UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

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Caracterização imuno-histoquímica do infiltrado inflamatório associado ao líquen plano oral e lesões liquenóides orais

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Orientadora: Profª Drª Elaine Maria Sgavioli Massucato
Coorientadora: Profª Drª Andreia Bufalino

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DADOS CURRICULARES

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Os discípulos perguntaram a Jesus:

Diga-nos: com o que se parece o Reino dos Céus?

Ele lhes disse:

É como a semente de mostarda – a menor dentre todas as sementes, mas, quando cai em terra fértil, dá origem a uma grande árvore, que se torna abrigo para todos os pássaros do céu.[*]

[*] OSHO. A Semente de Mostarda. São Paulo: Editora Icone; 1975.

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RESUMO

Este trabalho está dividido em 3 publicações cujos objetivos foram caracterizar o processo inflamatório de líquen plano oral e lesão liquenóide oral por meio da imuno-histoquímica em três grupos celulares a) células de Langherans, b) linfócitos e c) macrófagos. **Introdução:** células dendríticas (DCs) são células importantes na resposta imune inata com participação especial em eventos imunológicos na cavidade oral. Entre elas, as células de Langerhans (CLs) estão sendo associadas à patogênese do líquen plano oral (LPO) e das lesões liquenóides orais (LLO). Essas células juntas com os linfócitos e macrógafos são capazes de coordenar grande parte das respostas imunológicas. No entanto devido a presença de CLs, linfócitos e macrófagos em lesões reativas e traumáticas na cavidade oral, o real envolvimento dessas células na patogênese do LPO e do LLO deve ser melhor compreendido.

Objetivo: avaliar e comparar a densidade das CLs, linfócitos e macrofágos no LPO, LLO e na hiperplasia fibrosa inflamatória (HFI).

Metodologia: 14 casos de LPO, 14 casos de LLo e 14 casos de HFI foram selecionadas para análise de imuno-histoquímica com os seguintes anticorpos S100, CD1a, CD207, CD3, CD4, CD8, CD20, CD68 e CD163. A densidade celular foi calculada nas regiões intraepiteliais e subepiteliais. O grupo HFI foi subdivido de acordo com a presença de processo inflamatório liquenóide (HFIL) e com a ausência desse processo inflamatório (HFINL) para as análises de CLs e linfócitos. Para as análises estatísticas foi utilizado o software IBM SPSS 20.0.

Resultados: uma grande densidade de células S100 foi encontrada seguida de densidades similares de CD1a e CD207 localizadas no epitélio e no tecido conjuntivo. Houve diferença estatística entre células CD207 entre os grupos LLO e LPO (p=0,015) e entre células CD1a (p=0,024) e células CD207 (p=0,015) entre as regiões intraepiteliais e subepiteliais de todos os grupos. Para os linfócitos uma grande densidade de células CD4 foi encontrado no LPO e uma baixa densidade de células CD20 nos grupos LLO e LPO quando comparados ao grupo HFIL. Para os macrófagos, LPO foi o grupo que mais apresentou marcação positiva para CD68.

Conclusão: apesar da diferença estatística das células CD207 entre LLO e LPO, o resultado do presente trabalho pode ser mais bem explicado pela diferença existente entre epitélio e tecido conjuntivo entre todos os grupos. Células CD4 associadas com baixa densidade de CD20 podem sugerir como essas células participam da formação do processo inflamatório liquenóide. O LPO destaca-se pela grande presença de células CD68.

Palavras chave: Líquen plano. Células de Langherans. Linfócitos. Macrófagos.

Ferrisse TM. Immunohistochemical characterization of the inflammatory infiltrate associated with oral lichen planus and oral lichenoid lesions [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

ABSTRACT

This work is divided into 3 publications whose objectives were to characterize the inflammatory process of oral lichen planus and oral lichenoid lesion through immunohistochemistry in three cell groups a) Langherans cells, b) lymphocytes and c) macrophages. **Introduction:** Dendrict cells (DCs) are important cells of the innate immune system with essential participation in immunological events in oral cavity. Among them, the Langerhans cells (LCs) have been associated with oral lichen planus (OLP) and oral lichenoid lesions (OLL) pathogenesis. Together with the lymphocytes and macrophages, these cells are able to coordinate a large part of the immune response. However, due to present of LCs, lymphocytes and macrophages in reactive and traumatic lesions in oral cavity, the real involvement of these cells in LPO and LLO pathogenesis should better understood.

Objective: To evaluate and compared the density of LCs, lymphocytes and macrophages in oral lichen planus (OLP), oral liquenoid lesions (OLL) and oral inflammatory fibrous hyperplasia (OIFH).

Methodology: 14 cases of OLP, 14 cases of OLL and 14 cases of OIFH, were selected by immunohistochemical analysis for S100, CD1a, CD207, CD3, CD4, CD8, CD20, CD68 and CD163. Densities the cells were calculated in the intraepithelial and sub epithelial areas. The OIFH group was subdivided according to the presence (OIFHL n=14) and absence (OIFHNL n=14) of lichenoid inflammatory infiltrate in analyses involving the LCs and lymphocytes. The statistical analyses were performed by IBM SPSS Statistics 20.0

Results: a great deal of S100 + cells, followed by similar quantities of CD1a+ and CD207+ cells located at intraepithelial and sub epithelial areas were observed in all groups. There is statistical difference between CD207+ cells OLL against OLP (p=0.015) and among intraepithelial and sub epithelial areas to CD1a (p=0.024) and CD207 (p=0.015) to all groups. For the lymphocytes a large density of CD4 cells were observed in OLP and a low density of CD20 were found when compared OLL and OLP to the control group OIFHL. To macrophages the OLP was the group that presented more CD68+ cells.

Conclusion: despite of statistical difference in CD207+ cells in OLL and OLP, lichenoid diseases and reactive/traumatic lesions with lichenoid infiltrate (OIFHL) have a similar density of LCs. CD4 cell density associated with the low cell density of CD20 may suggest how these cells participate in the lichenoid inflammatory process. The LPO stands out for the great presence of CD68 cells. The role of these cells in the pathogenesis of the lesions needs to be better clarified

Keywords: Lichen planus. Langerhans cell. Lymphocytes. Macrophages.

SUMÁRIO

1 INTRODUÇÃO

As lesões liquenoides orais (LLOs) e o líquen plano oral (LPO) compreendem um grupo de doenças imunomediadas que clinicamente e histologicamente são semelhantes, mas com etiologia, tratamento e prognóstico distintos[1]. Atualmente os critérios histopatológicos para o diagnóstico de LPO utilizados na prática clínica, são os indicados pela Organização Mundial da Saúde (WHO, do inglês *World Health Organization*)[2]. No entanto, estes critérios não permitem a distinção entre as lesões de LPO e LLOs. Por esta razão, o termo LLO tem sido coletivamente utilizado como referência a ambas as patologias. Contudo, as LLOs usualmente possuem etiologia identificável, o que permite sua divisão em quatro tipos distintos, incluindo: (a) lesão liquenoide de contato ao amálgama, (b) lesão liquenoides a medicamentos, (c) doença do enxerto-*versus*-hospedeiro (GVHD – do inglês *graft-versus-host disease*), e (d) as lesões que têm um aspecto líquen plano-like, mas que falta um ou mais aspectos clínicos e histopatológicos característicos do LPO clássico. Por isso, os termos “reação liquenoide oral” e “estomatite liquenoide de contato”, são igualmente utilizados como referência as LLOs. Outro ponto importante de distinção entre LPO e LLOs, consiste na localização das LLOs que frequentemente ocorrem em regiões orais que são incomuns no LPO. Um exemplo é o desenvolvimento de LLOs no palato de pacientes com diagnóstico de GVHD. Desta forma, uma avaliação cuidadosa da história clínica e dos possíveis fatores etiológicos é fundamental para o estabelecimento do diagnóstico diferencial entre LLOs e LPO. Adicionalmente, a realização de biópsia deve ser considerada não apenas como medida de distinção entre LLOs e LPO, mas também para avaliar a presença de displasia epitelial ou mesmo carcinoma de células escamosas, quando os achados clínicos se aproximam daqueles observados em eritroleucoplasias[1,3].

Para auxiliar na distinção entre o LPO e LLOs e melhor entender a patogênese dessas condições, vários autores veem estudando a frequência e a densidade de certos grupos celulares distintos em ambas as condições. As células dendríticas apresentam grande papel na imunologia da cavidade bucal, sendo responsáveis pela apresentação antigênica aos linfócitos juntamente com os macrófagos[4]. A natureza e a severidade da resposta inflamatória mediada por células tipicamente T depende de qual receptor de células dendríticas reconhece o antígeno, assim como o tipo de estímulo recebido[5]. Desta forma, nos parece de

suma importância avaliar a densidade de células dendríticas e linfócitos T em LPO e LLOs. No entanto nenhum trabalho na literatura científica avaliou a densidade do processo inflamatório de LPO e LLO com grupo controle que não seja de mucosa normal ou uma doença não imunomediada.

O líquen plano (LP) foi descrito pela primeira vez em 1869, como uma doença sistêmica crônica de natureza imunomediada e que comumente envolve a cavidade bucal, mas pode envolver outros sítios, como a pele, mucosa vaginal e vulvar, glande do pênis, couro cabeludo e unhas[6]. A prevalência na população varia de 0,5% - 2,2%, principalmente entre a faixa etária de 30-60 anos, sendo as mulheres o gênero mais acometido. O LP quando envolve a mucosa oral normalmente se apresenta como lesões múltiplas, frequentemente bilaterais com distribuição simétrica. A apresentação clínica mais comum do LPO é a forma reticular, a qual é descrita como placa branco-acizentada em forma de linhas ou estrias, chamadas de estrias de Wickham[7]. Contudo, o LPO pode se apresentar com várias morfologias como reticular, papilar, tipo placa, atrófico/erosivo ou ulcerado e bolhoso[8]. Os sítios bucais mais comumente envolvidos são a mucosa jugal, dorso de língua, gengiva (gengivite descamativa), mucosa labial e vermelhão do lábio inferior[9,10]. Devido à grande variedade clínica do LPO, o diagnóstico diferencial deve incluir as LLOs, leucoplasia, leucoplasia verrucosa proliferativa, eritroleucoplasia, lúpus eritematoso, doenças vesículos bolhosas como o pênfigo vulgar, penfigóide e estomatite ulcerativa crônica[6]. Os aspectos microscópicos encontrados em amostras de LPO são relativamente característicos e incluem a presença de um epitélio em forma de “dentes de serra”, atrofia epitelial, acantose, degeneração hidrópica das células da camada basal, disceratose e infiltrado inflamatório em padrão “band-like” com predomínio de linfócitos tipo T na lâmina própria[11,12]. Portanto, o diagnóstico de LPO é feito usualmente pela combinação das características clínicas e histopatológicas das lesões[6,7]. Além disto, a análise histopatológica de lesões sugestivas de LPO é fundamental para a exclusão de condições malignas clinicamente semelhantes ao LPO, especialmente nas formas erosivas ou ulceradas[6]. Todavia, os achados histopatológicos de lesões sugestivas de LPO algumas vezes são imprecisos, em aproximadamente metade dos casos há uma pobre correlação clinicopatológica, o que dificulta a confirmação do diagnóstico final[11-13].

A etiologia do LP ainda é incerta, mas parece estar relacionada a uma resposta imunomediada que altera a fisiologia dos queratinócitos na camada basal

do epitélio, tornando-os suscetíveis às respostas imunológicas mediadas por células[14]. Consequentemente, ocorre a ativação de linfócitos CD4+ e CD8+ e a produção de citocinas como, interleucina-2 (IL-2), interferon-gama (IFN-γ) e fator de necrose tumoral (TNF) que promovem a apoptose dos queratinócitos[15,16]. Contudo, os fatores que desencadeiam todo este processo ainda continuam desconhecidos. Alguns estudos buscaram avaliar fatores como infecções virais e desordens psicológicas, como por exemplo, depressão, ansiedade e estresse com o possível desenvolvimento de LPO e exacerbação das lesões do mesmo, mas os resultados parecem conflitantes[17-19]. Foi demonstrada que a expressão de proteínas de choque térmico (HSP – do inglês *heat shock protein*) encontra-se aumentadas em paciente com LPO, porém outros fatores como as mudanças de temperatura, vários agentes externos, medicações, vírus e nutrientes, podem também alterar a expressão destas proteínas, o que torna difícil afirmar esta correlação[20-22].

O tratamento do LPO é baseado no alívio dos sintomas preconizando-se o uso tópico ou sistêmico de drogas imunossupressoras combinadas com drogas antimicrobianas e acompanhamento cuidadoso e rigoroso[23-26]. O uso de corticoesteróides sistêmicos é reservado para situações de falha da terapia tópica, sucessivas recidivas e espalhamento das lesões para sítios extra-bucais como a pele, região genital, couro cabeludo e esôfago[6].

As lesões liquenóides orais (LLO) pertencem ao grupo de doenças de hipersensitividade, e, portanto, suas manifestações clínicas assemelham-se a como áreas de placa e estrias brancas assintomáticas e erosões/ulcerações eritematosas com áreas focais de estrias brancas sintomáticas[1,7,27].

Quatro tipos de LLOs são descritas atualmente na literatura: lesão liquenoide de contato (sendo a restauração de amálgama a causa mais comum), lesão liquenoide a medicamentos (as lesões podem ocorrer em mucosa bucal associada ou não à lesão cutânea), GVHD e as LLOs líquen plano-like, como a estomatite ulcerativa crônica (EUC)[1,7,12,28].

Histopatologicamente a LLO é caracterizada por uma degeneração da camada basal do epitélio estratificado intimamente associada a um infiltrado de células T no tecido conjuntivo subjacente, sendo esta, a provável patogênese desta condição[29-32]. Outros achados histopatológicos descritos são o infiltrado inflamatório misto profundo espalhado na lâmina própria, áreas focais de paraqueratose,

interrupção focal da camada granular, corpos citoides e número aumentado de mastócitos nas áreas de degeneração da membrana basal[1].

O diagnóstico diferencial das LLOs deve envolver praticamente todas as lesões imunomediadas que apresentam repercussão bucal, incluindo o penfigóide das membranas mucosas, doença linear IgA, pênfigo vulgar, eritema multiforme, estomatite ulcerativa crônica e o líquen plano[33]. Portanto, o diagnóstico dessas lesões depende de uma associação dos achados clínicos e histopatológicos, baseado em uma anamnese bem conduzida[7,34]. Além disto, como estas lesões apresentam amplo espectro clínico, são de natureza imunomediada e muitas vezes os seus achados histopatológicos são inespecíficos, a imunofluorescência direta é requerida para o estabelecimento do diagnóstico definitivo[35,36].

A precisão do diagnóstico diferencial entre as LLOs é fundamental para a realização do tratamento adequado, pois esse é dependente do fator etiológico relacionado. Por exemplo, nos casos de lesão liquenoide ao amálgama o tratamento indicado é o polimento ou substituição do amálgama por outro material restaurador. Por outro lado, nos casos de lesão liquenoide a medicamentos, a substituição do medicamento causador das lesões seria o melhor tratamento, embora, em algumas situações é observada a permanência das lesões, mesmo após a suspenção do fármaco. Em outras situações, como na GVHD, o uso de corticoesteróides ou, imunomoduladores, são os tratamentos preferidos[1,7]. Adicionalmente, alguns estudos mostraram que as LLOs apresentam um potencial para a transformação maligna em carcinoma de células escamosas, sendo, portanto, o seu diagnóstico e tratamento fundamentais na prevenção do câncer bucal[12,37]. No entanto, mais estudos são necessários para o esclarecimento dessa hipótese.

As lesões liquenoides de contato caracterizam-se clinicamente como áreas de placas ou pápulas brancas associas a áreas erosivas ou ulceradas que podem afetar qualquer sítio na cavidade bucal[18,38]. Estas lesões são frequentemente unilaterais e de aspecto não simétrico, sendo observada uma relação topográfica do agente causador com o tecido bucal afetado[18,38,39] . Os sítios bucais mais comuns de acometimento das LLOs de contato incluem a mucosa jugal, lateral de língua, seguido de gengiva, palato duro e assoalho bucal[39]. Os materiais odontológicos representam a quase totalidade de agentes causadores da LLO por contato, os quais são capazes de alterar a antigenicidade dos queratinócitos da camada basal que passam a ser reconhecidos como antígenos pelas células do sistema

imunológico[21,40]. O amálgama é o material odontológico mais frequentemente associado ao aparecimento das LLOs de contato, no entanto, o ouro, paládio, níquel, cromo e cobalto, utilizados na reabilitação oral, também podem estar relacionados. As lesões bucais que se desenvolvem neste grupo de lesões, geralmente é uma consequência da hipersensibilidade a um dos componentes presentes nestes materiais odontológicos, como o mercúrio, cobre ou zinco[40-43].

Os estudos mostram que a lesão liquenoide ao amálgama é resultante de uma reação de hipersensibilidade do tipo IV e o desenvolvimento desta reação pode ocorrer após meses ou anos do contato como o material irritante[44,45]. Contrariamente aos outros tipos de hipersensibilidade que levam a produção de auto-anticorpos, nas LLOs a reação caracteriza-se por uma resposta imunológica mediada por células[39]. A patofisiologia da reação de hipersensibilidade tipo IV é complexa e envolve linfócitos CD8+ citotóxicas e CD4+ auxiliares que reconhecem o antígeno em qualquer um dos sistemas do complexo de histocompatibilidade. Macrófagos presentes do meio ambiente secretam Interleucinas que estimulam ainda mais a proliferação de linfócitos CD4+, estimulando a síntese de outras Interleucinas que mediam a resposta imune[39].

Os estudos mostram que a substituição das restaurações de amálgama dentário em pacientes com LLOs por resina composta pode levar a resolução parcial ou completa da condição clínica[46]. No entanto, biópsia incisional deve ser realizada para auxiliar no diagnóstico diferencial destas lesões e excluir a possibilidade de displasia associada[47,48]. Testes de sensibilidade cutâneos podem auxiliar no diagnóstico de pacientes que possuem hipersensibilidade a algum material, porém os estudos apresentam resultados conflitantes[49]. Provavelmente, isto é decorrente da falha no diagnóstico diferencial entre o LPO e outras LLOs[42,50].

Diversos medicamentos são capazes de induzir uma reação liquenóide de hipersensibilidade na cavidade bucal que clinicamente e histologicamente são indistinguíveis do LPO. As lesões envolvendo a pele manifestam-se clinicamente como pápulas ou placas queratóticas, pruriginosas, com ausência de estrias de Wickham e frequentemente estão localizadas no tronco e nas estremidades[51]. Na literatura, duas classes de medicamentos são frequentemente associadas ao desenvolvimento de lesões liquenóides a medicamentos, incluindo, os anti-inflamatórios não-esteroidais (AINES) e agentes anti-hipertensivos como os beta-bloqueadores, inibidoras de ECA e diuréticos[20]. Contudo, outros grupos de

medicamentos parecem estar relacionados com o desenvolvimento de LLOs, dentre os quais podemos citar os hipoglicemiantes, antifúngicos, anticonvulsivantes e drogas imunomoduladoras[32,52]. Foi proposto que a patogênese da lesão liquenóide a medicamentos está associada à presença de polimorfismos nas enzimas do citocromo P450, tornando os pacientes mais suscetíveis ao desenvolvimento da doença[53,54]. O diagnóstico desta condição é baseado na avaliação dos medicamentos utilizados pelo paciente, aparência clínica das lesões e achados histopatológicos[8,42]. Portanto, o diagnóstico final desta condição é difícil e a melhor conduta terapêutica é a substituição da medicação causadora das lesões. No entanto, a remissão completa das lesões geralmente é lenta ou não ocorre.

A doença de enxerto *versus* hospedeiro (GVHD) pode ser definida como uma reação imunomediada que ocorre em pacientes que receberam transplante de medula óssea alogênica (TMO)[55]. A fisiopatologia da GVHD envolve uma reação imunológica entre os linfócitos T imunocompetentes do doador que reconhecem e atacam antígenos de histocompatibilidade oriundos de tecidos do hospedeiro[55,56]. Neste contexto a GVHD é uma das principais complicações em pacientes que se submetem ao TMO alogênico[57,58]. Clinicamente a GVHD pode ser dividida em formas agudas, que ocorrem entre 50-70% dos casos de todos os pacientes com TMO alogênico, e formas crônicas, que representam um total de 30-50% dos casos[59]. A forma aguda da doença é potencialmente fatal e desenvolvem-se nos primeiros 100 dias após o TMO alogênico, tipicamente afetando a pele (exantema), trato gastrointestinal (dor abdominal, diarréia) e fígado (icterícia)[60]. A forma crônica da GVHD é uma síndrome multiorgânica, com características clínicas semelhantes às das doenças imunomediadas e do colágeno, como lesões que envolvem usualmente a cavidade bucal[61]. Essa última apresentação, pode se desenvolver após 3 anos do transplante e normalmente é precedido da forma aguda[60,61]. As lesões bucais são similares a lesões observadas em doenças imunomediadas como o LPO, LLOs e lúpus eritematoso. Estas lesões bucais caracterizam-se por placas hiperqueratóticas, restrição da abertura bucal, consequentemente gengivites, mucosites, eritemas e dor[62]. Existem relatos de que alguns fatores possam contribuir para o aumento do risco do desenvolvimento da GVHD crônica, incluindo a idade avançada, doador do sexo feminino para um receptor masculino e transplante de células do sangue periférico[63]. Histologicamente as amostras de tecido bucal caracterizam-se por células epiteliais disceratóticas, apoptose e infiltrado inflamatório

liquenóide abaixo da camada basal, formado por células CD3+, CD68+ e linfócitos T[64,65]. O tratamento desta condição envolve principalmente o uso de corticoesteróides e imunomoduladores tópicos, como por exemplo, dexametasona, predinisona, triancinolona, clobetasol, e tacrolimus. Outras modalidades de tratamento como terapia fotodinâmica e a talidomida podem ser utilizadas para acelerar o reparo tecidual[56,65].

Células dendríticas são células apresentadoras de antígeno (APC) que fazem a captura, o processo e a apresentação do antígeno para os linfócitos preferencialmente virgens, iniciando e regulando desta forma, a resposta imune adaptativa[66]. Apesar de haver várias formas de classificar as células dendríticas, evidencias atuais, preferem assim faze-lo associando sua origem ontogênica com os receptores celulares expressos na membrana plasmática[67]. Desta forma, há 3 tipos de células dendríticas, a saber: células derivadas de monócitos, células dendríticas convencionais e células dendríticas plasmacitóides. Células dendríticas residentes do epitélio oral, também denominadas por células de Langerhans (CL), são na sua maioria formadas por células dendríticas convencionais e expressam na sua superfície celular receptores CD1a, mais especificamente CD207 e menos especificamente o S100[67-69]. Estudos de ultraestrutura da mucosa oral indicam que as CL apresentam entre 5 a 9 dendritos localizados horizontalmente em relação ao epitélio oral cobrindo até 25% da área do mesmo epitélio[70]. Apesar de CL também estarem presentes em outros sítios como o a derme, na parede da artéria aorta, linfonodo e timo, essas CL apresentam preferência por epitélio escamoso estratificado, em que, sua densidade celular é em média de 160-550 células/mm^2. Em mucosa não queratinizada, como o palato mole, ventre de língua, lábios e assoalho bucal encontramos CL em maior quantidade[71]. Estudos veem mostrando o potencial envolvimento de CL na participação de doenças bucais, tais como a gengivite, periodontite, reação de hipersensibilidade ao contato, candidíase crônica hiperplásica, líquen plano, leucoplasia, doença do exerto *versus* hospedeiro, lesões herpéticas e o carcinoma de células escamosas[72].

Os linfócitos residentes da mucosa oral apresentam papel de destaque na imunidade e tolerância imunológica local. Devido a isso, a deficiência ou alguma alteração nesses linfócitos, principalmente os linfócitos T, está associado a grande quantidade de doenças. No entanto, o fenótipo e a exata função dos linfócitos na mucosa oral ainda permanecem um tema pouco estudado[73]. CL como mencionado

acima são APC, portanto, uma vez que capturem e processem o antígeno, migram para os folículos linfoides, locais esses onde se localizam os linfócitos T. Após a apresentação do antígeno ao linfócito T virgem, o mesmo começa sua maturação, tornando-o ativado e capaz de combater o antígeno[74]. A associação entre o antígeno e a vários tipos de citocinas produzidas por células dendríticas, macrófagos e outros linfócitos é responsável pela diferenciação e subdivisão dos linfócitos T virgens. Assim, a interleucina -12 (IL-12) é responsável pela diferenciação em Th1, IL-4 pelo Th2, TGF-β pelos linfócitos Treg, TGF-β + IL-6 pelo Th17, TGF-β + IL-4 pelo Th9, IL-6 pelo Th22 e IL-6 + IL-21+ interação com células B pelo Tfh[75-77]. Linfócitos Th1 e Th2 apresentam receptores de superfície celular CD4, assim como os linfócitos Treg (78), Th17 (79), Th9, Th22 (76) e o Tfh[76,77]. Na mucosa oral, há outro Imunofenótipo de linfócitos que apresentam na sua superfície receptores CD8 e são denominados linfócitos intra-epiteliais[74]. Especificamente, o subtipo Th2 produz IL-4 e IL13, essas interleucinas são capazes de induzir e ativar os linfócitos de tipo B a produzirem anticorpos, iniciando uma resposta imunológica humoral. Linfócitos B apresentam na sua superfície celular receptor CD20[80]. Como mencionado acima, linfócitos participam também das mesmas doenças que as CL, e não poderia ser diferente, tendo em vista que suas funções são dependentes uma das outras[73].

Assim como as células dendríticas, os macrófagos apresentam também a função de célula apresentadora de antígeno, além de atuar como fagócito. Portanto, esses tipos celulares atuam tanto na resposta imunológica inata quanto na resposta imune adaptativa[81]. Macrófagos são células grandes presentes em todos os tecidos com grande capacidade plástica e dinâmica. Assim, quando ativados, essas células modificam sua morfologia e expressão de proteínas rapidamente[82]. Estudos recentes nos mostram que os macrófagos podem apresentar duas origens, sendo a necessidade de uma fase intermediária em monócito o fato que as distingue[83]. Funcionalmente, os macrófagos podem ser divididos em dois grandes grupos, a saber; pro-inflamatórios (M1) e anti-inflamatórios (M2). Ainda os M2 podem ser subdivididos em mais 4 classes, a saber; M2a, M2b, M2c e M2d[84].

Uma vez que os monócitos sejam estimulados por IFN-gama e LPS ocorrem a diferenciação em M1, sendo responsável pela produção principalmente de TNF, IL-β, IL-6, IL-12, IL-23, CCL-5 e CCL-8. Já o monócito sendo estimulado por IL-4 e IL-13, ocorre à diferenciação em M2a produzindo principalmente TGF-β e arginase. Quando exposto em contato com complexos imunes, LPSe IL-1 o monócito se

diferencia em M2b e produz principalmente IL-10 e inibidores de IL-12. Já quando exposto a glucocorticóides, TGF-β, IL-10 e CCL-13 o monócito torna-se capaz de se diferenciar em M2c e produzir principalmente IL-10 e TGF-β. Finalmente, quando estimulado por IL-6 e adenosina A, o monócito é capaz de se diferenciar em M2d e produzir principalmente IL-10, TGF-β e VEGF, ou seja, apresentando uma capacidade pró- angiogênica[84]. Consequentemente, o equilíbrio entre macrófagos M1 e M2 contribui para o desenvolvimento e manutenção de doenças, especialmente as doenças autoimunes[84.85]. Como novamente mencionado acima, linfócitos participam também das mesmas doenças que as CL assim como os macrófagos, e não poderia ser diferente, tendo em vista que suas funções são dependentes uma das outras[85].

2 PROPOSIÇÃO

O objetivo deste trabalho foi:

1) Caracterizar por meio da imuno-histoquímica células de Langherans (CD1a, CD207 e S100) presentes no líquen plano oral e nas lesões liquenóides orais.
2) Caracterizar por meio da imuno-histoquímica linfócitos (CD3, CD4, CD8 e CD20) presentes no líquen plano oral e nas lesões liquenóides orais.
3) Caracterizar por meio da imuno-histoquímica macrófagos (CD68 e CD163) presentes no líquen plano oral e nas lesões liquenóides orais.

3 PUBLICAÇÕES

3.1 Publicação 1*

Density of Langerhans cells in oral lichen planus and oral lichenoid lesions: an immunohistochemical study.

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* O artigo segue as normas do periódico *Histopathology, para qual foi submetido.*

Abstract

Introduction: Dendrict cells are important cells of the innate immune system with essential participation in immunological events in oral cavity. Among them, the Langerhans cells (LCs) have been associated with oral lichen planus (LPO) and oral lichenoid lesions (LLO) pathogenesis. However, due to present of LCs other diseases that affect the oral cavity, the real involvement of LCs in LPO and LLO pathogenesis should better understood.

Objective: To evaluate and compared the density of LCs in oral lichen planus (OLP), oral liquenoid lesions (OLL) using as control group other diseases with inflammatory cells in yours composition. In the study we used the oral inflammatory fibrous hyperplasia (OIFH) as control group.

Methodology: 14 cases of OLP, 14 cases of OLL and 14 cases of OIFH, were selected by immunohistochemical analysis for S100, CD1a and CD207. Densities of LCs were calculated in the intraepithelial and sub epithelial areas. The OIFH group was subdivided according to the presence (OIFHL n=14) and absence (OIFHNL n=14) of lichenoid inflammatory infiltrate. The multivariate analyses was performed (Manova two-way) followed by correlation and linear regression analyses. The statistical analyses were performed by IBM SPSS Statistics 20.0

Results: a great deal of S100 + cells, followed by similar quantities of CD1a+ and CD207+ cells located at the intraepithelial and sub epithelial areas were observed in all groups. There is statistical difference between CD207+ cells OLL against OLP (p=0.015) and in the intraepithelial and sub epithelial areas to CD1a (p=0.024) and CD207 (p=0.015) to all groups. In OLL, the LCs appears to be more important in the immunopathogenesis.

Conclusion: despite of statistical difference in CD207+ cells in OLL and OLP, lichenoid diseases and reactive/traumatic lesions with lichenoid infiltrate (OIFHL) have a similar density of LCs. The role of LCs in the pathogenesis of these lesions needs to be better clarified.

Key-words: dendritic cells; Langerhans cells; oral lichen planus; oral inflammatory fibrous hyperplasia and immunohistochemistry.

Introduction

Lichen planus is a common chronic inflammation mucocutaneous disease affecting until 2% of the population when the oral mucosa is affected.[1] The disease most commonly occurs in oral cavity being buccal mucosa, tongue and gingiva (desquamative gingivitis) the most common oral sites.[2] The main profile of involvement is female gender with age average around 30-60 years.[3] Clinically, oral lichen planus (OLP) can be divided into six different forms: reticular type, papular type, plaque-like type, atrophic type, ulcerative type and bullous type.[4] Histopathologically, signs of liquefaction degeneration in basal cell layer, inflammatory cell infiltration mainly of lymphocytes and absence of epithelial dysplasia are the major aspects.[5] Until now the pathogenesis of oral lichen planus (OLP) remains poorly understood. It is considered to be T-cell–mediated chronic inflammatory tissue with both antigen-specific and nonspecific mechanisms hypothesized.[6]

Oral lichenoid lesions (OLL) are also considered chronic inflammatory disease of unknown etiology at some cases. OLL are frequently symptomatic and affect 1%-2% of the population.[7] The OLP and OLL are similar in their oral presentation and histopathological features, however the OLL in most cases have distinct causes, including reactions to amalgam restorations, drug involvement and as consequence of the chronic graft versus host disease (cGVHD). There is one last type of OLL named OLL lichen planus-like. It is diagnosed when there is no sign of distinct cause.[3] Reactive lesions of the oral cavity are clinically non-neoplastic nodular swellings that develop in response to chronic and recurrent tissue injury.[8] The most common reactive lesions of the oral cavity include pyogenic granuloma, peripheral giant cell lesion, peripheral ossifying fibroma, fibroma and oral inflammatory fibrous hyperplasia (OIFH).[9] OIFH typically occurs around the borders of ill-fitting complete denture in the maxilla and mandible. In microscopic, the OIFH can included hyperkeratotic epithelium, areas with acanthosis alternating with atrophic areas and the connective tissue of the lamina propria is usually grossly thickened and displays many areas of chronic inflammation coexisting with dense fibrosis. [10]

Dendritic cells (DCs) pays a central role in the oral mucosa immunology, so to the better understand the pathogenesis of OLP and OLL, some studies are evaluating that presence of the lymphocytic inflammatory infiltrate and consequently

locally generated cytokines by the quantity and concentration of the DCs.[11] These cells are antigen presenting cells endowed with ability to stimulate naïve T-cells and, depending on the DCs subset and type stimulus received, DCs takes control of the nature and severity of T-cell responses.[12] DCs can have 3 different origin according ontogeny classification that influence of DC subsets (i) monocyte derived cells (ii) conventional dendritic cells and (iii) plasmocytoids dendritic cells.[13]

Resident DCs of oral epithelium are composed mainly of conventional myeloid DCs from the Langerhans cells (LCs) subtype expressing CD1a and more specific CD207.[13,14] There is other DC subtype normally that normally express an unspecific marker S100 that may be retained in mature and immature DCs.[15] Only three studies in literature evaluated consecutively the association of LCs, OLP and OLL. The authors found that OLP and OLL has similar distribution and concentration of LCs and statistically different when compared to the control group formed by healthy oral tissue.[11, 16, 17] Thereby, the authors concluded that LCs may play a significant role on its pathogenesis. Despite these results are significant, no study until now evaluated the presence of LCs in OLP and OLL with other inflammatory disease non-immune-mediated.

Therefore, carrying out a comparative analysis and evaluation of OLP, OLL and oral inflammatory fibrous hyperplasia (OIFH) with presence (OIFHL) and absence liquenoid inflammation (OIFHNL) could help to understand better the profile of LCs in OLP and OLL.

Materials and Methods

Patient population

The design of the study was approved by the Ethics Committee of the São Paulo State University (UNESP), School of Dentistry, Araraquara. The present study was comprised of 42 patients diagnosed with OLP (n=14), OLL (n=14) and OIFH (n=14). The OLP and OLL were diagnosed based in the modified criteria of van der Meij et al[5] (2003). The modified criteria include clinical aspects and histopathologic aspects. Clinical aspects are presence of bilateral lesion, more or less symmetrical; presence of lace-like network of slightly raised gray-white lines and erosive, atrophic, bullous and plaque-like lesions are only accepted as a subtype in presence of reticular lesions. The histopathologic aspects are presence of a well-defined band-like zone of cellular infiltration that is confined to the superficial part of the connective

tissue, signs of “liquefaction degeneration” in the basal cell layer and absence of epithelial dysplasia. When all the criteria are present, the final diagnosis was OLP and when some criteria were not presence, the final diagnosis was OLL. The OIFH group was subdivided into two other groups, according with lichenoid inflammatory infiltrate presence, as OIFHL and as OIFHNL due to absence of lichenoid inflammatory infiltrate. The sample was randomly select to histopathologic and immunohistochemical staining procedure.

Histopathologic and immunohistochemical analysis

All tissue specimens were fixed in 10% neutral-buffered formalin for 24 hours at room temperature, embedded in paraffin at 55ºC and cut into consecutive parallel 3-µm thick sections. For immunohistochemistry, the slides were hydrated and treated with hydrogen peroxide. Immunohistochemical staining procedure was conducted in biopsy specimens involved the following LCs epitopes: CD1a, CD207 and S100. The tissue specimens were pretreated with 10mM sodium citrate buffer, pH 6.0, in a pressure cooker. The sections were then successively incubed with the primary antibodies CD1a (dilutions 1: 400, DakoCytomation ,Glostrup, Denmark), CD207 (dilutions 1:200, Monosan, Uden, the Netherlands) and S100 (dilutions 1:300, Leica Biosystems Newcastle,Ltd). Next, the sections were incubed with secondary antibodies conjugated with streptavidin-biotin-peroxidase (K0690; Universal Dako LSAB + Kit, Peroxidase, Carpinteria, California). The reactions developed with diaminobenzidine, and the sections counterstained with Carazzi hematoxylin. Results were then observed with optical microscope with magnification 200X (Leica DM 2500). Five strong staining in intraepithelial and sub epithelial areas were select for photomicrography (Leica Aplication Suite – LAS). Finally, the cells were counted used the ImagJ sotware.

Statistical analysis

Descriptive statistics was used to evaluate patient population and immunohistochemical findings. Statistical analysis was performed according with the study design. Thus to evaluated the normality distribution was performed the Shapiro-Wilk test. The presence of outliers was checked. To assess homoscedasticity was utilized Box’s Test of Equality of Covariance Matrices and the Levene’s Test of Equality Variances. Therefore the Multivariate Analysis of Variance

with two independent factors was performed (Manova two-way). Post-test of Tukey was chosen to evaluate the differences between the groups. In additional, we also conducted a correlation study and a linear regression study with the biomarkers mentioned above to OLP and to OLL. A *p* value of < 0.05 was regarded to be statistically significant. The effect factor was also calculated when *p* value was statistically significant. Statistical analysis was performed by IBM SPSS Statistics 20.0 and graphics image were built using GraphPad Prism version 6.0.

Results

Patient population

OLP. Fourteen patients were included in this group. 64.29% were female and 35.71% were male, the mean age was 48.64 (±13.80). Buccal mucosa was the most affected site (35%) following by tongue (27%), gingiva (19%) and lips (19%). The most prevalent clinical aspect was erosive associated with striated plate form (60%). Clinical images are illustrated in figure 1 and more clinical data is illustrated in table 1.

OLL. Fifteen patients were included in this group. 80.0% were female and 20.0% were male, the mean age was 53.22 (±6.28). Buccal mucosa was the most affected site (40%) following by tongue (36%), lips (14%) and gingiva (10%). The most prevalent clinical aspect was erosive associated with striated plate form (55%).

OIFH. Fourteen patients were included in this group. 85.71% were female and 14.29 were male, the age was 43.35 (±17.33). All patients presented the same clinical aspects as a hyperplasia.

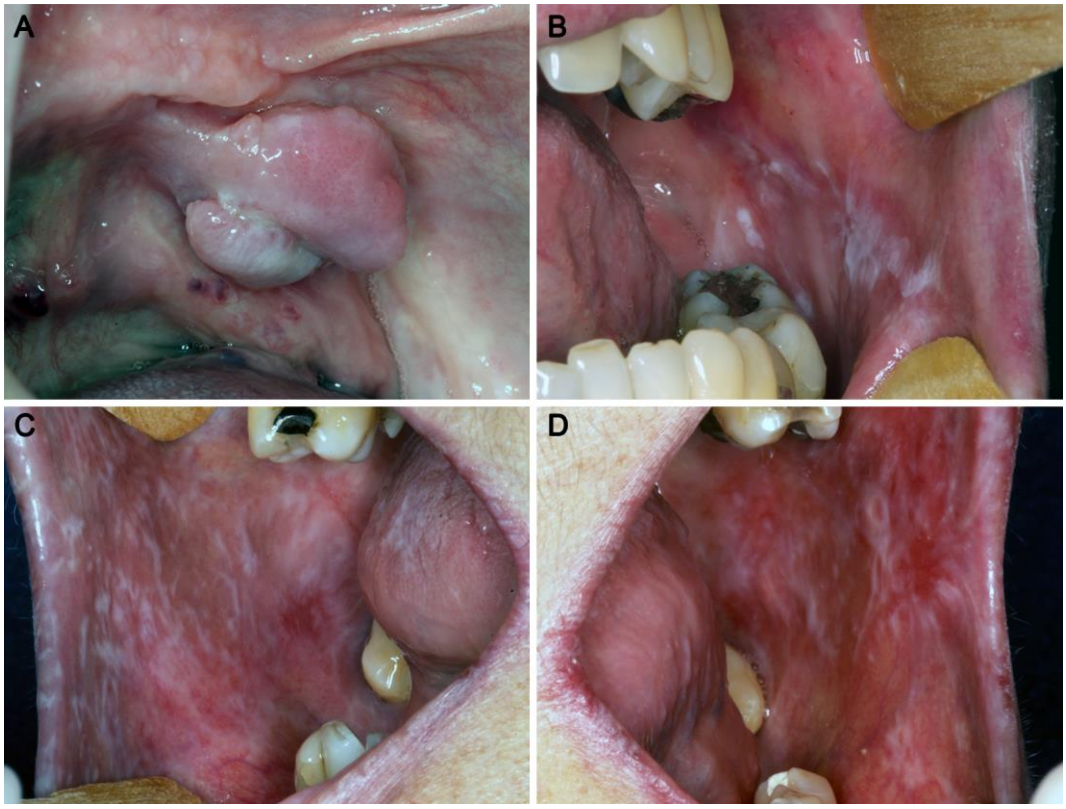


Figure 1. Clinical images representing the diseases that were evaluated in the present study. (A) Oral inflammatory fibrous hyperplasia (OIFH): hyperplasia located in the vestibule background of maxillae; (B) Oral liquenoid lesions (OLL): erosive areas associated with white plaques closely in the amalgam restoration located in buccal mucosa unilaterally; (C/D) Oral lichen planus (OLP): erosive areas associated with white plaques in striated form located in buccal mucosa bilaterally and symmetrically.

Table 1. Clinical data of patients involving in the present study.

Diagnosis	Gender	Age	Localization	Clinical Aspect
OLP	5M/9F	48.64±13.80	BM (35%); TON (27%); GIN (19%); LIP (19%)	E+S (60%); PL (20%) RT (14%); U+S (6%)
OLL	3M/12F	53.27±6.29	BM (40%); TON (36%); LIP(14%); GIN (10%)	E+S (55%), PL (17%); U+S (17%); U (11%)
OIFH	2M/13F	44.36±17.33	Buccal vestibule fund	Tissue hyperplasia

OLP (Oral lichen planus); OLL (Oral lichenoid lesions); F (feminine gender); M (male gender); BM (buccal mucosa); TON (Tongue); GIN (gingiva); LIP (Lips); E+S (erosive associated with striated plate form); RT (reticulate); PL (plaque); U+S (ulcerative associated with striated plate form); U (ulcerative).

Immunohistochemical results

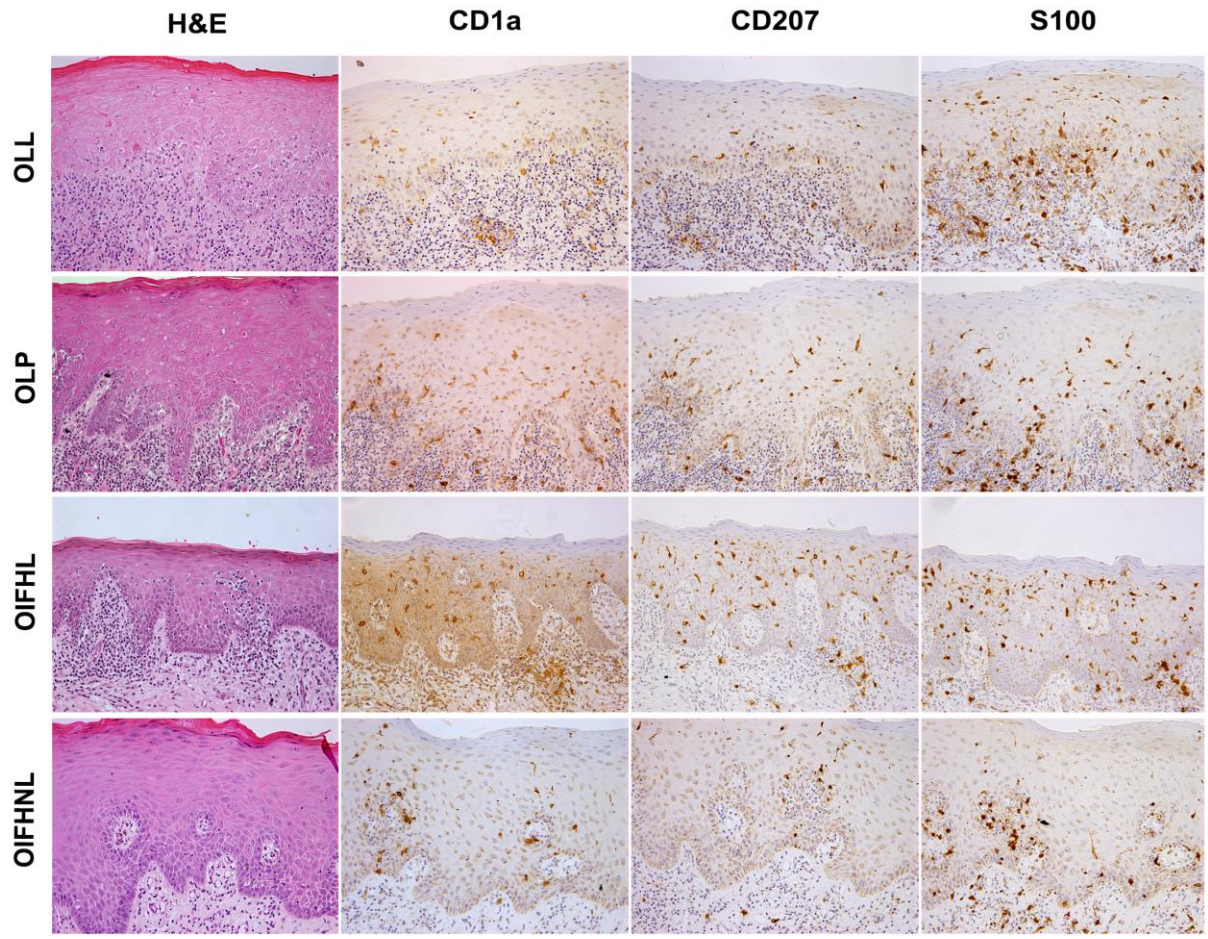
Descriptive analyses

In descriptive analyses was observed a higher number of Langerhans cells (LCs) on the intraepithelial areas than to sub epithelial areas in all groups for the CD1a and CD207 markers. In addition was also observed that CD1a and CD207 markers showed more positive cells in OLP followed by OIFHL. On the other hand, in the S100 marker was observed a higher number of LCs in the intraepithelial area of OIFHL and OIFHNL than sub epithelial area. However to the OLL and OLP groups the sub epithelial area was the tissue that showed more positive cells, to the biomarker S100, than intraepithelial area.

Inferential analyses

Multivariate Analyses of Variance with two independent factors (Manova two-way)

Multivariate Analysis of Variance (Manova) essentially tests whether or not the independent grouping variable explains a significant amount of variance in the canonical variate.[18] On the present study, the independent factors were groups (OLP, OLL, OIFHL and OIFHNL) and tissue (intraepithelial and sub epithelial). The dependent variates were LCs positive to CD1a, CD207 and S100. Thus, it is characterizing a Manova two-way study design. The microscopic images are illustrated in figure 2.



.Figure 2. Microscopy characteristics of the groups evaluated to each Langerhans cells biomarker (CD1a, CD207 and S100). H&E; hematoxylin and eosin: OLL: oral lichenoid lesions showing a sub epithelial lichenoid inflammatory infiltrate. OLP: oral lichen planus showing acanthosis and sub epithelial lichenoid inflammatory infiltrate with liquefaction degeneration in basal cell layer. OIFHL: oral inflammatory fibrous hyperplasia with lichenoid infiltrates showing sub epithelial lichenoid inflammatory infiltrate. OIFHNL: oral inflammatory fibrous hyperplasia without lichenoid infiltrates showing acanthosis and sub epithelial inflammatory infiltrate.

The distribution of data was normal and non-homoscedastic. Box's Test of Equality of Covariance Matrices and the Levene's Test of Equality Variances showed $p < 0.001$. Thus was utilized the statistics of Pillai's trace to evaluate the Manova two-way. The *p*-value of statistics of Pillai was 0.038 for group and 0.001 for tissue. The effect factor (η^2_p) to group was 0.053 that is considering as middle level of effect and to tissue, the effect factor (η^2_p) was 0.254 that is considering high effect. There was not statistically significant between the interaction of independent factors ($p = 0.115$). Statistical information is showed in table 2 and table 3

Table 2. Pillai's trace for multivariate analyses to the Langerhans cell.

Factors	Value	Hypothesis df	F	*p*-value	Observed Power	Partial eta square (η^2_p)
Groups	0.158	3.000	2.007	0.038	0.854	0.053
Tissue	0.254	3.000	12.043	0.001	1.000	0.254
Groups*tissue	0.127	9.000	1.595	0.115	0.743	0.042

*: association between independent factors (groups versus tissue); Groups: OLL, OLP, OIFHL and OIFHNL; Tissue: Intraepithelial and Sub-epithelial; F: F statistic; Hypothesis: number of dependent variables; df; degrees of freedom.

Table 3. Multivariate analyses with Langerhans cell for independent factors.

Factors	Langerhans cell	Sum of Squares	Mean Square	df	F	*p*-value	Observed Power	Partial Eta square (η^2_p)
Groups	CD1a	769.766	256.589	3	1.810	0.15	0.459	0.048
	CD207	10621.223	3540.589	3	3.644	0.015	0.785	0.092
	S100	2377.068	792.356	3	1.564	0.202	0.402	0.042
Tissue	CD1a	740.994	740. 994	1	5.228	0.024	0.620	0.046
	CD207	17968.528	17968.528	1	18.494	0.001	0.989	0.146
	S100	435.630	435.630	1	0.860	0.356	0.151	0.008

Groups: OLL, OLP, OIFHL and OIFHNL; Tissue: Intraepithelial and Sub-epithelial; F: F statistic; df; degrees of freedom.

Post-test of Tukey was performed and showed only statistically significant difference to the intraepithelial cells to the biomarker CD207, between OLL and OLP (p = 0.015). For the rest of all analyses among groups there were no statistically significant differences (p > 0.05) (figure 3). For analyses between intraepithelial and intraepithelial areas inside the groups there was statistically significant to CD1a+ LCs (p = 0.024) and to CD207+ LCs (p= 0.015) for all groups.

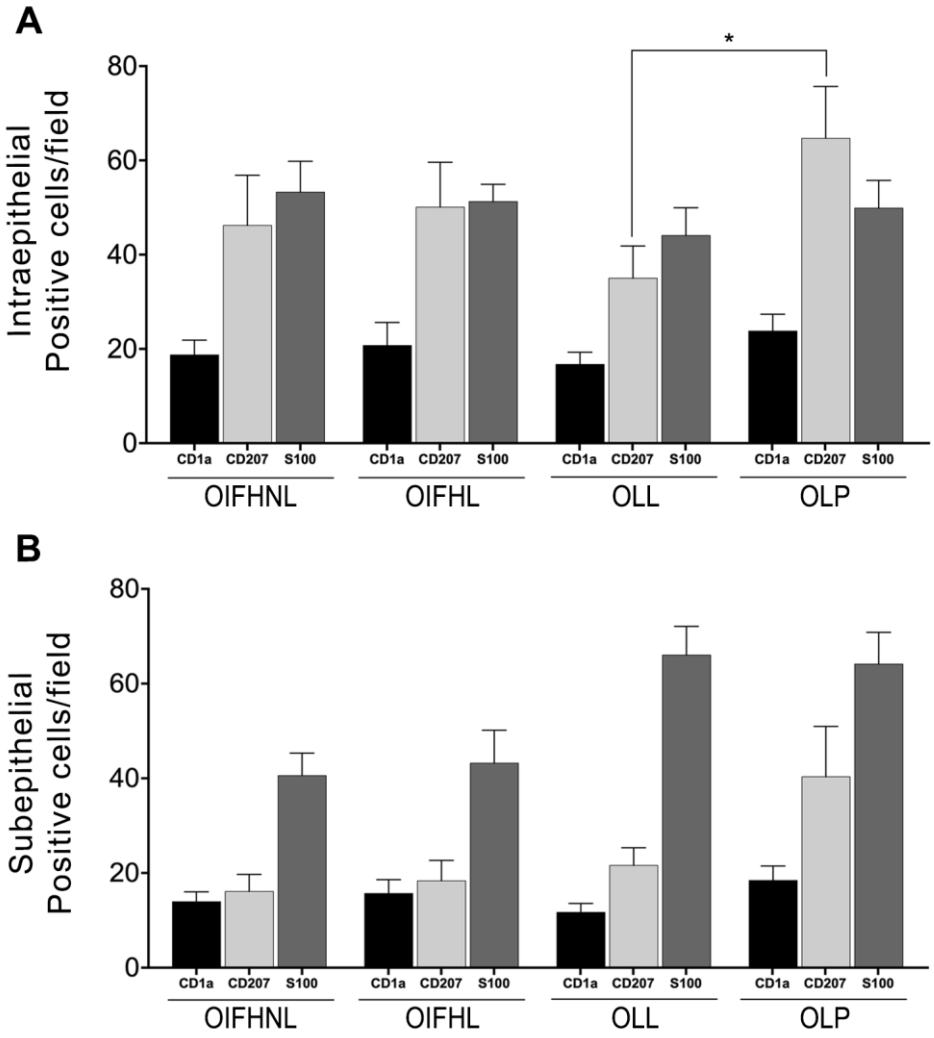


Figure 3. Illustrate the results of post-test of Tukey. OIFHNL: oral inflammatory fibrous hyperplasia without lichenoid inflammatory infiltrates. OIFHL: oral inflammatory fibrous hyperplasia with lichenoid inflammatory infiltrates. OLL: oral lichenoid lesions; OLP: oral lichen planus.*: statistically significant difference to the intraepithelial cells to the biomarker CD207, between OLL and OLP (p = 0.015).

Correlation and linear regression studies

In order to evaluate the magnitude and sense of association between two variables, was performed a correlation study to density of LCs positives to CD1a and to the CD207 using the Pearson's correlation coefficient. However, before the analyses were being done, all data was transformed in $\log_{10}$ to eliminate the non-homoscedastic distribution. There was a statistical significance between the biomarkers to the OLP (p = 0.0002) and to the OLL (p < 0.0001). Intriguingly, the correlation coefficient (r) to OLP was equal to 0.8035 that is classified as a strong

relation with determination coefficient (R^2) equal to 0.6457. To the OLL, the correlation coefficient (r) was equal to 0.9786 that is classified as a very strong relation with determination coefficient (R^2) equal to 0.9577. In additional, due to the fact the correlation study have statistical difference it was performed a simple linear regression to each conjunct of LCs biomarkers to OLP and OLL (table 4). To each conjunct of the biomarkers were observed a significate statistical difference ($p < 0.05$). Interestingly, we found different percentages in adjust determination coefficient. The linear models can be also being observed in the figure 4.

Table 4**.** Summary of simple linear regression of Langerhans cells biomarkers from OLP and OLL

Group	**Y**	**X**	***p*-value**	**R^2**	***p*-value (a)**	***p*-value (b)**	**Equation log Y = a + b · log X**	**CI (95%) (a)**	**CI (95%) (b)**
OLP	CD1a	CD207	0.0003	0.6204	0.3831	<0.0001	log Y = 0.20 + 0.65 log X	(-0.28) – 0.68	0.37 – 0.92
	CD207	CD1a	0.0003	0.6204	0.1453	<0.0001	log Y = 0.40 + 0.99 log X	-0.16 – 0.97	0.57 – 1.41
OLL	CD1a	CD207	<0.0001	0.9545	0.0069	<0.0001	log Y = 0.18 + 0.68 log X	0.06 – 0.31	0.60 – 0.77
	CD207	CD1a	<0.0001	0.9545	0.0553	<0.0001	log Y = -0.20+ 1.39 log X	(-0.40) – 0.01	1.21 – 1.56

OLP: oral lichen planus; OLL: oral lichenoid lesion; Y: dependent variable; X: independent variable; R^2: adjusted determination coefficient; (a): linear coefficient; (b): angular coefficient; CI: confidence interval. P-value <0.05 was considered as statistical significance.

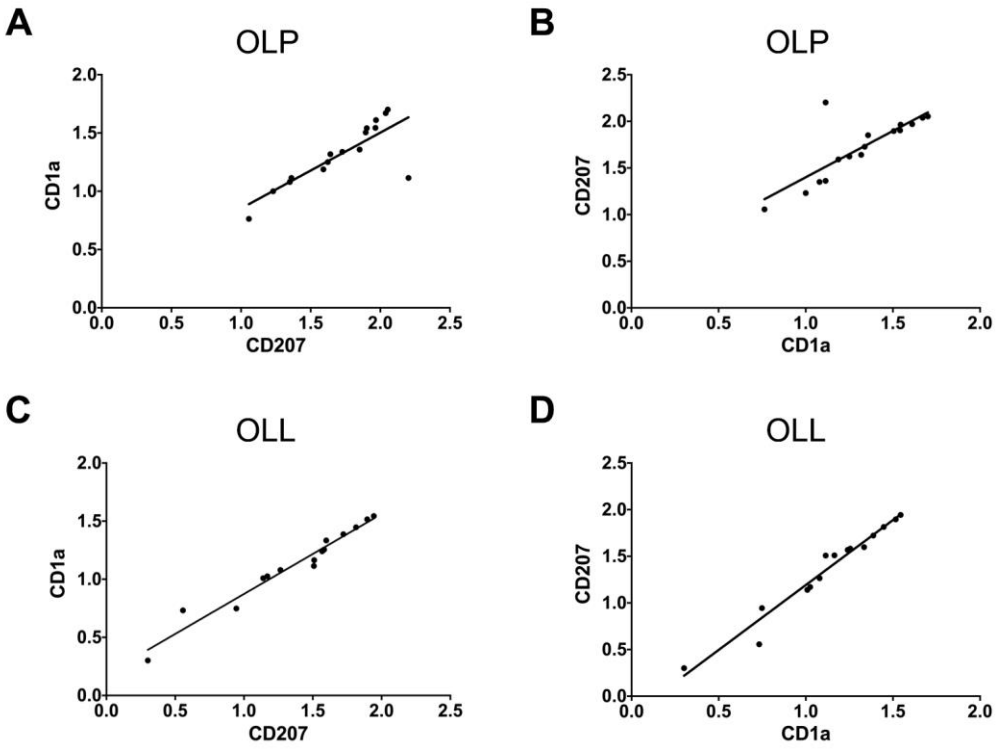


Figure 4. Illustrate the regression models to the OLP (A-B) and to the OLL (C-D). All regression models presented significance statistical ($p<0.0001$). The regression models are. (A): $\log Y = 0.20 + 0.65 \log X$; (B): $\log Y = 0.40 + 0.99 \log X$; (C): $\log Y = 0.18 + 0.68 \log X$; (D): $\log Y = -0.20 + 1.39 \log X$.

Discussion

Oral lichen planus (OLP) is one of the most common oral mucosal diseases but the etiology and pathogenesis remains unknown until now.[19] The associations of wide variety of differential diagnosis such as mucous membrane pemphigoid, lupus erythematosus, chronic ulcerative stomatitis and mainly with oral lichenoid lesions (OLL) and additionally with the controversy regarding malignant transformation become the OPL one of the most discussed disease in the field of oral and maxillofacial pathology.[19,20] OLP and OLL are lichenoid lesions which resemble and overlap clinical and histopathological aspects can explain the significant disagreements concerning the diagnosis among pathologists and clinicians.[5,19] Therefore due to this general overview researches that strive to respond these lacks in literature should be more strengthened.

The presence of interface mucositis with predominance of lymphocytes in OLP and OLL suggest the participation of LCs in the role of both pathogenesis. However these conditions presenting a different nature, immunomediated to OLP and cell-

mediated hypersensitivity to OLL.[17,21,22] The present results highlight that OIFH may be a good option by control group due to presence of inflammatory cells and lichenoid inflammatory infiltrate and because the OIFH is a common lesion.

Only the study of Upeniece et al.[23] (2016) in all literature evaluated the difference between the presence of to S100+ LCs in OLP and other mucosa not health and without lichenoid infiltrate, in this case with vulgaris psoriasis. The researchers concluded that there is no significant difference between the groups. This result is in agreement with our data although there is more LCs in experimental groups than control groups. Contrary, Santoro et al.[24] (2005) and Mega et al.[25] (2001) found statistically significant difference S100+ LCs from OLP and normal mucosa. In the present study, we did not use disease free mucosa as a control group. Thus, we prefer to use the presence of inflammatory cells as a control group. Mega et al.[25] (2001) still compared the presence S100+ LC between OLP and OLL. The authors found statistically significant difference. However the cells were positive to C- type HBV. In other words, the data can be influenced by the presence of the virus (25).

Of all biomarkers used to identify LCs, the CD1a is the most common used in the literature. Many studies evaluated the presence of CD1a+ LCs in OLP against normal mucosa. [11,16,17,24,26,27-29] The results are opposite to our due to we did not find significant difference between OLP and the control groups. Nevertheless in our study there are more CD1a+ LCs in OLP than all others groups. Souto et al.[17] (2016) evaluated the presence of CD1a+ LCs in OLP and OLL and found a significant difference in the groups. The authors concluded that LCs play a different role to the pathogenesis of the lesions. The data of Sato et al.[11] (2006) and Gueiros et al.[16] (2012) are affirmative to absence statistically difference between OLP and OLL. However there was statistically difference between the study groups and the normal mucosa utilized as control group. The authors concluded that CD1a+ LCs are responsible to pathogenesis of OLP and OLL. Our results differ among the others because we do not find differences in CD1a+ LCs among all groups studied. To the CD1a biomarker there only statistically difference between the intraepithelial and the sub epithelial areas to all groups.

CD207 (Langerin) is a membrane receptor associated with LCs that recognizes microbial mannose bearing glycoproteins and glycolipids that is found mainly in epidermis.[30] Few studies in literature evaluated the presence of CD207+ LCs in oral mucosa and in lichenoid lesions. The studies of Gustafson et al.[26] (2007),

Mukae et al.[27] (2009) and Wang et al.[29] (2018) were conclusive to difference between CD207+ LCs in OLP against normal mucosa. Ours results do not agree with that. When compared CD207 + LCs in OLP with control group were did not observe significant difference. There was only significant difference in comparison with CD207 + LCs in OLP and OLL with more density of cells in OLP. Perhaps, this density of CD207 + LCs in OLP and OLL can influence the role of pathogenesis of the lichenoid lesions.

The frequency distribution between LCs subsets can influence the T-cell activation outcome.[31-33] Thus, the characterization of OLP as $CD207^{high}$ and OLL as $CD207^{low}$ discovered in this present study might explain some aspects of the pathogenesis differences between OLP and OLL. In addition, the study of Lindenberg et al.[31] (2013) indicated that the presence of cytokine balance in the microenvironment can modify the T-cell activation outcome. It is possible that the different type of chronic inflammation in OLP and OLL can influence the microenvironment and influence the expression of CD207 in these diseases. There is scientific evidence that when a blood progenitor cell is exposed specific inflammatory mediators the frequency and density of CD207+ cells is modify. [33-37]

Although ours results are significant to difference in density of CD207 + LCs in OLP and OLL the effect factor (η^2_p) was 0.053 considering as middle level. On the other hand, the difference about tissue for all groups in CD1a + LC and CD207 + LC showed effect factor (η^2_p) equal to 0.254 considering as high level. The effect factor is a measure that allows the extrapolation of statistical results to the biological events. So ours results may be more explained about the density of LCs in intraepithelial against sub epithelial areas than the distinct groups. Thus it is necessary, additional studies that evaluate the functional aspects of the CD1+ LCs, S100+LCs and mainly for CD207+ LCs.

To help understand the differences in the role of LCs between OLP and OLL, we also performed a correlation study to each experimental group. Due to analyze of correlation coefficient (r) and determination coefficient (R^2) made clear that in OLL, the LC appears to have more impact in the role of development and maintain of the hypersensitivity disease than in autoimmune disease. Interestingly, with the linear regression model, we noticed that CD1a+ cells have more capability to influence the density of CD207+ cells in OLP and OLL than the other way around.

In accordance with results in correlation study, the $R^{2,}$ showed that in OLP the LCs have dependency to each other in 62.04% while in OLL this percentage rises to 95.45%. Thus we can suppose that the presence of LCs apparently are more important to the development and maintain of OLL than OLP. Probably, these differences might be explain due to distinct type of antigen that is recognizes to each LCs in each disease.[38]

The present study is the probably the first one in build a mathematical model about LCs in OLP and OLL. Additionally, we would like to highlight that the study of biomarkers should be also direct to the how and how many cells are involving in immunopathogenesis of each disorder. Therefore, we can expand the knowledge about it.

Finally, the OLP malignant transformation is controversy in the literature. A recent systematic review showed that the rate of malignant transformation was not significant in OLP patients.[39] Up to now any study evaluated the role of LCs in OLP malignant. Nevertheless, in oral squamous cell carcinoma (OSCC) authors have been found that a decrease of LC can occurred in OSCC [40] and the high expressions of LCs are responsible to better prognosis in patients with OSCC. [41] So, the high density of LCs found in the present study might be one indicator that upholds the theory OLP is not a potentially malignant disorder. However, further studies should be encouraged to clarify this topic.

In short, despite the higher density of CD207 found in OLP, more studies evaluating the relationship between LCs in OLP and OLL should be performed to better understand the role these cells in lichenoid diseases even as studies evaluating the functional aspects of LCs in these diseases.

Conclusion

The present study found many positive LCs to the S100+ cells followed by CD1a+ cells and CD207+cells in OLL, OLP, OIFHL and OIFHNL. Despite the statistically significant difference between CD207+ cells in OLL and OLP our results can be more explained due to the density of LCs in intraepithelial and sub epithelial areas of all groups. Thus there are almost similar density of LCs in OLL, OLP, OIFHL and OIFHNL. However, the LCs appears to be more participation in the immunopathogenesis of OLL. So the role of these cells in the immunopathogenesis of the lichenoid lesions needs, mainly in functional aspects, to be better clarified.

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3.2 Publicação 2*

Density of lymphocytes in oral lichen planus and oral lichenoid lesions: an immunohistochemical study

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* O artigo segue as normas do periódico *Histopathology, para qual foi submetido.*

Abstract

Introduction: Lymphocytes are cells that regulate the adaptive immune system with essential participation in immunological events in oral cavity. Among them, the CD3, CD4, CD8 and CD20 cells have been associated with oral lichen planus (LPO) and oral lichenoid lesions (LLO) immunopathogenesis. However, due to present of lymphocytes in other lesions that affected the oral cavity, the real involvement of these cells in LPO and LLO immunopathogenesis should better understood.

Objective: To evaluate and compared the density lymphocytes (CD3, CD4, CD8 and CD20) in oral lichen planus (OLP), oral liquenoid lesions (OLL), using as control group the oral inflammatory fibrous hyperplasia (OIFH).

Methodology: 14 cases of OLP, 14 cases of OLL and 14 cases of OIFH, were selected by immunohistochemical analysis for CD3, CD8 and CD20. The density of CD4 was calculated after. Densities lymphocytes were calculated in the intraepithelial and sub epithelial areas. The OIFH group was subdivided according to the presence (OIFHL n=14) and absence (OIFHNL n=14) of lichenoid inflammatory infiltrate. The Multivariate Analysis of Variance with two independent factors was performed (Manova two-way) even as a correlation and linear regression studies. The statistical analyses were performed by IBM SPSS Statistics 20.0

Results: More density of CD3+ cells was found in OLP against OLP ($p=0.013$). Similar quantities of CD4+ cells in OLP, OLL and OIFHL with difference between OLP and OIFHNL was found ($p=0.013$). More CD20+ cells was found in an OIFHL in comparison with OLP ($p=0.001$) and to OLP ($p=0.001$). The correlation and regression studies demonstrated that the lymphocytes biomarkers are dependent with each other ($p<0.0001$) with a very strong association and with different participations in OLP and OLL.

Conclusion: despite of statistical differences between the groups the density of each type of lymphocyte can influence the immunopathogenesis of OLP and OLL. More studies that evaluate density and functional capabilities of the lymphocytes in OLP and OLL should be done to clarify the role of these cells in immunopathogenesis of lichenoid diseases.

Key-words: lymphocytes; oral lichen planus; oral inflammatory fibrous hyperplasia and immunohistochemistry.

Introduction

Oral lichen planus (OLP) is a common immune mediated condition that occurs mainly in oral cavity and has been estimated to affect 1-2% of the population.[1] However in a recent systematic review conduct by Li et al.[2] (2020) the prevalence of OLP may be changed by the type of study. Thus, to observational study the prevalence of OLP ranged varies from 0.02% - 3.25% and to clinic-basic study the prevalence varies from 0.08% - 6.04%. The reported female/male sex ratio is 2 to 1 and the age of onset is generally between 30 and 60 years. [3] Clinically, the lesion of OLP is characterized as chronic, similar, bilateral, recurrent, in some cases painful and it can manifest as plaque, striated, erosive, atrophic, ulcerative and more rarely as bullous. Interestingly, white striated is also observed around the erosive, atrophic and ulcerative lesions. [4] In oral cavity, the buccal mucosa, tongue and gingiva are the most common sites. [5] Microscopic analyses of biopsy's sample of OLP is characterized with signs of liquefaction degeneration in basal cell layer, inflammatory cell infiltration mainly of lymphocytes and absence of epithelial dysplasia.[4] Until now, the etiology of OLP remains unclear, but it is believed that lymphocytes play a central role in the immunopathogenesis of OLP. [6]

Oral lichenoid lesions (OLL) even as OLP are lichenoid lesions although with different nature. OLL is defined was a chronic cell-mediated hypersensitivity. [7] Because of that, the OLL can be classified according the etiology in reactions to amalgam restorations, drug involvement and chronic graft versus host disease (cGVHD). However, when the etiology is not clear, the OLL is classified as lichen planus – like. [4, 8] Clinically and microscopically, the OLL and OLP are similar, being the absence of one aspect or more than one aspect that represents the OLP needed to diagnosis to OLL [4.8]. Therefore, the biopsy is necessary to the final diagnosis of these diseases. [9] The oral inflammatory fibrous hyperplasia (OIFH) is a reactive lesion resulting from chronic irritation due to the use of removable partial or complete prostheses. [10] The OIFH is one of the most frequent oral and maxillofacial lesions in order people (≥60 years). [11] Microscopically, OIFH can included hyperkeratotic epithelium, areas with acanthosis alternating with atrophic areas and the connective tissue of the lamina propria is usually grossly thickened and displays many areas of chronic inflammation coexisting with dense fibrosis. [12]

Resident lymphocytes of the oral mucosa play a prominent role in local immunity and immune tolerance and are responsible to acquired immunity. However,

the immunophenotype and the exact function of lymphocytes in the oral cavity until now remain unclear. [13] In general the beginning of T-lymphocyte maturation start after the interaction with an antigen presenting cells (APC) and functionally ending due to the interaction with antigen and variety of cytokines produced by dendritic cells, macrophages and other lymphocytes [14] This association is responsible to division of T- lymphocytes in regulatory, that normally express positive receptors to CD4 in the surface of the cell and intra-epithelial lymphocytes that normally express positive receptors to CD8 also in the surface of the cell. [14,15]

T-cells centrally contribute to the pathophysiology of autoimmune disease through the generation of specific cytokine, chemo attraction of additional inflammatory cells, and/ or the promotion of autoantibody production by B-cells. [16] The surface cell marker CD20 is expressed during B-cell development and on the maturation from pre-B cells to plasmablasts. Because of that, CD20 is typically considered as a specific B-cell marker. [17] Classically, the lymphocytes CD20+ are responsible to produce antibodies, initiating the humoral immune response.[18]

Therefore, the aim of the present study was to evaluate the density of lymphocytes (CD3, CD4, CD8 and CD20) in OLP, OLL and oral inflammatory fibrous hyperplasia with presence of liquenoid inflammation (OIFHL) and without liquenoid inflammation (OIFHL), hoping in this way, to better understand the role of these cells in the immunopathogenesis of the chronic diseases mentioned above.

Materials and methods

Study population

The methodology of the study was approved by the Ethics Committee of the São Paulo State University (UNESP), School of Dentistry, Araraquara. The study was comprised of 42 patients diagnosed with oral lichen planus (OLP/n=14), oral lichenoid lesions (OLL/n=14) and oral inflammatory fibrous hyperplasia (OIFH/n=14). The OLP and OLL were diagnosed according to the modified criteria of van der Meij et al.[4] (2003). These criteria are divided in clinical and histopathologic aspects. The clinical aspects are presence of bilateral lesion, more or less symmetrical; presence of lace-like network of slightly raised gray-white lines and erosive, atrophic, bullous and plaque-like lesions are only accepted as a subtype in presence of reticular lesions. The histopathologic aspects are presence of a well-defined band-like zone of cellular infiltration that is confined to the superficial part of the connective tissue,

signs of “liquefaction degeneration” in the basal cell layer and absence of epithelial dysplasia. If all the criteria are present, the final diagnosis will be OLP and whether some criteria were not presence, the final diagnosis will be OLL. The OIFH group was subdivided into two other groups due to the presence of lichenoid inflammatory infiltrate (OIFHL) and absence of lichenoid inflammatory infiltrate (OIFHNL). The sample was randomly select to histopathologic and immunohistochemically staining procedure.

Microscopic analysis

The tissue specimens were fixed in 10% neutral-buffered formalin during to 24 hours at room temperature then embedded in paraffin at 55ºC and finally cut into consecutive parallel 3-µm thick sections. In the immunohistochemistry, the slides were hydrated and treated with hydrogen peroxide. Immunohistochemical staining procedure was conducted in biopsy specimens involved the following lymphocyte’s epitopes: CD3, CD8 and CD20. The tissue specimens were pretreated with 10mM sodium citrate buffer, pH 6.0, in a pressure cooker. The sections were then successively put in contact with the primary antibodies CD3 (dilutions 1: 500 DakoCytomation,Glostrup, Denmark), CD8 (dilutions 1:400, DakoCytomation ,Glostrup, Denmark) and CD20 (dilutions 1:2000, DakoCytomation ,Glostrup, Denmark). After that, the sections were incubed with the secondary antibodies conjugated with streptavidin-biotin-peroxidase (K0690; Universal Dako LSAB + Kit, Peroxidase, Carpinteria, California). The reactions developed with diaminobenzidine, and the sections counterstained with Carazzi hematoxylin. Results were then observed with optical microscope with magnification 200X (Leica DM 2500). Five strong staining areas located at intraepithelial and sub epithelial were select for photomicrography (Leica Aplication Suite – LAS). For the cell counting was used the ImagJ sotware. The subtraction of the CD3 in relation to CD8 was used to quantify the presence of lymphocytes CD4.

Statistical analysis

Descriptive statistics was used to evaluate data from patient population and immunohistochemical results. To evaluate the normality distribution of the data was used Shapiro-Wilk test. The presence of outliers was checked. To verify the

homoscedasticity was utilized Box's Test of Equality of Covariance Matrices and the Levene's Test of Equality Variances. Thus the Multivariate Analysis of Variance with two independent factors was performed (Manova two-way). Post-test of Tukey was chosen to evaluate the differences between the groups. The effect factor was calculated when *p* value was statistically significant. Additionally, was performed a correlation study and a simple linear regression with the biomarkers to oral lichen planus (OLP) and oral lichenoid lesion (OLL). A *p* value of < 0.05 was regarded to be statistically significant. Statistical analysis was performed by IBM SPSS Statistics 20.0 and graphics image using GraphPad Prism version 6.0.

Results

Study population

In oral lichen planus (OLP) group 64.29% of the patient were female and 35.71 % were male, being the mean age equal to 48.64 (±13.80). The most affected site was buccal mucosa (35%) following by tongue (27%), gingiva (19%) and lips (19%). The most prevalent clinical aspect was erosive associated with striated plate form (60%). Details about the clinical data are showed in table 1.

In oral lichenoid lesion (OLL) group 80.0% of the patient were female and 20.0% were male being the mean age equal to 53.22 (±6.28). The most affected site was buccal mucosa (40%) following by tongue (36%), lips (14%) and gingiva (10%). The most prevalent clinical aspect was also the erosive form associated with striated plate form (55%).

In oral inflammatory fibrous hyperplasia (OIFH) group 85.71% of the patient were female and 14.29 were male, being the age equal to 43.35 (±17.33). All clinical aspects were the hyperplasia in the border background.

Table 1. Clinical data of patients involving in the present study.

Diagnosis	Gender	Age	Localization	Clinical Aspect
OLP	5M/9F	48.64±13.80	BM (35%); TON (27%); GIN (19%); LIP (19%)	E+S (60%); PL (20%) RT (14%); U+S (6%)
OLL	3M/12F	53.27±6.29	BM (40%); TON (36%); LIP(14%); GIN (10%)	E+S (55%), PL (17%); U+S (17%); U (11%)
OIFH	2M/13F	44.36±17.33	Buccal vestibule fund	Tissue hyperplasia

OLP (Oral lichen planus); OLL (Oral lichenoid lesions); F (feminine gender); M (male gender); BM (buccal mucosa); TON (Tongue); GIN (gingiva); LIP (Lips); E+S (erosive associated with striated plate form); RT (reticulate); PL (plaque); U+S (ulcerative associated with striated plate form); U (ulcerative).

Immunohistochemical results

Descriptive analyses

In descriptive analyses was observed a higher number of positive lymphocytes to all biomarkers used in the sub epithelial area for the oral lichen planus (OLP), oral lichenoid lesion (OLL) and to oral inflammatory fibrous hyperplasia with lichenoid inflammation (OIFHL). Only, in the oral inflammatory fibrous hyperplasia without lichenoid inflammation (OIFHNL) there was more positive cells to CD3 in the intraepithelial area than to sub epithelial area.

Between the groups, the OLP was the disease that has more positive T- cells in all biomarkers used. After that, to CD4 and CD8, OIFHL was the group with more positive cells following to OLL and OIFHNL. To CD20, there were more cells in the OIFHL, following by OIFHNL, OLP and OLL.

Inferential analyses

Multivariate Analyses of Variance with two independent factors (Manova two-way)

In our study, the independent factors were groups (OLP, OLL, OIFHL and OIFHNL) and tissue (intraepithelial and sub epithelial areas). The dependent variates were lymphocytes positive to CD3, CD4, CD8 and CD20. Thus, it is characterizing a Manova two-away study design. The immunohistochemical images are illustrated in figure 1.

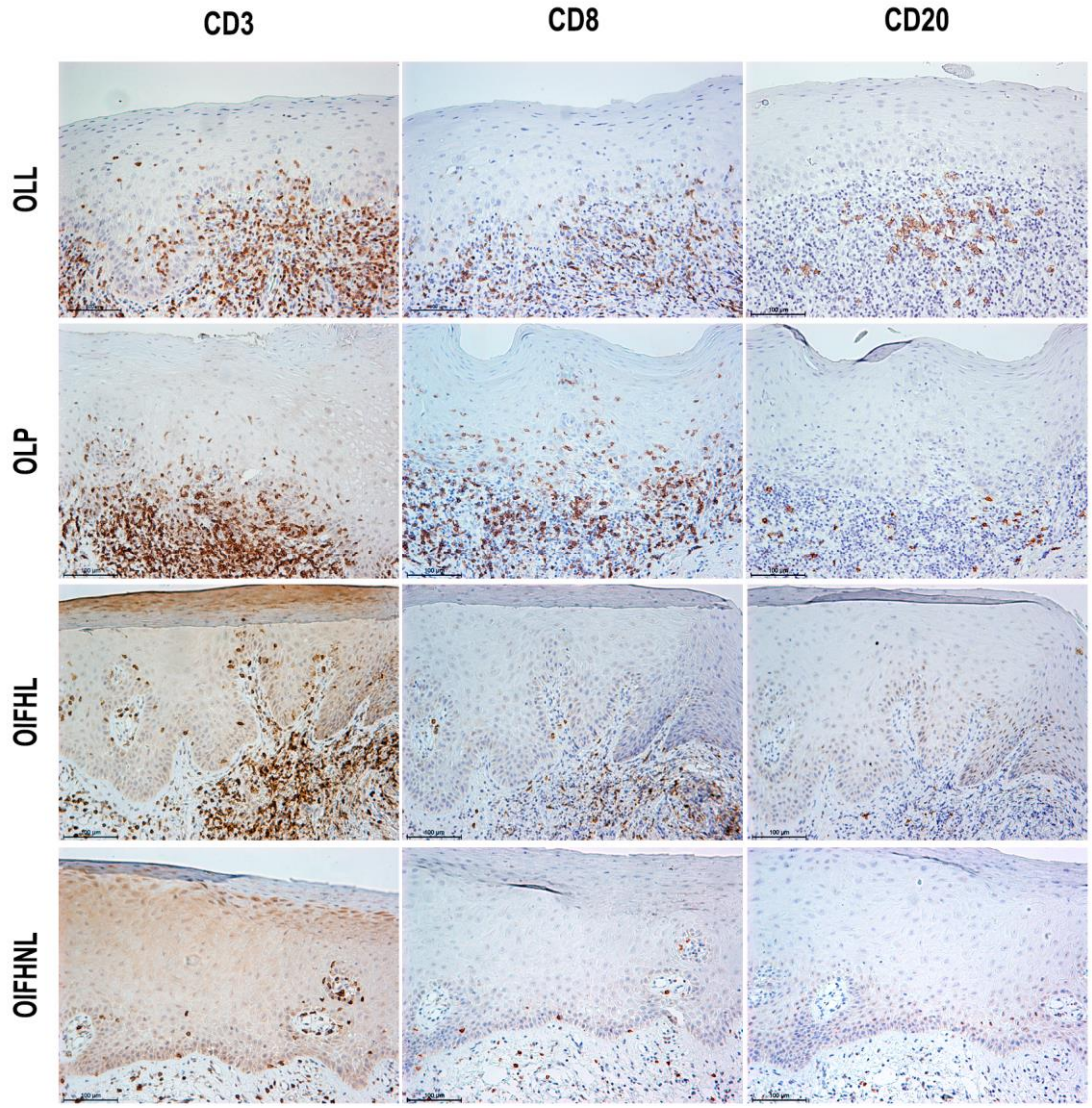

Figure 1. Immunohistochemical images showing positivity to CD3, CD8 and CD20 to the OLL (oral lichenoid lesions), OLP (oral lichen planus), OIFHL (oral inflammatory fibrous hyperplasia with lichenoid inflammatory infiltrate) and to OIFHNL (oral inflammatory fibrous hyperplasia without lichenoid inflammatory infiltrate).

The distribution of data was normal and no-homoscedastic. Box's Test of Equality of Covariance Matrices and the Levene's Test of Equality Variances showed $p < 0.001$. The Roy's Largest Root was utilized to evaluate the Manova two-way. There was a statistical significance in the interaction between groups and tissue (intraepithelial and sub epithelial areas) $p < 0.001$ with effect factor (η^2_p) = 0.193 that was considered a middle level (table 2). Thus, there were a statistical significance to the CD3 (p = 0.001/ η^2_p = 0.152), CD4 (p = 0.005/ η^2_p = 0.112) and to CD20 (p = 0.001/ η^2_p = 0.155) (table 3). All effect factors were also considered as a middle level. To CD8, there was not statistical significance ($p < 0.05$).

Table 2. Roy's Largest Root for multivariate analyses to the lymphocytes.

Factors	Value	Hypothesis df	F	*p*-value	Observed Power	Partial eta square (η^2_p)
Groups	0.268	4.000	7.180	<0.0001	0.994	0.212
Tissue	1.048	4.000	27.518	<0.0001	1.000	0.512
Groups*tissue	0.239	4.000	6.385	<0.0001	0.988	0.193

*: association between independent factors (groups versus tissue); Groups: OLL, OLP, OIFHL and OIFHNL; Tissue: Intraepithelial and Sub-epithelial; F: F statistic; Hypothesis df: degrees of freedom.

Table 3. Multivariate analyses with lymphocytes for independent factors.

Factors	Lymphocytes	Sum of Squares	Mean Square	df	F	*p*-value	Observed Power	Partial Eta square (η^2_p)
Groups*Tissue	CD3	302510.744	100836.915	3	6.453	<0.0001	0.965	0.152
	CD4	170396.645	56798.882	3	4.535	0.005	0.874	0.112
	CD8	24091.395	8030.465	3	2.198	0.092	0.545	0.058
	CD20	11295.696	3765.232	3	6.606	<0.0001	0.969	0.155

*: interaction between independent variables; Groups: OLL, OLP, OIFHL and OIFHNL; Tissue: Intraepithelial and Sub-epithelial; F: F statistic; df; degrees of freedom.

The post-test of Tukey was performed to evaluate to where differences between groups were located. Thus, to the CD3 biomarker there was a statistical significance difference between OLP and OLL (p = 0.014) and between OLP and OIFHNL (p = 0.001). In both situations, there was more density of CD3+ cells in OLP. Towards OIFHNL and OIFHL there was a significance difference (p = 0.013), being found more density of CD3+ cells in OIFHL (Figure 2).

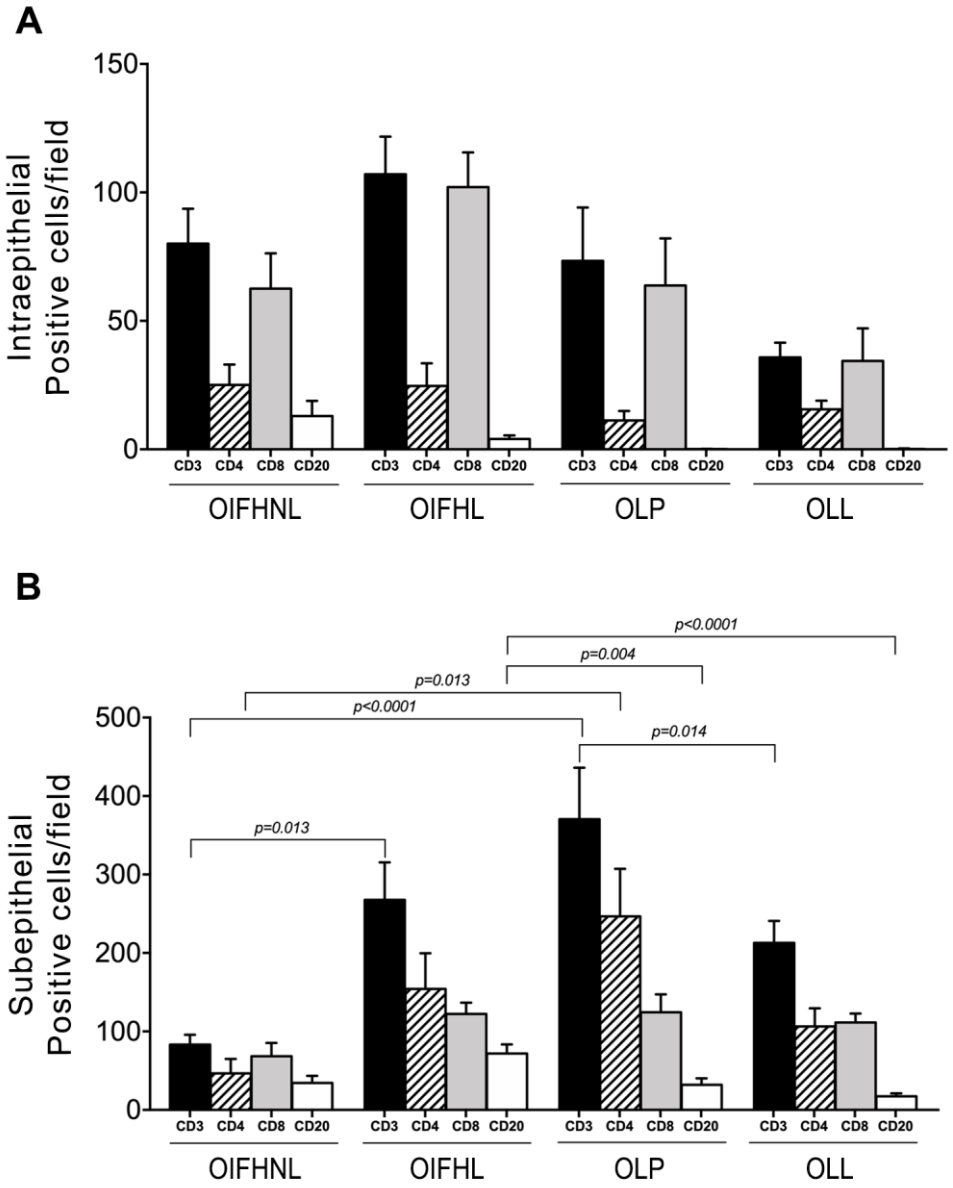

Figure 2. Illustrate the results of post-test of Tukey. OIFHNL: oral inflammatory fibrous hyperplasia without lichenoid inflammatory infiltrates. OIFHL: oral inflammatory fibrous hyperplasia with lichenoid inflammatory infiltrates. OLL: oral lichenoid lesions; OLP: oral lichen planus. Statistical significance was found only in types of lymphocytes located in sub epithelial areas.

To CD4 biomarker there was only a statistical significance difference between OLP and OIFHNL (p = 0.013), being found more density of CD4+ cells in OLP. Finally, to the biomarker CD20, there was a statistical significance difference among OLL and OIFHL (p = 0.001), and to the OLP and OIFHL (p = 0.001). In both situations there were more CD20+ cells in OIFHL.

Correlation and linear regression analyses

In order to evaluate the magnitude and sense of association between two variables, was performed a correlation study to each conjunct of lymphocytes biomarkers using the Pearson's correlation coefficient. However, before the analyses were being done, all data was transformed in log_{10} to eliminate the non-homoscedastic distribution of the data cited above. To each conjunct of lymphocytes biomarkers were observed a significate statistical difference ($p < 0.0001$). Interestingly, all correlation coefficient (r) can be classified as a very strong, and with the analyses of determination coefficient (R^2) we can suppose that lymphocytes have a great participation about the density of each other. More details about the correlation study were showed in table 4.

Table 4. Summary of correlation study of lymphocytes biomarkers from OLP and OLL

Groups	Biomarkers		*p*-value	CI (95%)	r (Pearson)	R^2
OLP	CD3	CD4	<0.0001	0.88 - 0.99	0.9605	0.9226
	CD3	CD8	<0.0001	0.86 - 0.98	0.9530	0.9083
	CD3	CD20	<0.0001	0.89 - 0.99	0.9616	0.9246
	CD4	CD8	<0.0001	0.96 - 1.00	0.9871	0.9743
	CD4	CD20	<0.0001	0.88 - 0.99	0.9602	0.9219
	CD8	CD20	<0.0001	0.89 - 0.99	0.9620	0.9255
OLL	CD3	CD4	<0.0001	0.92 - 0.99	0.9726	0.9460
	CD3	CD8	<0.0001	0.95 - 0.99	0.9815	0.9633
	CD3	CD20	<0.0001	0.82 - 0.98	0.9359	0.8759
	CD4	CD8	<0.0001	0.87 - 0.98	0.9559	0.9137
	CD4	CD20	<0.0001	0.90 - 0.99	0.9652	0.9317
	CD8	CD20	<0.0001	0.86 - 0.98	0.9495	0.9015

OLP: oral lichen planus; OLL: oral lichenoid lesion; CI: confidence interval; r: correlation coefficient; R^2: determination coefficient. P-value <0.05 was considered as statistical significance.

In additional, due to the fact of all correlation have statistical difference it was performed a simple linear regression as the same model to the correlation study. Thus, to each conjunct of the biomarkers were observed a significate statistical difference ($p < 0.0001$). With the analyses of adjust determination coefficient ($R^{2'}$) we can suppose that lymphocytes have a dependency to each other differently to OLP

and to OLL. More details about the regression study and the regression models can be observed in table 5.

Table 5**.** Summary of simple linear regression of lymphocytes biomarkers from OLP and OLL

Group	**Y**	**X**	***p*-value**	**R^2**	***p*-value (a)**	***p*-value (b)**	**Equation log Y = a + b · log X**	**CI (95%) (a)**	**CI (95%) (b)**
OLP	CD8	CD4	<0.0001	0.9565	0.0556	<0.0001	log Y = 0.21 + 0.81 log X	0.006 – 0.43	0.71 – 0.91
	CD20	CD4	0.0001	0.7169	0.0883	<0.0001	log Y = -0.56 + 0.83 log X	-1.22 – 0.09	0.53 – 1.13
	CD4	CD8	<0.0001	0.9565	0.2331	<0.0001	log Y = -0.17 + 1.18 log X	-0.46 – 0.12	1.03 – 1.33
	CD4	CD20	<0.0001	0.9159	0.4668	<0.0001	log Y = 19.03 + 7.09 log X	-35.80 – 73.87	5.85 – 8.33
OLL	CD8	CD4	<0.0001	0.9526	<0.0001	<0.0001	log Y = 1.46 + 0.30 log X	1.39 – 1.53	0.27 – 0.34
	CD20	CD4	<0.0001	0.8615	0.0155	<0.0001	log Y = -0.44+ 0.83 log X	(-0.78) – (-0.09)	0.65 – 1.01
	CD4	CD8	<0.0001	0.9526	<0.0001	<0.0001	log Y = -4.43 + 3.08 log X	(-5.20) - (-3.66)	2.70 – 3.46
	CD4	CD20	<0.0001	0.8615	<0.0001	<0.0001	log Y = 0.68 + 1.04 log X	0.41 – 0.95	0.81 – 1.27

OLP: oral lichen planus; OLL: oral lichenoid lesion; Y: dependent variable; X: independent variable; R^2: adjusted determination coefficient; (a): linear coefficient; (b): angular coefficient; CI: confidence interval. P-value <0.05 was considered as statistical significance.

Discussion

There are a few studies in the scientific literature that compared the density of the subpopulation of lymphocytes in oral lichen planus (OLP) and oral liquenoid lesion (OLL) with other disease characterized with the presence of lichenoid inflammation. [19,20] Omar et al.[20] (2009), evaluated the density of subpopulations of lymphocytes in OLP and chronic junctional stomatitis. The authors concluded that there are more CD4+ cells in OLP than chronic junctional stomatitis. In additional the author also evaluated the expression of CD4+ and CD8+ cells being more CD4+ cells found in OLP. These results are similar with the present study. Zhou et al.[21] (2016) evaluated the density of subpopulations of lymphocytes in clinical variations of lichen planus, however no difference between the lymphocytes was found. Our results are opposite, because we discovered differences among the lichenoid groups to the biomarkers CD3 and CD20, being more CD3+ cells in OLP and more CD20+ cells in OIFHL.

The etiology of OLP is not yet fully understand, although it is believe that CD8+ cells represents the majority of the cells involved in the pathogenesis of OLP.[21] However a recent study reported more CD4+ cells than CD8+ cells in this disease. The authors also concluded that low levels of CD8+ cells can be a diagnostic biomarker of the remission of OLP. [22] Additionally, CD4+ cells modify the cytotoxic of CD8+ cells and the increased number of CD4+ cells is may be associated with a good response to OLP treatment.[23] The present study did not have as aim the association between clinical and immunohistochemical aspects, but all OLP patients did not receive any local or systemic treatment before the final diagnostic and the major of them presented satisfactory results after local treatment with corticosteroids. Interestingly, in our data, we faced more CD4+cells than CD8+ cell in OLP group and appears that CD4+cells has great responsible to development of lichenoid infiltrate.

The CD4+ and CD8+ cells develop within thymus from double-positive precursor cells. After that, T-cells are released in the periphery as mature naïve (T_N) cells that working on between secondary lymphoid organs.[24] In contrast of CD8+ T_N cells, the CD4+ T_N cells can differentiated into several effect subsets after the APC stimulation, as Th1, Th2, Th17, Th22, T-tregs and Tfh, The factors in the local environment, such as cytokines and T-cell receptor (TCR) signaling strength are responsible to this differentiation .[25] Each CD4+ cell subset has yours own

transcription factor, which promote a lineage-specific gene expression and cytokine secretion, thus activing of many different ways in the immunological response [24]

Current scientific evidence suggest that multiple signals such as TCR, co-stimulation, inflammation and metabolic signals can orchestrate the CD8+ cells fate decisions.[26] Functionally, the CD8+cells has two great performances: cytotoxic and memory. However, there is evidence suggesting that CD8+cells can also act regulating the immunological process as Treg.[27] Further studies involving the relationship between these types of cell should be encouraged.

The Th1, Th2 and Tfn are subpopulations of CD4+ lymphocytes, and they are responsible to orchestrate the humoral adaptive response, though activating and inducing B-cells to product antibodies.[20] Ours results showed that OLP and OLL presented approximately the same density of CD20+ cells, being significance difference with OIFHL that presented more density of this cells. Among all lymphocytes biomarkers used to study the lichenoid diseases, the CD20 is the least employed, and their results about the cellular density are controversy.[19,20] Therefore more studies must be applied about the role of CD20+ cells in OLP and OLL.

With effect factor analyses we also can observed that the differences among the groups are considering as middle level.[30] In other words, only the density of different lymphocytes between the groups are not integrally responsible to the differences in clinical and microscopically aspects. Therefore, more studies involving the functional characteristics about lymphocytes, as inflammatory mediators, different interleukins and polymorphism should be done to better understand the exact role of the lymphocytes and other immunological cells in the immunopathogenesis of OLP and OLL.[9, 31]

With the correlation studies and subsequently linear regressions made clear that only the different densities of the lymphocytes in the groups cannot explain how these cells working on OLP and OLL. This is one the great advances of using a linear regression study. Just as, the results showed the exact density of the lymphocytes biomarkers that are important to the development and/or maintenance of the OLP and OLL and how these cells are impact the density of each other. Thus, we highlight that the smaller participation in the density of lymphocytes are between CD4+ cells and CD20+, being this percentage of participation to OLP equal to 71.69% and to the OLL, 86.15%.

B-cells can also regulate T and B responses including the maintenance of CD4 treg.[32,33] It is interesting to know, that in the linear regression analyses, for OLP, one little amount of CD20+ cells in able to influence the expression of a greater amount of CD4+cells, being responsible to the 91.59% ($R^{2'}$) of this expression. To OLL, this relation is significant, but CD20+cells can influence the less amount expression of CD4+ cells. So we highlight the importance of CD20+ cells may have to the development of OLP, maybe indicating that in OLP, humoral immune response and auxiliary T-cells response have great participation in immunopathogenesis. The present study was the first to build a mathematical model to evaluate how the densities of lymphocytes are related with others in OLP and OLL.

We also performed a linear regression with CD8+ cells influencing the density of CD4+cells, because there is growing the evidence of this interaction in context of hypersensitivity reactions, autoimmunity and infections.[34,35] In the analyses, we evidenced that the CD8+ cells can participate the influence the CD4+ cells in 95.65% ($R^{2'}$) to the OLP and to the OLL in 95,26% ($R^{2'}$), but with more impact in the OLP.

The exact cause of the different densities of lymphocytes in OLP and OLL are obscure. Maybe, the type and density of APC may influence the density of the lymphocytes modulating the inflammatory responses and consequently influencing the clinical as microscopical characteristics to OLP and OLL.[36, 37]

The possibility and the rate of OLP to malignant transformation are controversy in the literature. A recent systematic review conducted by Giuliane et al.[38] (2019) was performed to clarify this topic. The results showed that the rate was not significant, relegating any possibility of OLP malignant transformation. There are not studies that evaluate the role of lymphocytes in a possibility of malignance of OLP. However further researches should be direct to evaluate this deficiency, because in tumors, the infiltration of cytotoxic T-cells are associated with a good prognosis while regulatory T-lymphocytes suppress anti-tumor response.[39] The exact action of B-cells in tumor is still unfamiliar, but these cells can act as anti-tumorigenic or pro-tumorigenic depends on the type of tumor, activating way, relationship with other cells and inflammatory and immunologic mediators.[40]

In a nutshell more studies should be direct to analyses the density and functional of the lymphocytes employed in immunopathogenesis of OLP and OLL.

The use of OIFH demonstrated to be a good choice to control group due to the great prevalence in the population.

Conclusion

In OLP the presence of high levels CD3+ cells, CD4+ cells and low level of CD20 is possibility related with his immunopathogenesis while for OLL the expression of CD3+cells and CD4+ cells are in moderate level and low level to CD20+ cells. The exact density of the types of lymphocytes may explain the different in immunopathogenesis and subsequently in clinical and microscopically aspects. The great participation of CD20+cells in the density of CD4+ cells in OLP may indicate that in OLP, the humoral immune response as well as the auxiliary T-cells response have a great impact in the development and maintain this specific disease.

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3.3 Publicação 3*

Density of macrophages in oral lichen planus and oral lichenoid lesions: an immunohistochemical study

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Abstract

Introduction: The macrophages are phagocytic cells that act in innate and adaptive immune response with essential participation in immunological events in oral cavity. The macrophages can be classified in two main groups; pro-inflammatory (CD68) and anti-inflammatory (CD163). There are a few studies in literature that evaluate the density of these macrophages in oral lichen planus (OLP) and in oral lichenoid lesions (OLL).

Objective: To evaluate and compared the density macrophages (CD68 and CD163) in oral OLP, OLL using as control group the oral inflammatory fibrous hyperplasia (OIFH) by immunohistochemical staining.

Methodology: 14 cases of OLP, 14 cases of OLL and 14 cases of OIFH, were selected by immunohistochemical analysis to CD68 and CD163. Densities of macrophages were calculated in the intraepithelial and sub epithelial areas. The Multivariate Analysis of Variance with two independent factors was performed (Manova two-way) even as a correlation and linear regression studies. The statistical analyses were performed by IBM SPSS Statistics 20.0

Results: there was a statistical significance in the interaction between groups and tissue (intraepithelial and sub epithelial areas) (p = 0.008) being more positive cells found in sub epithelial areas. Based in analyses among groups, there is only a statistical difference to the CD68 biomarker (p = 0.013), being OLP the group with more positive cells. The correlation studied showed a very strong relationship between the macrophages types. The linear regression showed that to the OLP; CD163 is more responsible to density of CD68 and to the OLL; CD68 is more responsible to density of CD68.

Conclusion: despite the differences between the CD68+ cells, the density of each type of macrophage can influence the immunopathogenesis of OLP and OLL. More studies that evaluate density and functional aspects of the macrophages in OLP and OLL should be done to clarify the role of these cells in immunopathogenesis of lichenoid diseases.

Key-words: lymphocytes; oral lichen planus; oral inflammatory fibrous hyperplasia and immunohistochemistry.

Introduction

Oral lichen planus (OLP) is a chronic immunomediated mucocutaneous disorder that affected 0.08% to 6.04% of general population. [1] The etiology of OLP is unknown, but there is evidence that lymphocytes play a central role in pathogenesis. [2] Clinically, the vast majority of patients affected with OLP presented lesions only in oral mucosa. A small set of patients has oral lesions simultaneously with cutaneous regions.[3] Oral lesion are nearly always in bilateral, more or less symmetrical, being some lesions painful. Additionally, the most common sites affected are buccal mucosa, tongue and gingiva.[4] The clinical morphology can vary as plaque, striated, erosive, atrophic, ulcerative and more rarely as bullous forms. Curiously, white striated is also observed around the erosive, atrophic and ulcerative lesions.[4,5] Histopathological analyses of OLP's samples is characterized with signs of liquefaction degeneration in basal cell layer, inflammatory cell infiltration mainly of lymphocytes and absence of epithelial dysplasia.[5] For treatment the options are lifestyle modifications, use of topical and systemic corticosteroids and in most severe cases use of immunomodulators and biological agents can be administrated. [6]

Different to the OLP, oral lichenoid lesions (OLL) are hypersensitivity reactions directed mainly to the metallic structures presented in oral mucosa, after drugs administration and as a reaction that belongs to chronic graft versus host diseases.[4,5] Interestingly, OLP and OLL presented clinical and microscopic aspects that are similar between then, being the absence of one aspect or more than one aspect that represents the OLP needed to diagnosis to OLL [4,5] However, in some cases is very difficult to distinguish these diseases.[4,5] The oral inflammatory fibrous hyperplasia (OIFH) is a reactive lesion resulting from chronic irritation due to the use of removable partial or complete prostheses with adaptive problems. [7] Because of that, nowadays, the OIFH is more frequently seen in order population (≥60 years). [8] Histopathological analyses of OIFH's samples are characterized with hyperkeratotic epithelium, areas with acanthosis alternating with atrophic areas and the connective tissue of the lamina propria is usually grossly thickened and displays many areas of chronic inflammation coexisting with dense fibrosis.[9]

Macrophages are phagocytic cells derived from blood monocytes and they are most commonly known, as one of the cells that act as first line defense against antigens.[10] Monocytes differentiated in macrophages when in presence of inflammation in tissues. [11] However recent studies indicate that macrophages may

develop from embryonic leukocyte precursors without needed for a monocyte intermediate. [12] Macrophages have import functions in the organism such as recognizes and kill pathogens, initiate and resolve inflammation and heal and modulate adaptive immune system. [10] Besides that, macrophages can be divided according with your polarization. Thus there are two main groups: classically activated macrophages (M1), witch drive pro-inflammatory responses and alternatively activated macrophages (M2) which can act in control immune regulation and tissue remodeling.[12-14] Additionally, M2 can be subdivided into M2a, M2b, M2c, M2d based on transcriptional changes after the exposure of different stimuli.[13,14]

The exact role of macrophages in chronic inflammatory diseases still unfamiliar and can be different due to the macrophage polarization in each illness.[15] Therefore, to better understand the role of these cells in the immunopathogenesis of the OLP, OLL and OIFH we performed a immunohistochemical analyses using as biomarkers CD68 (M1) and CD163 (M2) to evaluate the density of these cells in the diseases mentioned above.

Materials and methods

Study population

The methodology of the study was approved by the Ethics Committee of the São Paulo State University (UNESP), School of Dentistry, Araraquara. The study was comprised of 42 patients diagnosed with oral lichen planus (OLP/n=14), oral lichenoid lesions (OLL/n=14) and oral inflammatory fibrous hyperplasia (OIFH/n=14). The OLP and OLL were diagnosed according to the modified criteria of van der Meij et al.[5] (2003). For performed the diagnostic, the modified criteria has been divided in clinical and histopathologic aspects. The clinical aspects are: presence of bilateral lesion, more or less symmetrical; presence of lace-like network of slightly raised gray-white lines and erosive, atrophic, bullous and plaque-like lesions are only accepted as a subtype in presence of reticular lesions. The histopathologic aspects are presence of a well-defined band-like zone of cellular infiltration that is confined to the superficial part of the connective tissue, signs of “liquefaction degeneration” in the basal cell layer and absence of epithelial dysplasia. If all the criteria are present, the final diagnosis was OLP and whether some criteria were not presence, the final diagnosis was OLL. The sample was randomly select to histopathologic and immunohistochemically staining procedure.

Histopathological analysis

The tissue specimens were fixed in 10% neutral-buffered formalin during to 24 hours at room temperature then embedded in paraffin at 55ºC and finally cut into consecutive parallel 3-µm thick sections. To prepare the immunohistochemical procedures, the slides were hydrated and treated with hydrogen peroxide. Immunohistochemical staining performance was conducted in biopsy specimens involved the following macrophages' epitopes: CD68 and CD163. The tissue specimens were pretreated with 10mM sodium citrate buffer, pH 6.0, in a pressure cooker. The sections were then successively put in contact with the primary antibodies CD68 (dilutions 1: 3000 DakoCytomation ,Glostrup, Denmark), CD163 (dilutions 1:500, Leica Biosystems Newcastle, UK). Thereafter, the sections were incubed with the secondary antibodies conjugated with streptavidin-biotin-peroxidase (K0690; Universal Dako LSAB + Kit, Peroxidase, Carpinteria, California). The reactions developed with diaminobenzidine, and the sections counterstained with Carazzi hematoxylin. Results were then observed with optical microscope with magnification 200X (Leica DM 2500). Five strong staining areas for epithelium and lamina propria were select for photomicrography (Leica Aplication Suite – LAS). ImagJ sotware has used to cell account.

Statistical analysis

Descriptive statistics was used to evaluate data from patient population and immunohistochemical results. After that, to evaluate the normality distribution of the data was used Shapiro-Wilk test. The presence of outliers was checked. The homoscedasticity has been checked according to the following tests: Box's Test of Equality of Covariance Matrices and the Levene's Test of Equality Variances. Thus the Multivariate Analysis of Variance with two independent factors was conducted (Manova two-way). Post-test of Tukey was chosen to evaluate the differences between the groups. The effect factor was calculated when *p* value was statistically significant. In additional, we also performed a correlation study and a simple linear regression with the biomarkers to oral lichen planus (OLP) and oral lichenoid lesion (OLL). A *p* value of < 0.05 was regarded to be statistically significant. Statistical analysis was performed by IBM SPSS Statistics 20.0 and graphics image with GraphPad Prism version 6.0.

Results

Study population

The oral lichen planus (OLP) group was comprised with 64.29% of the patient being female and 35.71 % being male. The mean age was equal to 48.64 (±13.80). The most affected site was buccal mucosa (35%) following by tongue (27%), gingiva (19%) and lips (19%). The most prevalent clinical aspect was erosive form associated with striated plate form (60%). Details about the clinical data are showed in table 1.

The oral lichenoid lesion (OLL) group was comprised of 80.0% of the patient being female and 20.0% being male. The mean age was equal to 53.22 (±6.28). The most affected site was buccal mucosa (40%) following by tongue (36%), lips (14%) and gingiva (10%). The most prevalent clinical aspect was also the erosive form associated with striated plate form (55%).

The oral inflammatory fibrous hyperplasia (OIFH) group was comprised of 85.71% of the patient being female and 14.29% being male. The mean age was equal to 43.35 (±17.33). All clinical aspects were the hyperplasia in the border background.

Table 1. Clinical data of patients involving in the present study.

Diagnosis	Gender	Age	Localization	Clinical Aspect
OLP	5M/9F	48.64±13.80	BM (35%); TON (27%); GIN (19%); LIP (19%)	E+S (60%); PL (20%) RT (14%); U+S (6%)
OLL	3M/12F	53.27±6.29	BM (40%); TON (36%); LIP(14%); GIN (10%)	E+S (55%), PL (17%); U+S (17%); U (11%)
OIFH	2M/13F	44.36±17.33	Buccal vestibule fund	Tissue hyperplasia

OLP (Oral lichen planus); OLL (Oral lichenoid lesions); F (feminine gender); M (male gender); BM (buccal mucosa); TON (Tongue); GIN (gingiva); LIP (Lips); E+S (erosive associated with striated plate form); RT (reticulate); PL (plaque); U+S (ulcerative associated with striated plate form); U (ulcerative).

Immunohistochemical results

Descriptive analyses

In descriptive analyses was observed a higher number of positive macrophages to both biomarkers used in the sub epithelial areas for the oral lichen planus (OLP), followed by the oral inflammatory fibrous hyperplasia (OIFH) and oral

lichenoid lesion (OLL). Between the groups, the OLP was the disease that has more positive macrophages in all biomarkers used. After that, to the biomarker CD68 was found more positive cells in OIFH followed by OLL and to the biomarker CD163 was found more positive cells in OLL followed by OIFH.

Inferential analyses

Multivariate Analyses of Variance with two independent factors (Manova two-way)

In our study, the independent factors were groups (OLP, OLL and OIFH) and tissue (intraepithelial and sub epithelial areas). The dependent variates were the density of macrophages positives to CD68 and CD163. Thus, it is characterizing a Manova two-away study design. The Immunohistochemical images are illustrated in figure 1.

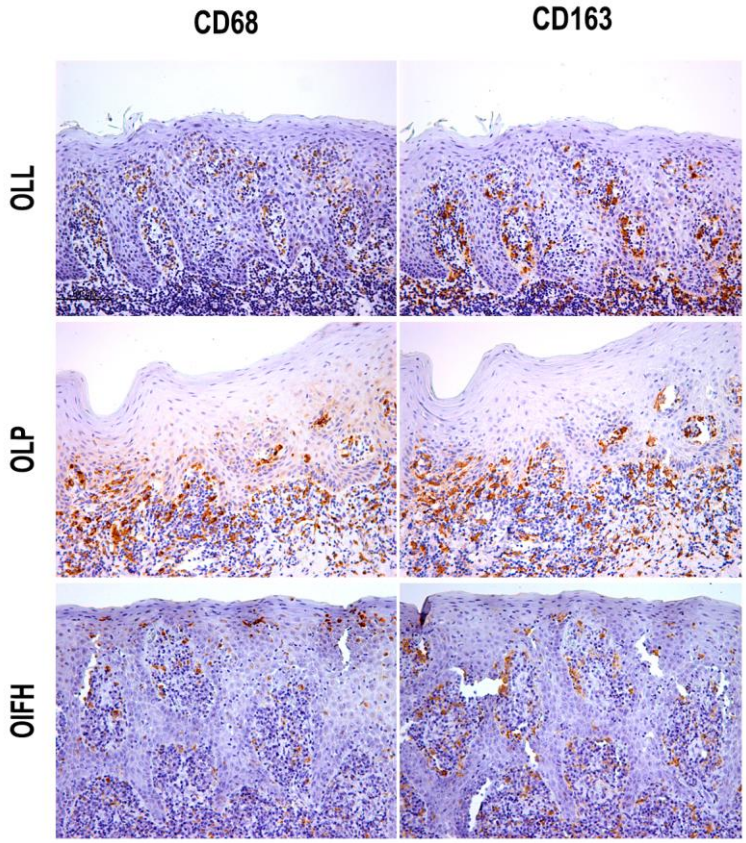

Figure 1. Immunohistochemical images showing positivity to CD68 and CD163 to the OLL (oral lichenoid lesions), OLP (oral lichen planus) and OIFH (oral inflammatory fibrous hyperplasia.

The distribution of data was normal and no-homoscedastic. Box's Test of Equality of Covariance Matrices and the Levene's Test of Equality Variances showed $p < 0.001$. Thus was utilized the statistics of Roy's Largest Root to evaluate the Manova two-way. There was a statistical significance in the interaction between groups and tissue (intraepithelial and sub epithelial areas) $p = 0.008$ with effect factor (η^2_p) = 0.135 that was considered as middle level (table 2). Thus, there were only a statistical significance to the biomarker CD68 $p = 0.013$ and $\eta^2_p = 0.123$ that was also considered as middle level (table 3).

Table 2. Roy's Largest Root for multivariate analyses to the macrophages.

Factors	Value	Hypothesis df	F	*p*-value	Observed Power	Partial eta square (η^2_p)
Groups	0.258	2.000	8.528	0.001	0.960	0.205
Tissue	0.813	2.000	26.407	<0.0001	1.000	0.448
Groups*tissue	0.156	2.000	5.153	0.008	0.809	0.135

*: association between independent factors (groups versus tissue); Groups: OLL, OLP, and OIFH; Tissue: Intraepithelial and Sub-epithelial; F: F statistic; Hypothesis df: degrees of freedom.

Table 3. Multivariate analyses with types of macrophages to independent factors.

Factors	Macrophages	Sum of Squares	Mean Square	df	F	*p*-value	Observed Power	Partial Eta square (η^2_p)
Groups*Tissue	CD68	4291.844	2145.922	2	4.629	0.013	0.793	0.123
	CD163	3281.791	1640.896	2	1.520	0.226	0.312	0.044

*: interaction between independent variables; Groups: OLL, OLP, and OIFH; Tissue: Intraepithelial and Sub-epithelial; F: F statistic; df; degrees of freedom.

The post-test of Tukey was performed to evaluate to where differences between groups were located. Thus, there is statistical significance in macrophages CD68+ between OLP and OLL ($p = 0.001$) and with OLP against OIFH ($p = 0.045$) (figure 2).

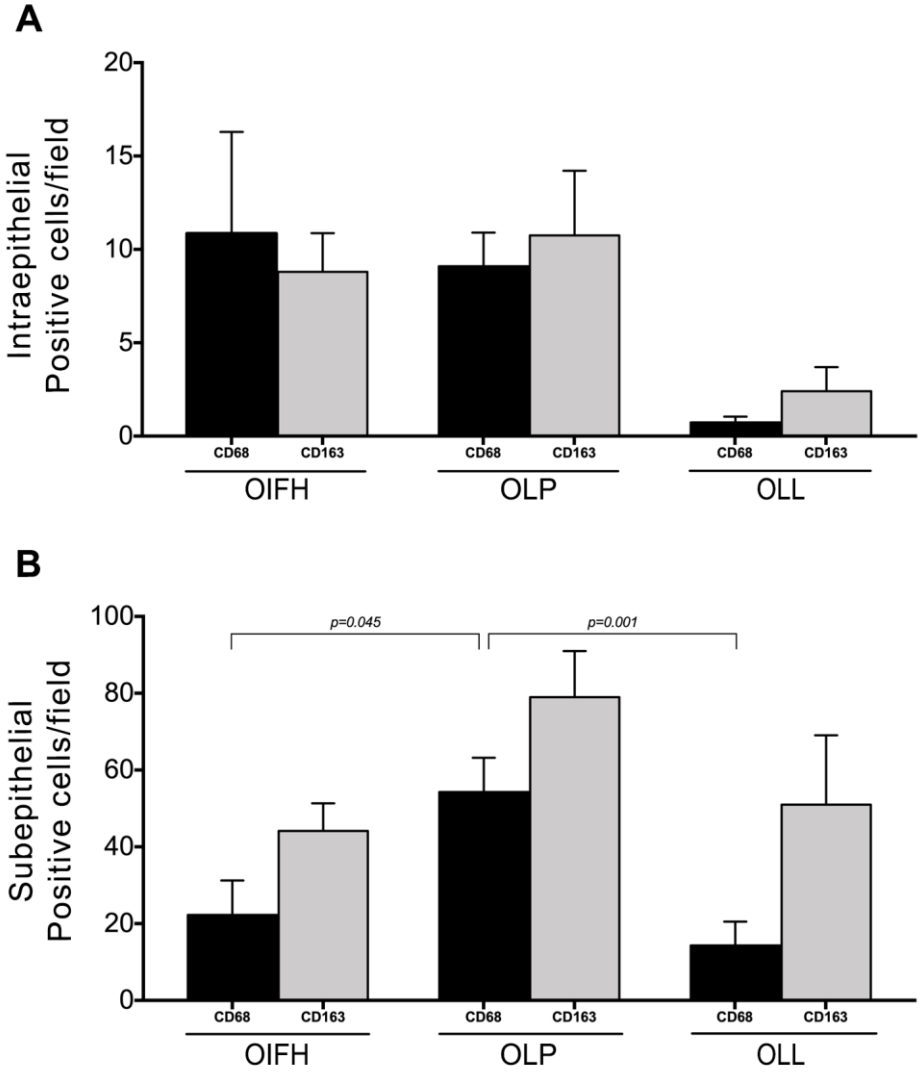

Figure 2. Illustrate the results of post-test of Tukey. OIFH: oral inflammatory fibrous hyperplasia. OLL: oral lichenoid lesions; OLP: oral lichen planus. Statistical significance was found only in types of macrophages located in sub epithelial areas.

Correlation study and linear regression model

In order to evaluate the magnitude and sense of association between two variables, was performed a correlation study to density of macrophages positives to CD68 and to the CD163 using the Pearson's correlation coefficient. However, before the analyses were being done, all data was transformed in $\log_{10}$ to eliminate the non-homoscedastic distribution of the data cited above. There was a statistical significance between the biomarkers (p < 0.0001). Intriguingly, the correlation coefficient (r) to OLP was equal to 0.9525 and to the OLL r = 0.9809 that are classified as very strong. The analysis of determination coefficient (R2) was equal

0.9076 to the OLP and 0.9622 to the OLL. Thus we can suppose that each type of macrophage have a great participation about the density of each other to the OLP and OLL.

In additional, due to the fact the correlation study have statistical difference it was performed a simple linear regression to each conjunct of macrophages biomarkers to OLP and OLL. To each conjunct of the biomarkers were observed a significate statistical difference ($p < 0.0001$). With the analyses of adjust determination coefficient ($R^{2'}$) and with the linear regression model we can suppose that different macrophages have a great dependency to each other and this relation is differently to OLP and to OLL (table 4 and figure 3).

Table 4**.** Summary of simple linear regression of macrophages biomarkers from OLP and OLL

Group	**Y**	**X**	***p*-value**	**$R^{2'}$**	***p*-value (a)**	***p*-value (b)**	**Equation log Y = a + b · log X**	**CI (95%) (a)**	**CI (95%) (b)**
OLP	CD68	CD163	<0.0001	0.8983	0.0069	<0.0001	log Y = -0.86 + 1.36 log X	(-0.14) – (0.29)	1.05 – 1.66
	CD163	CD68	<0.0001	0.8983	<0.0001	<0.0001	log Y = -0.56 + 0.83 log X	0.49 – 0.99	0.51 – 0.81
OLL	CD68	CD163	<0.0001	0.9584	0.5042	<0.0001	log Y = -0.04 + 0.68 log X	(-0.09) – 1.17	0.59 – 0.78
	CD163	CD68	<0.0001	0.9584	0.8134	<0.0001	log Y = -0.02+ 1.42 log X	(-0.21) – 0.17	1.20 – 1.59

OLP: oral lichen planus; OLL: oral lichenoid lesion; Y: dependent variable; X: independent variable; $R^{2'}$: adjusted determination coefficient; (a): linear coefficient; (b): angular coefficient; CI: confidence interval. P-value <0.05 was considered as statistical significance.

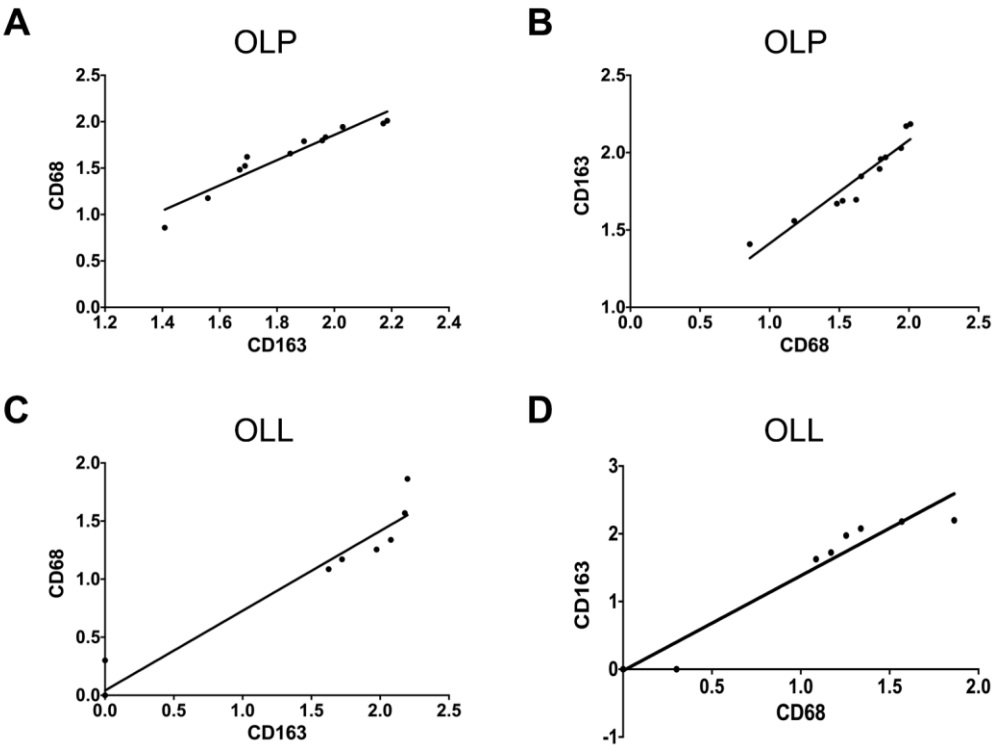

Figure 3. Illustrate the regression models to the OLP (A-B) and to the OLL (C-D). All regression models presented significance statistical ($p < 0.0001$). The regression models are. (A); log Y = -0.86 + 1.36 log X: (B); log Y = -0.56 + 0.83 log X; (C): log Y = -0.04 + 0.68 log X; (D): log Y = -0.02+ 1.42 log X.

Discussion

There are a few studies in a current literature that evaluate the density of macrophages in oral lichen planus (OLP) [16, 17] and in oral lichenoid lesions (OLL). [17] The first report to evaluate the density of CD163+ cells (M2) in OLP was in 2013 with the study of Vered et al.[16] (2013) The authors concluded that M2 cells are less expressed than others dendritic and lymphocytes pro-inflammatory biomarkers. However, M2 cells were more frequently expressed in patients with erosive form of OLP than patients with hyperkeratotic forms of OLP. [16] The study of Motta et al.[17] (2018) evaluate the density of chronic graft-versus-host disease against OLP; the authors concluded that no differences between the density of M2 cells was found.[17] This result is in agree with ours, due to we did not find difference in expression of CD163+ cells among the groups. Additionally, there are no currently studies that evaluate the expression of CD68+ cells (M1) in lichenoid lesions. However in OLP that is credited that CD68 is the biomarker more frequently associated than CD163.

CD68 is a heavily glycosylated type I transmembrane glycoprotein, which is mainly associated with endosomal and lysosomal compartment. CD68 is used to identify cells of macrophage linage such as histiocytes, multinucleated giant cells and osteoclasts.[19] Additionally, CD68 is not expressed by dendritic cells and this helps to immunohistochemically distinguish macrophages from dendritic cells, being both of them have antigen-presenting capabilities.[20] The exact role of CD68 in inflammation and immunity is still mysterious. [21] Interestingly, the study of Song et al.[22] (2011) found that CD68-deficient demonstrated non-affected capacity to present antigen and induce humoral immune responses. Furthermore, the authors observed that CD68-deficient mononuclear phagocyte in comparison with dendritic cells shown an increased ability to presentation to CD4+ T-cells suggesting that CD68 might negatively regulate either antigen uptake and major histocompatibility complex (MHC) of class II trafficking.

Curiously, ours results showed a high expression of CD68+ cells in OLP when comparing with OLL and with oral inflammatory fibrous hyperplasia (OIFH). Therefore we can suppose the CD68 is high expressed in autoimmune diseases when comparing in a hypersensitive and chronic reactional diseases.

The hemoglobin scavenger receptor or CD163 is a macrophage-specific membrane protein being high expression of CD163 in macrophages one of the characteristics of tissue responding to inflammation. [23] Even as the biomarker CD68, CD163 helps to immunohistochemically distinguish macrophages from dendritic cells, but with a little expression in dendritic cells too.[23] Although the CD163 represents an anti-inflammatory responses, there are scientific reports in literature with high levels of CD163 in chronic inflammatory diseases.[24,25] Additionally, the reduce of CD163 in autoimmune diseases development is controversy in literature.[26,27] Fascinating, our results showed approximately the same expression of CD163 in OLP , OLL and OIFH. Further studies should be conducted to clarify the role of CD163 in inflammatory diseases.

After the correlation study was performed, we notice that CD68+ and CD163+ cells are variables that rise together directly and positively. In other words, the density of CD68+ cells that is pro-inflammatory do not reduce the density of CD163+ cells that act as anti-inflammatory. The difference between these types of macrophages in OLP and OLL must be analyses with a linear regression model to evaluate if there are dependencies between the expressions of each other.

Thus, with the linear regression analyses we noticed that in OLL the dependency of CD68+ and CD163+ cells is higher in OLL than OLP ($R^{2'}$). However the dependency between the macrophages types in both diseases are considered as great ($R^{2'}$ > 89.0%). Interestingly, in OLP group we can highlight that the density of CD163+ cells can influence more the density of CD68+cell than the other way around. This fact associated with more CD163+ cells in OLP found in descriptive analyses may influence the statistical difference found in CD68+cells in comparison among groups. The difference among groups can be measure from the point of view of biological significance calculating the effect factor (η^2_p), which to CD68 was considering as middle level.[28] To the group OLL we can highlight that the density of CD68+ cells can influence more the density of CD163+ cells than the other way around. Again this fact can help understand the differences found in inferential analyses among the group's studies.

For the best we know, the present study was the first in build a mathematical model through linear regression analyses that explain how and how many density of macrophages are necessary to development and maintain of OLP and OLL. In additional, this model helps in explaining the relationship between CD68+ and CD163+ cells. Apparently, only the differences between groups are not being able to show the differences among groups. It is necessary studies to evaluate the relationship of the cells in immunological responses and their functional aspects.[29]

The possibility and the rate of OLP to malignant transformation are controversy in the literature. A recent systematic review performed by Giuliane et al.[30] (2019) showed that the rate was not significant, relegating any possibility of OLP malignant transformation. Until now any study evaluated the role of macrophages in OLP malignant. Nevertheless, in oral squamous cell carcinoma the higher concentration of CD68+ and CD163+ cells are related with worse prognosis.[31] Maybe studies involving the role of macrophages in OLP malignant transformation should be done to clarify this point.

In short, despite the higher density of CD68 found in OLP, more studies evaluating the relationship between CD68 and CD163 in OLP and OLL should be performed to better understand the role of macrophages in lichenoid diseases even as studies evaluating the functional aspects of macrophages in these diseases.

Conclusion

The biomarker CD68 presented higher levels when comparing with OLL and OIFH. This result can be explain due to the relationship between the CD163 influencing the density of CD68. In OLL and OIFH there were approximately the same density of CD68 and CD163. In OLL differently with OLP, the CD68 can influence more the density of CD163. The exact density of the macrophages types may explain the differences in immunopathogenesis and the development of the lichenoid diseases.

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4 CONCLUSÃO

As diferenças dos biomarcadores encontrados entre os diferentes grupos aliado ao estudo detalhado de como cada tipo de biomarcador está correlacionado com a dependecia de cada biomarcador para cada tipo de doença, é uma estratégia fundamental para o melhor entendimento das doenças estudadas. Estudos adicionais que apresentem como objetivo o estudo das funções das presentes células devem ser realizados encorajados.

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