.

In addition, it is known that macrophages are important APCs. It was demonstrate that these cells can contribute to innate immunity employing various cytokines and chemokines [13, 37]. However, it was not possible to evaluate macrophages in the present study. It was demonstrated that responses of the macrophages and DCs in the production of cytokines and chemokines were similar for the various microbial challenges [37]. Huang et al. [37] demonstrated that only response of immature DC to polymicrobial challenge could contribute to production of IL-6 [37]. Furthermore, others studies are necessary to understand mechanisms that affect the relation between immature DCs and IL-6.

IL-6 levels showed no increase when compared with NM, whereas the levels of IL-2, TNF-α, INF-ɣ, IL-10, and IL-17A did present a significant increase. It is well-known

that, when there is an absence of cytokines, such as TNF-α, IL-12, or IFN-ɣ, the IL-6 cytokine can favor immune tolerance, in turn contributing to homeostasis [34]. By contrast, as can be witnessed with other pro-inflammatory cytokines and in inflammatory conditions, IL-6 can aggravate the inflammatory disease pattern [34]. Therefore, it can be proposed that the increased expression of IL-2, TNF-α, INF-ɣ, IL-10, and IL-17A cytokines can influence the role of IL-6, in turn causing alterations in host immune responses, thus contributing to the pathogenesis of CP.

In line with these results, the IL-6 expression proved to be positively correlated with PD. Guillot et al. [38] and Baker, et al [39] reported on the role of IL-6 in the resorption of the alveolar bone in periodontal disease. IL-6 also induces bone resorption, both alone and together with other bone-resorption agents [40]. However, Moutsopoulos et al., [10] demonstrated that only IL-17 was positively correlated with bone loss in CP, suggesting that the Th17 subset may well drive or contribute to bone destruction. It could also be observed that female mice without an IL-17 receptor proved to be much more susceptible to bone loss in periodontal disease than in males, demonstrating a gender-dependent effect of IL-17 signaling [41]. In addition, estrogen loss resulted in an IL-6-mediated stimulation of osteoclastogenesis, which suggests a mechanism for the increased bone resorption in postmenopausal osteoporosis [42]. Nevertheless, although the present study's results demonstrated that IL-17A, IL-2, TNF-α, INF-ɣ, and IL-10 levels were higher in CP when compared to NM, it was impossible to demonstrate any form of altered phenotypes between genders when comparing cytokine levels, the density of inflammatory infiltrate cells, or the densities of immature and mature DCs.

Th1-related cytokines are related to the early process of periodontal disease [19,43], while Th2-related cytokines were related to the later stages of the periodontal disease [14,19]. In addition, Th17 cells amplified the inflammatory destruction through the recruitment of inflammatory cells to the target site [4,13,21]. In the present study, IL-2 was negatively related to CAL, which is related to advanced CP, resulting in a

greater severity of the disease [2]. Accordingly, our data showed that, in individuals presenting mild-moderate CP, the Th17 cytokine (IL-17) proved to be positively correlated with the Th1 (IL-2 and INF-ɣ) cytokine profile, while in individuals presenting advanced CP these correlations were not observed. This result suggests that the balance of Th17/Th1 cytokines is involved in the amplification of inflammatory reaction, especially in the early stages of periodontal disease.

Differences in cytokines levels can be observed in accordance with the applied methodology. Analysis of the IL-6 levels in gingival crevicular fluid (GCF) of patients with CP showed a decrease in disease sites, as compared to non-diseased sites [37,44]. However, an increase in IL-6 levels could be identified in the gingival connective tissue adjacent to the intra-bony pocket [38] and the GCF of sites with CP [45-47]. The present study's data showed no significant differences in IL-4 and IL-6 but did register a significant increase in TNF-α, IL-2, INF-ɣ, IL-10, and IL-17 levels in tissues with CP when compared to the NM. By contrast, IL-4 [29] and IFN-ɣ [29,47] levels showed a significant reduction in patients with CP, as compared to those of healthy gingival samples, while other studies demonstrated an increase in IL-4 [4] and IL-6 [44] after treatment, suggesting a protective role for these cytokines.

In most studies evaluating DCs in periodontal disease, an increase in these cells can be observed in diseased tissues, as compared to healthy samples, although a decrease in the later processes of the disease can also be seen [48-55]. In the present study, positive correlations could be identified between immature DCs and probing depth. Likewise, fewer immature DCs could be found in the group of individuals with advanced CP. Although no comparisons were performed between diseased tissue and NM, these data indicate that increased numbers of immature DCs are associated with the initial stage of periodontal disease. In addition, negative correlations could be observed between immature DCs and CAL>3, as well as between immature DCs and CAL>5, thus suggesting that these cells decrease with the severity of CP.

The present study found an increase in the percentage of sites with PD>4 and CAL >3, and IL-6 levels and immature DCs with higher inflammatory infiltrate. In a previous study by our research group, an increase in immature DCs with inflammatory infiltrate was identified, while mature DCs proved to be positively correlated with mild inflammatory infiltrate in samples presenting chronic gingivitis [23]. In this study, a decrease in mature DCs, with higher inflammatory infiltrate, could be observed. This observation was also associated with the absence of these cells in the SE, a region characterized by intense inflammatory infiltrate in the adjacent LP. Therefore, the inverse relation of mature DCs with inflammatory infiltrate may well explain their absence in the SE.

In conclusion, the IL-6 can contribute to the increase of the immature DCs in the CP, in turn impacting the inflammatory response. Higher levels of IL-2, TNF-α, INF-ɣ, IL-10, and IL-17A cytokines in the gingival tissue are related to human CP, while higher densities of inflammatory infiltrate and lower densities of immature and mature DCs may well result in much more severe degree of human CP.

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Figure Legend:

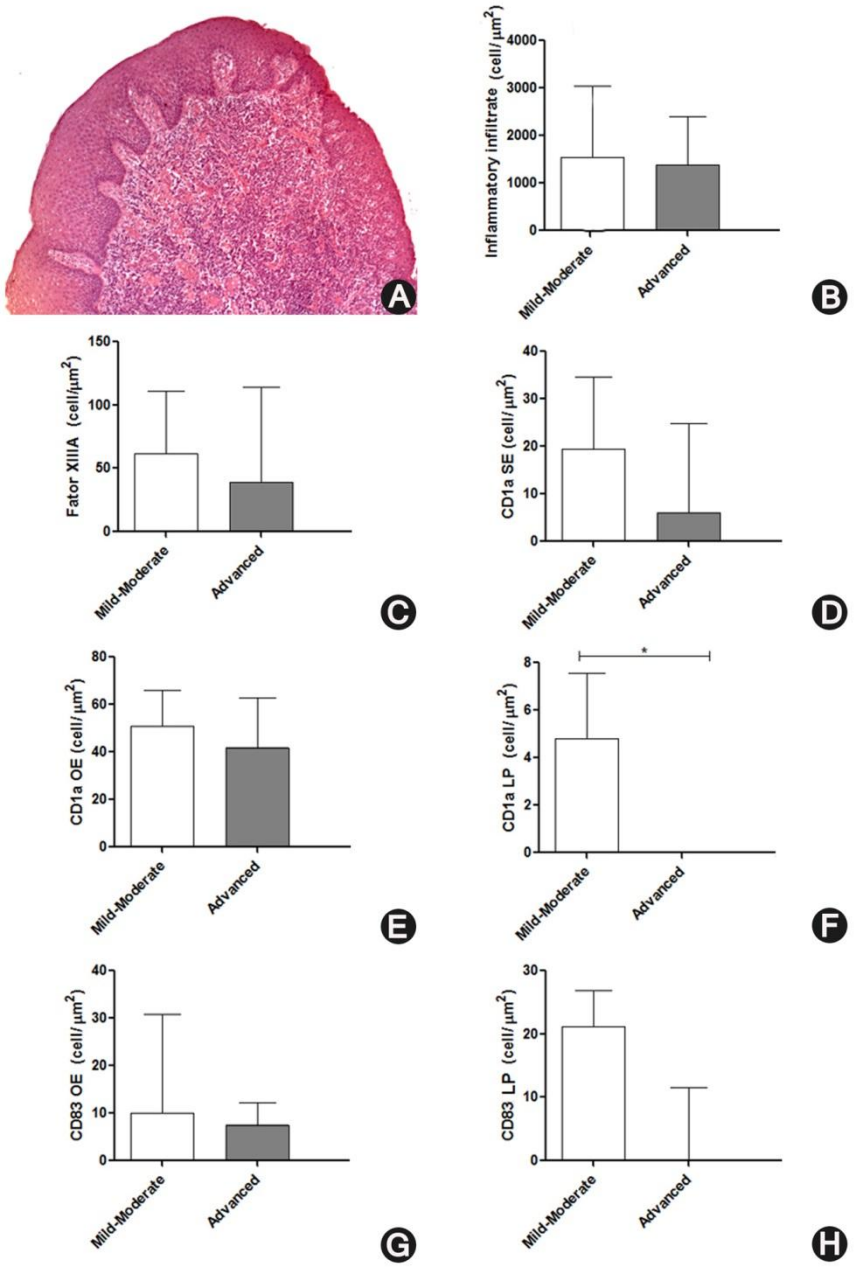

Figure 1 – Inflammatory infiltrate cells and dendritic cells (DCs) in individuals with mild-moderate or advanced chronic periodontitis A) Gingival tissue with intense inflammatory infiltrate on the lamina propria (LP) (haematoxilin-eosin, 50x original magnification). Distribution of the inflammatory infiltrate cells and DCs in groups of individuals with mild-moderate or advanced chronic periodontitis. B) Density of inflammatory infiltrate cells (cells/mm^2). C) Density of Factor XIIIa+ immature DCs

(cells/mm^2). Density of CD1a+ immature DCs (cells/mm^2) in the sulcular epithelium (D), oral epithelium (E), and LP (F). Density of CD83+ mature DCs (cells/mm^2) in the oral epithelium (G) and LP (H). *Mann-Whitney U test, statistically significant difference at $P<0.05$.

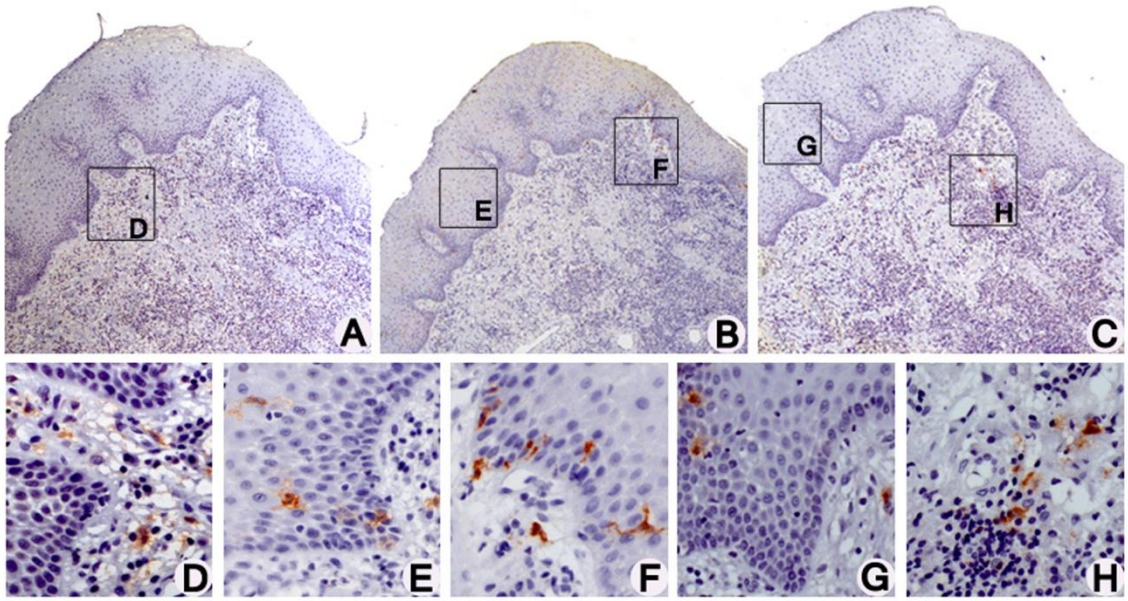

Figure 2 – Regions of the gingival tissue evaluated for immature and mature DCs. Immunohistochemical staining of Factor XIIIa+ immature DCs (A), CD1a+ immature DCs (B), and CD83+ mature DCs (C) (streptavidin-biotin, 50x original magnification). Factor XIIIa+ immature DCs could be observed in the lamina propria (LP) region (D). CD1a+ immature DCs could be observed in the oral epithelium (E), sulcular epithelium and LP regions (F). CD83+ mature DCs could be observed in the oral epithelium (G) and LP regions (H) (streptavidin-biotin, 400x original magnification).

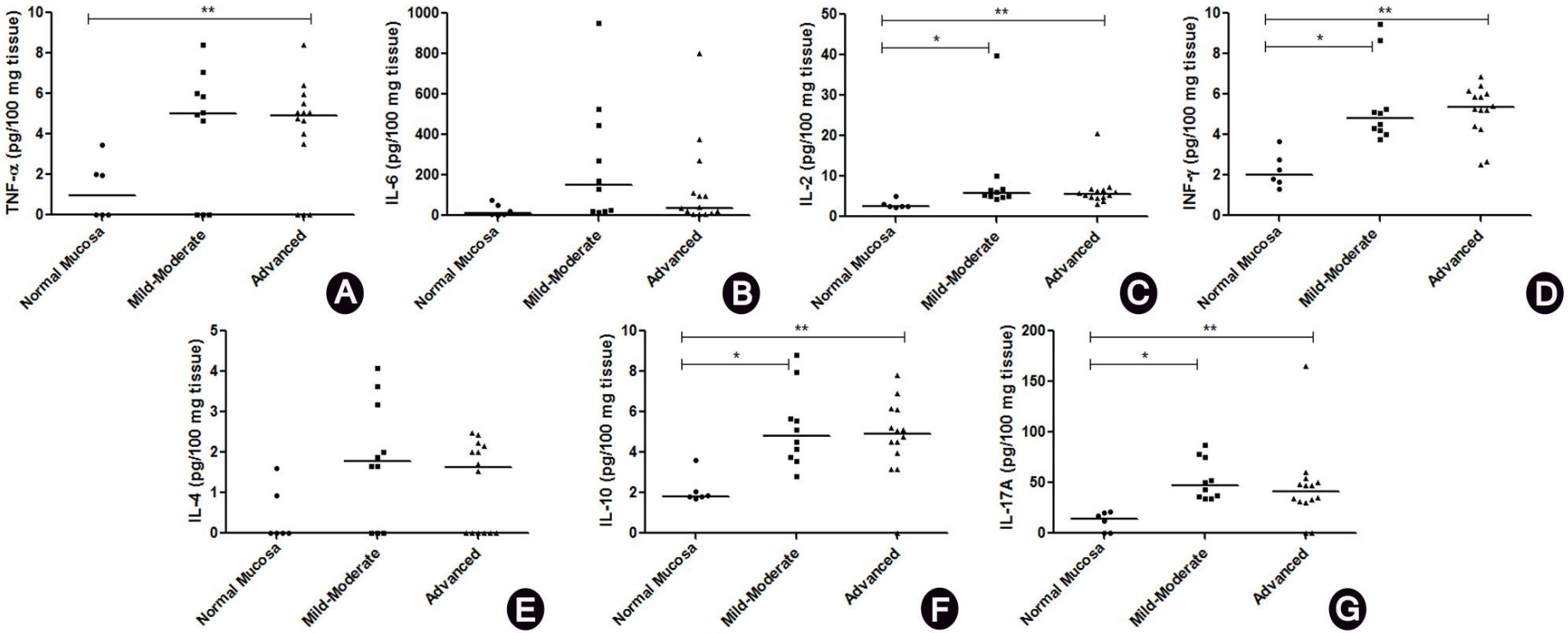

Figure 3 – Cytokine levels in individuals with mild-moderate or advanced chronic periodontitis and normal mucosa. Levels of TNF-α (A), IL-6 (B), IL-2 (C), INF-ɣ (D), IL-4 (E), IL-10 (F), and IL-17A (G). *Statistically significant difference at P<0.016 in normal mucosa versus mild-moderate chronic periodontitis. **Statistically significant difference at P<0.016, in normal mucosa versus advanced chronic periodontitis.Table 1 – Periodontal clinical parameters of samples (n=24)

Clinical Parameters	Median (minimum-maximum)
Number of teeth	16 (4-28)
Number of teeth PD>4mm	6 (4-21)
Number of teeth CAL>3mm	12 (4-27)
Number of teeth CAL>5mm	5 (1-18)
% sites PD>4mm	19.0 (4.0-100.0)
% sites CAL>3mm	80.0 (22.0-100.0)
% sites BOP	54.5 (18.0-100.0)

PD - probing depths; CAL - clinical attachment levels; BOP – bleeding on probing

Table 2 – Densities of dendritic cells (DCs) in gingival samples of individuals presenting mild and intense inflammatory infiltrate

DC (cells/mm^2)	mild inflammatory infiltrate (n=11)	intense inflammatory infiltrate (n=11)
Fator XIIIA*	38.64 (± 23.84)	99.60 (± 63.81)
CD1a SE	15.47 (± 14.88)	18.91 (± 23.42)
CD1a OE	50.91 (± 28.21)	48.87 (± 22.05)
CD1a LP	1.81 (± 2.62)	5.52 (± 7.08)
CD83 OE	11.18 (± 13.86)	17.02 (± 15.95)
CD83 LP	15.40 (± 21.72)	7.67 (± 11.11)

SE – sulcular epithelium; OE – oral epithelium; LP – lamina própria

* $p<0.05$, Student t test

Table 3 – Correlations between pro-inflammatory, Th1, Th2 and Th7 profiles of cytokines in all individuals with chronic periodontitis (CP), or in individuals with mild-moderate CP and individuals with advanced CP.

	Pro-inflammatory cytokines		Th1 cytokines		Th2 cytokines		Th17 cytokines
Individuals with CP (n=24)	TNF-α	IL-6	IFN- γ	IL-2	IL-4	IL-10	IL-17
TNF-α	1	-0.20	0.42*	0.65*	0.75*	0.70*	0.60*
IL-6		1	0.07	-0.09	0.03	-0.03	0.04
IFN-γ			1	0.59*	0.59*	0.56*	0.50*
IL-2				1	0.58*	0.73*	0.52*
IL-4					1	0.55*	0.63*
IL-10						1	0.50*
Mild-moderate CP group (n=10)	TNF-α	IL-6	IFN-γ	IL-2	IL-4	IL-10	IL-17
TNF-α	1	-0.05	0.47	0.76*	0.61	0.86*	0.65*
IL-6		1	0.51	0.00	0.23	0.20	0.34
IFN-ɣ			1	0.43	0.78*	0.67*	0.72*
IL-2				1	0.51	0.75*	0.75*
IL-4					1	0.61	0.58
IL-10						1	0.87*
Advanced CP group (n=14)	TNF-α	IL-6	IFN-γ	IL-2	IL-4	IL-10	IL-17
TNF-α	1	-0.24	0.27	0.52	0.86*	0.54*	0.64*
IL-6		1	-0.17	-0.11	-0.16	-0.21	0.15
IFN-ɣ			1	0.65*	0.37	0.38	0.44
IL-2				1	0.55*	0.60*	0.43
IL-4					1	0.42	0.66*
IL-10						1	0.40

* $p<0.05$, Spearman correlation coefficient

5.2 Artigo 2 – Submetido ao "Mediators of Inflammation"

TITLE: Effect of smoking on immunity in human chronic periodontitis

RUNNING TITLE: Smoking *versus* chronic periodontitis

KEYWORDS: Chronic periodontitis, Cytokines, innate immunology, smoking

Abstract

Objective: Evaluate the effects of smoking on dendritic cells (DCs), cytokines, clinical periodontal parameters, and number of teeth in samples of human chronic periodontitis (CP).

Background: Smoking is an important environmental risk factor associated with CP. The evaluation of smoking in inflammatory cells and mediators can contribute to more clarification about pathogenesis of human CP.

Methods: Gingival samples were obtained from 24 non-smokers and 21 smokers with CP. Periodontal examination was carried out. Immunohistochemical staining was performed to identify Factor XIIIa+ immature, CD1a+ immature, and CD83+ mature DCs. The inflammatory infiltrate was counted, and IL-2, IL-10, IL-4, IL-6, IFN-ɣ, TNF-α, and IL-17A were measured using the cytometric bead array (CBA). Inflammatory infiltrate, DCs, cytokines, classification of CP, clinical periodontal parameters, number of teeth, smoking habit in years (SH/years), and number of cigarettes smoked per day (C/day) were correlated and compared.

Results: CD83+ mature DCs decreased in the smokers group. Negative correlations could be observed between the number of C/day with levels of IL-17A and number of teeth. Correlations between smoking, periodontal disease status, and other cytokines were not observed.

Conclusions: Smoking decreases mature DCs in chronic periodontitis. Moreover, a dose-dependent relation can be observed between C/day and number of teeth and levels of IL17A observed. Smokers show a different modulation of the CP immune response.

Introduction

The response to periodontal pathogens is determined by the nature and control of both innate and adaptive immune responses. Host response factors, such as genetic and environmental factors, are essential in determining susceptibility to periodontal disease (1,2). Smoking has proven to be a major environmental risk factor associated with common forms of human chronic periodontitis (3,4). It has been reported that the smoking habit causes a reduction in the clinical signs of gingivitis (5). In a prior analysis, results demonstrated that smoking decreased both the inflammatory infiltrate and dendritic cells (DCs) in samples of chronic gingivitis (6). In addition, one study on chronic periodontitis identified a delayed neutrophil chemotaxis in smokers as compared to non-smokers (7).

The host immune response begins when DCs capture microbes and their antigens while in the immature state and stimulate a T-cell response to these antigens in their mature state (8,9). Mature DCs are involved in the production of inflammatory cytokines and in the polarized pattern of Th1/Th2/Th17 responses in periodontal disease (8,10). Moreover, DCs stimulate naive T cells to differentiate to effector T-cell subsets that may be actively involved in the immunopathogenesis of periodontal diseases (8,11).

In vitro studies demonstrate that DCs differentiated in the presence of nicotine and stimulated by lipopolysaccharide induced a differentiation of naive CD4 T cells into Th2 cells, whereas DCs differentiated without nicotine stimulated by LPS induced Th1 immune responses (11,12). Furthermore, studies evaluating venous blood samples (13,14) and gingival crevicular fluid (15,16) of individuals with chronic periodontitis verified that smoking increased the production of cytokines, such as IL-10 (13,14) and IL-1α, IL-1β, IL-6, IL-12 (p40), IL-8, MCP-1, MIP-1α, IL-2, IFN-γ, IL-3, IL-4, and IL-15 (16). A more pronounced Th2 response in smoking periodontitis patients was linked to an increased severity of the disease samples (13,14). However, the effect of smoking

on immature and mature DCs, cytokine production, clinical periodontal parameters, and number of teeth in gingival tissue samples of patients with chronic periodontitis needs to be better understood.

Therefore, the present study aimed to evaluate the effect of smoking on inflammatory infiltrate cells and immature and mature DCs, as well as in the production of pro-inflammatory (tumor necrosis factor (TNF)-α and IL-6); Th1 (IL-2 and interferon (IFN)-γ), Th2 (IL-4 and IL-10), and Th17 (IL-17A) cytokines; clinical periodontal parameters; and number of teeth in gingival tissue samples of individuals diagnosed with chronic periodontitis.

Material and methods

Patients and periodontal samples

The present study was approved by the Research Ethics Committee from Universidade Federal de Minas Gerais (UFMG), Brazil (423/11). Forty-five patients were recruitedand provided written informed consent to participate in this study. All individuals received a full-mouth periodontal examination in which probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were determined. Individuals presented proximal CAL ≥ 3mm in ≥ 2 non-adjacent teeth were diagnosed as chronic periodontitis. Individuals presenting proximal CAL ≥ 5mm in ≥ 30% of teeth were diagnosed as advanced chronic periodontitis, otherwise they were diagnosed as mild-moderate chronic periodontitis (17). Gingival samples were obtained during tooth extractions for prosthetic or endodontic reasons. The patients evaluated in this study reported no presence of systemic diseases or immunologic abnormalities.

Regarding the characterization of smoking, individuals were classified according to the criteria of Tomar and Asma (18,19) in non-smokers (NS) and smokers (S). The individuals were asked about the time of their smoking habit in years (SH/years) and how many cigarettes they smoked per day (C/day).

Two gingival samples were collected for each individual. The first sample was set in 10% buffered formalin, histologically processed, sectioned, and either stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry (IHC). The second sample was stored in buffer (0.4 mMNaCl, 10 mM NaPO4, pH 7.4) containing inhibitors of proteases (0.1 mM PMSF – phenylmethylsulfonyl fluoride – 0.1 mMbenzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4, at a ratio of 1 ml solution per 100 mg tissue to perform the cytometric bead array (CBA). The evaluated cytokines comprised mediators present in the secreted form (tissue cytokines) and previously intra-cellular cytokines. Due to losses while processing the samples, 45 samples were used in CBA; 43 samples were used to analyze the inflammatory infiltrate cells, Factor XIIIa+ immature DCs, and CD83+ mature DCs; and 42 samples were used to analyze CD1a+ immature DCs.

Inflammatory assessment

The inflammatory infiltrate in gingival tissues was measured using H&E-stained sections (Fig. 1A). The sections were digitized using a microscope (AxioScoup A1, Zeiss, Göttingen, Germany) at a magnification of 400x and interfaced to a computer. Cell counts were taken during the sectioning by a blinded examiner (GRS). The mean inflammatory infiltrate was determined by counting the number of inflammatory cells on the lamina propria (LP) in consecutive fields of all gingival samples (field area: 0.04652 mm^2) using the software Image Tool, version 3.0 (University of Texas Health Science Center, San Antonio, TX). The mean number of cells per unit area (cell number per square millimeters) was obtained. The S and NS groups were compared according to inflammatory infiltrate density.

Immunohistochemistry and cell counts

An immunohistochemistry (IHC) reaction was performed using the streptavidin-biotin standard protocol. Anti-Factor XIIIa and anti-CD1a are considered markers for immature DCs. However, only anti-CD1a is considered a marker of Langerhans cells (LC) located in the epithelium (20). Anti-CD83 is considered a marker for mature DCs

(21). Serial sections of 3µm in thickness from paraffin-embedded blocks were deparaffinized and dehydrated. Antigen retrieval was carried out using a Dewaxing & Antigen Retrieval Buffer 4, pH 9.0 (Spring bioscience, Pleasanton, CA, USA) for 12 minutes at 98^{o}C, for anti-CD1a and anti-CD83. Antigen retrieval was carried out with a 10-mM citrate buffer (Laboratory Synth, Diadema, SP, Brazil), pH =6.0, for 20 minutes at 98^{o}C for anti-Factor XIIIa. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Primary antibodies were incubated at room temperature for 1 hour. The following monoclonal antibodies were used: anti-CD1a (clone MTB1; BioSB, Santa Barbara, CA, USA) at a dilution of 1:20; anti-Factor XIIIa (clone AC-1A1; BioSB, Santa Barbara, CA, USA) at a dilution of 1:500; and anti-CD83 (clone 1H4b; Abcam, Cambridge, UK) at a dilution of 1:100. Detection was performed using the Advance HRP (Dako, Carpinteria, CA, EUA) for anti-CD1a, the LSAB system (Dako, Carpinteria, CA, EUA) for anti-FatorXIIIa, and the Reveal system (Spring bioscience, Pleasanton, CA, USA) for anti-CD83. In addition, 3.3'-diaminobenzidine tetrahydrochloridechromogen (DAB, Sigma-Aldrich, St. Louis, MO) and Mayer hematoxylin was used for counter-staining.

Densities of immunolabeled cells (cell number per square millimeters) were calculated for anti-Factor XIIIa, anti-CD1a, and anti-CD83. Positive cell counts were restricted to immunolabeled cells that exhibited well-defined cell nuclei. The slices were digitized with a microscope (AxioScoup A1, Zeiss, Göttingen, Germany) at a magnification of 400x and interfaced to a computer. Cell counts were performed throughout the sections by a blinded examiner (GRS). Areas were delineated using a mouse and measured using the software AxioVision (version 4.8, Zeiss). Factor XIIIa+ immature, CD1a+ immature, and cells CD83+ mature DCs were counted in the oral epithelium (OE), sulcular epithelium (SE), and LP regions (Fig. 1A). The densities of Factor XIIIa+ immature, CD1a+ immature, and CD83+ mature DCs were compared between the S and NS groups.

Detection of tissue cytokines

Multiple gingival tissue cytokines (TNF-α, IL-6, IL-2, IFN-ɣ, IL-4, IL-10, and IL-17A) were simultaneously measured by flow cytometry using the CBA Human Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA, USA) following manufacturer instructions. The limit of detection of each cytokine is 0.1 pg/ml (IL-2), 0.03 pg/ml (IL-4), 1.4 pg/ml (IL-6), 0.5 pg/ml (IFN-ɣ), 0.9 pg/ml (TNF-α), 0.8 pg/ml (IL-17A), 16.8 pg/ml (IL-10). Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument has been checked for sensitivity and overall performance with Cytometer Setup & Tracking beads (BD Biosciences, San Jose, CA, USA) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The S and NS groups were compared according to cytokine levels. According to the CBA manufacturer, this method presents a mean coefficient of inter-assay variation of ~5%.

Statistical analyses

SPSS Statistics software (SPSS Inc., version 17.0, Chicago, IL, USA) was used for statistical analyses. Normal distribution was tested using the Shapiro-Wilk test. In samples with a normal distribution, the Student-t test and Pearson correlations were applied. In samples that presented non-normal distributions, the Mann-Whitney U test was used for comparisons, and the Spearman correlation was applied. Differences between groups were considered significant when a probability of less than 5% significance ($P<0.05$) could be determined. Correlations were performed between SH/years and C/day with the number of teeth, percentage of sites with PD>4 mm and CAL>3 mm, cytokines, inflammatory infiltrate cells, and DCs. The correlation was graded according to the Cohen classification as weak (<0.30), moderate (0.30 to 0.50), or strong (>0.50) (22).

Sample calculation was performed and considered both type I and II errors. It was assumed a 95% confidence interval and 80% power of test. The parameters of cytokine levels and cell counting were obtained from the studies of Behfarnia (23) and Souto (6), respectively. From such studies, the number of patients is consistent with

the sample of the current manuscript. For cell counts, intraclass correlation was performed, and the value of the correlation proved to be significant (P<0.001).

Results

The samples included 24 NS (12 males and 12 females; mean age: 44.60 years (20-67)) and 21 S (11 males and 10 females; mean age: 46.70 years (28-62)). Clinical periodontal parameters and information of non-smoking and smoking individuals are presented in Table 1.

All regions of the gingival tissue were evaluated for each antibody. Cells in the LP that proved positive for Factor XIIIa showed several cytoplasmic processes (Fig. 1B), while cells that were positive for CD1a showed a rounded aspect (Fig. 1C). CD1a+ immature DCs detected in the SE and OE showed several cytoplasmic processes (Fig. 1C). Mature DCs were identified by their immunoreactivity for CD83, and round cells were detected in OE and LP (Fig. 1D).

The S group presented slightly less inflammatory infiltrate cells; Factor XIIIa+ immature DCs of the LP; CD1a+ immature DCs of the SE, OE and LP; and mature CD83+ DCs of the LP than did the NS group, although no statistically significant difference could be observed (Fig. 2A, 2B, 2C, 2E, 2G, respectively). Statistically significant difference could only be observed for CD83+ mature DCs of the OE (Fig. 2F). Cytokine analyses also demonstrated no significant differences between the NS and S groups (Fig. 3A, 3B, 3C, 3D, 3E, 3F, and 3G).

This study evaluated the correlation among the time of SH/years and C/day, number of teeth, percentage of sites with PD>4 mm and CAL>3 mm, cytokines, inflammatory infiltrate cells, and immature and mature DCs. Positive correlation was observed between SH/years with C/day ($r=0.50$; $p<0.05$). The correlation among the number of C/day and number of teeth, percentage of sites with PD>4 mm and CAL>3 mm, cytokines, inflammatory infiltrate cells, and immature and mature DCs was also evaluated. A negative correlation was found between the number of C/day with the number of teeth ($r= -0.51$; $p<0.05$) and with the IL-17A levels ($r= -0.50$; $p<0.05$). All

other correlations proved to be statistically insignificant and are presented in the table 2.

Discussion

Although a great number of studies report that in periodontal disease smoking directly affects various inflammatory cells, such as neutrophils (7,24-26), lymphocytes (27) and macrophages (28), the effect of smoking on DCs in gingival tissue samples of patients with chronic periodontitis has yet to be proven. DCs are the most important antigen-presenting cells (APCs), necessary in the interaction between innate and adaptive immunity. These cells stimulate naive T cells to differentiate in to effector T-cell subsets that may be actively involved in the immunopathogenesis of periodontal diseases (8,9,11,29). In addition, studies have shown that IL-6 plays a major role in maintaining immature DCs and in blocking DC maturation (30,31). One prior study reported that smoking decreases the inflammatory infiltrate cells and immature DCs in the gingival tissue of individuals with chronic gingivitis (6). In the present study, although only mature DCs demonstrated a statistically significant decrease in the S group, less inflammatory infiltrate cells and immature DCs could be identified in this group of patients.

DCs in the immature stage are capable of capturing microbes and their antigens, which induces phenotypic and functional changes and culminates in the transition to mature DCs (8,32). Mature DCs are involved in the production of pro-inflammatory cytokines and a polarized pattern of Th1/Th2/Th17 response in periodontal disease (8,10). It could be observed that a differentiation of LPS-stimulated DCs in the presence of nicotine induced lower levels of pro-inflammatory cytokines (11) and altered Th1 immunity (12). Likewise, *in vitro* studies also revealed that cigarette smoke constituents induced or attenuated the production of the TNF-α, IL-6, IFN-ɣ, IL-12, and IL-23 cytokines by DCs (33-36). Therefore, the present study sought to evaluate whether or not smoking provoked alterations in the production of cytokines in gingival tissue samples of individuals with chronic periodontitis. However, no significant

differences between NS and S groups have been observed. These results are in contrast with previous studies that evaluated the cytokine production in *ex vivo* whole blood cell cultures and demonstrated a more pronounced Th2 response in smoking chronic periodontitis patients (13,14). Differences between prior studies and the present study's results may well be related to methodological differences. In this *in vivo* study, it was impossible to control the individual differences between patients, the period of exacerbation of periodontal disease, or the time of smoking before performing the surgery to collect the sample, whereas in *ex vivo* studies, it is possible to use more controlled methods.

Despite the apparent reduced inflammatory status, it could be observed that S individuals presented increased periodontal attachment loss, deeper periodontal pockets, and greater gingival recession when compared to individuals who were former smokers or who had never smoked (37-41). In the present study, contrary to what was expected, smokers presented lower percentages of CAL>3 when compared with non-smokers. However, no statistically significant difference could be observed. These findings may well be related to the lower number of individuals evaluated in the present study.

Previous studies have demonstrated that evaluations considering the number of cigarettes smoked per day are a valid measure of nicotine dependence (13,41,42). In the same context, one recent published study showed that although during the gestation nicotine metabolism increased, a significant positive correlation among blood and/or salivary cotinine levels and self-reported number of cigarettes could also be observed (43). In the current study, correlations between quantity of C/day with IL-17A levels and number of teeth could be identified.

A negative and moderate correlation could be identified between the quantity of C/day and levels of IL-17A. Th17 cells are CD4 T cells that produce IL-17, IL-21, and IL-23, as well as the transforming growth factor-β (TGF-β) cytokines (44,45). Elevated levels of IL-17, TGF-β, IL-1 β, IL-6, and IL-23 messenger RNA and protein were

demonstrated in diseased tissues from patients with periodontitis when compared to healthy tissues (46). In addition, Th17 cells are highly pro-inflammatory and can mediate autoimmune diseases (45). Kazantseva (47) sought evidence for a possible mechanism linking smoking, the function of DCs, and their effects on IL17A expression. In accordance with the present study's results, Kazantseva's findings showed that rheumatoid arthritis patients who smoked presented a reduced expression of IL17A and impairment in the activation of a subset of synovial DCs, suggesting an effect of smoking on Th17 cell systems that involve changes in DC responses. In periodontal disease, the role of IL-17 seems to be controversial. Most studies suggest that the higher levels of IL-17A are associated with worse clinical periodontal parameters (48-50). In contrast, Yu (51) reveal a protective role for IL-17 in inflammatory bone loss induced by the oral pathogen *Porphyromonasgingivalis*.

In addition, a dose-dependent relation between both the amount of cigarette smoking and time of smoking habit with a development of chronic periodontitis has been demonstrated (52). In the present study, individuals who smoked greater quantities of C/day presented a high rate of tooth loss. Although it was not determined whether or not the tooth loss occurred because of the periodontal disease, the tooth loss was considered to be an important marker to determine a worse outcome for chronic periodontitis (53). Thus, it is possible to suggest that the number of C/day is associated with a worse outcome for chronic periodontitis.

In conclusion, the present study shows that the smoking habit decreases mature DCs in chronic periodontitis. A dose-dependent relation could be observed between increased C/day and decreased number of teeth and levels of IL17A. Smokers demonstrated a different modulation of the CP immune response.

Table 1 – Clinical periodontal parameters, number of teeth, and information from non-smoking and smoking individuals.

Clinical Periodontal Parameters	Non-Smoking (n=24) Mean (SD)	Smoking (n=21) Mean (SD)
Number of teeth	15.5 (±7.8)	18.5 (±7.3)
Number of teeth PD>4mm	6.8 (±4.1)	8.4 (±5.0)
Number of teeth CAL>3mm	13.1 (±6.4)	14.8 (±6.1)
Number of teeth CAL>5mm	6.5 (±4.6)	8.6 (±5.3)
% sites PD>4 mm	25.0 (±26.0)	25.0 (±25.0)
% sites CAL>3 mm	72.0 (±26.8)	55.0 (±25.0)
% sites BOP	63.0 (±28.0)	59.0 (±24.0)
Time of smoking habit/years	-	28.0 (±11.0)
Number of cigarettes/day	-	18.0 (±9.0)

SD – Standard Deviation; PD - probing depths; CAL - clinical attachment levels; BOP= bleeding on probing

Table 2 – Correlations between smoking habit in years (SH/years) and cigarettes smoked per day (C/day) with number of teeth, percentage of sites with PD>4 mm and CAL>3 mm, cytokines, inflammatory infiltrate cells, and DCs

Individuals with CP (n=24)	SH/years	C/day
Number of teeth	-0.32	-0.51[#]
% of sites with PD>4 mm	0.02	-0.14
% of sites with CAL>3 mm	0.00	-0.17
Cytokines		
TNF-α	0.36	-0.26
IL-6	0.41	0.12
IFN-ɣ	0.25	-0.30
IL-2	0.19	0.06
IL-4	-0.10	-0.22
IL-10	0.12	-0.15
IL17-A	-0.13	-0.50*
Inflammatory infiltrate cells	0.00	-0.04
Factor XIIIa+ immature DCs	-0.16	-0.06
CD1a+ immature DCs of the SE	-0.08	0.19
CD1a+ immature DCs of the OE	0.03	0.05
CD1a+ immature DCs of the LP	-0.09	0.31
CD83+ mature DCs of the OE	0.05	0.04
CD83+ mature DCs of the LP	0.22	0.38

CP – chronic periodontitis * $p<0.05$, Pearson correlation coefficient; [#] Spearman correlation coefficient.

Figure Legend:

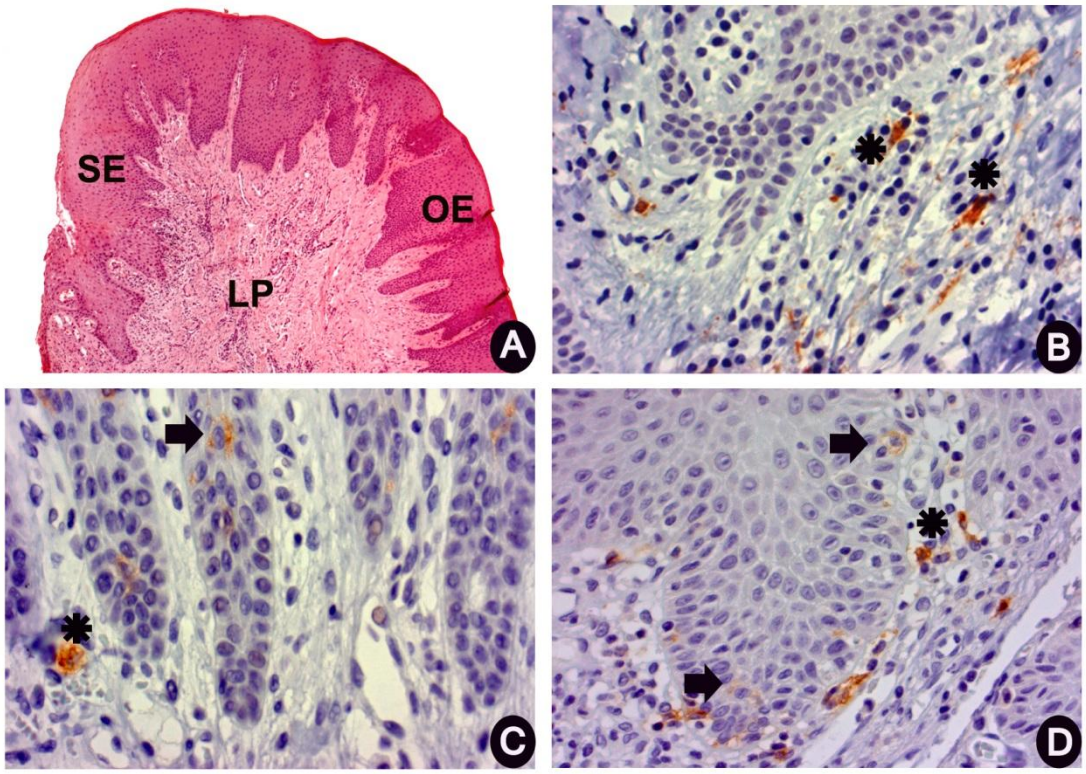

Fig. 1 – A) Gingival tissue with moderate inflammatory infiltrate on lamina propria (LP).View of sulcular epithelium (SE) and oral epithelium (OE) (haematoxilin-eosin, 50x original magnification). B) Immunohistochemical staining of Factor XIIIa+ immature dendritic cells in the LP on the asterisk. C) Immunohistochemical staining of CD1a+ immature dendritic cells in OE (arrow) and LP (asterisk). D) Immunohistochemical staining of CD83+ maturedendritic cells in OE (arrow) and LP (asterisk) (streptavidin-biotin, 400x original magnification).

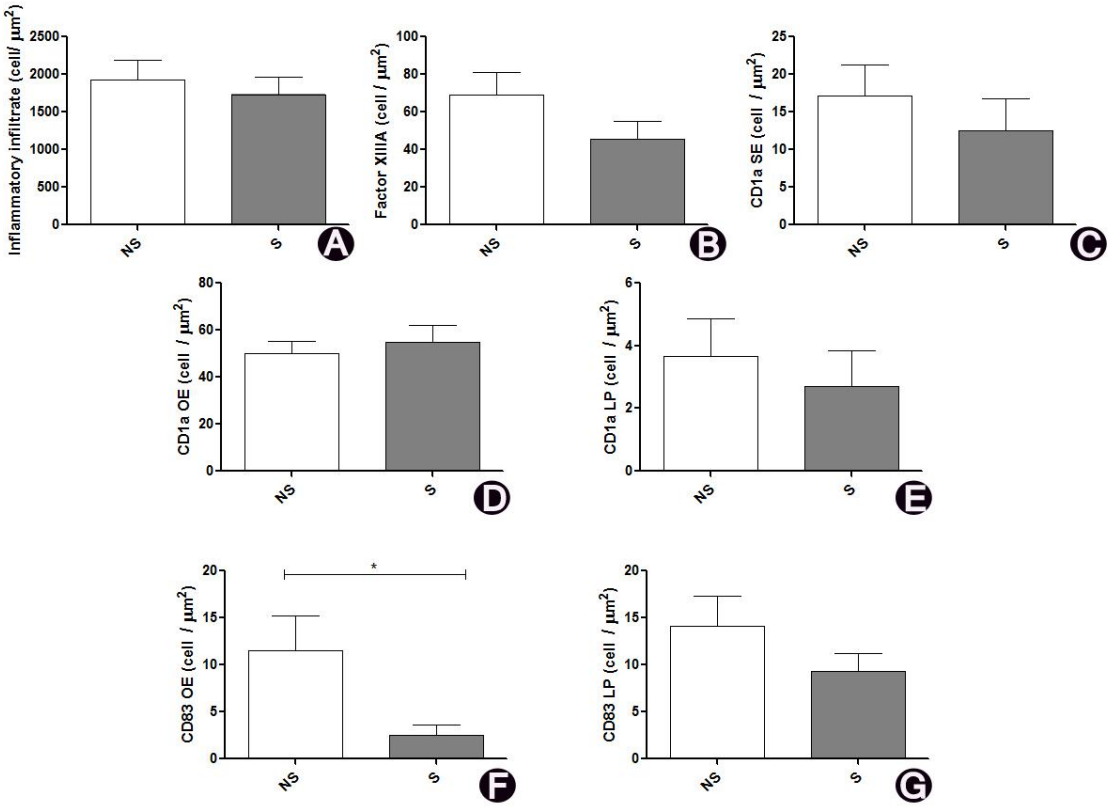

Fig. 2 – Distribution of the inflammatory infiltrate cells and DCs in NS and S with chronic periodontitis. A) Density of inflammatory infiltrate cells (cells/mm^2) in NS and S. B) Density of Factor XIIIa+ immature DCs (cells/mm^2) in NS and S.Density of CD1a+ immature DCs (cells/mm^2) in the sucular epithelium (SE) (C), oral epithelium (OE) (D) and lamina propria (LP) (E) in NS and S. Density of CD83+ mature DCs (cells/mm^2) in the OE (F) and LP (G) in NS and S. *Statistically significant difference at P<0.05.

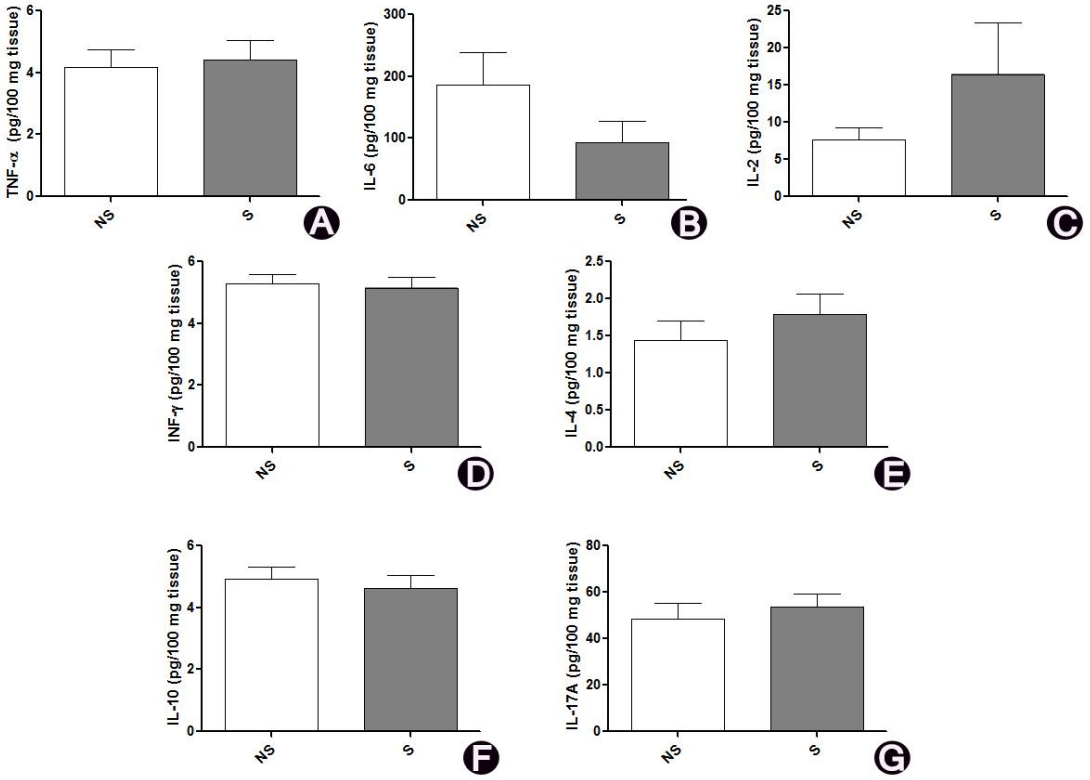

Fig. 3 – Levels of TNF-α (A), IL-6 (B), IL-2 (C), IFN-ɣ (D), IL-4 (E), IL-10 (F), and IL-17A (G) in the gingival samples of NS and S with chronic periodontitis.

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6.3 – Artigo 3 – Aceito para publicação no "Journal of Periodontology"

TITLE: Relationship between chemokines and dendritic cells in human chronic periodontitis

MANUSCRIPT CONTENT: 2724 words, three figures, and two tables.

RUNNING TITLE: Chemokines versus dendritic cells

SENTENCE SUMMARY: The increase of CD1a+ immature DCs is related to the expression of CCL2 and CCL20 in the chronic periodontitis. In addition, levels of CCL3 and CXCL8 are related to a greater severity of the disease.[*]

[*]

ABSTRACT

Aim: To evaluate the relationship between chemokines and dendritic cells (DCs) in human chronic periodontitis (CP).

Material and Methods: Gingival samples were obtained from 23 individuals with human chronic periodontitis and six samples of normal mucosa (NM) overlapping the third molar were used to control the chemokine levels. Periodontal examination was conducted. Immunohistochemistry was performed for Factor XIIIa+ and CD1a+ immature DCs, and CD83+ mature DCs. The levels of the chemokines CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 were measured in gingival tissues using enzyme-linked immune sorbent assay (ELISA). Inflammatory infiltrate, DCs, chemokines, classification of human chronic periodontitis, and clinical parameters were correlated and compared.

Results: The expression of CCL2 and CCL20 was positively correlated with increased densitites of CD1a+ DCs. CCL3 and CXCL8 were positively related to the clinical attachment level. The CCL3, CCL5, CCL19, and CXCL8 levels increased in the gingival samples of chronic periodontitis patients when compared to NM, while CCL20 levels increased in advanced CP as compared to mild-moderate CP.

Conclusion: An increased number of CD1a+ immature DCs is related to CCL2 and CCL20. CCL3 and CXCL8 chemokines are related to a greater severity of human chronic periodontitis.

Key words: Periodontitis, Inflammation and innate immunity, chemokine, dendritic cells, ELISA, immunohistochemistry

Introduction

Chronic periodontitis results from interactions between bacterial biofilm and the host inflammatory response. [1-3] This interaction triggers a cascade of inflammatory events, which result in tooth loss, [4] and is a modifying factor of the patient's systemic health. [5, 6] Therefore, as a result of the interplay between the bacterial biofilm and immune cells, the host adaptive response begins with the recognition of the putative pathogens by antigen-presenting cells, such as dendritic cells (DCs).[7] In addition, immune products are synthesized and released locally in the periodontal tissue.[8]

Chemokines or chemotactic cytokines are proteins of 8 to 10kDa that regulate the migration and activation/differentiation of leukocytes, such as DCs.[9] DCs migrate from bone marrow, through peripheral blood, to non-lymphoid tissues where they become resident cells. The recruitment of DCs from circulation to the tissues occurs in response to the production of chemokines in the inflammatory region and the expression of different chemokine receptors.[10, 11] It has been demonstrated that immature DCs respond *in vitro* to the CCL3, CCL4, CCL5, CCL7,[12] and CCL20 chemokines.[13] These chemokines transmit signals to various receptors, such as CCR1,[12, 14] CCR2,[14] CCR5, [12, 14] and CCR6.[13] Upon maturation, induced by either TNF-α, lipopolysaccharide, or CD40L, DCs lose their response to these chemokines when they acquire a sustained responsiveness to a single other chemokine, CCL19, which signalizes receptor CCR7.[13, 14]

In addition to the migration of DCs induced by a chemokine gradient under inflammatory conditions,[10, 11] it has also been demonstrated that DCs secrete higher levels of CCL2 and CXCL8 in the presence of immune-complexes. This could explain the elevated levels of these two chemokines in autoimmune disorders and their role in the recruitment of leukocytes for the involved tissues.[15] In fact, CXCL8 is intimately related to the recruitment of neutrophils for inflammatory sites, and these cells play a critical role in the innate immune response, related to the severity of chronic

periodontitis.[16] In this regard, sub-populations of peripheral neutrophils in patients with chronic periodontitis are more responsive to CXCL8 when compared to control individuals.[17]

Studies have demonstrated an increased number of DCs in gingival tissues of patients with chronic gingivitis and periodontitis.[18-24] Furthermore, increased levels of CCL2, [25, 26] CCL3, [27-29] and CCL5 [27, 30] have been observed in chronic periodontitis. These findings lead to the hypothesis of a possible correlation between chemokines and the maturation stage of DCs in samples of gingival tissues diagnosed with chronic periodontitis. In addition, chemokines may well be correlated with clinical periodontal parameters. Considering the important participation of DCs and chemokines in the pathogenesis of periodontal diseases, the core aim of the present study was to investigate the correlation among the expression of CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 chemokines with immature and mature DCs and the periodontal parameters of patients with chronic periodontitis.

Material and Methods

Patients and periodontal samples

This study was approved by the Committee of Ethics in Research from Universidade Federal de Minas Gerais, Brazil (423/11). Thirty patients provided written informed consent to participate in this study. The studied population presented a mean age of 46±9.2 years (12 males, mean age of 46; 11 females, mean age of 47). All individuals received a full-mouth periodontal examination. Probing was performed in a circumferential mode, in four sites per tooth in all teeth, by a single trained examiner (GRS). The analyzed parameters included: probing depth (PD) – distance from the gingival margin to the base of the gingival sulcus or periodontal pocket, clinical attachment level (CAL) – distance from the cementoenamel junction to the base of the gingival sulcus or periodontal pocket, and bleeding on probing (BOP) – bleeding

obtained up to 10 seconds after probing. Individuals who presented proximal CAL ≥ 3mm in ≥2 non-adjacent teeth were diagnosed with chronic periodontitis. Individuals presenting proximal CAL ≥5mm in ≥30% of their teeth were diagnosed with advanced chronic periodontitis, otherwise they were diagnosed with mild-moderate chronic periodontitis.[4] Individuals with a smoking habit were excluded.[31] The patients evaluated in this study reported no presence of systemic diseases or immunologic abnormalities but did present untreated chronic periodontitis. Samples were obtained during tooth extractions for prosthetic or endodontic reasons. Two samples were collected for each individual. The first sample was collected to perform the enzyme-linked immuno sorbent assay (ELISA) to detect chemokines. The second sample was set in 10% buffered formalin, histologically processed, sectioned, and stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry (IHC). Due to losses during the processing of samples, 23 samples were used for analyses of chemokines, while 21 samples were stained in H&E and subjected to IHC.

Six samples were collected from the normal mucosa (NM) overlapping the third molar that had been recommended for extraction. These samples were used to control the chemokine levels.

Detection of tissue chemokines

Collected samples were weighed and homogenized in a buffer (0.4 mM NaCl, 10 mM NaPO4, pH 7.4) containing inhibitors of proteases (0.1 mM PMSF – phenylmethylsulfonyl fluoride – 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4, at a ratio of 1 ml solution per 100 mg tissue. The homogenate was centrifuged (8946 g) at 4ºC for 10 min. The supernatant was then collected and used for the quantification of chemokines. The chemokine levels of CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 were evaluated by ELISA, using commercially available kit†. All assays were carried out according to

† R&D Systems, Minneapolis, MN

manufacturer instructions. The results were expressed as picograms of chemokine/100 mg of tissue.

Inflammatory assessment

The inflammatory infiltrate was measured using H&E stained sections (Fig. 1A). The sections were digitized using a microscope[‡] at a 400x magnification and interfaced to a computer. Cell counts were taken during the sectioning by a masked examiner (GRS). The mean of inflammatory infiltrate was determined by counting the number of inflammatory cells on the lamina propria (LP) in consecutive fields of all gingival samples (field area: 0.04652 mm^2) using the Image Tool[§] software. The mean number of cells per unit area (cell number per square millimeters) was obtained. The results for each sample were grouped in crescent order, and the data were dichotomized by medians. Two groups were formulated: (1) individuals with samples that presented mild inflammatory infiltrate and (2) individuals with samples that presented intense inflammatory infiltrate.[31] Periodontal indexes, densities of DCs, and chemokine levels were compared between mild and intense inflammatory infiltrate groups. Furthermore, inflammatory infiltrate density was correlated with periodontal indexes, densities of DCs and chemokine levels.

Immunohistochemistry

An immunohistochemical reaction was performed using the streptavidin-biotin standard protocol. Anti-CD1a and anti-Factor XIIIa are considered markers for immature DCs, [32] while anti-CD83 is considered a marker for mature DCs.[33] Serial sections of 3µm in thickness from paraffin-embedded blocks were deparaffinized and dehydrated. Antigen retrieval was performed using the Dewaxing & Antigen Retrieval Buffer 4[‖], pH=9.0, for 12 minutes at 98°C for anti-CD1a and anti-CD83. Antigen retrieval was carried out using 10-mM citrate buffer,[¶] pH=6.0, for 20 minutes at 98°C for anti-Factor XIIIa.

[‡] Axio Scoup A1, Zeiss, Göttingen, Germany
[§] version 3.0, University of Texas Health Science Center, San Antonio, TX
[‖] Spring bioscience, Pleasanton, CA
[¶] Laboratory Synth, Diadema, SP, Brazil

Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Primary antibodies were incubated at room temperature for 1 hour. The following monoclonal antibodies were used: anti-CD1a (clone MTB1)[#] dilution of 1:20 and specific for immature LC; anti-Factor XIIIa (clone AC-1A1)[**] dilution of 1:500 and specific for immature DC of LP; and anti-CD83 (clone 1H4b)[††] at a dilution of 1:100 and specific for mature DC. Detection was performed using the Advance HRP[‡‡] for CD1a, LSAB system[‡‡], for Fator XIIIa, and Reveal system[§§] For CD83. Both 3.3'-diaminobenzidine tetrahydrochloride chromogen[‖‖] and Mayer hematoxylin were used for counter-staining.

Immunoexpression analysis and cell counts

Densities of immunolabeled cells (cell number per square millimeters) were calculated to determine CD1a, Factor XIIIa and CD83. Positive cell counts were restricted to immunolabeled cells that exhibited well-defined cell nuclei. The slices were digitized with a microscope[¶¶] at a 400x magnification and interfaced to a computer. Cell counts were performed throughout the sections by a masked examiner (GRS). Areas were delineated using a mouse and measured using the AxioVision[##] software.

Statistical Analyses

SPSS statistic software[***] was used for statistical analyses. Normal distribution was tested using the Shapiro-Wilks procedure. In samples with a normal distribution, the Student-t test and Pearson correlations were applied. Kruskal–Wallis and Mann-Whitney U tests were used for analyses of the samples with non-normal distributions. After appling the Bonferroni correction, the statistical significance was achieved when P values were <0.016. The correlation was graded according to the Cohen classification as weak (<0.30), moderate (0.30 to 0.50), or strong (>0.50).[34] Chemokine levels

[#] BioSB, Santa Barbara, CA
[**]BioSB, Santa Barbara, CA
[††]Abcam, Cambridge, UK
[‡‡] Dako, Carpinteria, CA
[§§] Spring bioscience, Pleasanton, CA
[‖‖] DAB, Sigma-Aldrich, St. Louis, MO
[¶¶] Axio Scoup A1, Zeiss, Göttingen, Germany
[##] version 4.8, Zeiss
[***] SPSS Inc., version 17.0, Chicago, IL

expressed as picograms of chemokine/100 mg of tissue were correlated with the number of DCs expressed in densities (cells/mm^2). Chemokine levels were compared between individuals diagnosed with mild-moderate and advanced chronic periodontitis, and NM. Chemokine levels were compared between individuals with mild and intense inflammatory infiltrate in gingival samples.

Sample calculation was performed and considered both type I and II errors. A 95% confidence interval and an 80% power of test were also applied. The parameters of chemokine levels and cell counts were determined according to findings from Tymkiw et al. [35] and Souto et al. [31]

Results

Periodontal clinical parameters of the samples are presented in Table 1.

Immunohistochemistry was performed and all gingival regions were evaluated for each antibody. Factor XIIIa+ immature DCs could be observed in the LP region, CD1a+ immature DCs in the oral epithelium (OE), sulcular epithelium (SE), and LP regions, and CD83+ mature DCs in the OE and LP (Fig. 1A). Cells that proved to be immunoreactive for anti-Factor XIIIa were observed as rounded cells on the LP (Fig. 1B). Anti-CD1a cells showed cytoplasmic processes on the OE (Fig. 1C) and SE, whereas rounded cells were observed on the LP. For anti-CD83, rounded cells were observed on the LP (Fig. 1D) and occasionally on the OE. There were positive correlations between CCL2 and CD1a+ immature DCs in the OE ($P<0.05$), and between CCL20 and CD1a+ immature DCs in the SE and LP ($P<0.05$) (Table 2).

The chemokine levels of CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 were correlated among themselves, and results were presented in the table 2.

In accordance with periodontal parameters, for chemokine analysis, individuals were classified as presenting mild-moderate chronic periodontitis (n=10) or advanced chronic periodontitis (n=13). The chemokine levels were compared among the groups with mild-moderate and advanced chronic periodontitis, and NM (n=6) (Fig. 2). CCL3

and CCL5 levels were higher (P<0.05) in the individuals presenting mild-moderate and advanced chronic periodontitis when compared to NM (Fig.2B and 2C, respectively). CCL19 and CXCL8 levels were increased in the individuals presenting advanced chronic periodontitis as compared to NM (Fig. 2D, and 2F, respectively). CCL20 levels were increased in the individuals presenting advanced chronic periodontitis when compared to mild-moderate chronic periodontitis (Fig. 2E). In addition, there were positive correlations between the percentage of sites with CAL>3 and the chemokine levels of CCL3 and CXCL8 (P<0.05).

No differences could be observed when chemokine levels were compared between samples with mild and intense inflammatory infiltrate (Fig. 3).

Discussion

Chemokines are small molecular mass proteins (8–10 kD) that regulate leukocyte migration and activation.[9] They are secreted by activated leukocytes themselves, as well as by endothelial and epithelial cells upon inflammatory stimuli.[13, 36] It has been demonstrated in an *in vitro* study that DCs migrate in response to chemokines, such as CCL2, CCL3, CCL19, and CCL20, indirectly initiating the immune response. [36] Therefore, specific chemokines could determine the migration of the DCs at different maturation stages. However, which chemokines are related to the concentrations of immature or mature DCs in gingival tissues diagnosed with chronic periodontitis could not be determined. In the current study, the hypothesis proposed in the introduction proved to be affirmative when positive correlations could be observed between CCL2 and CCL20 with CD1a+ immature DCs.

CCL2 is secreted at sites of antigenic stimulation and is capable of recruiting monocytes and T-lymphocytes from the bloodstream into inflammatory lesions. [37, 38] The production of high levels of CCL2 by basal keratinocytes is capable of stimulating the recruitment of DCs, in special Langerhans cells, in the presence or absence of inflammation.[39] In addition, CCL2 influences the expression of cytokines related to T

helper responses. [38, 40] Therefore, CCL2 influences both innate immunity, through effects on monocytes, and adaptive immunity, through the control of T helper cell polarization. [40] By contrast, CCL20 is produced constitutively by human keratinocytes and is involved in the chemoattraction of immature DCs. [41] It has been demonstrated that CCL20 was expressed on the basal layer of gingival epithelial cells, microvascular endothelial cells, and areas of inflammatory cells and may also play a role in T-cell recruitment to periodontal diseased tissue.[42] Although it has been demonstrated that CCL2 and CCL20 transmit signals to different receptors, such as CCR2 [14] and CCR6 [13], respectively, in the present study, positive correlations between CCL2 and CCL20 were observed. However, no differences could be observed in the levels of CCL2 and CCL20 among individuals who presented chronic periodontitis and NM. In contrast, levels of CCL20 proved to be higher in individuals diagnosed with advanced chronic periodontitis when compared to individuals diagnosed with mild-moderate chronic periodontitis. Therefore, it is possible to suggest that both CCL2 and CCL20 contribute to the migration of immature DCs in healthy gingival tissues or diagnosed with chronic periodontitis. However, only CCL20 increases with a greater severity of the disease. In addition, it can be inferred that CCL20 plays a more important role in the migration of dendritic cells in advanced stages of chronic periodontitis. However, other studies are warranted to better understand and certificate these mechanisms.

Chemokine responsiveness appears to be one important functional feature that characterizes the stage of DC maturation. An *in vitro* study demonstrates that immature DCs respond to CCL3, CCL5, and CCL20. Upon maturation, DCs lose their responsiveness to these chemokines to become sensitive to a single chemokine, CCL19. [13] Therefore, it was hypothesized that a correlation between CCL19 and CD83+ mature DC in the gingival tissue diagnosed with chronic periodontitis could be achieved. However, in this study, although CCL19 increases in advanced chronic periodontitis, as compared to NM, it was not possible to demonstrate correlations between CCL19 and CD83+ mature DC in the gingival tissue of individuals presenting

chronic periodontitis, suggesting that the effects of CCL19 on mature DCs may well occur in other sites and in different stages of migration. Further studies to evaluate the relation between CCL19 and other markers of mature DCs are also warranted.

In samples of individuals diagnosed with chronic periodontitis, a higher messenger RNA (RNAm) expression of the CCL2, CCL5, CCL19, and CCL20 chemokines, as compared to NM could be identified.[28] In samples of gingival crevicular fluid (GCF), higher CCL2 levels could be observed in patients with chronic periodontitis, when compared to healthy patietns, which correlated positively with the severity of the disease and decreased after treatment.[25, 26] In the present study, a higher expression of CCL3, CCL5, CXCL8, and CCL19 chemokine levels, when analyzed by ELISA, could be observed in chronic periodontitis as compared to NM. However, when evaluating the severity of the disease, no differences could be observed between mild-moderate and advanced chronic periodontitis for these chemokines.

Attachment loss is a gold-standard parameter to measure past experiences of periodontitis.[4] Furthermore, attachment loss evaluated through CAL>3 is an accepted measurement of the cumulative lifetime experience of periodontitis, and the increase of CAL, mainly at the proximal level, is an accepted measure of severity for chronic periodontitis. [4] The present study evalutated a possible correlation among CAL, chemokine levels, and DC densities. Positive correlations could be found between the percentage of sites with CAL>3 and those with CCL3 and CXCL8. CXCL8 is released by phagocytes and a wide variety of tissue cells upon exposure to inflammatory stimuli. It is one of the main chemoattractants for neutrophils. [43] Many studies have demonstrated an increase in CXCL8 levels in chronic periodontitis, as compared to the controls, in the GCF[44] as well as in the mRNA of gingival samples.[45] The present study also verified an increase in CXCL8 levels in chronic periodontitis. In the same context, positive correlations could be observed between CXCL8 and BOP, PD, and CAL, [44] which is in accordance with findings from the present study that identified a positive

correlation of CXCL8 and CAL>3. In contrast, this study has been unable to demonstrate correlations between DC and CXCL8. Blengio,[46] however, observed that DC maturation results in gene coding for various members of the neutrophil-attracting CXC chemokine family, such as CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8.

Additionally, CCL3 is a biologically active chemokine that is primarily known as a chemotactic agent for monocytes. CCL3 is secreted by inflammatory cells, but it also acts to stimulate osteoclasts.[47] CCL3 increases motility of mature osteoclasts, but it does not alter osteoclast resorption activity, adhesion, or survival.[48] Longitudinal studies have demonstrated that CCL3 can be used as a salivary biomarker of bone loss. Individuals with bone loss showed either a 50-fold [49] or an 18-fold increase [29] in CCL3 when compared to healthy individuals. In addition, increased CCL3 levels correlated with an increase in PD. [29, 49] Therefore, these studies may well explain the relation between CCL3 and the clinical attachment level found in the present study.

In the present investigation, both CCL3 and CCL5, when compared to NM, increased in chronic periodontitis. Positive correlations could also be observed between CCL3 and CCL5. A previous study suggested the relative redundancy of the chemokine system, such as CCL4 and CCL5, which share the CCR1 and CCR5 receptors with CCL3, presenting a similar kinetics of expression with CCL3. Absence of CCL3 in mice does not affect the development of experimental periodontal disease, most likely due to the presence of homologous chemokines, such as CCL4 and CCL5. However, the absence of chemokine CCR1 and CCR5 receptors attenuate inflammatory bone resorption.[50] By contrast, in the present study, only CCL3 correlated with CAL>3, suggesting a more important role of CCL3 in the severity of human chronic periodontitis.

Conclusions

In conclusion, an increased number of CD1a+ immature DCs is related to CCL2 and CCL20. CCL3 and CXCL8 chemokines are related to a greater severity of human chronic periodontitis.

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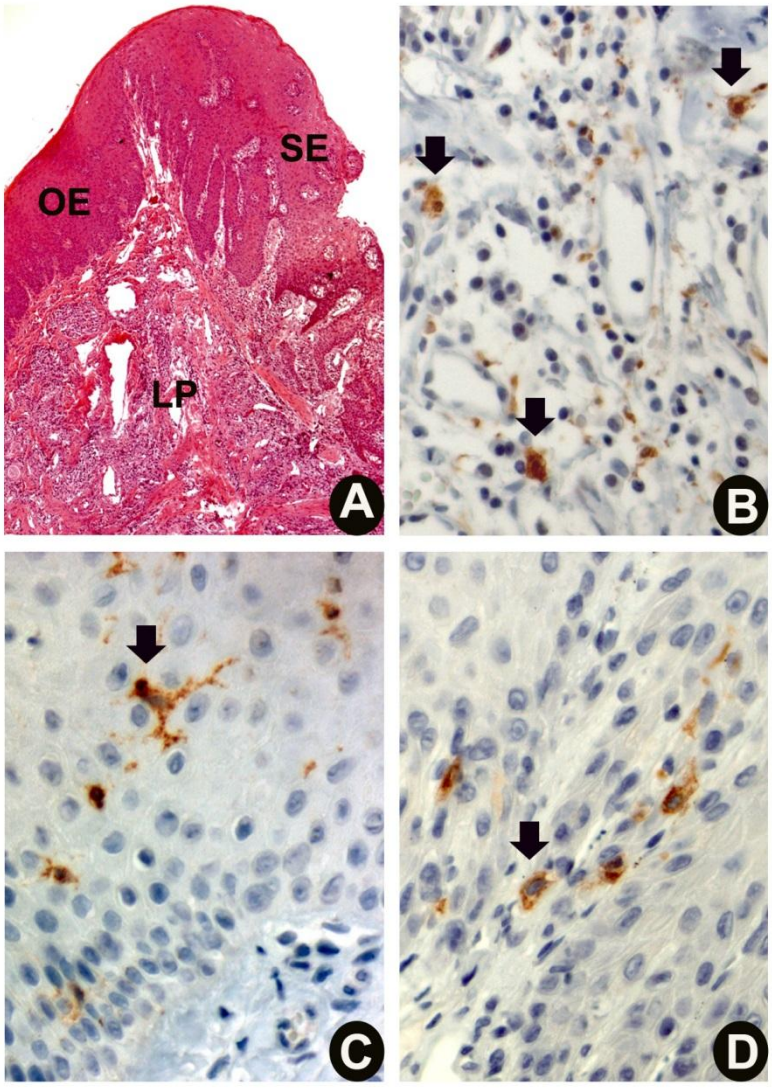

Fig. 1 – A) Gingival tissue with view of sulcular epithelium (SE), oral epithelium (OE), and intense inflammatory infiltrate on lamina propria (LP) (haematoxilin-eosin, 50x original magnification). B) Immunohistochemical staining of Factor XIIIa+ immature dendritic cells in the LP (arrow). C) Immunohistochemical staining of CD1a+ immature dendritic cells in OE (arrow). D) Immunohistochemical staining of CD83+ mature dendritic cells in LP (arrow) (streptavidin-biotin, 400x original magnification).

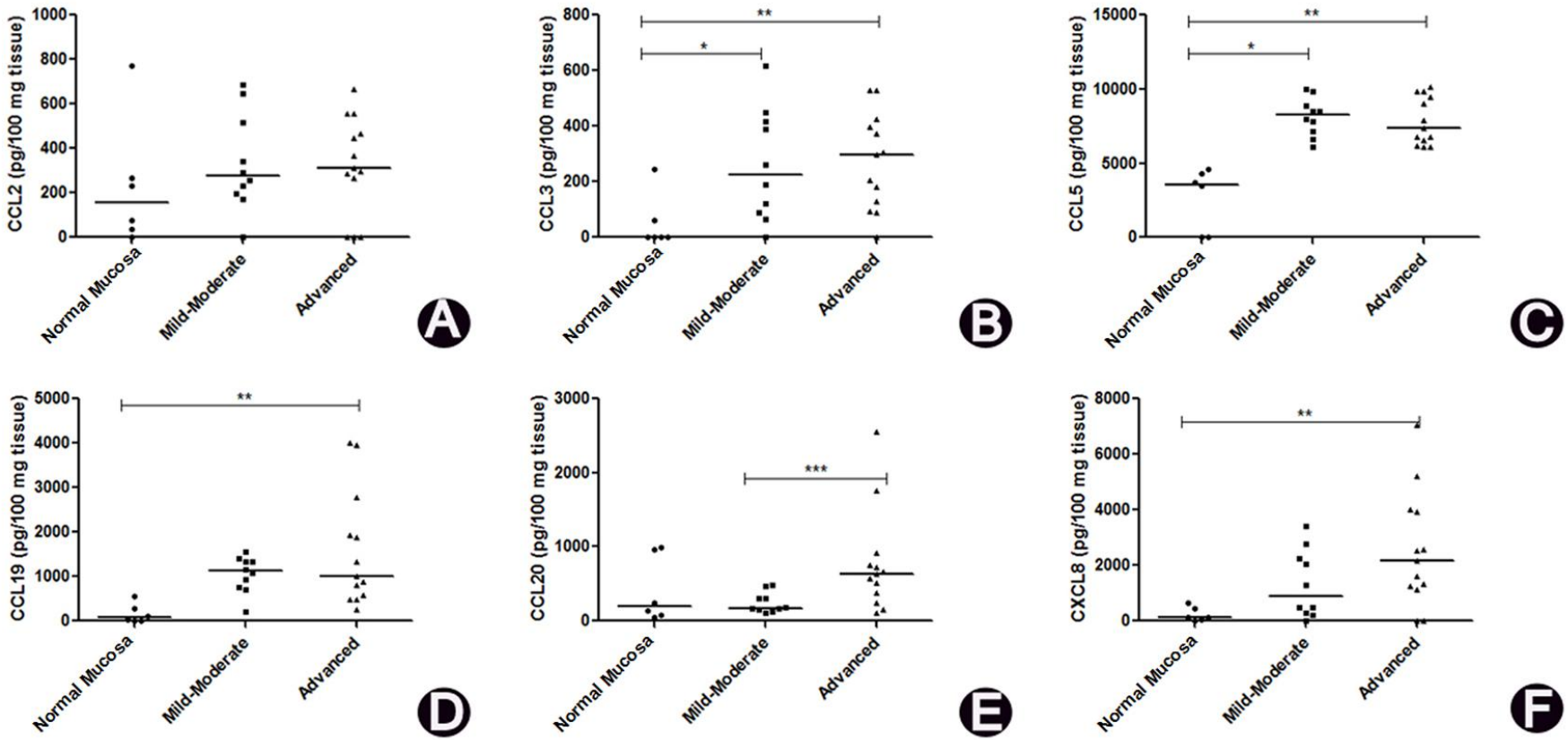
CCL2 (pg/100 mg tissue)
1000
800
600
400
200
0
Normal Mucosa
Mild-Moderate
Advanced
A
CCL3 (pg/100 mg tissue)
800
600
400
200
0
**
*
Normal Mucosa
Mild-Moderate
Advanced
B
CCL5 (pg/100 mg tissue)
15000
10000
5000
0
**
*
Normal Mucosa
Mild-Moderate
Advanced
C
CCL19 (pg/100 mg tissue)
5000
4000
3000
2000
1000
0
**
Normal Mucosa
Mild-Moderate
Advanced
D
CCL20 (pg/100 mg tissue)
3000
2000
1000
0
***
Normal Mucosa
Mild-Moderate
Advanced
E
CXCL8 (pg/100 mg tissue)
8000
6000
4000
2000
0
**
Normal Mucosa
Mild-Moderate
Advanced
F

Fig. 2 – Chemokines levels in the gingival sample of individuals with mild-moderate or advanced chronic periodontitis and normal mucosa. Levels of CCL2 (A), CCL3 (B), CCL5 (C), CCL19 (D), CCL20 (E), and CXCL8 (F). *Statistically significant difference at P<0.05 in normal mucosa *versus* mild-moderate chronic periodontitis. **Statistically significant difference at P<0.016, in normal mucosa *versus* advanced chronic periodontitis. ***Statistically significant difference at P<0.016, in mild-moderate *versus* advanced chronic periodontitis.

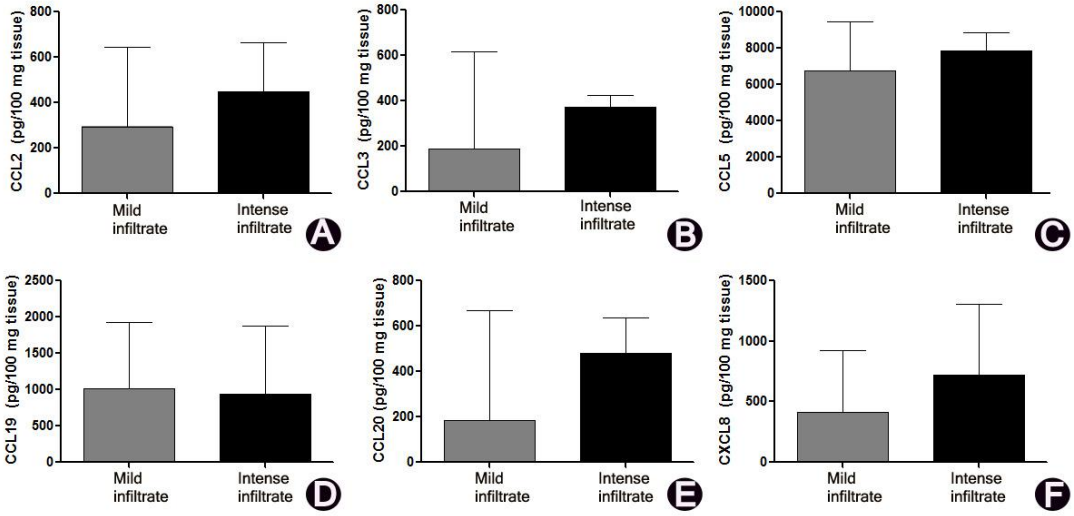

Fig. 3 – Chemokine levels in individuals with mild or intense inflammatory infiltrate in gingival samples with chronic periodontitis. Levels of CCL2 (A), CCL3 (B), CCL5 (C), CCL19 (D), CCL20 (E), and CXCL8 (F).

Table 1 – Periodontal clinical parameters of samples (n=23)

Clinical parameters	Mean (SD*)
Number of teeth	15,0 (±7,5)
Number of teeth PD>4mm	6.2 (±2.8)
Number of teeth CAL>3mm	12.5 (±5.8)
Number of teeth CAL>5mm	6.0 (±4.2)
% sites PD>4 mm	25.0 (± 27.0)
% sites CAL>3 mm	73.0 (±27.0)
% sites BOP	63.0 (±18.0)

* Standard Deviation

Table 2 – Correlations between chemokines, inflammatory infiltrate cells, and dendritic cells.

Individuals with chronic periodontitis	**Chemokine levels**					
Chemokine levels (n=23)	CCL2	CCL3	CCL5	CCL19	CCL20	CXCL8
CCL2	1	0.29	0.10	0.11	0.57*	0.52*
CCL3		1	0.72*	-0.14	-0.02	0.53*
CCL5			1	0.26	-0.17	0.42*
CCL19				1	0.30	0.13
CCL20					1	0.29
CXCL8						1
Density (cel/mm^2) (n=21)						
Inflammatory infiltrate	0.20	0.11	0.00	-0.13	-0.11	0.41
Factor XIIIa	0.18	0.12	-0.14	-0.12	-0.31	0.38
CD1a SE	0.39	-0.11	-0.30	0.19	0.52*	0.09
CD1a OE	0.48*	-0.02	-0.04	0.06	0.26	0.20
CD1a LP	0.37	-0.05	-0.29	0.06	0.53*	-0.04
CD83 OE	-0.20	-0.18	-0.08	0.09	0.06	-0.22
CD83 LP	-0.13	-0.30	-0.27	0.09	0.01	-0.03

* P<0.05 Spearman correlation coefficient

6.4 – Artigo 4 – Aceito para publicação na revista “Immunobiology”

TITLE: Smoking effect on chemokines of the human chronic periodontitis

RUNNING TITLE: Smoking, chemokines and periodontitis

KEYWORDS: Periodontal disease, chemokines, smoking

List of abbreviations:

CP – chronic periodontitis

S – smokers

NS – non-smokers

C/day – cigarette per day

SH/years – smoking habit in years

DCs – dendritic cells

PD – probing depth

CAL – clinical attachment level

BOP – bleeding on probing

ELISA – enzyme-linked immunosorbent assay

ABSTRACT

Aim: Evaluate the effects of smoking on chemokines of the human chronic periodontitis (CP).

Material and Methods: Gingival samples were obtained from 23 smokers (S) and 20 non-smokers (NS) diagnosed with CP. Periodontal examination was performed. The CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 chemokine levels were measured in gingival tissues using enzyme-linked immune sorbent assay. Chemokines were compared between S and NS, and were correlated with the numbers of cigarettes per day (C/day) and time of the smoking habit in years (SH/years).

Results: CCL3 and CXCL8 of S were significantly smaller than that found in NS subjects, whereas the CCL5 levels increased in the S group. Negative correlations could be observed between CCL19 levels and SH/year.

Conclusion: Smoking suppresses the immune response which may contribute to an increased susceptibility to periodontal disease in smokers.

Introduction

Periodontal disease is an infectious condition triggered by bacteria that colonize the tooth surface and gingival sulcus. Nevertheless, the host response plays an essential role in the destruction of connective tissue and bone (Graves, 2008). In this context, a group of inflammatory chemotactic cytokines, known as chemokines, are known to be involved in the immunopathogenesis of periodontal disease (Repeke et al., 2010; Fredriksson, 2012). Chemokines bind to specific receptors and selectively attract different cell subsets to the inflammatory site. They can also interact with classic cytokines and modulate the local immune response (Garlet et al., 2003). Although chemokines have been historically identified as leukocyte products, many of them are also produced by other cells, including keratinocytes, resident mesenchymal cells (fibroblasts and osteoblasts), dendritic cells (DCs), and endothelial cells (Graves, 2008). During periodontal disease, chemokines drive the migration and the maintenance of inflammatory cell, such as polymorphonuclear leukocytes, DCs, natural killer cells, macrophages, and subsets of lymphocytes in the gingival tissues (Garlet et al., 2003). These also stimulate monocytes and/or osteoclast progenitor cells to become active osteoclasts (Terpos et al., 2005; Al-Sabbagh et al., 2012).

Despite the clear involvement of the host inflammatory response in periodontal disease, it is known that several modifying factors can modulate such a response. In this regard, smoking is a significant and well known risk factor for chronic periodontitis (CP) (Persson et al., 2001). Tobacco use induces alterations in microbial populations (Machtei et al., 1999; Haffajee and Socransky, 2001), as well as alters the host vascular function, polymorphonuclear neutrophil phagocytosis, and the production of IL-1 (Genco and Borgnakke, 2013). In prior studies, the present research group demonstrated that smoking is related to a decrease in immature DCs densities in chronic gingivitis (Souto et al., 2011).

Considering the importance of smoking on periodontal diseases, the aim of this study was to evaluate the CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 levels in samples of gingival tissues of non-smokers (NS) and smokers (S) diagnosed with chronic periodontitis. In addition, there was also an analysis of the correlation between chemokine levels and the number of cigarettes per day (C/day) and the time of the smoking habit in years (SH/year).

Material and Methods

Patients and periodontal samples

This study was approved by the Research Ethics Committee from Universidade Federal de Minas Gerais, Brazil (423/11). Forty-three patients were recruited and provided written informed consent to participate in this study. All individuals received a full-mouth periodontal examination in which probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were determined. Individuals presenting proximal CAL≥3mm in ≥2 non-adjacent teeth were diagnosed with CP (Tonetti and Claffey, 2005). The patients reported no presence of systemic diseases or immunologic abnormalities, but they did present untreated CP. Gingival samples were obtained during tooth extractions for prosthetic or endodontic reasons.

Patients were classified according to the criteria of Tomar and Asma (Tomar and Asma, 2000; Demoor et al., 2009) in non-smokers (NS) and smoker (S). The individuals were asked about the SH/years and the C/day.

Detection of tissue chemokines

Gingival tissue samples were collected, weighed and homogenized in a buffer (0.4 mM NaCl, 10 mM NaPO4, pH 7.4) containing protease inhibitors (0.1 mM PMSF – phenylmethylsulfonyl fluoride – 0.1 mM benzethonium chloride, 10 mM EDTA and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4 at a ratio of 1 ml solution per 100 mg tissue. The homogenate was centrifuged (8946 *g*) at 4ºC for 10 min. The supernatant was collected and used to quantity the chemokines. The CCL2, CCL3,

CCL5, CCL19, CCL20, and CXCL8 chemokine levels were evaluated by double-ligand enzyme-linked immunosorbent assay (ELISA), using commercially available kits (R&D Systems, Minneapolis, MN, USA). All assays were performed according to manufacturer instructions. The results were expressed as picograms of chemokine/100 mg of tissue.

Statistical Analyses

The SPSS statistics software (SPSS Inc., version 17.0, Chicago, IL, USA) was used for statistical analyses. Normal distribution was tested using the Shapiro-Wilks test. In samples with a normal distribution, the Student-t test and Pearson correlations were applied. In samples that presented non-normal distributions, the Mann-Whitney U test was used for comparisons, and the Spearman correlation was applied. Differences between NS and S were considered significant when a probability of less than 5% significance ($P<0.05$) could be determined. Correlations were performed between SH/years and C/day with levels of chemokines. The correlation was graded according to the Cohen classification as weak (<0.30), moderate (0.30 to 0.50), or strong (>0.50) (Cohen, 1988).

Sample calculation was performed considering both type I and II errors. A 95% confidence interval (CI) and an 80% power of test were applied. The parameters of chemokine levels were obtained from findings from Tymkiw et al. (2011).

Results

The samples included 23 NS {12 males and 11 females; mean age: 45.0 years (range 17-67)} and 20 S {10 males and 10 females; mean age: 46.5 years (range 28-62)} with CP. Clinical periodontal parameters and information of NS and S individuals are presented in Table 1.

The CCL3 and CXCL8 levels fell significantly in the S group when compared to the NS group (p=0.026 and p=0.047, respectively) (Figure 1B and 1F). By contrast, the

CCL5 chemokine levels increased in the gingival tissues of S subjects when compared to NS patients (p=0.047) (Figure 1C).

The correlation coefficients between the CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 chemokines and C/day or SH/years in S subjects are presented in Table 2. A significant moderate and negative correlation (r=-0.45; p=0.030) could be observed between the CCL19 chemokine levels and SH/years.

Discussion

Prior studies have reported associations between smoking and tooth loss, periodontal attachment loss, deeper periodontal pockets and more extensive alveolar bone loss (Bergstrom, 2003; Radvar et al., 2011). Radvar et al. (2011) demonstrated that the destructive effects of smoking on periodontal tissues may well be a systemic effect on the body's immune and inflammatory responses, although additional local effects, which include vasoconstriction caused by nicotine and decreased oxygen tension, may be present. These authors also suggested that further investigations were needed to assess the underlying mechanism of the local effects of smoking on periodontal tissues. Tymkiw et al. (2011) showed a decrease in IL-6, IL-7, IL-15, CXCL-8, IP10, IL-12(p40), CCL2, CCL3, and CCL5 levels, when evaluating the gingival crevicular fluid (GCF) of smokers with severe CP. In the present study, the effect of smoking on chemokine levels was also observed, although the evaluation was performed in gingival tissue samples.

The CCL3 chemokine is a protein associated with important biological phases of bone remodeling. It stimulates monocytes and/or osteoclast progenitor cells to become active osteoclasts (Terpos et al., 2005; Al-Sabbagh et al., 2012; Taddei et al., 2013). Salivary levels of CCL3 proved to be significantly increased in subjects with CP, and showed a strong correlation with the clinical parameters of periodontal disease, such as BOP, PD≥4mm, PD≥5mm, and percentage of CAL (Al-Sabbagh et al., 2012). Given the association between smoking and extensive alveolar bone loss (Radvar et

al., 2011), higher CCL3 levels were expected in the current gingival samples of S subjects. However, lower levels of this chemokine were detected, as compared to the increased CCL5 levels in the S group. CCL5 is a chemokine related to the recruitment of leukocytes at inflammatory sites (Blengio et al., 2013). In a study from Gamonal et al. (2001) CCL5 was detected in CP patients and presented reduced levels after periodontal therapy. In line with the present findings, experimental periodontitis in mice demonstrated a cooperative role of the CCL3, CCL4, and CCL5 chemokines and their CCR1 and CCR5 receptors in *Aggregatibacter actinomycetemcomitans* induced alveolar bone loss. These authors observed that the absence of CCL3 did not affect the development of experimental periodontitis, possibly due to a redundant role of CCL4 and CCL5 chemokines, which share the binding to CCR1, and CCR5 with CCL3 (Repeke et al., 2010). These findings corroborate with the concept of the complexity of the chemokine/chemokine receptor system and suggest that, in the S group, the increase in CCL5 levels may well play a compensatory role due to the decrease in CCL3 levels, which can also be verified in human CP.

CCL2 is a chemokine responsible for the chemotaxis of monocytes and is involved in monocyte infiltration in inflammatory disease as CP (Pradeep et al., 2009). An *in vitro* study by Yanagita et al. (2012) demonstrated an increase in the production of CCL2 by monocyte-derived DCs in the presence of nicotine. By contrast, smokers diagnosed with severe CP displayed decreased amounts of CCL2 in GCF (Tymkiw et al., 2011). However, in the present study, no significant difference could be observed in the CCL2 levels in the gingival tissue of S and NS patients. Contrary to observations from Tymkiw et al. (2011) regarding the effect of smoking in CCL2 levels in GCF, the results of the present study suggest that smoking does not affect the CCL2 expression in this sample's local tissue.

Levels of CXCL8, a neutrophil chemotactic and activating factor (Blengio et al., 2013), were detected in the GCF of CP patients, and presented reduced levels after periodontal therapy (Gamonal et al., 2001). Neutrophils constitute the first line of

defense (Fredriksson, 2012), and play a critical role as part of the innate immune response (Bhadbhade et al., 2012). Significantly reduced chemotaxis in peripheral blood neutrophils on individuals with gingivitis, periodontitis, and healthy periodontal tissues was observed in smokers (Srinivas et al., 2012). These findings corroborate with the present study, which detected lower CXCL8 levels in the gingival tissue samples of the S patients. Therefore, lower CXCL8 levels may well be associated with a deficiency in the inflammatory response of neutrophils in S diagnosed with CP, suggesting an effect of smoking on the inflammatory response against oral pathogens.

The chemokine CCL20 is produced by the epithelial cells of inflamed epithelial tissues and is the most potent chemokine for the selective attraction of immature DCs *in vitro* through an interaction with the CCR6 receptor (Charbonnier et al., 1999; Dieu-Nosjean et al., 1999). In addition, one study using gingival samples of smokers with chronic gingivitis observed a decrease in immature CD1a+ DCs (Souto et al., 2011). Therefore, an association between the lower CCL20 levels and smoking was expected. However, in the present study, this relation was not confirmed. These findings suggest that smoking habits do not affect CCL20 levels or the migration of immature DCs CD1a+ in response to CCL20 in human CP.

Maturation of DCs and the migration to lymph nodes are crucial steps in the triggering of specific immune responses (Banchereau and Steinman, 1998; Sallusto et al., 1999). CCL19 represents the combination of CCR7 ligands linked to the migration of mature DCs. A deficiency of CCR7 or its ligands, CCL19 and CCL21, leads to impaired DCs migration into draining lymph nodes (Forster et al., 1999). Another *in vitro* study demonstrated that DCs conditioned with cigarette smoke extract (CSE) suppressed maturation-associated CCR7 expression (Vassallo et al., 2005). In the present study, although CCL19 did not show a statistically significant decrease in S, negative correlations could be observed between CCL19 levels and SH/years. It has been suggested that smoking results in immunosuppressive or immunostimulatory effects and depends directly upon dose and duration of exposure (Palmer et al., 2005).

The correlation between CCL19 and SH/years supports the notion that the negative effects of smoking on periodontal health also appear to be dose-related.

In conclusion, the results of the present study show that the effect of the smoking habit on periodontal tissues can be demonstrated locally by evaluating the CCL3, CCL5, CXCL8, and CCL19 chemokines. Except for CCL5, smoking suppresses the immune response and may thus contribute to an increase in the susceptibility to periodontal disease among smokers.

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Figure Legend:

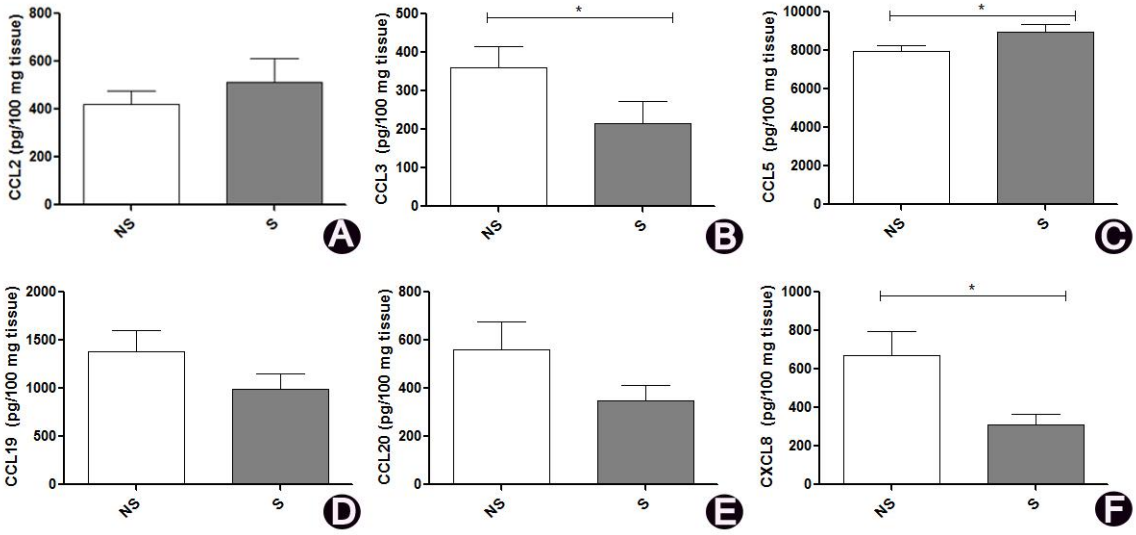

Fig. 1 – CCL2 (A), CCL3 (B), CCL5 (C), CCL19 (D), CCL20 (E), and CXCL8 levels in the gingival samples of NS and S with CP. *Mann-Whitney U test, statistically significant difference at $P<0.05$.

Table 1 – Clinical periodontal parameters of non-smokers and smokers

Clinical periodontal parameters	Non-smokers (n=23) Mean (SD)	Smokers (n=20) Mean (SD)
Number of teeth	15.0 (±7,5)	18.5 (±7.3)
Number of teeth PD>4mm	6.2 (±2.8)	8.4 (±5.0)
Number of teeth CAL>3mm	12.5 (±5.8)	14.8 (±6.0)
% sites PD>4 mm	25.0 (±7.0)	25.0 (±25.0)
% sites CAL>3 mm	39.7 (±19.4)	55.0 (±25.0)
% sites BOP	64.0 (±28.5)	60.0 (±24.0)
Time of smoking habit/years	-	30 (10-50)
Number of cigarettes/day	-	20 (3-40)

PD - probing depths; CAL - clinical attachment levels; BOP= bleeding on probing

Table 2 – Correlation coefficients between CCL2, CCL3, CCL5, CCL19, CCL20, and CXCL8 chemokine levels in smokers with the number of cigarettes per day (C/day) and time of smoking habit in years (SH/years).

Smoker (n=20)	C/day	SH/year
CCL2	-0,13	-0,00
CCL3	-0,09	-0,04
CCL5	-0,30	-0,19
CCL19	-0,01	-0,46*
CCL20	-0,15	-0,06
CXCL8	0,06	0,18

* $p<0.05$, Pearson correlation coefficient

7 – Considerações Finais

Considerando o efeito do hábito de fumar, observou-se que a densidade de CDs maduras CD83+, níveis de CCL3 e CXCL8 diminuíram no grupo de indivíduos fumantes. Enquanto os níveis de CCL5 estavam aumentados nos indivíduos fumantes. Além disso, negativas correlações foram observadas entre o número de C/dia com níveis de IL-17A e o número de dentes, e entre o tempo do HF/anos e níveis de CCL19.

No presente estudo observa-se que as expressões de IL-6, CCL2 e CCL20 foram positivamente correlacionadas com o número de CDs imaturas CD1a+. No entanto, verificou-se que estavam aumentados somente os níveis de IL-2, TNF-α, IFN-ɣ, IL-10, IL-17A, CCL3, CCL5, CCL19 e CXCL8 no tecido com PC quando comparados com MN.

Verificou-se ainda que a porcentagem de sítios com NIC≥3mm foi positivamente correlacionada com densidade do infiltrado inflamatório, níveis de expressão de CCL3 e CXCL8, e negativamente correlacionada com densidade de CDs imaturas CD1a+ e níveis de IL-2.

8 – Conclusões

Conclui-se que o hábito de fumar afeta células e mediadores inflamatórios da resposta imune de indivíduos diagnosticados com periodontite crônica. Além disso, o aumento das CDs imaturas pode estar associado com maiores níveis de IL-6, CCL2 e CCL20, embora somente os níveis de IL-2, TNF-α, IFN-ɣ, IL-10, IL-17A, CCL3, CCL5, CCL19 e CXCL8 apresentaram aumentados nos tecidos gengivais com periodontite crônica. Maior densidade de infiltrado inflamatório, níveis de CCL3 e CXCL8, e menor densidade de CDs imaturas CD1a+ e níveis de IL-2 parecem estar associados com maior gravidade da doença.

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ANEXOS

Anexo 1 – COEP

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Projeto: CAAE – 0423.0.203.000-11

Interessado(a): Prof. Ricardo Alves de Mesquita
Depto. de Clínica, Patologia e Cirurgia Odontológicas
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 22 de novembro de 2011, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Efeito do tabagismo nas células dendríticas e no padrão de expressão de citocinas e quimiocinas na periodontite crônica"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amarai
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2º andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

Anexo 2 – termo consentimento

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE ODONTOLOGIA
DEPARTAMENTO DE CLÍNICA, PATOLOGIA E CIRURGIA ODONTOLÓGICAS

Prezado paciente,

Estamos realizando uma pesquisa para avaliar a inflamação na gengiva de indivíduos com periodontite crônica, fumantes e não fumantes. Para isso precisamos analisar pequenos pedaços de gengiva que são removidos durante a cirurgia de dentes com indicação de extração. Gostaríamos de contar com sua colaboração, esclarecendo que:

A pesquisa consiste em preenchimento de prontuário odontológico onde deverão constar radiografias recentes do elemento indicado para exodontia.

A remoção da gengiva em excesso após a cirurgia de exodontia é necessária para realizar a sutura da ferida. Estes pedaços de gengiva que seriam jogados fora é que irão fazer parte da pesquisa.

Não há qualquer risco na coleta do material uma vez que não se trata de nova técnica sendo pois um tratamento de rotina na clínica de cirurgia. O pacientes estará sujeito aos riscos inerentes ao procedimento cirúrgico como dor, inchaço e sangramento no pós-operatório.

O atendimento será com material estéril, seguindo o protocolo de esterilização do serviço odontológico da Faculdade de Odontologia da UFMG.

Não há nenhum gasto por conta do paciente e também nenhuma remuneração.

Todos os participantes poderão, a qualquer momento, desistir de fazer parte dessa pesquisa, e ainda assim continuarão a receber o tratamento proposto.

Todos os seus dados serão confidenciais, sua identidade não será revelada publicamente, em hipótese alguma, e somente os pesquisadores envolvidos neste projeto terão acesso a estas informações, que serão utilizadas somente para fins de pesquisa.

Também os dados coletados de cada paciente poderão ser excluídos, a qualquer momento, a critério do sujeito dessa pesquisa.

Desde já agradecemos sua colaboração.

Belo Horizonte,____de _____ de 20___.

_____
Assinatura do paciente ou responsável

Documento de Identidade:_____

_____ _____
Ricardo Alves de Mesquita Giovanna Ribeiro Souto

Os Telefones dos Pesquisadores para quaisquer esclarecimentos são

- Giovanna Ribeiro Souto 97692709; - Ricardo Alves de Mesquita 3409-2499

- Comitê de Ética em Pesquisa da UFMG – Av. Presidente Antônio Carlos, 6627 – Unidade Administrativa II - 2° andar – sala 2005, CEP: 31270-901 – BH-MG Telefax (031) 3409-4592 email:coep@prpq.ufmg.br

Anexo 3 – Periodontograma

Nome:_____ Idade:_____

Fumante:_____Tempo de hábito_____Número de cigarros por dia_____

	Profundidade de sondagem				Nível de inserção clínica				Sangramento			
Dente	M	V	L	D	M	V	L	D	M	V	L	D
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