



UNESP - Universidade Estadual Paulista

“Júlio de Mesquita Filho”

Faculdade de Odontologia de Araraquara



Darcy Fernandes

Estudo *ex vivo* e *in vitro* da modulação da imunidade inata e adaptativa por queratinócitos displásicos em leucoplasia oral e leucoplasia verrucosa proliferativa

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Tese apresentada ao programa de Pós-Graduação em Ciências Odontológicas Área de Diagnóstico e Cirurgia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista para título de Doutor em Ciências Odontológicas.

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Darcy Fernandes

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"Se as coisas são inatingíveis... ora!

Não é motivo para não querê-las...

Que tristes os caminhos, se não fora

A mágica presença das estrelas!"

Mário Quintana*

* Quintana, M. Espelho Mágico. Porto Alegre: Editora Globo; 1951.

Fernandes D. Estudo *ex vivo* e *in vitro* da modulação da imunidade inata e adaptativa por queratinócitos displásicos em leucoplasia oral e leucoplasia verrucosa proliferativa [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

RESUMO

O carcinoma espinocelular (CEC) representa mais de 95% de todas as neoplasias malignas que acometem a cavidade oral e muitas vezes estes tumores são precedidos por alterações clínicas que apresentam um evidente potencial de transformação maligna, as quais são chamadas de distúrbios potencialmente malignos orais (DPMOs). Dentre estas, a leucoplasia oral (LO) possui taxa de transformação maligna que varia de 0,2% até 17,5%; contudo, uma outra DPMO conhecida como leucoplasia verrucosa proliferativa (LVP) apresenta um comportamento persistente e progressivo para malignidade, com taxa de transformação maligna maior que 70%. Diferente da LO, fator de risco como tabaco, álcool e noz de areca não parecem estar associados com o desenvolvimento da LVP. Adicionalmente, a LVP frequentemente apresenta resposta inadequada a todas as modalidades de tratamento, sofre rápida disseminação pelos sítios orais e muitas vezes demonstra recorrência. Estudos recentes sugerem que o infiltrado inflamatório associado às lesões leucoplásicas de paciente com LVP pode estar relacionado a etiologia e/ou comportamento clínico agressivo desta DPMO. Assim, foi realizada uma análise comparativa entre amostras de LO e LVP que consistiu em: (1) avaliar a porcentagem e identificar os subtipos de linfócitos T auxiliares e estado de ativação de linfócitos T citotóxicos, (2) avaliar a densidade e estado de ativação das células dendríticas, e (3) determinar o efeito de produtos solúveis de células displásicas na modulação da imunidade inata e/ou adaptativa e se tal modulação confere uma vantagem para a progressão do tumor. Para alcançar estes objetivos, a expressão imuno-histoquímica de marcadores linfócitos T e B, para células dendríticas (CD) de Langerhans, CD submucosas imaturas e maduras, CD plasmocitóides, foram utilizados. Adicionalmente, ensaios com cultura de células foram realizados para acessar o potencial modelador de fatores solúveis produzidos por culturas celulares derivadas de PVL sobre células mononucleares do sangue periférico (PBMCs). Estes ensaios incluíram a avaliação das taxas de apoptose e imunofenotipagem dos PBMCs após contato com meios de cultura condicionados de diferentes culturas celulares, incluindo queratinócitos normais (HOK), displásicos (DOK e DOK-PVL) e tumorais (SCC-25). Os resultados da análise imuno-histoquímica mostraram uma redução global de células T e B acompanhada de uma inibição da ativação de células Th17 e consequente indução de uma resposta imune mediada por células Treg na LVP, parece favorecer o comportamento mais agressivo desta DPMO em relação a LO. Adicionalmente, CD de Langerhans, maduras e plasmocitóides estão reduzidas nos compartimentos epitelial e subepiteliais na LO e LVP em relação ao controle. Por outro lado, CD imaturas encontram-se aumentadas nos compartimentos epitelial e subepiteliais destas duas DPMOs. Juntos, estes achados favorecem uma apresentação inadequada dos antígenos pré-tumorais facilitando a tolerância imunológica. Os experimentos com cultura de células revelaram ainda, que os produtos solúveis de queratinócitos displásicos tem capacidade de alterar o fenótipo dos PBMCs favorecendo a transformação maligna de DPMOs. Assim, os resultados deste estudo indicam que os produtos solúveis dos queratinócitos derivados de LVP são capazes de modular de forma distinta a resposta imunológica desta DPMO em relação à LO, sendo esta modulação acompanhada do ganho de vantagens que favorecem o processo de tumorigênese.

Palavras-chave: Leucoplasia. Sistema imunitário. Neoplasias bucais. Técnicas de cultura de células.

Fernandes D. Ex vivo and in vitro study of the modulation of innate and adaptive immunity by dysplastic keratinocytes in oral leukoplakia and proliferative verrucous leukoplakia [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2020

ABSTRACT

Oral squamous cell carcinoma (SCC) represents more than 95% of all malignant neoplasms that affect the oral cavity and these tumors are often preceded by clinical changes that have an evident potential for malignant transformation, which are called potentially malignant oral disorders (OPMDs). Among these, oral leukoplakia (OL) has a malignant transformation rate that varies from 0.2% to 17.5%; however, another OPMD known as proliferative verrucous leukoplakia (PVL) has a persistent and progressive behavior for malignancy, with a malignant transformation rate greater than 70%. Unlike OL, a risk factor such as tobacco, alcohol and areca nut does not seem to be associated with the development of PVL. Additionally, PVL often presents an inadequate response to all treatment modalities, suffers rapid dissemination through oral sites and often shows recurrence. Recent studies suggest that the inflammatory infiltrate associated with leukoplakic lesions in a patient with PVL may be related to the etiology and / or aggressive clinical behavior of this OPMD. Thus, a comparative analysis was performed between OL and PVL samples that consisted of: (1) evaluating the percentage and identify subtypes of auxiliary T lymphocytes and activation status of cytotoxic T lymphocytes, (2) evaluating the density and activation status of dendritic cells, and (3) determine the effect of soluble products of dysplastic cells on the modulation of innate and / or adaptive immunity and whether such modulation confers an advantage for progression of the tumor. To achieve these goals, immunohistochemical expression of markers for Langerhans dendritic cells (DC), immature and mature submucous DC, plasmacytoid DC, T and B lymphocytes were used. In addition, cell culture assays were performed to access the potential modeling of soluble factors produced by PVL-derived cell cultures on peripheral blood mononuclear cells (PBMCs). These trials included the assessment of apoptosis and immunophenotyping rates of PBMCs after contact with conditioned culture media from different cell cultures, including normal keratinocytes (HOK), dysplastic (DOK and DOK-PVL) and tumor (SCC-25). The results of the immunohistochemical analysis showed a global reduction in T and B cells accompanied by an inhibition of Th17 cell activation and the consequent induction of an immune response mediated by Treg cells in PVL, seems to favor the more aggressive behavior of this OPMD in relation to OL. In addition, Langerhans DC, matures and plasmacytoids are reduced in the epithelial and subepithelial compartments in the OL and PVL in relation to the control. In contrast, immature DCs are increased in the epithelial and subepithelial compartments of these two OPMD. Together, these findings favor an inadequate presentation of pre-tumor antigens, facilitating immunological tolerance. The experiments with cell culture also revealed that the soluble products from dysplastic keratinocytes may to change the PBMCs phenotype favoring the malignant transformation of OPMD. Thus, the results of this study indicate that soluble product from PVL-derived keratinocytes are capable of differently modulating the immune response of this DPMO in relation to OL, and this modulation is accompanied by the gain of advantages that favor the tumorigenesis process.

Keywords: Leukoplakia. Immune System. Mouth Neoplasms. Cell Culture Techniques.

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1 INTRODUÇÃO

O carcinoma espinocelular (CEC), também denominado de carcinoma de células escamosas ou carcinoma epidermóide, representa mais de 95% de todas as neoplasias malignas que acometem a cavidade oral¹. Apesar dos recentes avanços no entendimento e nos protocolos de tratamento em diferentes tipos de tumores malignos, a taxa de sobrevida de 5 anos para pacientes com CEC oral permanece entre 50 e 60%^{1,2}. Portanto, prevenir e diagnosticar a doença nos estágios iniciais, é indispensável para um melhor prognóstico. Partindo deste princípio, um melhor entendimento das desordens potencialmente malignas da mucosa oral (DPMOs) representa uma estratégia interessante para elucidar mecanismos moleculares relacionados com a carcinogênese oral. As DPMOs são apresentações clínicas que possuem maior potencial para se transformar em uma neoplasia maligna, quando comparadas com a mucosa clinicamente normal. Neste contexto, são consideradas como as principais DPMOs a leucoplasia, eritroplasias, líquen plano, queilite actínica; além de outras como a fibrose submucosa, xeroderma pigmentoso e anemia de Fanconi^{3,4}.

A leucoplasia oral (LO) é a principal entre as DPMOs, com uma taxa de incidência igual a 3,4% e taxa de transformação maligna que varia de 0,2% até 17,5%^{4,5}. Segundo a Organização Mundial da Saúde (OMS), a LO é definida como "uma placa branca de risco questionável definida após a exclusão de outras doenças ou distúrbios conhecidos que mimetizam este aspecto, mas que não carregam nenhum risco conhecido para o câncer"⁶. Portanto, LO é considerado atualmente um termo clínico usado temporariamente como hipótese diagnóstica que implica na exclusão de outras lesões de aspecto similar. A LO é mais frequente entre homens caucasianos acima de 40 anos, e sua prevalência aumenta com o avanço da idade⁷. A etiopatogenia da LO abrange duas grandes categorias, incluindo: LO de etiologia desconhecida ou idiopática e LO associada ao uso do tabaco⁸. As LOs podem exibir características clínicas similares entre si, mas apresentam um considerável grau de heterogeneidade microscópica. Segundo a OMS (2005), a OL pode se apresentar microscopicamente com aspectos que variam de uma hiperqueratose sem ou com variados graus de displasia (leve, moderada ou severa) até um carcinoma in situ; sendo assim, a realização de uma biópsia é mandatória para o estabelecimento do diagnóstico definitivo. Atualmente estes achados microscópicos são utilizados para prever o risco de transformação maligna deste grupo de lesões. No entanto, não há métodos objetivos ainda disponíveis para tipificar lesões displásicas e permitir resultados consistentes e reprodutíveis, uma vez que nem todas as lesões orais com displasia progridem

para uma neoplasia maligna⁹. Além disto, a utilidade desta subdivisão de displasia tem sido questionada devido à grande variabilidade inter e intra observador no que diz respeito a avaliação de presença/ausência e grau de displasia¹⁰. A maioria das OLs convencionais permanecem benignas ao longo do tempo, caracterizando-se por acantose ou hiperqueratose com um pequeno grupo de lesões progredindo para displasias de alto grau ou carcinoma francamente invasivo^{3,4}. Assim, um dos maiores desafios entre os casos de OLs é o dilema de tentar prever quais lesões irão de fato progredir para uma neoplasia maligna, em particular o CEC oral.

Interessantemente, existe uma variante menos conhecida entre as leucoplasias que apresenta um comportamento único de persistente progressão para malignidade, conhecida como leucoplasia verrucosa proliferativa (LVP). O termo LVP foi definido pela primeira vez por Hansen e colaboradores em 1985, que descreveram uma desordem agressiva de etiologia desconhecida e tendência a se tornar multifocal ao longo do tempo¹¹. A LVP é caracterizada por placas brancas difusas e múltiplas, com prevalência quatro vezes maior no gênero feminino, sendo frequentemente diagnosticada a partir da sexta década de vida em indivíduos usualmente não fumantes e apresentando alta taxa de recorrência e malignização, a qual pode variar de 40 a 70%^{12,13}. Devemos destacar que o perfil epidemiológico da OL convencional se aproxima do observado no CEC oral, sendo associado ao uso do tabaco e apresentando maior incidência entre os homens. Em contrapartida na LVP isso parece não ocorrer, demonstrando um possível padrão biológico distinto entre LO e LVP. Esta apresentação clínica distinta associada ao comportamento biológico agressivo permitem que a LVP seja classificada como uma entidade distinta da LO convencional, sendo descrita pela OMS como a DPMO com maior risco de progressão para o CEC oral. Contudo, o diagnóstico definitivo da LVP deve ser realizado de forma retrospectiva com base na observação temporal das lesões, devido a inexistência de características clínicas e microscópicas específicas da doença nas fases iniciais. Assim, um particular desafio tem sido encontrado para realizar um diagnóstico diferencial e precoce entre os casos de LO convencional e LVP.

Ainda, a classificação proposta pela OMS enfatiza os critérios microscópicos para definir o estágio clínico das DPMOs, no entanto, pouca importância tem sido dada à caracterização das células do sistema imune nestas desordens⁶. Neste contexto, a utilização de técnicas de biologia molecular em estudos com LO e LVP poderiam melhorar acentuadamente a detecção de alterações que são invisíveis ao microscópio, melhorando a eficácia na identificação dos pacientes com maior risco de transformação maligna¹⁴. Assim, o

entendimento mais aprofundado da natureza molecular e do comportamento destas DPMOs é fundamental para que seja possível o desenvolvimento de potenciais ferramentas diagnósticas e prognósticas em casos de LO e LVP. Além disto, a LVP frequentemente apresenta resposta inadequada a todas as modalidades de tratamento e muitas vezes sofre recorrência. Então, o melhor entendimento dos mecanismos moleculares relacionados a LVP, incluindo os mecanismos relacionados à imunidade inata e adaptativa, podem auxiliar no desenvolvimento de medidas terapêuticas mais eficazes.

No modelo atual de oncogênese, o câncer surge a partir de uma sequência de alterações celulares resultantes de fatores intrínsecos e extrínsecos; sendo bem estabelecido e aceito que existem seis alterações fundamentais na fisiologia celular que determinam o início da oncogênese¹⁵. Segundo este conceito as células tumorais caracteristicamente fornecem seus próprios sinais de crescimento, ignoram os sinais inibidores do crescimento, evitam a morte celular (evasão da apoptose), replicam-se de forma ilimitada, sustentam a angiogênese e invadem os tecidos através da membrana basal e da parede dos capilares (invasão tecidual e metástase). Além disso, como recentemente proposto, evitar a vigilância imunológica pode ser a sétima característica do câncer^{16,17}. Este conceito mostrou que apesar das evidências a favor do papel fundamental do sistema imunológico no combate à formação do câncer (“imunovigilância”), as células e fatores que compõem este sistema podem agir selecionando ou editando os tumores emergentes¹⁸. Neste contexto, surgiu o conceito da “imunoedição” que é composto por três fases conhecidas como os três “Es” da imunoedição: (I) eliminação, (II) equilíbrio e (III) escape^{16,18}. Recentemente e baseado no conceito da imunoedição, foi reconhecido também que as células tumorais têm a interessante capacidade de utilizar a presença do infiltrado inflamatório tumoral em seu favor. Processo chamado de cooptação e subversão do sistema imunológico^{19,20}.

O conceito de imunovigilância tumoral presume que o sistema imunológico pode reconhecer precursores de câncer e, na maioria dos casos, destruir esses precursores antes de se tornarem clinicamente aparentes. Se a imunovigilância tem um papel importante na supressão de tumores, então seria de esperar que pacientes com lesões potencialmente malignas apresentassem respostas imunológicas vigorosas. Há, de fato, várias linhas de evidência que indicam que este pode ser o caso. Por exemplo, pacientes com gamopatia monoclonal, que é uma lesão potencialmente maligna, mostram uma vigorosa resposta inflamatória mediada por linfócitos T (LT) contra linfócitos B (LB) pré-malignas autólogas, enquanto tais respostas não

são encontradas em pacientes com mieloma múltiplo, que é uma condição maligna²¹. Contudo, outros estudos têm demonstrado que a resposta imune associada ao tumor não é exclusivamente antitumoral e a evasão da resposta imune adaptativa pelos tumores pode ocorrer por meio de dois possíveis mecanismos: 1) depleção de células T, inibindo a detecção de células tumorais; e 2) indução de imunotolerância²²⁻²⁵. Em suporte ao primeiro mecanismo, o aumento da produção de Fas ligante por células tumorais pode induzir apoptose de células da resposta imune²⁶ e, de fato, linfócitos T expostos a lesões de HNSCC metastáticas são mais susceptíveis à apoptose²⁷. Contudo, a falta de eficiência de abordagens terapêuticas de CECs de cabeça e pescoço em modelos pré-clínicos utilizando a estimulação da resposta imune por citocinas e da transferência (transplante) de células T citotóxicas juntamente com as evidências que demonstram que as células T presentes no microambiente tumoral se tornam suprimidas ou inativadas sugerem a possibilidade do segundo mecanismo (imunotolerância) também estar envolvido^{28,29}. Diante do exposto, um estudo detalhado da resposta imune nas DPMOs, pode contribuir para elucidação de tais eventos na fase inicial da oncogênese.

Os estudos que avaliam a participação da imunidade inata e adaptativa em DPMOs são escassos e não incluem amostras de LVP. Um estudo preliminar realizado por Bondad-Palmario (1995) avaliou superficialmente o fenótipo e a distribuição de células da imunidade em amostras de LO, revelando que a diminuição da razão entre linfócitos T auxiliares/citotóxicos (CD4/CD8), aumento das células natural killer (NK) CD57+ e expressão do receptor HLA-DR por queratinócitos ocorreram simultaneamente em casos de OL com displasia severa³⁰. Posteriormente, Gannot e colaboradores (2002) mostraram que a intensidade de células inflamatórias é maior em LOs com displasia moderada e severa (170 ± 27 e 176 ± 30 células por $0,04\text{mm}^2$, respectivamente) quando comparadas com LO sem ou com grau leve de displasia (102 ± 15 e 82 ± 24 células por $0,04\text{mm}^2$, respectivamente). A caracterização superficial desta população de células inflamatórias, mostrou que linfócitos B foram significativamente mais proeminentes em casos de LO com displasia moderada e severa³¹.

Outro estudo mostrou que células de Langerhans (CLs) e LTCD8+ são mais abundantes em OLs com displasia em comparação com LOs sem células displásicas³². Em CECs orais o número de CLs e LT são aumentados em comparação com os casos de LOs³². Estes resultados suportam os resultados apresentados por Syafriadi e colaboradores (2005), que encontraram uma correlação significativa entre o número aumentado de células LTCD8+ e o grau de severidade da displasia³³. Deve-se destacar que moléculas CD8 não são expressas

exclusivamente em LT, uma vez que células NK e células dendríticas (CD) plasmocitóides expressam moléculas CD8 α ^{34,35}. Consequentemente, é possível que algumas das células CD8+ registradas nestes estudos possam ser em parte células NK ou CD plasmocitóides. Posteriormente, um estudo realizado por Öhman e colaboradores (2015) revelou que pacientes com LO que evoluíram para CEC oral apresentavam uma redução significativa no número de LTCD3+ em comparação com os casos que não desenvolveram um CEC³⁶. Estes estudos suportam a hipótese da imunovigilância, mas uma caracterização detalhada dos subtipos de LT se faz necessária para uma exploração adequada desta hipótese.

Um estudo recente investigou o padrão de expressão do genes da IL-1 β , IL-6, IL-8, TNF- α e COX-2 em casos de LOs, sugerindo que estes fatores da inflamação aparentemente não contribuem para a progressão destas lesões³⁷. Segundo os resultados deste estudo, houve apenas uma redução na expressão da citocina próinflamatória IL-1 β em amostras de LO quando comparadas ao tecido gengival normal.

Woodford e colaboradores (2014) mostraram mudanças dinâmicas na resposta imunológica de DPMOs, revelando um aumento proeminente da IL-17 nestas lesões e redução desta mesma citocina em CECs³⁸. O estudo demonstrou ainda que um declínio na produção de IL-23 e o aumento na produção TGF- β por células de CEC são os prováveis contribuintes na polarização de Th17 para Treg no ambiente tumoral. Contudo, nenhuma diferença na produção de IL-2, IL-6 e IFN- γ foi observada nos grupos estudados³⁸. Resultados semelhantes foram observados em um estudo que mostrou uma redução gradual na expressão sorológica de IL-17 de pacientes saudáveis, pacientes com LO para casos de CEC oral³⁹. Em contraste, a concentração de TNF- α , IL-6 e IL-8 no soro e saliva de pacientes com LO foi apontada como significativamente aumentada em relação ao grupo de pacientes saudáveis⁴⁰.

Outro estudo recente, avaliou a relação entre o perfil de LT e a polarização de macrófagos (M1/M2) em amostras de LO e encontraram uma correlação positiva entre o número de LTCD4+ e macrófagos CD163+. Curiosamente, os LTCD4+ revelaram um perfil Th1 (CXCR3+ e CCR5+), principal subtipo celular produtor de IFN, enquanto o fenótipo Th2 foi escasso nestas lesões. Estes resultados demonstram que embora o CD163 tenha sido considerado um marcador de macrófagos M2 em muitos tumores sólidos, os macrófagos CD163+ nas LOs parecem possuir um fenótipo M1 caracterizado pela expressão de produtos gênicos induzidos por IFN^{41,42}. Como revelado pela dupla marcação que demonstrou que os macrófagos CD163+ coexpressavam STAT1 ativo (pSTAT1).

A maioria dos estudos indica que DPMOs provavelmente representam um estado de equilíbrio entre ativação do sistema imune e a proliferação de células displásicas, como originalmente sugerido por Dunn^{16,18}. Contudo, um estudo molecular mais profundo sobre as células imunes e citocinas relacionadas com LO e LVP representa uma estratégia de pesquisa interessante para a elucidação da etiologia destas DPMOs. Assim, os resultados deste estudo podem indicar potenciais novas ferramentas para prever de forma mais confiável quais as lesões que sofrerão transformação maligna.

2 PROPOSIÇÃO

2.1 Objetivo Geral

Comparar o perfil da resposta imune inata e adaptativa entre casos de LO e LVP; além de avaliar o impacto desta interação no processo de transformação maligna.

2.2 Objetivos Específicos

Comparar a porcentagem e estado de ativação dos subtipos de linfócitos T auxiliares e citotóxicos em lesões leucoplásicas de LO e LVP.

Avaliar comparativamente a densidade e estado de ativação das CDs por meio da expressão imuno-histoquímica de marcadores que caracterizam CDs imatura, maduras e plasmocitóides em lesões leucoplásicas de LO e LVP.

Determinar os efeitos promovidos pelos produtos solúveis oriundos de células normais, displásicas e neoplásicas na apoptose de PBMCs e modulação de linfócitos T auxiliares (Th1, Th2, Th17 e Treg) por meio de análise *in vitro*.

3 PUBLICAÇÕES

3.1 Publicação 1*

LYMPHOCYTE PROFILE IN ORAL LEUKOPLAKIA AND PROLIFERATIVE VERRUCOUS LEUKOPLAKIA

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ABSTRACT

Background: The oral leukoplakia (OL) has a malignant transformation rate ranging from 0.2% to 17.5%, whereas proliferative verrucous leukoplakia (PVL) presents persistent and progressive malignant behavior, with a malignant transformation rate greater than 70%. We aimed to characterize and compare the lymphocyte profile in the inflammatory infiltrate associated with PVL and OL lesions, as well as the NF- κ B expression.

Methods: The profile of T- cells (CD3, CD4, and CD8) and B-cells (CD20, CD138) were analyzed by immunohistochemistry, while cytokine and chemokine profiles were assessed using a bead-based multiplex assay to identify differences in immune signaling pathways between OL and PVL.

Results: An overall reduction of T-cells and B-cells in OL and PVL groups, compared to the control group. Moreover, the cytokines and chemokines profiles of the T cells are different between these oral potentially malignant disorders (OPMDs).

Conclusion: There is a general suppression of T cells in the OPMDs. Moreover, the cytokines profiles observed in PVL probably favors the polarization of CD4+ T cells to Tregs when compared to OL. Also, the cytotoxic activity of T cells is reduced in OL and PVL. The process of carcinogenesis in PVL may be mediated by TNF- α -induced activation of the NF- κ B signaling pathway.

KEYWORDS: Oral leukoplakia; Proliferative verrucous leukoplakia; Oral cancer; Immune system; Lymphocytes.

INTRODUCTION

In the oral oncogenesis, the oral squamous cell carcinoma may arise from histologically normal oral mucosa or from oral potentially malignant disorders (OPMDs), which include oral leukoplakia (OL) that presents a malignant transformation rate ranging from 0.2% to 17.5%.¹⁻³ Lower T-cell, monocytic, and myeloid dendritic cells immune infiltration as associated with oral cancer development in OPMDs.⁴ Also, the development of epithelial abnormalities from oral dysplasia to carcinoma is accompanied by an increased number of CD163+ cells and immunoexpression of PD-L1 and decreased number of CD3+ and CD8+ cells.^{5,6}

Another interesting and rare OPMDs is the proliferative verrucous leukoplakia (PVL), which presents persistent and progressive malignant behavior, with a malignant transformation rate greater than 70%.¹⁻³ Until now, there was no study which investigated the influence of inflammatory cells in the PVL. Thereby, we aimed to characterize and compare the lymphocyte profiles and their cytokines and chemokines involved in the inflammatory infiltrate of PVL and OL lesions.

METHODS

Samples

This study was conducted in full accordance with ethical principles, including the World Medical Association (Declaration of Helsinki) and after approval by local ethics committee. All participants agreed to participate in the study and provided written informed consent. Biopsies samples from patients diagnosed clinically and pathologically with OL (n=23) and PVL (n=15) were obtained. The diagnostic criteria for OL and PVL was based on the WHO Classification of Head and Neck Tumours.⁶ In addition, samples from patients with oral squamous cell carcinoma (OSCC, n=12) and inflammatory fibrous hyperplasia (IFH n=14) were included as neoplastic and non-neoplastic groups when necessary. After the sample collection, the fragmented tissue was divided into two parts. One half was fixed in 10% formalin and prepared for histopathological and immunohistochemical analyses. The other half was immediately frozen and stored at -80°C.

Histopathological

We evaluated the intensity of the inflammatory infiltrate using hematoxylin and eosin-stained tissue sections. Five representative fields were photographed with Olympus DP25 camera (Olympus, Center Valley, PA, USA) attached to Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) utilizing 200× magnification. The inflammatory cells were counted using the Image J software (version 1.52, NIH, Bethesda, MD, USA), and we determined the mean cell count for each sample. The dysplasia was grouped by low-grade and high-grade following the last criteria of WHO.¹ These analyses were made by two independent calibrated examiners (kappa = 0.87) and supervised by a third examiner.

Immunohistochemistry

Briefly, consecutive serial sections were obtained and submitted to the antigen were retrieval. Then the slides were incubated separately with primary antibodies against CD3, CD8, CD20, CD138 (#GA503, #IR649, #GA604, #IS642 respectively, Dako, Carpinteria, CA, USA) and CD4 (CD4-368-L-CE, Leica Biosystems, Wetzlar, HE, Germany), followed by the LSAB method (#K0675, Dako, Carpinteria, CA, USA) and the reactions were developed by incubating the sections with 0.6mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Saint Louis, MO, USA). Five representative fields were photographed from the intraepithelial and the subepithelial areas separately with an Olympus DP25 camera (Olympus, Center Valley, PA, USA) attached to Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) utilizing 20× objective. The positive cells were counted by two independents calibrated (kappa = 0.84) examiners using the Image J software (version 1.52, NIH, Bethesda, MD, USA).

Cytokines and Chemokines Assays

Total proteins were extracted from the frozen tissue samples using a detergent-based extraction buffer T-PER – Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Indianapolis, IN, USA). A cytokines/chemokines panel to assess CD4+ cell subsets (#EPX180-12165-901, ThermoFisher Scientific, Waltham, MA, USA), and the activation status of CD8+

cells (#HCD8MAG-15K, Merck Millipore Corporation, Billerica, MA, USA) was performed. A total of 26 cytokines/chemokines, including GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, TNF- α , Granzyme A, Granzyme B, Perforin, MIP-1 α , MIP-1 β , sCD137, sFas, and sFasL, were tested using the xMAP MAGPIX instrument (Merck Millipore Corporation, Billerica, MA, USA).

Statistical analyses

Differences between the groups were tested by Mann–Whitney U test or Student T-test and by Kruskal-Wallis followed by Dunn’s test with a correction or one-way ANOVA followed by Tukey’s test. Only the results with $P < 0.05$ were considered statistically significant. All the statistical analyses were performed using GraphPad Prism Software version 7 (GraphPad, La Jolla, CA, USA).

RESULTS

Chronic lymphoplasmacytic infiltrate was more often in PVL than OL

The epidemiological profile of the patients included in this study revealed that most patients in the OL group were male ($n = 13$; 56.5%) and smokers (13 patients; 56.5%), with a mean age of 55 years (range 30 to 79 years). The vast majority of OL patients revealed lesions with a total size of less than 3 cm ($n=20$; 86.9%). All OL patients required a single biopsy and most of them were histopathologically classified as low-grade ($n=15$, 65.2%). Analyzing the clinical-pathological characteristics of the PVL group, we observed that most patients were female ($n=9$; 60.0%), with a mean age of 64.6 years (range 41 to 79 years) and were smokers (10 patients; 66.6%). All patients in the PVL group had lesions at multiple oral sites with a total lesion size greater than 3 cm. Some PVL patients ($n=7$, 46.6%) presented lichenoid characteristics associated with dysplastic lesions. All PVL patients presented multifocal lesions during the disease development. The mean number of biopsies of the PVL patients was equal to 2.3, and the majority was histopathologically classified as high-grade ($n=26$, 74.2%). The frequency of subepithelial inflammatory cells was higher in PVL than OL ($p = 0.0197$), usually containing chronic lymphoplasmacytic infiltrate (Figure 1A). The OL group with high-grade dysplasia

shows an increase in the number of inflammatory cells infiltrated. Unlikely, there is a high density of inflammatory cells regardless of the dysplasia grade in the PVL samples (Figure 1B).

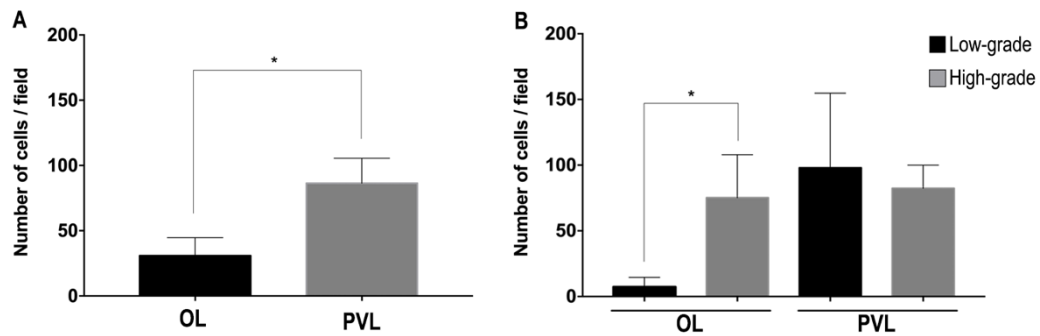


Figure 1. Number of the inflammatory infiltrate associated with PVL and OL. A. Mean number of immune cells counted per field in paraffined tissue samples of PVL and OL. B. * $p < 0.05$.

Number of lymphocytes is lower in OL and PVL

There was an overall reduction of the lymphocytes population on the OL and PVL groups when compared to the non-neoplastic group (IFH). T cells constitute the majority of the infiltrating lymphocytes observed in OL and PVL, being found under the epithelial basal layer. On the other hand, B cells were observed deeply in the connective tissue. When these cells were found in the intraepithelial area, they were more present in the basal layer and the spinous layer. In this area, there was no difference between the groups for the CD3+CD4+ cells. However, we found a reduction of CD3+CD4+ cells in the subepithelial area of PVL and OL. CD3 and CD8 positive cells were found reduced in both intraepithelial and subepithelial areas for both OPMDs. Figure 2 shows the statistical difference observed between the groups for the T cells markers. Similarly, the CD20+ and CD138+ cells were reduced only in the subepithelial area of PVL and OL (Figure 3).

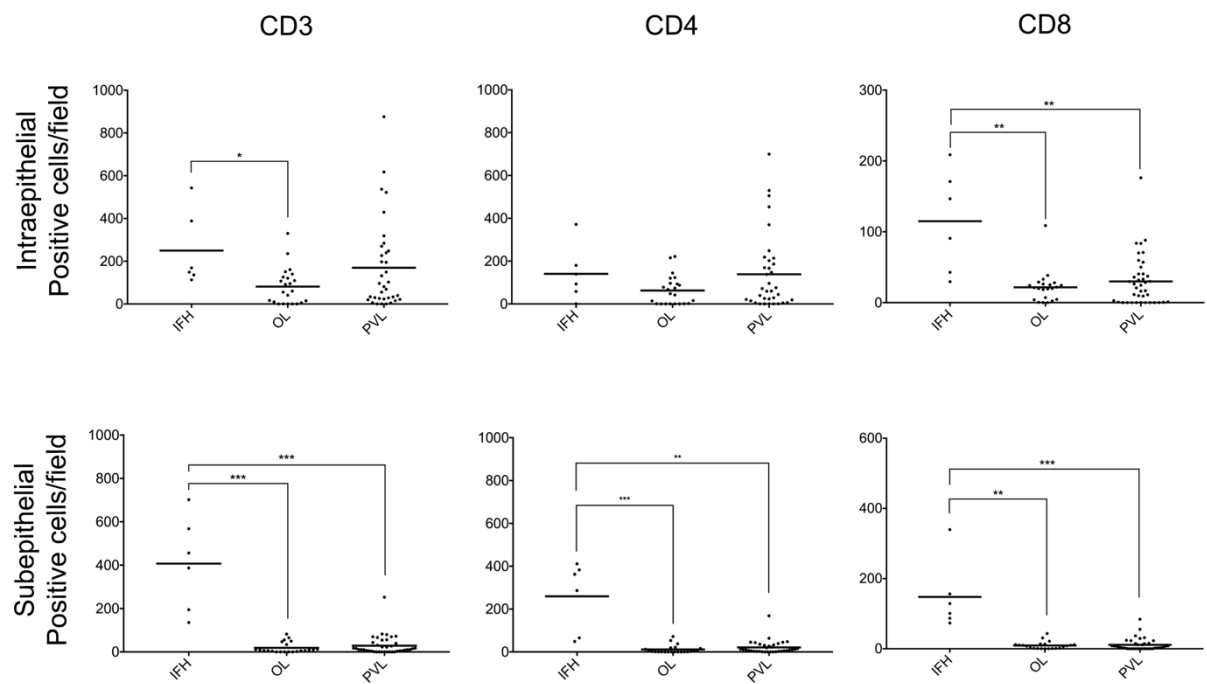


Figure 2. Number of T cells associated with IFH (non-neoplastic group), OL and PVL identified by immunohistochemistry. Mean number of CD3+, CD4+ and CD8+ cells counted in the epithelial and subepithelial area per field. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

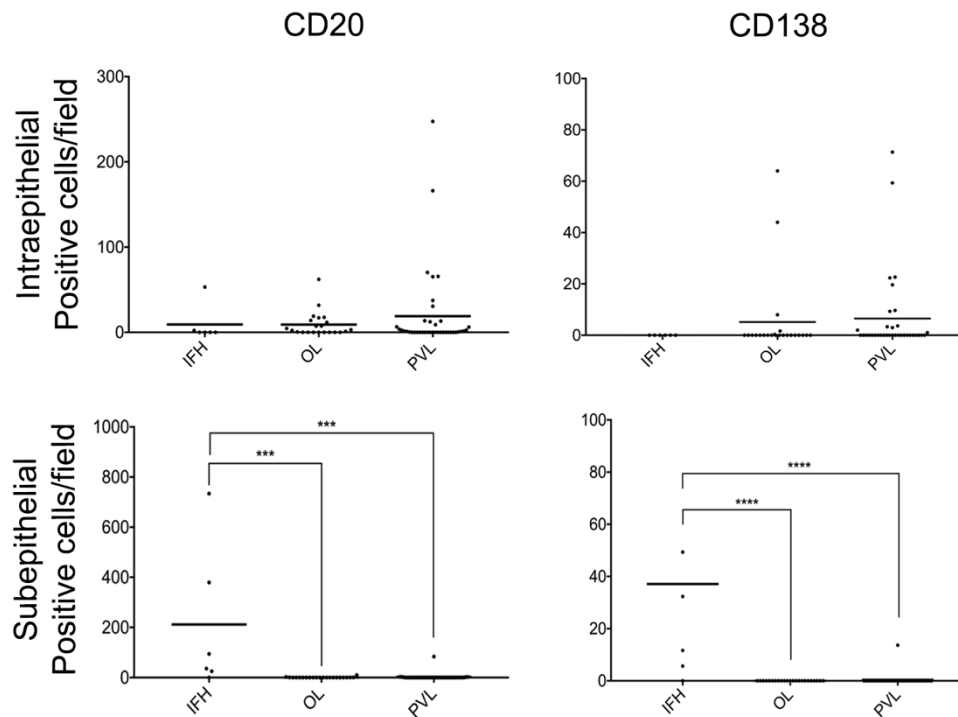


Figure 3. Number of B cells associated with IFH (non-neoplastic group), OL and PVL identified by immunohistochemistry. Mean number of CD20+ and CD138+ cells counted in the epithelial and subepithelial area per field. *** $p < 0.001$.

Cytokines and chemokines profiles in OPMDs and OSCC

The multiplex assay did not reveal any statistical difference in the levels of IFN- γ , IL-10, IL-12p70, IL-13, IL-17A, IL-23, IL-27, Granzyme A, Perforin, MIP-1 α , and sFasL between the groups. Nonetheless, the levels of IL-4, IL-18, and IL-21 are decreased in PVL than OSCC. There is also a decrease in the levels of GM-CSF, IL-6, IL-1 β , and sCD137 in PVL and OL compared to OSCC. The levels of granzyme B and IL-9 are reduced in both OPMDs and control compared to the OSCC. We found an increase in the levels of IL-2 and TNF of PVL comparing to the non-neoplastic group, whereas the sFAS levels are reduced. Moreover, the levels of IL-5 are increased in PVL when compared to OL and the control group (Figure 4). Based on these results we included the analysis by immunofluorescence of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation which may be involved in the TNF pathway. We observed that the tissue levels of NF- κ B are increased in malignant lesions from PVL patients when compared with lesions from the same patients without malignant transformation (Figure 5).

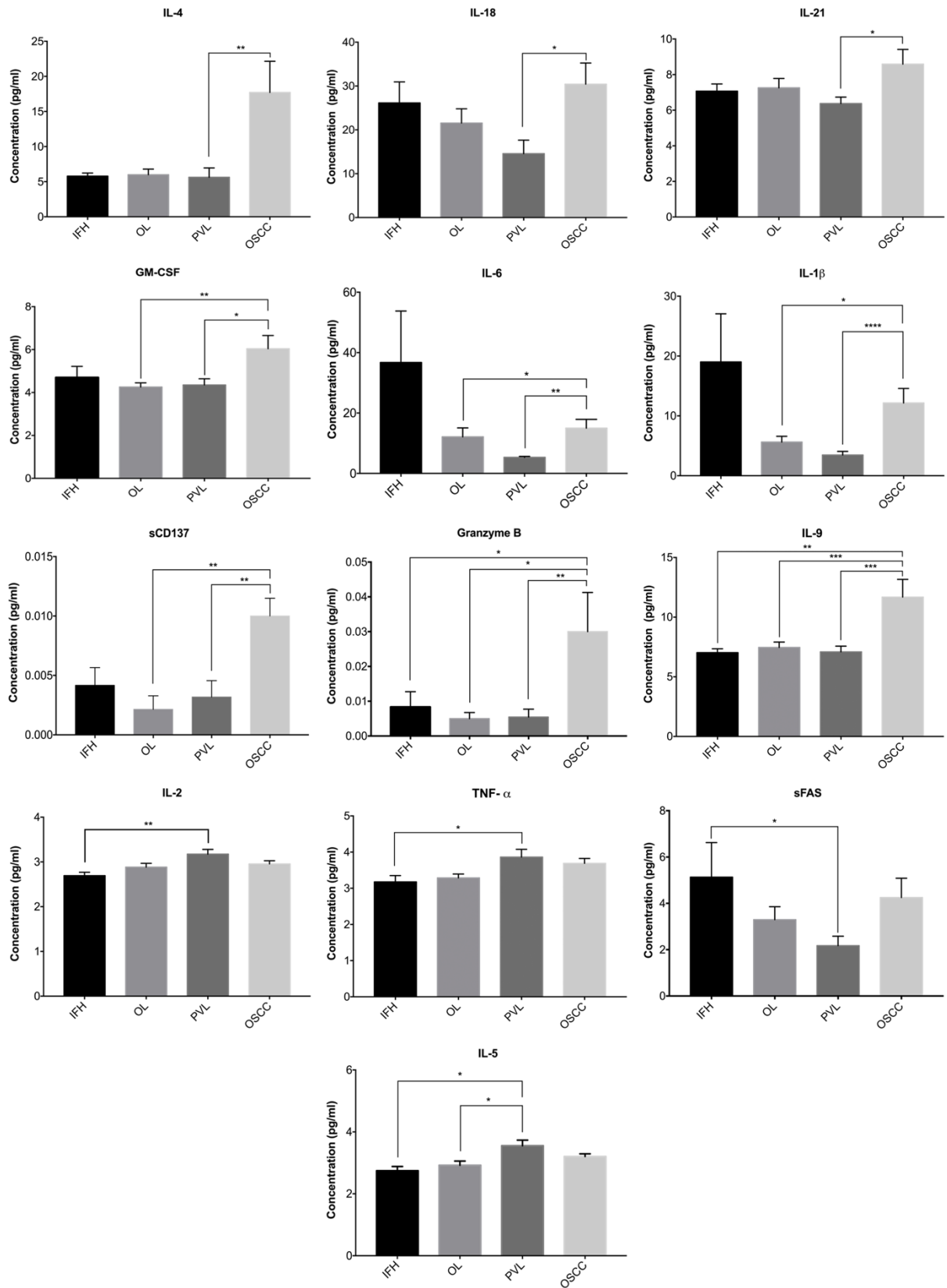


Figure 4. Cytokines and chemokines profile in OPMDs and OSCC using the multiplex assay with frozen tissue samples. Levels of IL-4, IL-18, IL-21, GM-CSF, IL-6, IL-1 β , sCD137, granzyme B and IL-9, IL-2, TNF, sFAS and IL-5 between IFH (non-neoplastic group), OL, PVL and OSCC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

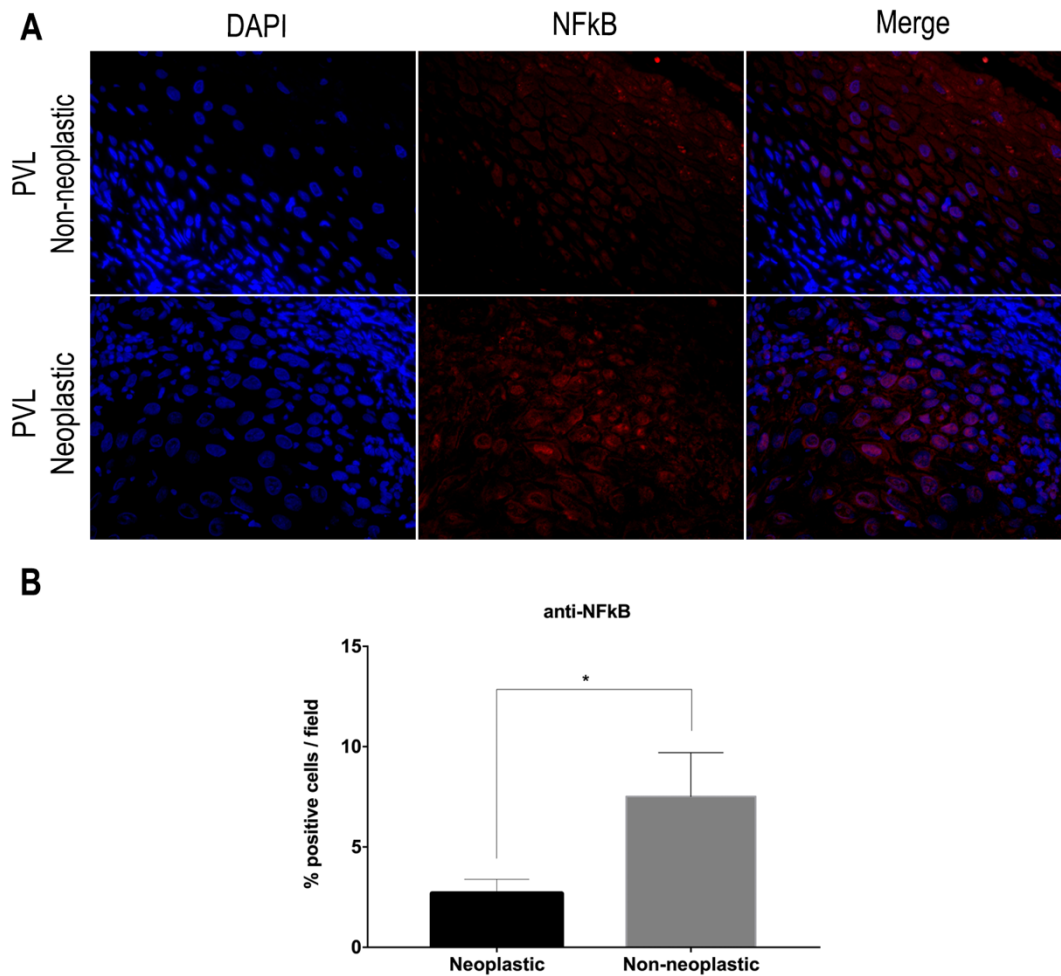


Figure 5. NF- κ B tissue expression in non-neoplastic and neoplastic samples of PVL by immunofluorescence. A. Immunofluorescence pattern of anti-Phospho-NF- κ B p65 antibody (red labeling). DNA was identified using Hoechst 33342 (blue labeling) in 200x magnification. B. Percentage of positive cells per field between non-neoplastic and neoplastic samples of PVL. * $p < 0.05$.

DISCUSSION

To the best of our knowledge, our study was the first to compare the lymphocytic infiltrate profiles in OL and PVL. Despite the difference in the overall inflammatory cell density observed in hematoxylin and eosin staining between PVL and OL, we could not find a difference between them when we analyzed the inflammatory cells subtypes by immunohistochemistry. Our results showed a decrease in the number of T-cells, as well as B-cells in the inflammatory infiltrate associated with PVL and OL when compared with a non-neoplastic lesion. In a previous study, OL lesions with records of malignant transformation had a reduced number of CD3+ T-cells when compared with patients without malignant transformation.⁷ Similarly, high stromal and parenchymal CD8+ T-cell density in OSCC is associated with improved overall survival and disease-specific survival.⁸ Another study showed that Langerhans cells (LC) and CD8+ T-cells are more abundant in dysplastic OL compared to non-dysplastic OL. In OSCCs the number of LCs and T-cells is increased when compared to cases of OLs.^{7,8} It was already demonstrated that the intensity of B-cells is higher in OL with moderate and severe dysplasia when compared with OL without or with mild dysplasia.⁹ In our study, we did not find any relationship between malignant transformation and the frequency of lymphocytes in OL and PVL. Notwithstanding, we show that the intensity of the inflammatory infiltrate is higher in OL with high-grade than low-grade dysplasia. These results may explain why dysplasia levels are not always efficient in predicting the malignant transformation in OL. Differently, the intensity of the inflammatory infiltrate in PVL is independent of the dysplasia grade, once it shows a higher density of immune cells infiltrated even in mild dysplasia. Nonetheless, the T-cells subsets characterization in OPMDs have been underexplored until now.

Based on the theory of “immunoediting”,¹⁰ we think that the factors produced by the epithelial cells in PVL have a higher capability of recruiting and subverting the inflammatory cells in its favor. Considering this, we analyzed the chemokine and cytokine profile related to cytotoxic and helper T-cells in OL and PVL comparing with non-neoplastic lesions and OSCC. In this analysis, we found that the concentrations of IL-4, IL-18, IL-21, GM-CSF, IL-6, IL-1 β , sCD137, granzyme B, and IL-9 are higher in OSCC. Specifically, the concentrations of IL-4, IL-18, and IL-21 are decreased in PVL when compared with OSCC. The high levels of IL-4 have been associated with several tumors including colon, breast, and lung cancer.¹¹⁻¹⁴ However, this interleukin may also present an antitumoral effect, being its true effect dependent on the

microenvironment.¹⁵ IL-18 is mediated through induction of interferon- γ (IFN- γ) secretion from T helper (Th1) cells. IL-18 in combination with IL-12 can activate CD8⁺ cells, to produce IFN- γ and, therefore, may contribute to tumor immunity. The biological activity of IL-18 is not limited to these cells, but it also plays a role in the development of Th17 cell responses. IL-18 synergistically with IL-23 can induce IL-17 secretion from Th17 cells.¹⁶ Thus, IL-21 can provide a signal required by naive CD8⁺ T cells to differentiate in response to Ag and costimulation, and the resulting effector cells represent a unique effector phenotype with highly effective cytolytic activity, but insufficient capacity to secrete IFN-gamma. In addition to its costimulatory functions on Th1 cytokines, IL-18 helps activate NK cells to express Fas ligand. These may explain the reduced concentration of IFN- γ , and FasL observed in the PVL group compared to the other groups. However, we did not find sufficient evidence of difference in the levels of IFN- γ between the groups. We also found reduced levels of sFAS and IL-21 in PVL, and this fact may be related to a reduction of the CD8⁺ cells and/or deficiency in the activity of these cells. In fact, we found a reduction in the levels of Granzyme B, which is secreted by the CD8⁺ cells and promotes caspase activation cascade inside the target cells. Moreover, increased production of Fas ligand by tumor cells may induce apoptosis of immune cells, and indeed *in vitro* T cells exposed to metastatic HNSCC cell lines are more susceptible to apoptosis.^{16,17} On the contrary, the addition of sFas perpetuates the proliferation of stimulated T cells in culture.¹⁸ It may be related to our results since we found an increased number of inflammatory cells even in low-grade dysplasia of PVL.

In addition, the PVL group showed higher concentrations of IL-2, IL-5, and TNF- α than the non-neoplastic group. Some studies show that IL-2 regulates survival, proliferation, and differentiation of activated T cells and NK cells through the transcription factor STAT5. Interestingly, IL-2 presents a paradoxical activity stimulating or suppressing the effector lymphocyte responses, specifically in CD4⁺ Foxp3⁺ regulatory T cells (Tregs). This subtype of T cells may be induced at the periphery by TGF- β and antigens resulting in inducible Tregs. Indeed, TGF- β may induce both forkhead box P3 (Foxp3) and retinoic acid receptor-related orphan nuclear receptor gamma t (ROR γ t), essential transcriptional factors to Tregs and Th17 cells, respectively.¹⁹ However, in the absence of IL-6, the ROR γ t is suppressed, favoring the Treg polarization. In this context, the polarization of Th17 cells also occurs in the presence of IL-21, and these cells are responsible for the production, not only of IL-17A and IL-17F but also of IL-21, IL-22 and granulocyte-macrophage colony-stimulator factor (GM-CSF).²⁰ These subtypes of T cells produce IL-10 and TGF- β that suppress the function of other effector T cells

and antigen-presenting cells inducing peripheral tolerance.²¹ Moreover, it was already reported that the frequency of Tregs is higher in the peripheral blood of patients with several types of cancer, including HNSCC.²² A study with OL showed that there is a gradual increase in Foxp3+ in parallel with the progression of dysplasia.²³ Another study observed a higher level of TGF- β 1 in OSCC samples when compared with OL, suggesting that this cytokine plays a role in the acquisition of a malignant phenotype in the early stages of oral carcinogenesis. Our data suggest that in the PVL group, there is a shift in the balance of Th17 to Tregs since we observed a reduction of IL-6, IL-21, and GM-CSF. Besides this, we have an increase in IL-2, which is important for Tregs survival and homeostasis. Differently, Woodford et al.²⁴ showed dynamic changes in the immune response of OPMDs, revealing a noticeable increase of IL-17 in these lesions and a reduction of this same cytokine in OSCC. The study also demonstrated that IL-23 is a critical mediator derived from OPMDs and promotes the Th17 phenotype. Moreover, a decline in IL-23 production and an increase in TGF- β production by OSCC cells are likely contributors to polarization from Th17 to Treg in the tumor microenvironment. However, no differences in IL-2, IL-6, and IFN- γ production were observed in the groups studied.²⁴ Similar results were observed in a study that showed a gradual decrease in IL-17 serological levels in healthy patients and OL patients when compared with OSCC patients.²⁵ In contrast, the concentration of TNF- α , IL-6, and IL-8 in serum and saliva of patients with OL was reported to be significantly increased than the healthy patient's group.²⁶

IL-5, together with IL-10, IL-4, IL-13, and IL-6, are produced by the subset Th2 cells, while IL-2, TNF, and IFN are produced by Th1.²⁷ However, the function of IL-5 depends on its origin and in the microenvironment context. Recently this interleukin has been reported as major factor in eosinophil differentiation and proliferation.²⁸ In addition, studies have reported high levels of IL-5 in small-cell lung carcinoma, but its role in cancer biology remains a controversy.²⁹ Our results showed lower levels of IL-4 and IL-6, and high levels of IL-2 and TNF- α in PVL, favoring the Th1 phenotype cells. Thus, we hypothesize that high levels of IL-5 in PVL may be produced by other cells, and it may be favoring the tumor transformation. In agreement with these findings, we identified low levels of IL-9, an interleukin mainly secreted by Th2 and Th9 cells, in PVL when compared to OSCC. Differently from the IL-5, we believe that the IL-9 may not contribute to the malignant transformation of PVL and OL.

A recent study investigated the pattern of IL-1 β , IL-6, IL-8, TNF- α , and COX-2 gene expression in cases of OL, suggesting that these inflammation factors do not contribute to

pathogeneses of these lesions.³⁰ According to this study, there was only a reduction in IL-1 β proinflammatory cytokine expression in OL samples when compared to normal gingival tissue. We found a reduction of IL-1 β in OL and PVL when compared to OSCC and the PVL group revealed the higher TNF- α concentration. The TNF- α has a dual role in the regulation of the inflammatory process associated with the tumor development through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) or the c-Jun N-terminal kinase (JNK). The NF- κ B may be activated by TNF- α to promote tumor cell proliferation and to inhibit the apoptosis, besides favoring the tumor angiogenesis, invasion, and potential metastasis.

CONCLUSION

In conclusion, we showed that there is a general suppression of T cells in the OPMDs. Therefore, depletion of the immune response observed in the OPMDs probably contributes to the malignant transformation of these lesions. Moreover, the cytokines and chemokines profile observed in PVL probably favors the activation of Tregs when compared to OL. We also showed that the activation of the NF- κ B pathway might mediate the process of carcinogenesis in PVL, and we suggest that high levels of TNF- α probably activate it. Also, the cytotoxic activity of T cells is reduced in OPMDs. Further studies are necessary to confirm the role of these immune cells and its factor in the pathogenesis of PVL, since these may be possible therapeutic targets.

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CONFLICT OF INTEREST

The authors disclose no potential conflicts of interest.

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3.1 Publicação 2*

DEPLETION OF THE DIFFERENT SUBTYPES OF DENDRITICS CELLS IN ORAL LEUKOPLAKIA AND PROLIFERATIVE VERRUCOUS LEUKOPLAKIA

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ABSTRACT

Objectives: This study aimed to evaluate the density and state of activation of the dendritic cells (DCs) in oral leukoplakia (OL) and proliferative verrucous leukoplakia (PVL) by immunohistochemical analysis. **Methods:** We analyzed the paraffined tissue samples of PVL (n=35), OL (n=23), and inflammatory fibrous hyperplasia (n=14) as a control group. The immunomarkers for conventional Langerhans cells (CD1a, CD207 and S100), submucosal mature DCs (CD83 and CD208), submucosal immature DCs (CD209 and factor XIIIa) and plasmacytoid DCs (CD123). A quantitative analysis of positive cells in the epithelial and the subepithelial areas were determined. **Results:** The frequency of CD1a and CD207 is reduced in the subepithelial area of PVL and OL compared with the control. S100 is reduced in the intraepithelial area of PVL and OL, as well as the CD207 in OL in the same area. CD208 is reduced in OL and PVL in both areas. Factor CD209 in the intraepithelial area and factor XIIIa in the subepithelial area are both increased in PVL. However, OL presents an increase in CD209 and a decrease of CD123 in the subepithelial area. **Conclusions:** There is general immunosuppression of the different types of DCs in both OL and PVL. A reduction of LCs and mature submucosal DCs occurred, while an increase of immature submucosal DCs in both OPMDs was observed. These findings indicate that the immune response is altered in OL and PVL, and it may influence its pathogenesis.

KEYWORDS: Oral leukoplakia; Proliferative verrucous leukoplakia; Oral cancer; Immune system; Dendritic cells.

INTRODUCTION

Potentially malignant disorders of the oral cavity (OPMD) are a group of mucosal lesions associated with an increased risk of malignant transformation. Oral leukoplakia (OL) is one of the major OPMDs, being the mean worldwide malignant transformation rate equal to 3.5% (Warnakulasuriya & Ariyawardana, 2016). Another OPMD, rarer, is the proliferative verrucous leukoplakia (PVL) which have a high rate of malignant transformation (63,9%) combined with a high rate of recurrence and resistance to all treatment modalities. (Hansen et al., 1985; Zakrzewska et al., 1996; Silverman & Gorsky, 1997). Differently from OL, PVL is not related to tobacco, it is more common in elderly women, and until now its etiology was not found (Gouvêa et al., 2013).

Dendritic cells (DCs) are immune cells, with high phagocytic activity as immature cells and high cytokine-producing capacity as mature cells (Banchereau & Steinman, 1998). A relationship between DCs and prognosis in patients affected by different cancers has been previously reported (Bennaceur et al., 1998; Upadhyay et al., 2012). Moreover, DCs are potentially good candidates for use in therapeutic cancer vaccines (Palucka et al., 2011; Zhang et al., 2016). Langerhans cells (LCs) may be considered immature DCs when express CD1a (Bennaceur et al., 2008; Austyn, 1993; Gondak et al., 2012), a protein that is responsible for presenting antigens to T lymphocytes (Gondak et al., 2012; Coventry & Heinzel, 2004).

Few studies have explored the role of the dendritic cells in OPMDs; moreover, none of them included PVL samples. Langerhans cells (LC) and CD8+ T-cells are more abundant in dysplastic OL compared to non-dysplastic OL (Öhman et al., 2012). In OSCCs, the number of LCs and T-cells is increased when compared to cases of Ols (Öhman et al., 2012; Shimizu et al., 2019). Therefore, this study aimed to evaluate the density and state of maturation of the dendritic cells (DCs) in oral leukoplakia (OL) and proliferative verrucous leukoplakia (PVL) by immunohistochemical analysis, in order to understand the more aggressive behavior of PVL.

MATERIALS AND METHODS

Samples

This study was conducted in full accordance with ethical principles, including the World Medical Association (Declaration of Helsinki) and after approval by the local ethics committee. All participants agreed to participate in the study and provided written informed consent. Biopsies samples from patients diagnosed clinically and pathologically diagnosed with OL (n=23) and PVL (n=35) were obtained. The diagnose criteria for OL and PVL was based on the 2017 WHO Classification of Head and Neck Tumours (El-Naggar et al., 2017). In addition, samples from patients with inflammatory fibrous hyperplasia (IFH n=14) were included as a non-neoplastic group (control).

Histopathological and Immunohistochemical analyses

We evaluated the dysplasia grade using hematoxylin and eosin stained tissue sections. The dysplasia was grouped in the binary system by low-grade and high-grade (Kujan et al., 2006). To the immunohistochemical analysis, we obtained consecutive (serial) cuts of 3- μ m in the paraffined tissue block. Briefly, after deparaffinization and rehydration in ethanol solutions, the antigens were retrieved. Afterward, we blocked endogenous peroxidase activity with 0.1% H₂O₂ for 5 min at room temperature. The slides were incubated separately with primary antibodies against, CD1a (#010, Dako, Carpinteria, CA, USA), S100, CD83, CD123, Factor XIIIa (Polyclonal, #1H4B, #BR4MS, #E980.1, respectively, Leica Biosystems, Wetzlar, HE, Germany), CD207 (#12D6, Monosan, Uden, Netherlands), CD208 (#1010E.101, Dendritics, Lyon, France), CD209 (#EPR5588, Epitomics, CA, USA) followed by the LSAB method (#K0675, Dako, Carpinteria, CA, USA) and the reactions were developed by incubating the sections with 0.6mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Saint Louis, MO, USA). The slides were counterstained with Carazzi's Hematoxylin. Negative controls were obtained by omitting primary antibodies. Five representative fields were photographed from the intraepithelial and the subepithelial areas separately with an Olympus DP25 camera (Olympus, Center Valley, PA, USA) attached to Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) utilizing 200 $\times$ magnification. The positive cells were counted by three independent calibrated examiners (kappa = 0.85) using the Image J software (version 1.52, NIH, Bethesda, MD, USA). All the immunohistochemical staining patterns for the primary antibodies were presented in Supplementary Figure 1.

Statistical analyses

Descriptive statistics were used to evaluate data from patient population and immunohistochemical results. To evaluate the normality distribution of the data was used Shapiro-Wilk test and the following the descriptive aspects: asymmetry and kurtosis. 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). The post-test of Tukey was chosen to evaluate the differences between the groups. The effect factor was calculated when the p-value was statistically significant. Additionally, it was performed a correlation study and a simple linear regression with the markers for OL and PVL. A p-value < 0.05 was regarded to be statistically significant. Statistical analysis was performed by IBM SPSS Statistics 20.0.

RESULTS

Our analysis was performed using paraffin blocks from 23 patients with the previous diagnosis of conventional OL and 35 paraffin blocks obtained at different times of the clinical follow-up of 15 patients diagnosed with PVL. The 35 paraffin blocks belonging to the PVL group were obtained over an average follow-up period of 88 months, with an average of 2 biopsies per individual. In this analysis, only samples that presented microscopical aspects ranging from hyperkeratosis without or with varying degrees of dysplasia (mild, moderate, or severe) were included. The epidemiological profile of the patients included in this study revealed that the majority of individuals in the OL group were male (n = 13; 56.5%), smokers (13 patients; 56.5%), with a mean age of 55 years (30 to 79 years). In addition, all individuals in the OL group had a single lesion with the vast majority showing a lesion with a total size of less than 3 cm (n = 20; 86.9%). All patients with OL underwent a single biopsy, and most of them were classified histopathologically as low grade (n = 15, 65.2%). Analyzing the clinical-pathological characteristics of the PVL group, we observed that most patients were female (n = 9; 60.0%), smokers (10 patients; 66.6%), and with a mean age of 64.6 years (41 to 79 years old). All patients in the PVL group had lesions in multiple oral sites with a total lesion size greater than 3 cm. All individuals classified as PVL had lesions that spread and affected new sites in the oral cavity. The average number of biopsies of patients with PVL was 2.3, with a total of 35 paraffinized samples. The majority of PVL samples were classified histopathologically as high grade (n = 26, 74.2%). The malignant transformation rate in our population was 13.0% for OL

and 46.6% for PVL. The recurrence rate found was 0% and 73.3%, for OL and PVL, respectively. All the clinicopathological data from the participants are presented in Table 1.

Table 1. Clinicopathological features of the OL and PVL cases included in this study.

Clinicopathological features		OL (n=23)	PVL (n=15)
		n (%)	n (%)
Average age (years)		55.0	64.6
Gender			
	Male	13 (56.5)	6 (40.0)
	Female	10 (43.5)	9 (60.0)
Ethnicity			
	White	18 (78.2)	15 (100)
	Nonwhite	5 (21.8)	0 (0.0)
Tobacco			
	No	10 (43.5)	5 (33.3)
	Yes	13 (56.5)	10 (66.7)
Alcohol			
	No	20 (86.9)	13 (86.6)
	Yes	3 (13.1)	2 (13.4)
Size of lesions			
	Less than 3 cm	20 (86.9)	0 (0.0)
	More than 3 cm	3 (13.1)	15 (100)
Spreading			
	No	23 (100.0)	0 (0.0)
	Yes	0 (0.0)	15 (100)
Dysplasia grade			
	Low grade	15 (65.2)	9 (25.8)
	High grade	8 (34.8)	26 (74.2)
Malignant transformation			
	No	20 (87.0)	8 (53.4)
	Yes	3 (13.0)	7 (46.6)
Recurrence			
	No	23 (100)	4 (26.7)
	Yes	0 (0.0)	11 (73.3)

We found that the subpopulations of immature LCs analyzed by CD1a+, CD207+, and S100+ presented a homogenous tissue distribution in the epithelial and subepithelial areas. For these markers, there is an overall reduction in OL and PVL compared with the non-neoplastic group. In the epithelial area, a significant reduction of CD207+ and S100+ cells in the OL group

compared with the non-neoplastic group (both markers, $p=0.01$) was observed. Regarding the PVL group, we found a reduction of S100+ in the same area compared with the non-neoplastic group ($p=0.004$). In the subepithelial area, we found a similar reduction, comparing the non-neoplastic group with OL and PVL, for the marker CD1a+ (OL, $p=0.004$; PVL=0.001) as for CD207+ (OL, $p=0.01$; PVL, $p=0.04$). The average, the standard deviation and statistical differences for CD1a, CD207, and S100 are presented in Figure 1.

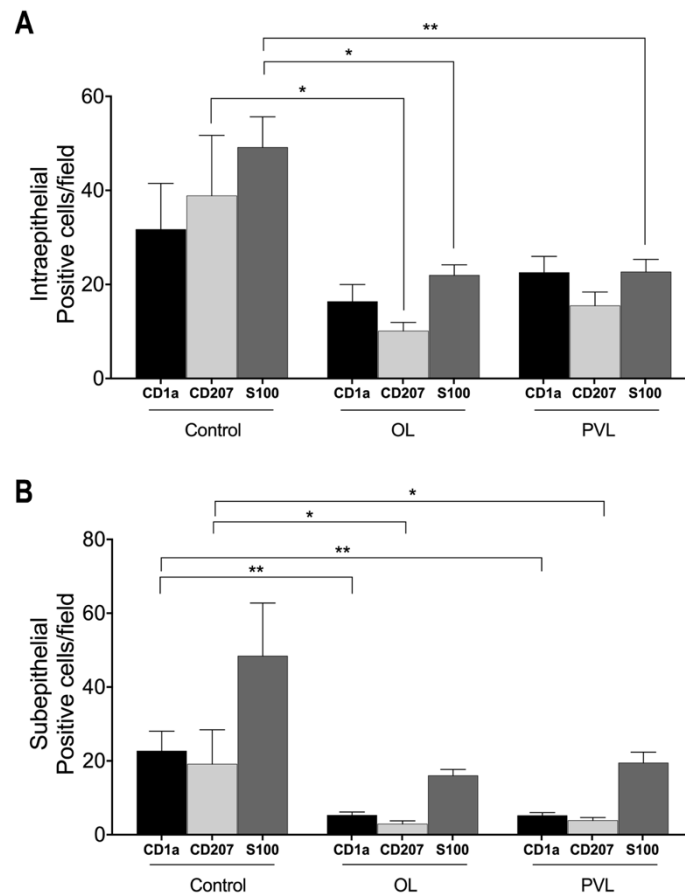


Figure 1. Average of LCs positive cells for CD1a+, CD207+ and S100+ in the intraepithelial area (A) and subepithelial area (B) for the control (inflammatory non-neoplastic tissue), OL and PVL groups; * $p = 0.01$ and ** $p = 0.001$.

In contrast, the mature DCs, CD83+, and CD208+ cells, were more present in the subepithelial area as round-shaped cells. The CD208+ cells were more frequent in the non-neoplastic group than the other groups in both areas, epithelial (OL, $p=0.007$; PVL, $p=0.0009$) and subepithelial (OL, $p=0.003$; PVL, $p=0.001$). There was no statistical difference for the CD83 marker. The

average, the standard deviation and statistical differences for CD83+ and CD208+ count are presented in Figure 2.

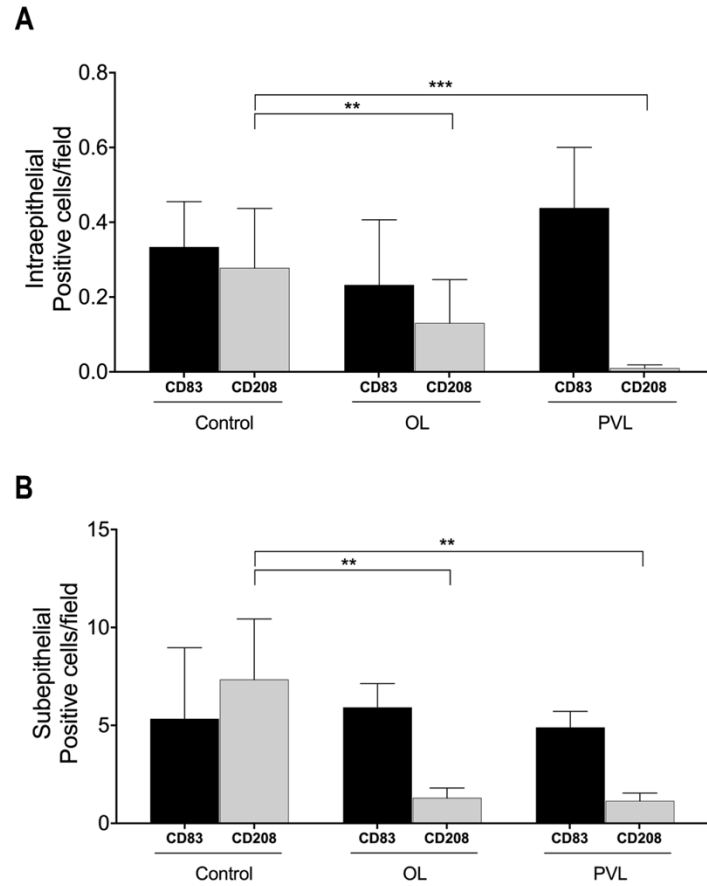


Figure 2. Average of submucosal (mature) DCs CD83+ and CD208+ in the intraepithelial area (A) and subepithelial area (B) for the control (inflammatory non-neoplastic tissue), OL and PVL groups; ** p = 0.001 and *** p = 0.0001.

Analyzing the markers for immature submucosal DCs, the CD209+ and factor XIIIa subpopulations show a pattern of tissue expression with evident abundance in the DPMO comparing with the pattern of mature DCs (CD83+ and CD208+). We found that factor XIIIa+ cells were more frequent in the epithelial area of PVL when compared with the non-neoplastic group (p=0.01). Furthermore, the CD209+ cells were found in a higher number in the subepithelial area of OL and PVL (both, p<0.0001) compared with the non-neoplastic group (Figure 3).

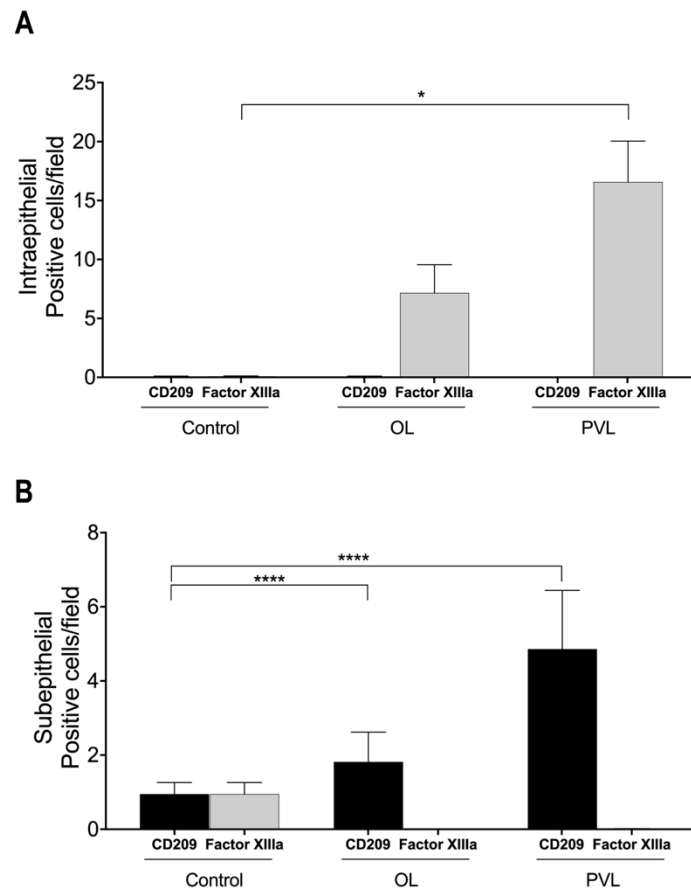


Figure 3. Average of submucosal (immature) DCs CD209+ and factor XIIIa+ in the intraepithelial area (A) and subepithelial area (B) for the control (inflammatory non-neoplastic tissue), OL and PVL groups; * $p = 0.01$.

Regarding the plasmacytoid DC, the CD123+ cells were more frequently found in the control group than the DPMOs. We found a reduction in the CD123+ cells count in the subepithelial area of OL compared with the non-neoplastic ($p=0.01$) and are presented in Figure 4.

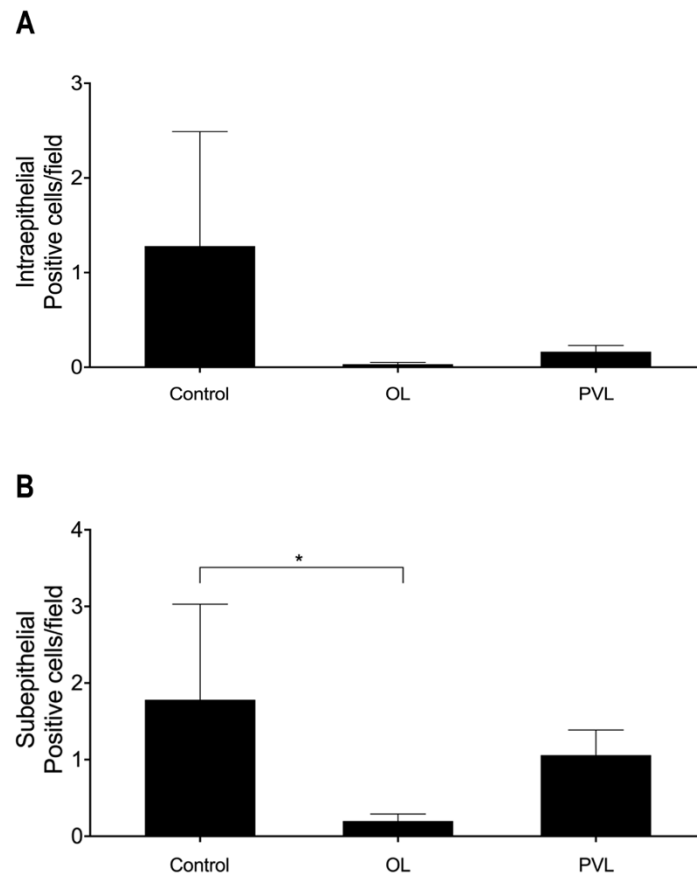


Figure 4. Average of plasmacytoid DCs (pDCs) CD123+ in the intraepithelial area (A) and subepithelial area (B) for the control (inflammatory non-neoplastic tissue), OL and PVL groups; * $p = 0.01$.

Despite these findings, we did not find any correlation between the number of different DCs subpopulations with the malignant transformation. Also, in the simple regression analysis, we found that the markers for immature LCs in OL and PVL (CD1a and CD207) have a dependent relationship in our population. Similar results were observed for mature DCs (CD83 and CD208) and submucosal DCs markers (CD209 and factor XIIIa) in both conditions. All the data from the simple linear regression are presented in Table 2.

Table 2. Summary of simple linear regression of dendritic cells biomarkers from OL and PVL

Group	Y	X	<i>p</i> -value	R ²	<i>p</i> -value (a)	<i>p</i> -value (b)	Equation log Y = a + b. log X	CI (95%) (a)	CI (95%) (b)
OL	CD1a	CD207	<0.0001	0.7888	<0.0001	<0.0001	log Y = 0.41 + 0.80 log X	0.21 – 0.61	0.62 – 0.99
	CD207	CD1a	<0.0001	0.7888	0.1385	<0.0001	log Y = -0.21 + 0.98 log X	-0.50 – 0.07	0.76 – 1.21
	CD83	CD208	<0.0001	0.6825	<0.0001	<0.0001	log Y = 0.46 + 1.04 log X	0.35 – 0.57	0.72 – 1.37
	CD208	CD83	<0.0001	0.7124	0.0016	<0.0001	log Y = -0.28 + 0.74 log X	(-0.45) – (-0.12)	0.53 – 0.94
	CD209	XIIIa	<0.0001	0.7531	0.0014	<0.0001	log Y = -0.16 + 0.66 log X	(-0.26) – (-0.06)	0.56 – 0.76
	XIIIa	CD209	<0.0001	0.7531	<0.0001	<0.0001	log Y = 0.37 + 1.14 log X	0.27 – 0.47	0.96 – 1.31
PVL	CD1a	CD207	<0.0001	0.9484	<0.0001	<0.0001	log Y = 0.26 + 0.93 log X	0.17 – 0.36	0.85 – 1.01
	CD207	CD1a	<0.0001	0.9484	<0.0001	<0.0001	log Y = -0.21 + 1.01 log X	(-0.33) – (-0.10)	0.93 – 1.10
	CD83	CD208	<0.0001	0.4946	<0.0001	<0.0001	log Y = 0.43 + 1.00 log X	0.30 – 0.55	0.65 – 1.36
	CD208	CD83	<0.0001	0.4946	0.0544	<0.0001	log Y = -0.12 + 0.50 log X	-0.26 – 0.00	0.33 – 0.68
	CD209	XIIIa	<0.0001	0.6788	0.0182	<0.0001	log Y = -0.21 + 0.69 log X	(-0.40) – (-0.04)	0.52 – 0.85
	XIIIa	CD209	<0.0001	0.6788	<0.0001	<0.0001	log Y = 0.50 + 0.99 log X	0.34 – 0.65	0.76 – 1.23

OL: leucoplakia; PVL: proliferative verrucous leucoplakia; Y: dependent variable; X: independent variable; R²: adjusted determination coefficient; (a): linear coefficient; (b): angular coefficient; CI: confidence interval. P-value <0.05 was considered as statistical significance.

DISCUSSION

The immune system plays an important role in tumor progression since it commands a complex signaling network to induce immune tolerance. Thus, there are multiple mechanisms by which head and neck squamous cell carcinoma (HNSCC) may suppress the immune system (Young, 2006). Several studies have addressed the importance of DCs for the prognosis in patients affected by different cancers (Bennaceur et al., 2008; Upadhyay et al., 2012; Jardim et al., 2018; Kindt et al., 2016). The DCs are antigen-presenting cells to CD4+ and CD8+ T cells in the lymph nodes and since the DCs act at the interface of the innate and adaptive immune systems, they may be used as targets in cancer immunotherapy (Datta et al., 2015). Overall, the distribution of DC subtypes indicated that ineffective DC response to tumor is a failure of DC function rather than recruitment. The dendritic cell suppression may be explained by a change in the cytokine balance within the tumor that shifts the immune system from an effective to an ineffective response. Until now, most studies have addressed the function of DCs in already established tumor diseases, but few studies explored the role of these leucocytes in the OPMDs. Therefore, our study is the first to investigate the presence of the different subpopulations of DCs in OL and PVL samples.

Our study revealed a general decrease of mature DCs in OL and PVL patients as well as an increase in the number of immature DCs compared with the inflammatory non-neoplastic lesion. Nonetheless, the results found in the current literature about the DC density and its correlation with dysplastic lesions are controversial. Of note, the majority of studies that investigate the role of DCs in the oral dysplastic and neoplastic lesions use normal tissue without inflammation as a control group. Some of these studies shown that depletion of CD1a+ cells in the tumor stroma is a strong independent prognostic factor for OSCC, predicting higher recurrence rates and lower survival outcomes independent of tobacco, alcohol, and human papilloma virus status (Jardim et al., 2018; Kindt et al., 2016). Interestingly, a reduction in the S100+ LCs count in dysplastic lesions may suggest the partial loss of immunosurveillance ability favoring the malignant transformation process (Wang et al., 2017). It was found an increase in CD83+ cells in OL larger lesions (>10mm) and with epithelial dysplasia, but could not find any relationship with CD1a+ count (Souto et al., 2018). Indeed, some groups reported a significant relationship between high-grade dysplasia and decrease of CD1a+ LC density (Upadhyay et al., 2012; Souto et al., 2018), whereas others have observed an increase of CD1a in dysplastic tissue (Bondad-Palmario, 1995). We did not find any association between any

marker with the dysplasia grade in PVL samples. Following our results, a previous study could not find any difference in the CD1a+ frequency when compared OL samples with and without dysplasia (Ohman et al., 2015).

In addition, we found a depletion of CD208+ cell, a marker for mature DCs, in the OL and PVL group when compared with the control group. Thus, we show that CD208 may be a marker of immune suppression in OL and PVL independent of the dysplasia grade. In a previous study with OSCC samples found that the number of CD208+ cells is higher in the tumor stroma and the tumor nest when compared with the non-neoplastic tissue (Ni et al., 2014). Also, they found that only the number of CD208+ in the tumor stroma was associated with lymph node positivity in OSCC patients, despite this, it did not correlate with the patient's survival time (Ni et al., 2014). O'Donnel et al. in 2007 found that a high numbers of CD207+ cells are not correlated with better prognosis, while the CD209+ DCs are associated with poor survival.

Also, some authors found that the number of mature CD83+ DCs was decreased in OSCC compared with dysplastic lesions, and the numbers of CD303+ pDC were significantly increased in the OSCC group compared with the dysplastic group (Pellicioli et al., 2017). Interestingly, our results show an reduction in the number of CD123+ pDCs in OL. However, this imbalance of pDCs does not occur in PVL. Although we did not find any correlation with the clinicopathological features, this distinct results found between OL and PVL suggests a different role of this DC subtype in the pathogenesis of these DPMOs.

It was already demonstrated that the density of DCs might be influenced by the smoking habit, being found a decrease of CD1a+ and CD83+ DCs density in smoker patients with oral leukoplakia (Souto et al., 2018). Since the majority of our patients were smokers, including the PVL populatiton (which usually are non smokers), our results are in agreement with these findings. Thus, the controversy about the density of DCs in oral epithelial dysplasia found in the literature may be related to the differences of each population, including the habits, as well as the high variability inter- and intra-observer related to the establishment of the dysplasia grade. Furthermore, our study is first to investigate a wide range of DCs subtypes simultaneously in OL and PVL, showing that there is a dependent relationship of all the DCs cell markers in our population.

We concluded that there is a general immunosuppression of the different types of DCs in both OL and PVL compared to the inflammaotory non-neoplastic lesion. We found a reduction in

the number of LCs and mature submucosal DCs, as well as an increase of immature submucosal DCs in both OPMDs. Also, we found that the participation of pDCs in PVL seems to be distinct from the observed in OL. However, , overall, the subpopulations of DCs are not different enough to justify the more aggressive behavior observed in PVL when compared to OL. More studies are necessary to elucidated if the DCs may promote or inhibit the tumor progression in the OL and PVL.

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CONFLICT OF INTEREST

The authors disclose no potential conflicts of interest.

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SUPPLEMENTARY

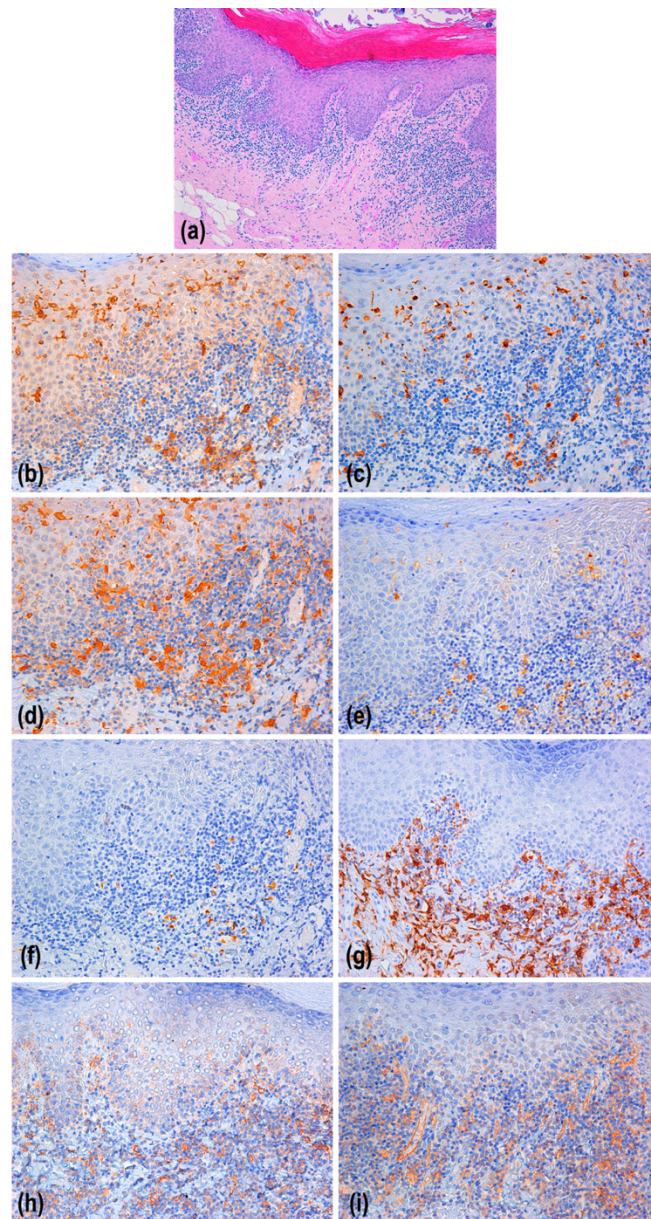


Figure 1S. Representative immunostaining pattern of the markers for the different subpopulations of DCs in a PVL sample. A. Hematoxylin and eosin staining (100x original magnification); B. CD1a+ staining pattern (200x original magnification); C. CD207+ staining pattern (200x original magnification); D. S100+ staining pattern (200x original magnification); E. CD83+ staining pattern (200x original magnification); F. CD208+ staining pattern (200x original magnification); G. CD209+ staining pattern (200x original magnification); H. Factor. XIIIa+ staining pattern (200x original magnification); I. CD123+ staining pattern (200x original magnification).

3.3 Publicação 3*

SOLUBLE FACTORS PRODUCED BY DYSPLASTIC KERATINOCYTES INHIBIT THE ACTIVATION OF TH17 CELLS AND THE APOPTOSIS OF PBMCs

SOLUBLE FACTORS PRODUCED BY PVL KERATINOCYTES ARE ABLE TO INHIBIT APOPTOSIS OF PBMCs AND PROMOTE A TREG/TH17 IMBALANCE FAVOURING THE MALIGNANT TRANSFORMATION

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ABSTRACT

We aimed to investigate whether the soluble products of dysplastic keratinocytes from proliferative verrucous leukoplakia (PVL) and other dysplastic cell lines were able to modulate the PBMCs to a phenotype that favours the carcinogenesis and influence the apoptosis of PBMCs. Conditioned medium of PVL and HOK (human oral keratinocytes) primary cell culture, and cell lines as DOK (dysplastic oral keratinocytes) and SCC25 (oral squamous cell carcinoma) cell lines were obtained and used to cultivate PBMCs. The immunophenotypic characterization of the different T helper lymphocytes and the apoptosis index was assessed through flow cytometry. Therefore, we conclude that the soluble factors produced by PVL keratinocytes may inhibit the activation of Th17 cells *in vitro* and the apoptosis of PBMCs *in vitro*.

KEYWORDS: Oral cancer; Oral leukoplakia; Proliferative verrucous leukoplakia; Immune system.

INTRODUCTION

The immune system has a vital role in the development, establishment, and progression of squamous cell carcinoma (SCC). Immune evasion of cancer cells leads to the progression of SCC. Improved understanding of tumor-immune system interactions has provided promising therapeutic options that are based on the rescue and enhancement of the anti-tumoral response. It has been shown that different cancer cell lines secrete soluble factors, as chemokines and cytokines, to modulate the immune response in order to subvert it and promote tumor progression.¹

Potentially malignant disorders of the oral cavity (OPMD) are a group of mucosal lesions associated with an increased risk of malignant transformation. Oral leukoplakia (OL) is one of the major OPMDs, being the worldwide average of malignant transformation rate equal to 3.5%.² Another OPMD is the proliferative verrucous leukoplakia (PVL), a rare and aggressive multifocal form of oral leukoplakia that presents high rates of recurrence, with a malignant transformation rate greater than 70% and unknown etiology.³ The role of inflammation and the development of SCC in OPMD has been investigated, indicating that OPMD probably represent a state of balance between activation of the immune system and the proliferation of dysplastic cells.⁴⁻⁸ Nevertheless, the role of secreted inflammatory factors that are produced by dysplastic keratinocytes modulating the immune response has not been determined. A consistent molecular study on the immune cells and cytokines related to OL and PVL represents an interesting research strategy to elucidate the etiology of these OPMDs. Thus, the results of this study offer potentially promising goals for the establishment of new tools that can reliably predict which lesions will undergo malignant transformation.

This study aimed to investigate if the soluble products of dysplastic and neoplastic keratinocytes may to influence the apoptosis index of peripheral blood mononuclear cells (PBMCs) and polarization of the T-helper cells in order to favour the carcinogenesis. To the best of our knowledge, this is the first study that compared the ability to modulate T-helper cells from soluble factors of dysplastic keratinocytes derived from OL and PVL.

MATERIALS AND METHODS

Primary cell culture and cell lines

This study was conducted in full accordance with ethical principles, including the World Medical Association (Declaration of Helsinki) and after approval by the local ethics committee. All participants agreed to participate in the study and provided written informed consent. Since there is no cell line derived from PVL described in the literature, we decided to cultivate the dysplastic oral keratinocyte from proliferative verrucous leukoplakia (DOK-PVL) primary cells *in vitro*. The donor patient had a previous diagnosis of PVL following the WHO description⁹ with more than 5 years of follow-up. The cells were derived from a white lesion located on the lower alveolar ridge of a 76-year-old man, and the degree of dysplasia was described as mild/moderate. In order to obtain primary cultures of the normal oral mucosa (human oral keratinocytes - HOK) as a control group, fragments of gingival tissue were also collected from health volunteer patients who underwent crown lengthening surgery.

Briefly, fragments of tissue measuring approximately 1.5 to 2.0 cm were divided into two parts, one of which was subjected to routine histopathological examination to confirm the histopathological degree and the other fragment was immediately conserved in EpiLife culture medium with 60M of calcium, supplemented with EDGS (ThermoFisher Scientific, USA), Gentamicin 50 mg/mL and Fungizone 250 μ g/mL. Subsequently, in a 12-well plate, 2 mL of EpiLife medium was added per well, and the fragment was washed for 5 min. The fragment was transferred to another well of the plate containing 2 mL of wash solution (PBS + Gentamicin 50 mg/mL + Fungizone 250 μ g/mL) and washed for 5 min; this step being was performed twice. The fragment was transferred to another well on the plate containing 2mL of 0.05% trypsin-EDTA, keeping the fragment in the solution for 8 hours at room temperature. Subsequently, the fragment was transferred to another well containing 2 mL of trypsin inhibitor (# R-007-100, ThermoFisher Scientific, USA) and washed for 5 min. After washing, the fragment was mechanically fragmented for 5 min in EpiLife medium and subsequently centrifuged at 4000xg for 2 min. The precipitate was resuspended in 6 mL of EpiLife medium supplemented with EDGS and Gentamicin / Fungizone and transferred to a 25 cm² flask. After 24 hours, cell adhesion was observed and was waited 48 hours before the first change of the culture medium. The HOK and DOK-PVL cultures were later used in the production of conditioned medium, as described below.

Additionally, the dysplastic keratinocyte cell line (DOK; CVCL-1180) from human tongue was obtained commercially (#94122104, ECACC - European Collection of Authenticated Cell Cultures, London, UK). This cell line originated from a white lesion in a 57-year-old man, and the degree of dysplasia was described as mild/moderate. The cell line SCC-25 (CRL-1628) came from OSCC of a human tongue and was purchased from the American Type Culture Collection (ATCC, USA). Appropriate culture medium for each cell line was used following the manufacturer's guidelines, supplemented with 10% fetal bovine serum (FBS; Cultilab Ltda, Brazil) and antibiotics (Invitrogen, USA). All cell lines were kept at 37°C in an atmosphere containing 5% CO₂ and 95% humidity.

Peripheral Blood Mononuclear Cells (PBMCs)

In order to obtain PBMCs, peripheral blood from four healthy donors was collected. The blood was diluted in PBS pH 7.2 in a 1:1 ratio and transferred to conical tubes containing Ficoll-Hypaque (GE Healthcare). After centrifugation, at 400xg for 30 minutes at 18°C, the PBMCs layer obtained between the Ficoll-Hypaque mixture and the plasma was removed, deposited in new conical tubes and washed with twice its volume of PBS. After the supernatant was discarded, the PBMCs was resuspended in RPMI 1640 culture medium (Invitrogen, USA) supplemented with L-Glutamine, 10% fetal bovine serum (FBS; Cultilab Ltda, Brazil), 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 200U/mL of Penicillin/Streptomycin (Invitrogen, USA).

Conditioned Medium (CM)

The CM of the cell cultures HOK, DOK, DOK-PVL, and SCC-25 were prepared to determine whether soluble factors in these cells have different effects on PBMCs in terms of the modulation of the Th phenotype and apoptosis. Briefly, 1x10⁶ cells from each of the four cell lines were cultivated on 100 mm² plates and maintained in culture until they reached approximately 80% confluence. Subsequently, a process of adaptation of cell cultures to the culture medium RPMI 1640 (without antibiotics) was carried out employing progressive changes of the culture medium in concentrations of 25%, 50%, and 75% every 12h. At the end of the adaptation process, 4 mL of RPMI 1640 culture medium were added to each plate, which were collected after 24h and immediately aliquoted and kept in a -20C freezer until use. For carrying out the experiments, the PBMCs were grown in the proportion of 1:1 (vol:vol) of CM and non-conditioned culture medium RPMI 1640, with the proportion of 0:1 being used as a

control. PBMCs were kept in culture for different experimental periods, according to the assay being performed as described below.

Apoptosis in PBMCs

To access the rates of cell death due to apoptosis, 3×10^5 PBMCs were cultured in 12-well plates in the presence of CM from the HOK, DOK, DOK-PVL, and SCC-25 cell cultures or RPMI 1640 non-conditioned culture medium, in the times of 24 and 48h. Then, the cells were transferred to sterile tubes and centrifuged for 4 min at 400xg. The precipitated cells were resuspended in 100 μ L of binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) containing 5 μ L of annexin-V PE and 5 μ L of 7-AAD (BD Pharmingen, USA). The mixture was gently stirred and incubated for 15 min in the dark at room temperature. After incubation, 400 μ L of binding buffer was added, and the percentage of positive cells was determined by reading 10,000 events on a FACSVerse flow cytometer (BD Biosciences, USA), followed by analyzing the data in the FlowJo software (version 10.0.7, Tree Star). Dot plot type graphs were used for quantification, and positive cells for apoptosis or doubly positive annexin-V and 7-AAD (late apoptosis) were considered positive for apoptosis.

Helper T cells (Th) immunophenotyping by flow cytometry

Immunophenotyping of Th cells (Th1, Th2, Th17 and Treg) via flow cytometry was performed using two commercially available kits (#560751 and #560762; BD Biosciences, USA). The phenotyping kits used provided a cocktail of primary antibodies for the detection of human CD4, IFN- γ (for Th1), IL-4 (for Th2), IL-17A (for Th17), and FoxP3 (Treg), which allowed to identify and characterize the nature of these Th cells by flow cytometric analysis. Briefly, PBMCs were grown in RPMI 1640 or CM produced by different cell cultures (HOK, DOK, DOK-PVL, and SCC-25), both supplemented with 10% FBS, for a period of 96h in a CO_2 oven with 5% humidity at 37°C. Additionally, an experimental group composed under the same conditions described above was cultured in the presence of the mitogen phorbol myristate acetate (PMA; 25 ng/mL) and ionomycin (1 μ g/mL). Monensin (10 μ g/mL) was added for 4 hours before the end of the culture to ensure the retention of cytokines inside the cell. After incubation, approximately 3×10^6 PBMCs were collected and centrifuged at 500xg for 5 min. The cells were then washed using a saline solution containing 1% FBS and 0.01% sodium azide (solution called stain buffer), centrifuged and then the PBMCs were fixed according to the guidelines of each kit used. Then, the cells were incubated for 30 minutes, at room temperature,

with each of the cocktails. After incubation, the cells were washed with stain buffer and centrifuged at 500xg for 5 minutes. Negative controls, calibration, and compensation of the flow cytometer were performed in the most appropriate way for each assay. A total of 30,000 events were acquired within the CD4 positive population on an LSR Fortessa flow cytometer (BD biosciences), and the data were analyzed using FACSDiva software (version 8.0.1, BD biosciences).

Statistical analyses

Descriptive statistics were used to evaluate data. To evaluate the normality distribution of the data was used Shapiro-Wilk test and the following the descriptive aspects: asymmetry and kurtosis. The presence of outliers was checked. The Box's Test of Equality of Covariance Matrices and the Levene's Test of Equality Variances were used to verify the homoscedasticity. Thus, the Analysis of Variance with one independent factor was performed (ANOVA ONE-WAY). The post-test of Tukey was chosen to evaluate the differences between the groups. The effect factor was calculated when p-value was statistically significant. A p-value of < 0.05 was regarded to be statistically significant. Statistical analysis was performed using the IBM SPSS Statistics 20.0.

RESULTS

The epithelial origin of the isolated cells of PVL-DOK and HOK was confirmed by immunofluorescence with antibody to human cytokeratin AE1/AE3 (#ab27988, Abcam) and it is presented in Figures 1 and 2, respectively.

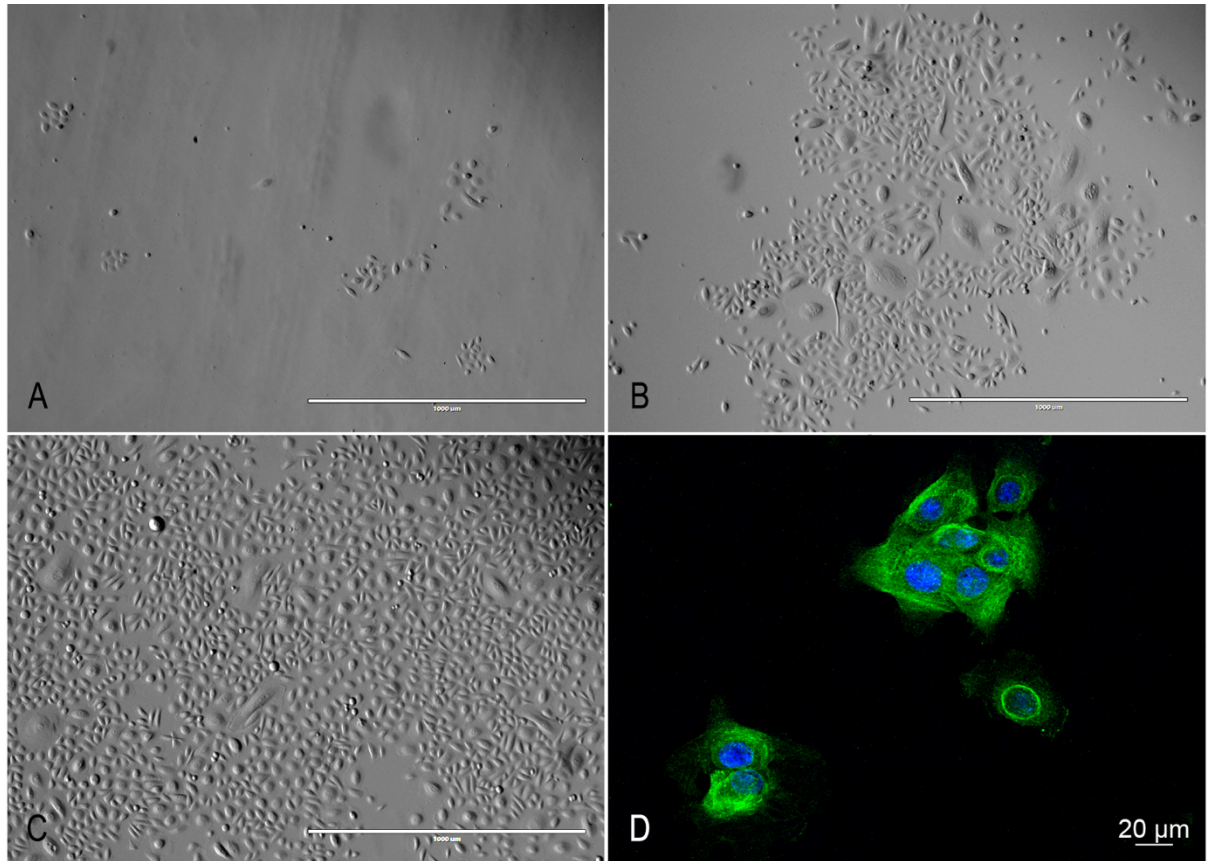


Figure 1. Representative image of primary keratinocytes isolated from a leukoplastic lesion in a patient with LVP (DOK-PVL3). At 7 days of cultivation in Epilife medium supplemented with EDGS, the formation of small islands of polygonal cells (A) was observed, with 14 days the expansion of colonies made up of cells of variable morphology (B) is observed and after 30 days the cells reached complete confluence (C). Immunofluorescence for human cytokeratin AE1/AE3 confirmed the epithelial origin of the primary culture DOK-PVL (D).

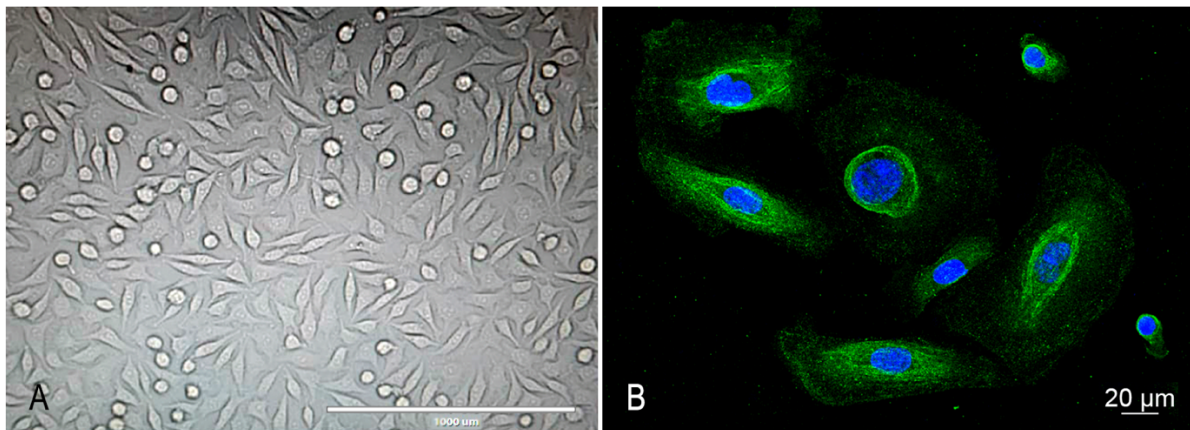


Figure 2. Representative image of primary keratinocytes isolated from normal oral mucosa (HOK). After 30 days, the cells showed complete confluence (A) and immunofluorescence for human cytokeratin AE1/AE3 confirmed the epithelial origin of the primary culture (B).

Apoptosis cell death rates were investigated by double staining with annexin V-PE and 7AAD with subsequent detection by flow cytometry. The results revealed that the soluble products contained in the CM derived from the primary culture DOK-PVL, and the cell line SCC-25 were able to significantly inhibit the apoptosis of PBMCs, versus the CM produced by the primary cultures HOK (Figure 3). PBMCs in the presence of CM derived from DOK-PVL or SCC-25 within 24h had an approximately 5-fold reduction in apoptosis rates compared to HOK ($p = 0.04$ and $p = 0.03$, respectively). In addition, a significant reduction was observed for PBMCs in the presence of SCC-25 CM over 48h, which revealed an approximately 3-fold reduction in the number of PBMCs in apoptosis ($p = 0.01$) (Figure 4).

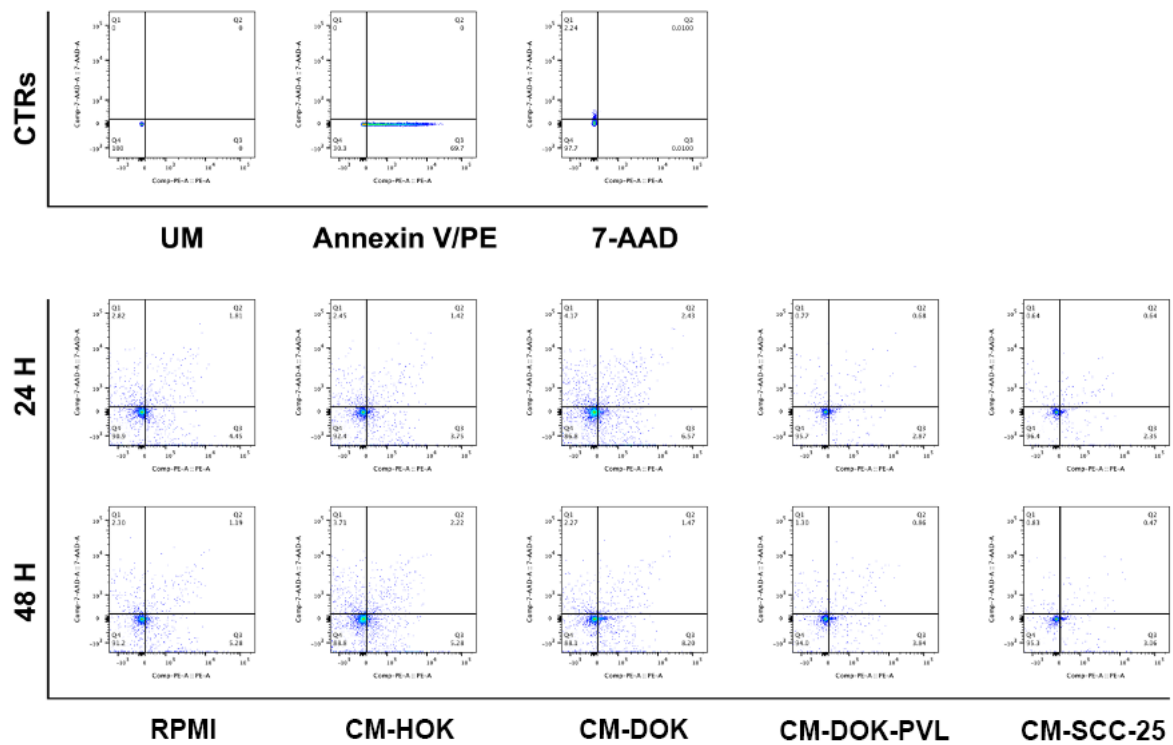


Figure 3. Graphs representative of the inhibitory potential of apoptosis promoted by CMs derived from all cell cultures. CTRs: Controls; ONE: non-marked PBMCs.

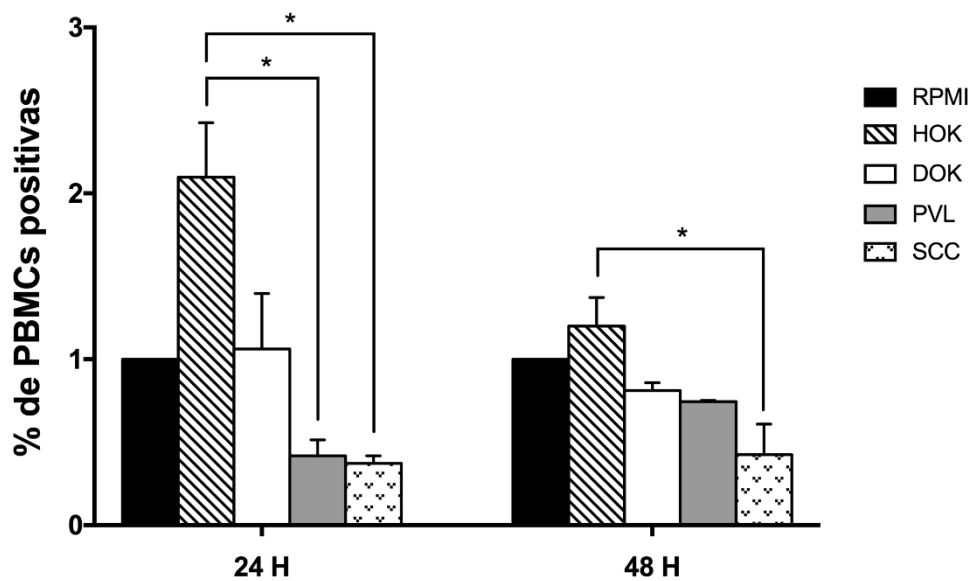


Figure 4. Apoptosis index by annexin V and 7-AAD labeling. Exposure of PBMCs to soluble products derived from cell cultures promoted the inhibition of apoptosis. * $p < 0.05$.

The modulation of the Th phenotype was accessed through the exposure of PBMCs to the different CMs derived from HOK, DOK, DOK-PVL, and SCC-25. The unconditioned RPMI medium was used as a reference sample. Performing a general assessment of the relative quantity of PBMCs expressing the explored markers, it can be observed that T CD4⁺ FoxP3⁺ cells are predominant in all tested CM groups.

Statistical analysis of the data showed that in the absence of chemical stimulators, the CD4⁺ IL-17A⁺ population (for Th17) was significantly reduced in the CM groups derived from DOK-PVL and SCC-25 versus the reference sample ($p = 0.01$), presenting a two and 3-fold reduction in the Th17 population for DOK-PVL and SCC-25, respectively (Figure 5A). Additionally, we observed that even in the presence of chemical stimulators, the CD4⁺ IL-17A⁺ population continued to be reduced, showing a 1.5-fold reduction in this T cells population in the DOK, DOK-PVL and SCC-25 groups in relation to the reference sample ($p = 0.001$) (Figures 5B).

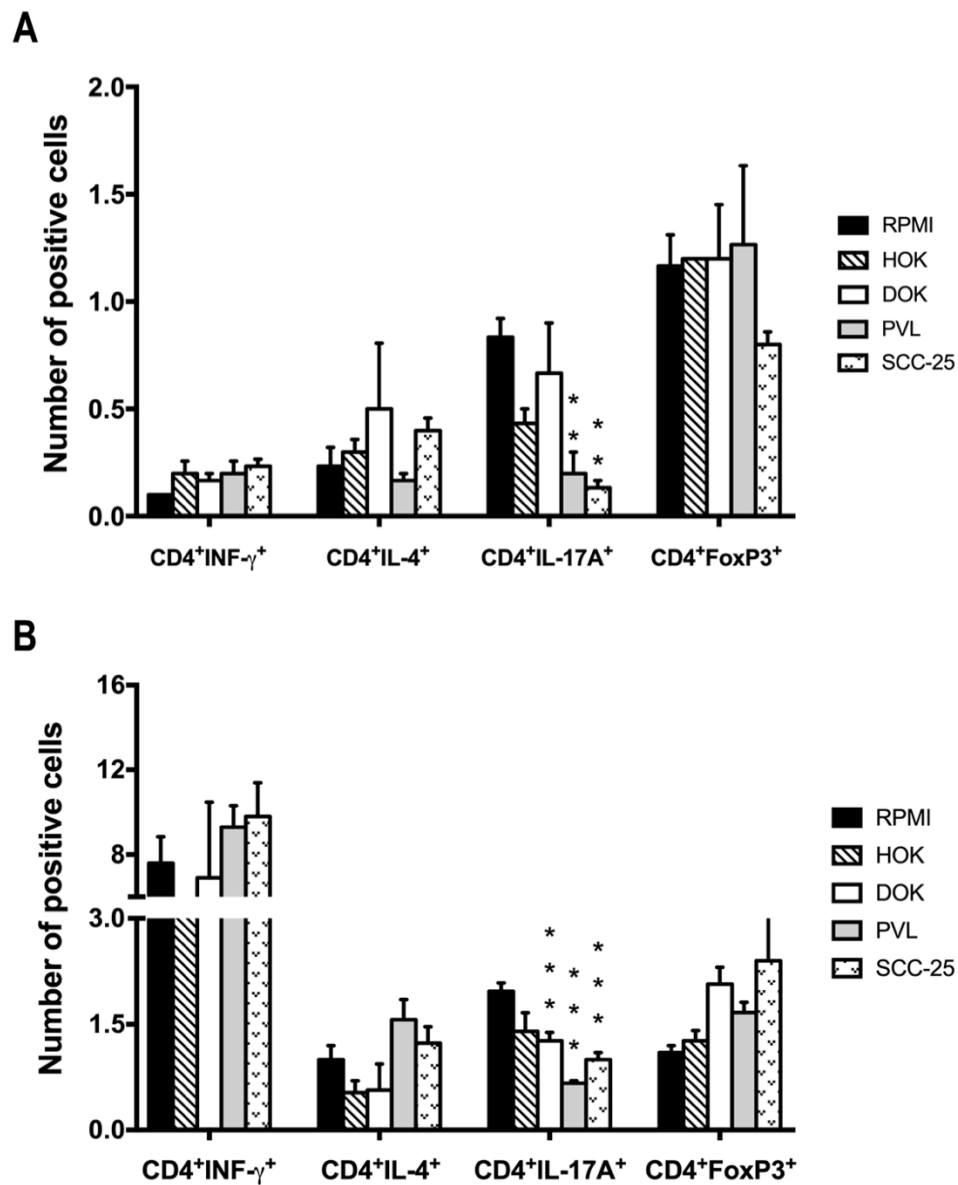


Figure 5. Modulation of the Th1, Th2, Th17 and Treg phenotype by contact with CM derived from HOK, DOK, DOK-PVL and SCC-25. Relative quantity of CD4⁺ IFN- γ ⁺, CD4⁺ IL-4⁺, CD4⁺ IL-17A⁺ and CD4⁺ FoxP3⁺ cells in the absence (A) and presence (B) of 25 ng/mL PMA and 1 μ g/mL of ionomycin. ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

Our study was the first to investigate the influence of the conditioned medium of dysplastic oral keratinocytes from OL and PVL in the PBMCs apoptosis index and the immunomodulation of T helper cells. After the activation, initial T helper cells may differentiate under the influence

of the microenvironment into many subsets. According to cell function, CD4⁺ T cells may be divided into Th1, Th2, Th17, Tregs and other T helper subtypes. The interactions between all the subsets cells are necessary to coordinate the inflammatory process. In this context, it was already demonstrated that the expression of anti-inflammatory or pro-inflammatory cytokines are regulated explicitly during development from dysplastic lesions to OSCC.¹⁰

Overall, we found that the CM derived from dysplastic keratinocytes inhibits the apoptosis index of PBMCs and is capable of modulating the T helper phenotype by creating an imbalance of Th17/Threg in the PVL and OSCC. However, when these PBMCs are simultaneously stimulated with PMA (25 ng/mL) and ionomycin (1 µg/mL), there is an overall increase in CD4⁺ IFN-γ⁺ cells (for Th1). Accompanied by this, we noticed an increase in the amount of CD4⁺ FoxP3⁺ cells (for Treg) in dysplastic and neoplastic keratinocytes. Tregs and Th17 cells, two subtypes of CD4⁺ T cells, play opposite roles in immune regulation, even though they share a common differentiation pathway, being the main producers of TGFβ and IL17, respectively.¹¹ Alterations in this complex cytokine network may lead to immune functions, uncontrolled cell growth, chronic inflammation, and carcinogenesis.¹²

In this context, the Treg/Th17 imbalance has been observed in colon cancer, bladder carcinoma, breast cancer and oral cancer.¹²⁻¹⁵ Our results show that the exposure to secreted products from PVL primary cells used in this study consistently and significantly inhibited the proportion of cells polarized to the Th17 phenotype. Controversial results have been shown in the literature about the role of Th17 in the carcinogenesis. Some studies show that higher expression of IL-17 is associated with worse prognosis of OSCC.^{9, 16, 17} However, a reduction in the prevalence of Th17 cells has been related to the development of metastasis in HNSCC.¹⁸ In contrast, it was demonstrated that an increase of Treg cells is associated with worse prognosis in HNSCC.¹⁹⁻²¹ The prevalence of Tregs is also correlated with the stage of HNSCC, as there is a higher prevalence of circulating Treg cells in patients with late-stage laryngeal tumors, in comparison with patients with earlier stages and patients with oropharyngeal tumors.²² Moreover, an in vitro study using the co-culture of immune and liver cancer cells shown that immune cells induce tumor cells to produce soluble factors capable of favoring the immunosuppressive response led by an increase of the Treg population in the tumor microenvironment.²³ It is important to note that the proportion of Treg/Th17 in tumor microenvironment may influence the tumor progression. Studying the imbalance between these T-cell subsets might help understand the tumor progression.

In addition to the Treg/Th17 imbalance, the tumor microenvironment has a complex network of immune cells, including T-cells subsets such as Th1 and Th2 cells. Th2 cytokines (IL-4, perhaps IL-5, and IL-10) are classified as anti-inflammatory ones, and are often related to pro-tumoral activities. In contrast, most Th1 cytokines, represented by IFN- γ , are classified as pro-inflammatory cytokines, and are associated with, in general, a good prognosis.¹² However, Th2 cells secrete IL4, IL5, IL6, IL10, and IL13, which mediate the anti-inflammatory humoral response and immune-suppression by inhibiting Th1 cytokine production.¹² Serum levels of IL-17A, TGF- β 1, IL-4, and IL-10 were significantly higher in OSCC patients. At the same time, IL-2 and IFN- γ were relatively low in OSCC patients when compared to controls.²⁴ An interesting aspect observed in this analysis is related to the IL-4 expression induced by the DOK-PVL CM. The CD4⁺ IL-4⁺ population (for Th2) was reduced in the absence of non-antigen-dependent chemical stimulators (PMA and ionomycin) for the DOK-PVL group. In contrast, the presence of inducing agents, these levels increased substantially. Interestingly, it is known that the topical application of PMA causes intense local inflammation that facilitates the growth of pre-malignant lesions on the skin after the subsequent exposure to carcinogenic agents, through the activation of immunostimulatory mechanisms.²⁵ This finding suggests that the CM derived from DOK-PVL has a low capacity to promote the differentiation of naive CD4⁺ T cells in cells producing cytokines with a Th2 profile (IL-4), however, the presence of intense local inflammation may favor plasticity for a pro-tumorigenic Th2 profile.

Our study is the first to cultivate primary cell culture derived from PVL, such a rare and peculiar disease, and to investigate the influence of the soluble secreted factors on the modulation of CD4⁺ cells. We used primary cells from PVL and HOK, one OSCC cell lines, and a dysplastic epithelial cell line derived from the human oral mucosa, which needs to be considered given the heterogeneity that is peculiar to HNSCC. The controversies found in the literature must be critically analyzed. A large number of experimental variables may influence the conflicting information. In this context, some of the essential variables in cytokine biology include the species surveyed, as well as, distinguish whether the study is carried out *in vivo*, *ex vivo*, or *in vitro*. The most recent research has proven that much of that earlier work is highly simplistic or inaccurate. Thereby, we conclude that the soluble factors produced by PVL keratinocytes may inhibit apoptosis of PBMCs and promote a Treg/Th17 imbalance favouring the malignant transformation of PVL.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors disclose no potential conflicts of interest.

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4 CONCLUSÃO

Por meio desse trabalho chegamos então as seguintes conclusões:

A presença de infiltrado inflamatório moderado/intenso, displasia de alto risco, altas taxas de recidivas e taxas elevadas de transformação maligna são fortemente correlacionadas com o diagnóstico de LVP;

Redução de CD de Langerhans, maduras e plasmocitóides nos compartimentos epitelial e subepiteliais na LO e LVP em relação ao controle, sugere uma apresentação inadequada de antígenos pré-tumorais pelas CDs;

Aumento de CD imaturas nos compartimentos epitelial e subepiteliais na LO e LVP em relação ao controle, pode favorecer a tolerância imunológica em relação às células pré-tumorais;

Redução global de células T e a atividade citotóxica das células T é reduzida em LO e LVP;

O processo de carcinogênese na LVP passa pela ativação da via NF- κ B e provavelmente esta via é ativada por altos níveis de TNF- α ;

O perfil de citocinas e quimiocinas observado em lesões leucoplásicas de LVP provavelmente favorecem a ativação dos Tregs quando comparados a LO;

Os fatores solúveis produzidos por queratinócitos de LVP são capazes de inibir a apoptose de PBMCs em um modelo in vitro;

Fatores solúveis produzidos por queratinócitos de LVP são capazes de inibir a ativação de células Th17 em um modelo in vitro, provavelmente promovendo uma resposta imune mediada por Treg.

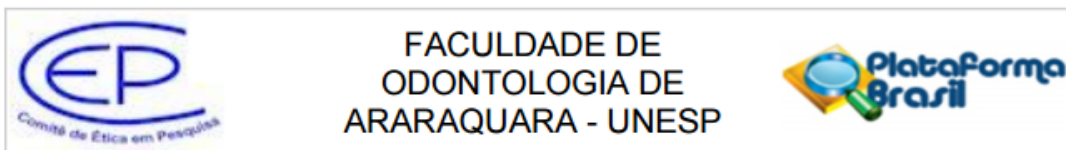
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ANEXO A – COMPROVANTE DO CEP**COMPROVANTE DE ENVIO DO PROJETO****DADOS DO PROJETO DE PESQUISA**

Título da Pesquisa: ESTUDO DA IDENTIFICAÇÃO DE POTENCIAIS BIOMARCADORES PARA LEUCOPLASIA ORAL E LEUCOPLASIA VERRUCOSA PROLIFERATIVA
Pesquisador: Andreia Bufalino
Versão: 2
CAAE: 34361814.9.0000.5416
Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

DADOS DO COMPROVANTE

Número do Comprovante: 065565/2014
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO

FACULDADE DE
ODONTOLOGIA DE
ARARAQUARA - UNESP



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO DA IDENTIFICAÇÃO DE POTENCIAIS BIOMARCADORES PARA LEUCOPLASIA ORAL E LEUCOPLASIA VERRUCOSA PROLIFERATIVA

Pesquisador: Andreia Bufalino

Área Temática:

Versão: 2

CAAE: 34361814.9.0000.5416

Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

Patrocinador Principal: FUNDAÇÃO DE AMPARO A PESQUISA DO ESTADO DE SÃO PAULO

DADOS DO PARECER

Número do Parecer: 822.355

Data da Relatoria: 07/10/2014

Apresentação do Projeto:

O Projeto de Pesquisa apresenta uma introdução que descreve a importância de identificar biomarcadores tumorais para leucoplasia oral e leucoplasia verrucosa proliferativa para auxiliar no diagnóstico e tratamento destas doenças que acometem um grande número de pessoas.

Objetivo da Pesquisa:

Identificar potenciais biomarcadores tumorais para leucoplasias e leucoplasia verrucosa proliferativa que possam futuramente serem auxiliares no diagnóstico, prognóstico e tratamento destas doenças.

Avaliação dos Riscos e Benefícios:

Os riscos que os pacientes estarão sujeitos, segundo o Pesquisador Responsável, poderão ocorrer durante a realização da biópsia e dor após a cirurgia.

Em relação aos benefícios, de acordo com o diagnóstico da biópsia, os pacientes receberão o tratamento adequado.

Comentários e Considerações sobre a Pesquisa:

A Pesquisa irá utilizar material biológico obtido de pacientes que procuram tratamento de suas

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Continuação do Parecer: 822.355

lesões na Faculdade de Odontologia de Araraquara - UNESP e, para validação por imuno-histoquímica utilizará amostra de tecidos parafinizados e culturas de linhagens de células para validação em culturas, ambos "in vitro".

Considerações sobre os Termos de apresentação obrigatória:

O Termo de Consentimento Livre e Esclarecido foi alterado, agora escrito em linguagem para leigo, no singular referindo-se somente ao Paciente que irá participar ou não da pesquisa. Os riscos e benefícios referentes à biópsia estão claros, bem como todos os cuidados que serão tomados antes e após a cirurgia.

Recomendações:

Corrigir algumas palavras digitadas erradas e algumas frases sem concordância no projeto completo.

Conclusões ou Pendências e Lista de Inadequações:

As recomendações e dúvidas relacionadas pelo Parecerista foram atendidas. Sendo assim, aprovo a realização do Projeto de Pesquisa.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Protocolo APROVADO em reunião de 7 de outubro de 2014.

ARARAQUARA, 07 de Outubro de 2014

Assinado por:
Maurício Meirelles Nagle
(Coordenador)

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Araraquara, 13 de março de 2020.

DARCY FERNANDES