UNIVERSIDADE FEDERAL DE MINAS GERAIS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

DEPARTAMENTO DE MORFOLOGIA

Janine Mayra da Silva

PARTICIPAÇÃO DA QUIMIOCINA CCL3 E SEUS RECEPTORES CCR1 E CCR5 E DO RECEPTOR ATÍPICO ACKR2 NA CARCINOGÊNESE BUCAL

Belo Horizonte

Junho

2016

Janine Mayra da Silva

PARTICIPAÇÃO DA QUIMIOCINA CCL3 E SEUS RECEPTORES CCR5 E CCR1 E DO RECEPTOR ATÍPICO ACKR2 NA CARCINOGÊNESE BUCAL

Tese apresentada ao Colegiado do Programa de Pós-Graduação em Biologia Celular do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Doutor em Biologia Celular.

Orientadora: Prof[a]. Tarcília Aparecida da Silva

Departamento de Clínica, Patologia e Cirurgia Odontológicas – Faculdade Odontologia/UFMG

Co-orientador: Prof. Remo de Castro Russo

Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas/UFMG

Belo Horizonte

Junho

2016

Este trabalho foi desenvolvido nos seguintes Laboratórios:

-Laboratório de Patologia Bucal Experimental, Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia-UFMG;

-Laboratório de Patologia Bucal, Departamento de Estomatologia (Patologia Oral), Faculdade de Odontologia-UFG;

-Laboratório de Interação Microrganismo-Hospedeiro, Departamento de Microbiologia, ICB-UFMG;

-Laboratório de Imunofarmacologia, Departamento de Bioquímica e Imunologia, ICB-UFMG;

-Laboratório de Imunologia e Mecânica Pulmonar, Departamento de Fisiologia e Biofísica, ICB-UFMG;

-Laboratório de Marcadores e Sinalização Celular no Câncer, Departamento de Análises Clínicas, Toxicologia e Ciência dos Alimentos, Faculdade de Ciências Farmacêuticas de Ribeirão Preto-USP.

Este projeto recebeu **apoio financeiro** do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Processos 474106/2012-8 e 455334/2014-5), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

DEDICATÓRIA

"Dedico este trabalho a todas as pessoas que acreditam e valorizam a ciência neste país. A pesquisa científica feita por pessoas comprometidas serve como agente transformador na vida econômica, política, tecnológica, social e cultural de milhões de brasileiros e brasileiras. Sinto orgulho de poder contribuir e escrever um pequeno capítulo desta história."

Janine M. Silva

Maio 2016.

AGRADECIMENTOS

A Deus, razão primária de todas as coisas. Pela proteção, amor, graça e misericórdia sempre presentes.

A minha família, estrutura a qual sempre suportou todas as minhas decisões, por maior a carga que elas implicassem. É um privilégio partilhar a minha vida com vocês. Quanto mais me doo, mais eu recebo em troca e cresço diante de minhas adversidades.

Ao meu amor, Eduardo, obrigada por topar embarcar comigo nessa longa jornada. Mesmo sem saber ao certo o desfecho de tudo, seu apoio em vários momentos tornou minha carga mais leve. Minhas dificuldades em expressar meus sentimentos pela escrita me impedem expressar o quanto quero o seu bem. Obrigada por tudo.

A Tarcília, que me proporcionou o que pôde para a construção da profissional que me tornei hoje. São muitos anos de convívio, desde a sua chegada à FO/UFMG quando me tornei monitora da patologia. Sempre me esforcei para dar o meu melhor para os meus mestres, com você não foi diferente. Obrigada por abrir portas, pela acessoria sempre presente, pelo aprendizado adquirido.

Aos meus parceiros de laboratório atuais e aos que já tive o prazer de conviver. São tantos ao longo destes 8 anos de laboratório que não ouso mencioná-los especificamente. Não quero retirar o brilho de ninguém, cada um tem seu valor guardado aqui comigo. Muitos tornaram-se parceiros para a vida, isso é um legado e tanto! Um alô especial aos queridos alunos de iniciação científica, os quais pude orientar. Digo a vocês que ninguém sabe ou faz nada sozinho. Aprendi muito, muito, com vocês. Divido com todos mais esta conquista de vida.

Um agradecimento para os professores da Patologia da FO e professores parceiros que me proporcionaram ferramentas (técnicas, ideológicas e muito espaço!)

para a realização deste trabalho. Profs. Mauro Teixeira, Daniele Batista, Aline Batista, Milene Rachid, Gustavo Garlet, Andréia Leopoldino, e ao meu co-orientador, prof. Remo Russo, muito obrigada por tudo.

Aos funcionários Inês, Domênico, Dani, Frank e Ilma pelos imensos “galhos quebrados”, pela amizade e boas gargalhadas e pelo ótimo trabalho técnico prestado sempre.

As agências CAPES, CNPq e FAPEMIG pelo apoio financeiro, sem o qual este trabalho não teria saído do papel. Espero poder devolver os frutos colhidos à sociedade.

Aos meus queridos amigos, sempre contribuindo com momentos de diversão, “relax”, “tin-tins” e muita energia boa. Obrigada pela acolhida de sempre e pelas lágrimas enxugadas também.

“Seria mais fácil fazer como todo mundo faz

O caminho mais curto, produto que rende mais

Seria mais fácil fazer como todo mundo faz

Um tiro certeiro, modelo que vende mais

Mas nós dançamos no silêncio

Choramos no carnaval

Não vemos graça nas gracinhas da TV

Morremos de rir no horário eleitoral

Seria mais fácil fazer como todo mundo faz

Sem sair do sofá, deixar a Ferrari pra trás

Seria mais fácil, como todo mundo faz

O milésimo gol sentado na mesa de um bar

Mas nós vibramos em outra frequência

Sabemos que não é bem assim

Se fosse fácil achar o caminho das pedras

Tantas pedras no caminho não seria ruim”

Outras Frequências,

Engenheiros do Hawaii.

RESUMO

Quimiocinas e receptores de quimiocinas têm assumido relevância no contexto da carcinogênese devido ao seu potencial de ativação de vias "pró-tumorigênicas". A expressão da quimiocina CCL3 e seus receptores (CCR1 e CCR5) foi demonstrada no carcinoma de células escamosas de boca (CCEB), contudo, suas funções ainda não foram definidas. Neste estudo, as funções do eixo CCL3/CCR1/CCR5 na carcinogênese oral quimicamente induzida foram avaliadas em camundongos geneticamente deficientes dessas moléculas. Avaliou-se também a participação do receptor atípico de quimiocinas ACKR2 – um "sequestrador" de quimiocinas CC que atuaria reduzindo a disponibilidade de CCL3 e outras quimiocinas no sítio tumoral. Foram também realizados experimentos *in vitro* utilizando linhagens de CCEB para analisar possíveis efeitos diretos de CCL3 nestas células. Os resultados mostraram que lesões de CCEB quimicamente induzidas exibiram expressão aumentada de CCL3. Camundongos deficientes para CCL3 ($CCL3^{-/-}$) e CCR5 ($CCR5^{-/-}$) tratados com o carcinógeno químico 4-Nitroquinolina-1-óxido (4NQO) apresentaram reduzida formação de tumores linguais comparados aos selvagens (WT) e animais deficientes para CCR1 ($CCR1^{-/-}$). Consistentemente, a análise microscópica demonstrou atenuada atipia citomorfológica e reduzida proliferação nas lesões de animais $CCL3^{-/-}$ e $CCR5^{-/-}$ tratados. Verificou-se que lesões de animais $CCL3^{-/-}$ exibiram expressão reduzida dos fatores Epidermal growth factor (EGF), Fibroblast growth factor-1 (FGF-1), Transforming growth factor-β1 (TGF-β1), Vascular endothelial growth factor-a (VEGFa) e VEGFb, as citocinas Interleucina-6 (IL-6) e Tumor necrosis factor-α (TNF-α), moléculas de adesão Integrina-4 (ITGA-4) e Vitronectina (VTN) e metaloproteinases de matriz MMP-1a, MMP-2 e MMP-9. *In vitro*, CCL3 foi capaz de induzir a invasão e liberação de CCL5,

IL-6, e MMP-2, MMP-8 e MMP-9 pelas células neoplásicas. O bloqueio de CCL3, utilizando anticorpo anti-CCL3 reduziu a invasão das células neoplásicas.

Os animais deficientes do receptor ACKR2 ($ACKR2^{-/-}$) tratados exibiram similaridade na formação de tumores linguais, e em parâmetros microscópicos quando comparados aos animais selvagens. Além disso, observou-se uma expressão aumentada das quimiocinas CCL3, CCL4, CCL5, CCL12 e dos receptores CCR1, CCR2 e CCR5, além de citocinas pró-inflamatórias, fatores angiogênicos, moléculas de adesão e metaloproteinases de matriz nas lesões de ambos os grupos tratados em comparação aos controles. Entretanto, expressão aumentada de CCL2, IL-6 e IL-17 foi verificada nas lesões linguais dos animais $AKCR2^{-/-}$ em relação ao grupo WT.

Coletivamente, os dados sugerem a relevância do eixo CCL3/CCR5 na carcinogênese oral, sendo que CCL3 é capaz de afetar diretamente as células tumorais e o microambiente tumoral. O receptor ACKR2 não modificou o processo da carcinogênese bucal. Estes resultados revelam potenciais efeitos protetores do bloqueio de CCL3 no carcinoma de células escamosas de boca.

***Palavras-chave**: Quimiocinas, CCL3, CCR1, CCR5, ACKR2 e CCEB.*

ABSTRACT

Chemokines and chemokine receptors have assumed relevance in the context of oral carcinogenesis due to its potential activation of "pro-tumorigenic" pathways. The expression of CCL3 and its receptors (CCR1 and CCR5) have been demonstrated in oral squamous cell carcinoma (OSCC), however, their roles were not defined yet. In this study, the roles of CCL3/CCR1/CCR5 axis in chemically-induced oral carcinogenesis were evaluated in genetically-deficient mice of these molecules. It was also evaluated the roles of the atypical chemokine receptor ACKR2 – a scavenger CC chemokine receptor that could reduce CCL3 and other chemokines availability at tumor sites. *In vitro* experiments were also performed employing OSCC lineages to analyze possible CCL3 direct effects in these cells. The results showed that chemically-induced OSCC lesions exhibited increased expression of CCL3. Deficient mice for CCL3 ($CCL3^{-/-}$) and CCR5 ($CCR5^{-/-}$) treated with the chemical carcinogen 4-Nitroquinoline-1-oxide (4NQO) presented decreased tongue tumor formation when compared to *wild type* (WT) and CCR1 ($CCR1^{-/-}$) deficient mice. Consistently, microscopical analysis demonstred attenuated cytomorphological atypia and reduced proliferation in lesions of $CCL3^{-/-}$ and $CCR5^{-/-}$ treated mice. It was verified that $CCL3^{-/-}$ mice lesions exhibited reduced expression of factors Epidermal growth factor (EGF), Fibroblast growth factor-1 (FGF-1), Transforming growth factor-β1 (TGF-β1), Vascular endothelial growth factor a (VEGFa) and VEGFb, the adhesion molecules Integrin-4 (ITGA-4) and Vitronectin (VTN) and matrix metalloproteinases (MMP-1a, MMP-2 and MMP-9). *In vitro,* CCL3 was able to induce invasiveness and release of CCL5, IL-6, and MMP's 2, 8 and 9 by neoplastic cells. Blockage of CCL3 using anti-CCL3 antibody reduced invasion of neoplastic cells.

Deficient mice for receptor ACKR2 (ACKR2$^{-/-}$) treated exhibited similarity in tongue tumor formation and in microscopical parameters when compared with *wild type* mice. Moreover, it was observed increased expression of chemokines CCL3, CCL4, CCL5, CCL12 and the receptors CCR1, CCR2 and CCR5, as well as pro-inflammatory cytokines, angiogenic factors, adhesion molecules and matrix metalloproteinases in lesions of both treated groups in comparison with controls. However, increased expression of CCL2, IL-6 and IL-17 were observed in tongue lesions of ACKR2$^{-/-}$ in relation to WT group.

Collectively, the data suggest the relevance of CCL3/CCR5 axis in oral carcinogenesis once CCL3 is able to directly affect neoplastic cells and the tumor microenvironment. The ACKR2 receptor did not modify the course of oral carcinogenesis. These results reveal potential protective effects of CCL3 blockage in OSCC.

Keywords: *Chemokines, CCL3, CCR1, CCR5, ACKR2 and OSCC.*

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS

4HAQO – 4-hidroxiaminoquinolina-1-óxido

4NQO – 4-nitroquinolina-1-óxido

8OHdG – 8-hidroxideoxiguanosina

ACKR'S – Do inglês "atypical chemokine receptors"

ACTB – Beta-actina

ADAMs – Do inglês "A disintegrin and metalloproteinase"

ADAMTSs – Do inglês "ADAMs with thrombospondin motif"

AKT/PKB – Do inglês "protein-kinase b"

AMD3100 – Do inglês "1,10-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride"

AOM-DSS – Azoximetano e sulfato de sódio dextrano

C – Do inglês "C chemokine"

CAF's – Fibroblastos associados a carcinomas

CBA – Linhagem de camundogos na qual animais albinos Bagg foram cruzados com animais CC. A linhagem resultante foi chamada de A, por isso CBA

CC – Do inglês "CC chemokine"

CCEB – Carcinoma de células escamosas de boca

CCR – Do inglês "CC chemokine receptor"

CDKN2A – Do inglês "cyclin-dependent kinase inhibitor 2a"

CEBIO – Centro de bioterismo

COL1a1 – Do inglês "collagen 1a1"

CX3C - Do inglês "CX3C chemokine"

CXC - Do inglês "CXC chemokine"

DMBA – 7,12-Dimetil-benz[a]antraceno

DMSO – Dimetil sulfóxido

DNA – Ácido desoxirribonucléico

EGF – Fator de crescimento epidérmico

EGFR – Receptor do fator de crescimento epidérmico

EMT – Transição epitélio-mesênquima

ERK – Do inglês "extracellular signal regulated kinase"

FGF – Fator de crescimento de fibroblastos

GAPDH – Do inglês "glyceraldehyde-3-phosphate dehydrogenase"

GST-P – Do inglês "glutathione-S tranferase pi"

HGF – Fator de crescimento de hepatócitos

HN – Do inglês "head and neck"

Hprt1 – Do inglês "hypoxanthine phosphoribosyltransferase 1"

HPV – Do inglês "human papilloma virus"

HUVEC – Do inglês "human umbilical vein endothelial cells"

IL – Interleucina

INCA – Instituto Nacional do Câncer

ITGA-4 – Integrina-4

LO – Leucoplasia Oral

LPS – Lipopolissacarídeo

MIP-1α – Do inglês "macrophage-inflammatory protein-1α"

MMP's – Metaloproteinases de matriz

NF-kB – Do inglês "nuclear factor kappa b"

NK – Células "natural killer"

NOTCH1 – Do inglês "neurogenic locus notch homolog protein1"

OMS – Organização Mundial da Saúde

p16 – Do inglês "cyclin-dependent kinase inhibitor 2a"

p21 – Do inglês "cyclin-dependent kinase inhibitor 1A"

p53 – Do inglês"phosphoprotein 53"

PCNA – Antígeno celular de proliferação nuclear

PCR – Reação em cadeia da polimerase

PI_3K – Fosfatidilinositol-4,5-bisfosfato 3 quinase subunidade catalítica alfa

RAS – Do inglês "retrovirus associated virus sequences isolated from rat sarcoma viruses"

RNA – Ácido ribonucleico

SCC-9 – Linhagem de CCEB, do inglês "squamous cell carcinoma-9"

SK – Sarcoma de Kaposi

SNAIL – Do inglês "snail family transcriptional repressor 1"

STAT3 – Do inglês "signal transducer and activator of transcription 3"

TAM's – Macrófagos associados a tumores

TGF-β – Do inglês "transforming growth factor-β"

TIMP's – Do inglês "tissue inhibitor of metalloproteinases"

TNF-α – Fator de necrose tumoral-α

TP53 – Do inglês "tumor protein-53"

TP63 – Do inglês "tumor protein-63"

TWIST – Do inglês "twist family bHLH transcription factor 1"

VEGF – Fator de crescimento vascular endotelial

VTN – Vitronectina

WT – Selvagem, do inglês "wild type"

SUMÁRIO

1 - REVISÃO DE LITERATURA

1.1 – O Carcinoma de células escamosas de boca – CCEB: aspectos clínico-epidemiológicos

O Carcinoma de células escamosas de boca (CCEB) ou carcinoma epidermóide é a neoplasia epitelial mais comum dentre as malignidades que acometem a cavidade bucal (FERLAY *et al*., 2015; WARNAKULASURIYA, 2009). O CCEB caracteriza-se por ter um comportamento altamente infiltrativo para estruturas vitais da região de cabeça e pescoço, com alta tendência para metástases linfonodais (CONTALDO *et al*., 2013). Esse comportamento biológico do CCEB tem sido reportado como um dos fatores responsáveis pelos altos índices de recidivas e altas taxas de morbidade e mortalidade (CONTALDO *et al*., 2013; KO *et al*., 2016). O tamanho do tumor e profundidade de invasão também podem ser considerados fatores relacionados a um pior desfecho da doença (FELLER; LEMMER, 2012; MASSANO *et al*., 2006).

Segundo levantamento realizado pelo Instituto Nacional do Câncer (INCA) para o biênio 2016-2017 foram estimados cerca de 15.490 novos casos de câncer de boca entre homens e mulheres, em todo o território nacional (INCA, 2015). O CCEB é mais comum entre homens do que em mulheres com uma distribuição estimada proporcional de 1,5 homens para cada 1 mulher (WARNAKULASURIYA, 2009) ou ainda, de 3,46 casos em homens para cada 1,76 casos em mulheres/100.000 habitantes (INCA, 2015).

O CCEB acomete principalmente a borda lateral e posterior de língua e assoalho bucal, mas outros sítios como gengiva, palato e mucosa jugal também podem ser afetados em menor frequência (BAGAN *et al*., 2010; FELLER; LEMMER, 2012). Clinicamente as lesões podem apresentar-se como leucoplasias, eritroleucoplasias, eritroplasias, úlceras ou lesões exofíticas (FELLER; LEMMER, 2012).

Dentre os fatores de risco ambientais mais comumente relacionados com a sua patogênese destacam-se o uso do tabaco e o consumo de álcool. De forma isolada, estes fatores já representam um risco, mas quando estão associados apresentam ação sinérgica aumentando consideravelmente o risco do desenvolvimento de CCEB (HASHIBE *et al*., 2009; SCULLY; BAGAN, 2009). Outro fator de risco extrínseco como a infecção das células epiteliais pelo vírus do papiloma humano - *Papilloma human virus* (HPV), principalmente os subtipos 16 e 18, tem sido correlacionados com a ocorrência de tumores de orofaringe (MELCHERS *et al*., 2015) e CCEB (BROUWER *et al*., 2016).

Fatores, intrínsecos relacionados ao CCEB têm sido descritos na literatura (ROTHENBERG; ELLISEN, 2012). Entre eles, o acúmulo de mutações em proto-oncogenes (ex. *PI_3KC, TP63, RAS, EGFR*) e em genes supressores de tumor (ex. *TP53, CDKN2A, NOTCH1*) nas células epiteliais. De um modo geral, os estudos mostram que tais mutações afetam a regulação de circuitos que estão estritamente interligados com mecanismos que regem a proliferação, diferenciação, sobrevivência, apoptose, capacidade de invasão e metástases das células tumorais (ROTHENBERG; ELLISEN, 2012; SCULLY; BAGAN, 2009).

Com relação ao tratamento, a cirurgia com ressecção ampla das margens com ou sem quimioterapia/radioterapia adjuvante pós-operatória ainda são as opções terapêuticas mais usuais no manejo do CCEB, contudo, a escolha do tipo de tratamento depende do estadiamento clínico do tumor e da sua localização (SHAH; GIL, 2009). Neste sentido, o atraso no diagnóstico e tratamento estão fortemente correlacionados com um pior estadiamento da doença e piores taxas de sobrevida e para os pacientes (WARNAKULASURIYA, 2009).

1.2 – Modelos experimentais de carcinogênese bucal

Um dos métodos para estudo dos mecanismos envolvidos na carcinogênese oral é a utilização de modelos experimentais. Atualmente existem alguns modelos animais bem estabelecidos que podem ser utilizados para a indução do CCEB em roedores (incluindo hamster, ratos, e camundongos) (DERKA *et al*., 2006; RIBEIRO *et al*., 2004; SCHOOP *et al*., 2009; TANG *et al*., 2004). Em geral, o método de indução destes tumores é baseado na utilização de compostos químicos sintéticos carcinogênicos. Dentre os carcinógenos mais comumente utilizados destacam-se o 7,12-Dimetil-benz[a]antraceno (DMBA), muito utilizado para induzir lesões na mucosa da bochecha "*cheek pouch*" de hamsteres Sírios ou tumores mamários em ratas (DERKA *et al*., 2006; SOUDA *et al*., 2009) e o 4-Nitroquinolina-1-óxido (4NQO).

O DMBA é um hidrocarboneto aromático policíclico carcinogênico que pode ser dissolvido em veículo oleoso como o dimetil sulfóxido (DMSO), parafina líquida ou em acetona. O modo mais comum de administração é o pincelamento diretamente sobre a pele ou mucosa, mas a injeção por via intraperitoneal e gavagem também já foram descritos em alguns estudos para indução de carcinomas (DERKA *et al*., 2006; SOUDA *et al*., 2009; VAIRAKTARIS *et al*., 2008). Os principais questionamentos do modelo DMBA em "*cheek pouch*" para estudar a carcinogênese oral é se ele é um modelo de carcinogênese verdadeiramente intra-oral, uma vez que esta estrutura anatômica pode não sofrer as mesmas influências do restante da cavidade oral, e ainda, que humanos não possuem anatomicamente estrutura semelhante (revisto por BOYD; READE, 1988). Outra desvantagem é em relação ao método de aplicação, sendo que o pincelamento deve ser realizado mais de uma vez por dia, durante repetidas semanas para que ocorra a indução das lesões (TAKE *et al*., 1999). Contudo, Nagini *et al*. (2009) estudando comparativamente as alterações histológicas e moleculares em carcinomas humanos e

induzidos por DMBA em hamsteres demonstrou a indução de carcinomas bem diferenciados e apresentando alterações moleculares semelhantes às lesões humanas com relação à expressão de PCNA, GST-P, NF-KB, p21 e p53 (NAGINI *et al*., 2009).

Outro modelo de carcinogênese descrito na literatura emprega o 4NQO, um composto aromático, derivado das quinolonas cujas propriedades carcinogênicas foram descritas pela primeira vez em 1957 (NAKAHARA *et al*., 1957). A ação carcinogênica inicia-se com a redução enzimática do seu grupo nitro, formando um composto intermediário chamado 4-hidroxiaminoquinolina-1-óxido (4HAQO). Este produto metabólico, por sua vez, liga-se a resíduos de guanina formando adutos (8-hidroxideoxiguanosina - 8OHdG) no DNA (ARIMA *et al*., 2006; KANOJIA; VAIDYA, 2006). Estudos ultraestruturais mostraram em culturas de fibroblastos humanos um aumento nos níveis de 8OHdG de maneira dose-dependente após a administração de 4NQO (ARIMA *et al*., 2006). Além disso, o 4NQO e seus metabólitos induziram intenso estresse oxidativo intracelular gerando espécies reativas de oxigênio e aumentando significativamente os níveis dos radicais superóxido e peróxido de hidrogênio, causando danos secundários no próprio DNA que podem ser irreparáveis (ARIMA *et al*., 2006; NUNOSHIBA; DEMPLE, 1993). Kim *et al*. (2006) objetivando estudar a formação de adutos de DNA induzida por 4NQO demonstraram um mecanismo de reparo mitocondrial defeituoso em duas linhagens de células de CCEB testadas e indicaram que esses defeitos podem permitir a iniciação das células (KIM *et al*., 2006). Entretanto, 4NQO administrado na dose de 15 micromolar por uma hora mostrou ser bem tolerado *in vitro*, onde 90% dos fibroblastos permaneceram viáveis (ARIMA *et al*., 2006).

O 4NQO é o carcinógeno de escolha em muitos estudos por suas propriedades de solubilidade em água e pelo fato de induzirem tumores preferencialmente em sítios

como a língua e esôfago (KANOJIA; VAIDYA, 2006; LU *et al*., 2006; TANG *et al*., 2004), diferentemente do modelo DMBA previamente descrito. Além disto, o dano provocado ao DNA pelo 4NQO ocorre de modo muito similar ao provocado pelo uso do tabaco, uma vez que seus metabólitos também formam compostos que se ligam em resíduos de guanina no DNA (BALBO *et al*., 2014; KANOJIA; VAIDYA, 2006). O 4NQO induz a iniciação, primeiro estágio da carcinogênese. A progressão deste processo resulta em mudanças moleculares e morfológicas de forma semelhante àquelas observadas na carcinogênese oral em humanos (BOYD; READE, 1988, KANOJIA; VAIDYA, 2006; NAUTA *et al.*, 1996; RIBEIRO *et al*., 2004).

Microscopicamente as lesões induzidas pelo tratamento com 4NQO apresentam-se como um aumento focal na espessura epitelial (hiperplasia), com ou sem perda de organização dos diferentes estratos (displasia/carcinoma *in situ*) ou carcinomas invasivos (GANNOT *et al*., 2004; TANG *et al*., 2004). Clinicamente as lesões em língua podem se apresentar como exofíticas ou papilomatosas, em forma de placa ou por vezes ulceradas, de colocação esbranquiçada e base ampla na superfície ventral, dorsal ou lateral e em terço médio e posterior da língua (IDE *et al*., 2001; TANG *et al*., 2004).

Corroborando achados clínico-microscópicos, alterações cromossômicas frequentemente observadas no CCEB em humanos (ex. *p53, p16* e caderinas) também foram demonstradas em carcinomas induzidos por 4NQO em camundongos (HONG *et al*., 2008; MINICUCCI *et al*., 2011; OKAZAKI *et al*., 2002; SAKAKI *et al*., 2003).

Com relação à via de administração, estudos prévios mostraram que o 4NQO induziu uma frequência de mutagenicidade significativamente aumentada no gene *lacZ* em camundongos transgênicos quando administrado por via oral (dose 200 mg/kg), afetando significativamente o fígado, pulmões e estômago quando comparado com

administração via intraperitoneal (7,5 mg/kg) (SUZUKI *et al*., 1999). Posteriormente, Tang *et al*. (2004) avaliaram comparativamente a indução de carcinomas em língua de camundongos *"CC x Bagg, A Strain"* (CBA) e C57/BL6 e demonstraram uma produção maior de tumores em camundongos que receberam o 4NQO diluído na água diariamente (100 µg/mL) em comparação com a técnica de pincelamento do produto (5 mg/mL) diretamente na mucosa da língua. Após um período de 28 semanas, observou-se incidência de apenas 20% de indução de papilomas e 5% de carcinomas invasivos nos animais CBA, utilizando a técnica do pincelamento. Além disso, dentre os animais que sobreviveram até a vigésima oitava semana do experimento com a dose de 100 µg/mL na água de beber, todos apresentaram 100% de incidência de carcinomas na língua e no esôfago nas duas linhagens desafiadas (TANG *et al*., 2004).

No que se refere à dose, os estudos mostram bons resultados com o 4NQO diluído nas concentrações de 50 e 200 µg/mL (LI *et al*., 2013; TANG *et al*., 2004 e SILVA *et al*., 2014 – ANEXO A). No entanto, a literatura mostra que o fator tempo é importante para permitir a maior gravidade das lesões, com um período que varia entre 4 a 50 semanas (IDE *et al*. 2001; LI *et al*., 2013; MINICUCCI *et al*., 2011; RIBEIRO *et al*., 2004; TANG *et al*., 2004; VERED et *al*., 2007).

Recentemente, foi descrito um modelo de carcinogênese em língua de camundongos BALB/c capaz de induzir a formação de metástases em linfonodos utilizando-se uma alta concentração do 4NQO (LI *et al*., 2013). Neste estudo, animais foram tratados com o 4NQO na dose de 200 µg/mL na água de beber por 20 semanas. O 4NQO foi então substituído por água filtrada e os animais acompanhados por períodos de observação de 25 a 32 e de 33 a 40 semanas. Os resultados mostraram que os animais que foram acompanhados pelo período mais longo (33-40 semanas),

apresentaram 100% de incidência de CCEB e 100% de metástases nos linfonodos submandibulares (LI *et al*., 2013).

1.3 – O sistema quimiocinas/receptores: aspectos gerais e efeitos no câncer

Quimiocinas são proteínas de baixo peso molecular (8-10 kilodaltons), com atividade qumiotática, ou seja, possuem a função de coordenar a migração de células durante processos como organogênese, na manutenção da homeostase tecidual e também em processos inflamatórios (BALKWILL, 2004). Essa migração é resultante da ligação da quimiocina em seu receptor específico ao longo de um gradiente químico, onde células expressando receptores movem-se em direção a locais com altas concentrações de quimiocinas (BALKWILL, 2004).

A Interleucina-8 (IL-8, hoje renomeada CXCL8) foi a primeira citocina com atividade quimiotática descrita e caracterizada (BAGGIOLINI *et al*., 1989). Desde então, aproximadamente 50 quimiocinas já foram descritas (ALLEN *et al*., 2007; BACHELERIE *et al*., 2014). Segundo a nomeclatura vigente, as quimiocinas podem ser divididas em quatro subfamílias, de acordo com a posição das primeiras duas cisteínas adjacentes ao domínio N-terminal, -CC -CXC -C e -CX3C (BACHELERIE *et al*., 2014). A letra “L” seguida por um número denota uma quimiocina específica, por exemplo, CCL3 e CXCL12. As quimiocinas exercem seus efeitos nas células via interação específica restrita a subfamílias de receptores – proteínas com 7 domínios transmembrana acoplados à proteína G (ALLEN *et al*., 2007; BACHELERIE *et al*., 2014). Para os receptores, a letra “R” seguida de um número denota o respectivo ligante, como CCR1 e CXCR4. Aproximadamente 20 receptores de quimiocinas já foram descritos até o presente momento (ALLEN *et al*., 2007; BACHELERIE *et al*.,

2014). Desta forma, nota-se que existem mais quimiocinas do que receptores, logo, o sistema possui certa seletividade, mas é redundante, onde mais de uma quimiocina pode se ligar ao mesmo receptor, e ainda, a mesma quimiocina pode compartilhar receptores diferentes com maior ou menor afinidade (ALLEN *et al*., 2007; BACHELERIE *et al*., 2014; BALKWILL, 2004). A tabela a seguir apresenta uma compilação do sistema quimiocinas/receptores convencionais já identificados (TAB. 1).

Tabela 1 *– O sistema quimiocinas e receptores.*

Quimiocinas	Sinônimo	Receptores	Alvos principais
Quimiocinas CC			
CCL1	I-309 e TCA-3	CCR8	Linfócitos T e Tregs
CCL2	MCP-1 e JE	CCR2	Monócitos, linfócitos, NK
CCL3	MIP1α	CCR1 e CCR5	Macrófagos, monócitos, basófilos e NK
CCL4	MIP1β	CCR5 e CCR8	
CCL5	RANTES	CCR1, CCR3 e CCR5	
CCL6	C10 e MRP-1	Desconhecido	Desconhecido
CCL7	MARC e MCP-3	CCR1, CCR2, CCR3 e CCR5	Monócitos, neutrófilos
CCL8	MCP-2	CCR1, CCR2, CCR3 e CCR5	Linfócitos T
CCL9/CCL10	MRP-2, CCF18 e MIP1γ	CCR1	Desconhecido
CCL11	*Eotaxin*	CCR2, CCR3 e CCR5	Eosinófilos e basófilos
CCL12	MCP-5	CCR2	Monócitos
CCL13	MCP-4 e NCC-1	CCR1, CCR2 e CCR3	Linfócitos T
CCL14	HCC-1, MCIF e NCC-2	CCR1 e CCR5	Desconhecido
CCL15	*Leukotactin-1*, MIP5, HCC-2 e NCC-3	CCR1 e CCR3	
CCL16	LEC, NCC-4 e LMC	CCR1, CCR2, CCR3 e CCR5	
CCL17	TARC e ABCD-2	CCR4 e CCR8	Linfócitos T
CCL18	PARC, DC-CK1, AMAC-1 e MIP4	Desconhecido	
CCL19	ELC, *Exodus-3* e MIP3b	CCR7	Linfócitos T e B, células dendríticas
CCL20	LARC, *Exodus-1* e MIP3a	CCR6	Macrófagos, linfócitos Tregs e monócitos
CCL21	SLC, *6Ckine*, *Exodus-2* e TCA-4	CCR7	Linfócitos T e células dendríticas
CCL22	MDC	CCR4	Macrófagos, células dendríticas

CCL23	MPIF-1	CCR1	Desconhecido
CCL24	*Eotaxin-2* e MPIF-2	CCR3	Eosinófilos e basófilos
CCL25	TECK	CCR9	Linfócitos T
CCL26	*Eotaxin-3*, MIP4α, IMAC e TSC-1	CCR3 e CCR10	Eosinófilos e basófilos
CCL27	CTACK e ILC	CCR10	Linfócitos T
CCL28	MEC	CCR3 e CCR10	
Quimiocinas CXC			
CXCL1	Gro-α, GRO1, NAP-3 e KC	CXCR2	Neutrófilos
CXCL2	Gro-β, GRO2 e MIP2α	CXCR2	
CXCL3	Gro-γ, GRO3 e MIP2β	CXCR2	
CXCL4	PF-4	CXCR3	Plaquetas, Neutrófilos e monócitos
CXCL5	ENA-78 e LIX	CXCR1 e CXCR2	Neutrófilos
CXCL6	GCP-2 e LIX	CXCR1 e CXCR2	
CXCL7	NAP-2, CTAPIII e PEP	CXCR2	
CXCL8	IL-8, NAP-1, MDNCF e GCP-1	CXCR1 e CXCR2	
CXCL9	MIG e CRG-10	CXCR3	Linfócitos e NK
CXCL10	IP-10 e CRG-2	CXCR3	
CXCL11	ITAC e IP-9	CXCR3	
CXCL12	SDF-1 e PBSF	CXCR4	Linfócitos e monócitos
CXCL13	BCA-1 e BLC	CXCR5	Lnfócitos T e B
CXCL14	BRAK	Desconhecido	Linfócitos B e monócitos
CXCL15	*Lungkin*e e WECHE	Desconhecido	Neutrófilos
CXCL16	SRPSOX	CXCR6	NKT e células linfóides inatas
CXCL17	DMC e VCC-1	Desconhecido	Desconhecido
Quimiocinas C			
XCL1	*Lymphotactin α*	XCR1	Linfócitos e NK
XCL2	*Lymphotactin β*	XCR1	
Quimiocina CXXXC			
CX3CL1	*Fractalkine* e *neurotactin*	CX3CR1	NK, monócitos e linfócitos T

Fonte: Russo et al. (2010), adaptado.

Embora as quimiocinas e seus receptores foram primariamente associadas com a função de "atrair" leucócitos, participando em processos como a organogênese e manutenção da homeostase tecidual (BAGGIOLINI *et al*., 1989; GRIFFITH *et al*., 2014; ZOU *et al*., 1998), estudos posteriores revelaram também sua atuação durante a carcinogênese, devido aos seus efeitos na angiogênese, proliferação, ganho de

motilidade e invasão das células neoplásicas (BALKWILL, 2004; CHOW; LUSTER, 2014 e SILVA *et al*., 2016 – ANEXO A).

A carcinogênese é um processo complexo que envolve o acúmulo de múltiplas alterações genéticas e epigenéticas, as quais resultam na disfunção de vias que controlam a proliferação, diferenciação e sobrevivência celular (BALKWILL, 2004, 2012; MANTOVANI *et al*., 2008; HANAHAN; COUSSENS, 2012). As células neoplásicas em proliferação estão inseridas em um estroma constituído por uma população heterogênea de células (fibroblastos, miofibroblastos, linfócitos, macrófagos, células dendríticas, *natural killer* (NK), além de nutrientes e moléculas sinalizadoras como fatores de crescimento, citocinas, quimiocinas e receptores. Neste cenário, denominado microambiente tumoral, as células neoplásicas interagem de forma contínua e dinâmica com as diferentes populações e moléculas presentes no estroma (BALKWILL, 2004, HANAHAN; COUSSENS, 2012).

Diversos estudos tem mostrado a presença de um heterogêneo infiltrado inflamatório no estroma tumoral, e destacado sua íntima conexão com os eventos da tumorigênese (MANTOVANI *et al*., 2008; SICA *et al*., 2008; GRIVENNIKOV; KARIN, 2010). Células estromais expressam receptores específicos na membrana plasmática, e são recrutadas para os sítios tumorais atraídas por gradiente químico – processo regulado pela ação de quimiocinas e receptores de quimiocinas (BALKWILL, 2004).

A definição do papel das quimiocinas em tumores iniciou-se com estudos demonstrando a sua expressão pelas células neoplásicas e sua interação com células do estroma tumoral. No câncer de mama, por exemplo, as quimiocinas CCL2 e CCL5 expressas pelas células neoplásicas foram identificadas como importantes reguladores da infiltração de macrófagos associados a tumores (TAM's). Juntas, células tumorais e

TAM's expressando CCL2 e CCL5 foram capazes de induzir a proliferação de células endoteliais (promovendo a angiogênese) e também atuando na atividade metastática das células tumorais e promover invasão das células neoplásicas para os tecidos adjacentes (SORIA; BEN BARUCH, 2008). Outra evidência advém de estudos que observaram a expressão aumentada de CCL2 em tumores mama que foi coincidente com estágios mais avançados da doença e pior sobrevida (UENO *et al*., 2000). A expressão de CXCL1, CXCL5 e CXCL8 e seu receptor CXCR2 foi identificada em espécimes de câncer de próstata e em linhagens tumorais, sendo sua ação angiogênica demonstrada *in vivo* e *in vitro* (MATSUO *et al*., 2009). Neste último estudo, após verificar a ausência de efeitos autócrinos das quimiocinas -CXC sobre linhagem de célula de tumor de próstata (confirmada pela deficiência na expressão de CXCR2) estas células foram mantidas em cocultivo com células endoteliais (*human umbilical vein endothelial cells* - HUVEC) (MATSUO *et al*., 2009). Verificou-se que as quimiocinas –CXC produzidas no sobrenadante afetaram a proliferação, invasão e a formação de brotamentos nas HUVEC, processo característico do início da formação vascular. Além disso, a administração de anticorpo anti-CXCR2 *in vivo* mostrou reduzir a formação desses brotamentos vasculares, e a expressão do Fator VIII ou "*von Willebrand factor"* (MATSUO *et al*., 2009).

Outro eixo implicado na patogênese de tumores é o sistema CXCL12/CXCR4. Estudos prévios mostraram sua participação na indução de metástases no câncer de ovário, onde células tumorais transfectadas com o receptor CXCR4 tiveram sua capacidade de invasão aumentada, e, quando injetadas em camundongos imunossuprimidos induziram o desenvolvimento de metástases peritoneais nesses animais quando comparado aos animais controles (GUO *et al*., 2014). Além disso, estudos posteriores demonstraram que a sinalização CXCL12/CXCR4 em células

tumorais induz um fenômeno conhecido como transição epitélio-mesênquima (EMT), no qual a célula tumoral perde aderência com as células vizinhas e adquire propriedades fenotipicamente mesenquimais. Yao *et al*. (2015) verificaram que a administração de CXCL12 em culturas de células de glioblastoma induziu, por exemplo, a superexpressão de TWIST – um dos fatores de transcrição chave para ocorrência de EMT – e aumentou o potencial invasivo e migratório dessas células. Além disso, o silenciamento genético de TWIST nas células tumorais resultou na reversão desse fenótipo *in vitro.* Ainda, camundongos imunossuprimidos injetados subcutaneamente com as células silenciadas mostraram formação reduzida de tumores em comparação aos controles (YAO *et al*., 2015).

1.3.1 – A quimiocina CCL3, seus receptores CCR1 e CCR5 e o receptor ACKR2: aspectos gerais e efeitos no câncer

A quimiocina *Macrophage inflammatory protein-1 alpha* (MIP-1α), também chamada CCL3 foi isolada pela primeira vez a partir da estimulação de culturas de macrófagos tumorais (RAW-264.7) com endotoxinas. Neste ensaio realizado em 1988, após ser injetada na pata de camundongos, CCL3 induziu um rápido influxo de neutrófilos (WOLPE *et al*., 1988). Conforme revisado no estudo de Mentem *et al*. (2002), a quimiocina CCL3 murina está localizada no cromossomo 11, contém três *éxons* e dois *íntrons* e codifica uma pré-proteína de 92 aminoácidos. A contraparte humana, é composta por três variações genéticas descritas – LD78α (69% de similaridade na sequência de nucleotídeos em relação a proteína murina), LD78β (94% de similaridade com LD78α) e LD78γ (descrito como um "pseudogene" - forma truncada 5' que não é transcrita), todas presentes no cromossomo 17 (MAURER;

STEBUT, 2004; MENTEM *et al*., 2002). Estudos estruturais demonstraram que CCL3 (murina e humana) é fisiologicamente encontrada na sua forma monomérica, contudo, um processo dinâmico e reversível entre auto-agregação e desagregação também foi descrito (VOLPE *et al*., 1988). Tais agregados parecem não influenciar a sua atividade biológica, mas podem refletir na sua capacidade de ligação aos receptores CCR1 e CCR5 (MENTEM *et al*., 2002). CCL3 é constitutivamente expressa em baixos níveis por monócitos e macrófagos, entretanto, um aumento significativo na sua produção por monócitos, macrófagos, linfócitos, neutrófilos, células dendríticas, fibroblastos e células epiteliais foi observado após serem estimulas com lipopolissacarídeo (LPS), TNF-α ou IL-1 (MAURER; STEBUT, 2004; MENTEM *et al*., 2002). Estudos posteriores implicaram sua participação em diferentes condições inflamatórias como em modelo de cicatrização de feridas, fibrose pulmonar induzida por bleomicina e em doenças como asma, artrite e psoríase, sendo um importante indutor do recrutamento de leucócitos para os sítios afetados (revisado por MAURER; STEBUT, 2004).

A expressão de CCL3 e seus receptores CCR1 e CCR5 também foi observada em tumores hepáticos humanos e experimentalmente induzidos (LIDA *et al*., 2008; LU *et al*., 2003; YANG *et al*., 2006), tumores de mama (NATH *et al*., 2006), leucemia mielóide crônica (BABA *et al*., 2013; SIVINA *et al*., 2011), mieloma múltiplo (LENTZSCH *et al*., 2003; VALLET *et al*., 2011), em carcinomas intestinais associados a colite (SASAKI *et al*., 2014) e em modelo de metástase pulmonar (WU *et al*., 2008). De um modo geral, as evidências associadas à participação de CCL3 na tumorigênese estão relacionadas às suas propriedades pró-inflamatórias como o recrutamento de leucócitos para o sítio tumoral, o que direta ou indiretamente poderia resultar na liberação de moléculas que favoreceriam a progressão tumoral. Estudos *in vitro* mostraram que CCL3 foi capaz de induzir a migração de células de tumor hepático pela

mudança na concentração intracelular níveis de cálcio (YUAN *et al*., 2010). CCL3 foi também capaz de aumentar a proliferação de fibroblastos associados a carcinomas (CAF's) (SASAKI *et al*., 2014). De modo importante, a atividade pró-tumorigênica de CCL3 agindo via CCR1 ou CCR5 foi demonstrada por alguns estudos. Nesse sentido, o eixo CCL3/CCR1 foi implicado na progressão da hepatocarcinogênese, sendo que animais deficientes para CCL3 e CCR1 apresentaram menor incidência na formação de tumores, menor vascularização, menor infiltrado de células de *Kupffer* e menor expressão de MMP-9 em relação aos animais do grupo selvagem (YANG *et al*., 2006). Outro estudo mostrou a atividade pró-metastática do eixo CCL3/CCR5 em modelo de metástase pulmonar induzida por injeção de células de carcinoma renal em camundongos. Neste estudo, animais deficientes para CCL3 e CCR5 tiveram reduzida formação de metástases e tumores metastáticos apresentando menor infiltrado de leucócitos e fibroblastos, menor vascularização e expressão de MMP-9 em relação aos animais selvagens (WU *et al*., 2008). Em modelo de carcinoma intestinal associado à colite induzida por azoximetano e sulfato de sódio dextrano (AOM-DSS), Sasaki *et al*. (2014) também observaram formação de tumor atenuada com menor acúmulo de leucócitos e fibroblastos em lesões intestinais de animais deficientes de CCL3 e CCR5 (SASAKI *et al*., 2014).

Recentemente, foi caracterizada mais uma classe de receptores de quimiocinas – os ACKR's. Tais receptores tem merecido considerável atenção porque possuem propriedades de capturar e internalizar quimiocinas, funcionando como "armadilhas" e reduzindo sua biodisponibilidade nos tecidos (BACHELERIE *et al*., 2014; MASSARA *et al*., 2016). Essa internalização do complexo ligante/receptor para compartimentos intracelulares, no entanto, resulta na degradação da quimiocina e posterior reciclagem do receptor, que retorna a membrana. Por este motivo, estas moléculas são ditas

"atípicas", por não ativarem sinalização intracelular como ocorre com os receptores convencionais (BACHELERIE *et al*., 2014; FRA *et al*., 2003; MASSARA *et al*., 2016). Até o momento, foram descritos seis ACKR's, os quais são: ACKR1 (previamente chamado *Duffy Antigen Receptor for chemokines*), ACKR2 (D6, CCBP2), ACKR3 (CXCR7), ACKR4 (CCRL1 e CCX CKR), ACKR5 (CCRL2) e ACKR6 (CCL18, *PARC receptor*) (BACHELERIE *et al*., 2014).

Dentre os ACKR's, o papel do receptor ACKR2 (previamente conhecido por D6) tornou-se alvo deste estudo pelo fato de afetarem principalmente a disponibilidade de quimiocinas CC. A expressão de ACKR2 foi demonstrada em diferentes tecidos e tipos celulares como em vasos linfáticos, macrófagos da mucosa do cólon, em pele e também em células tumorais (JAMIESON *et al*., 2005; NIBBS *et al*., 2001, 2007). Dados prévios demonstraram que ACKR2 liga-se com grande afinidade a quimiocinas da subfamília –CC. O papel desse receptor tem sido estudado em diferentes condições inflamatórias como em modelo de inflamação cutânea induzida quimicamente (JAMIESON *et al*., 2005) e na artrite (HANSELL *et al*., 2015). No estudo de Jamieson *et al*. (2005), por exemplo, ACKR2 foi demonstrado limitar a resposta inflamatória *in vivo*, na qual animais deficientes para ACKR2 apresentaram um atraso na resolução da resposta inflamatória devido ao acúmulo significativo de quimiocinas como CCL3, CCL5 e maior infiltrado de macrófagos, linfócitos e mastócitos na pele em relação aos animais selvagens (JAMIESON *et al*., 2005). De fato, pode-se observar que os animais desenvolveram sinais clínicos exacerbados, caracterizados por doença psoríaseforme na ausência de ACKR2, o que não foi observado nos animais selvagens (JAMIESON *et al*., 2005). A partir desses resultados, sugeriu-se a participação de ACKR2 na carcinogênese cutânea. De fato, essa hipótese foi posteriormente confirmada por estudos utilizando modelos de carcinogênese cutânea quimicamente induzida em camundongos

deficientes deste receptor (BORDON *et al*., 2009; NIBBS *et al*., 2007; SAVINO *et al*., 2014; SCHNEIDER *et al*., 2012; VETRANO *et al*., 2010). Demonstrou-se que camundongos ACKR2$^{-/-}$ apresentaram um aumento significativo na formação de tumores na pele após aplicação tópica de forbol-éster e DMBA (NIBBS *et al*. 2007). Juntamente com a maior indução de tumores, foi observado maior índice proliferativo, maior recrutamento de linfócitos CD3^{+}, mastócitos e maior expressão de CCL3 nas lesões. Além disso, utilizando camundongos transgênicos para expressar ACKR2 em queratinócitos da camada basal da epiderme, os pesquisadores conseguiram demonstrar um papel protetor de ACKR2, onde reduzida formação de tumores e reduzida expressão de CCL3 foi observada nesses animais (NIBBS *et al*., 2007).

Um papel protetor para ACKR2 também foi reportado em adenocarcinomas de cólon, onde sua expressão reduzida foi correlacionada com aumento na expressão de CCL22 e piores características clínico-patológicas, como profundidade de invasão e a localização dentro do cólon (proximal, média, distal) (LANGENES *et al*., 2013). Em tumores de mama, a expressão de ACKR2 nas células tumorais inibiu a proliferação e invasão *in vitro*, além da formação de tumores e desenvolvimento de metástases *in vivo* (WU *et al*., 2008). Neste último estudo, essa inibição mediada por ACKR2 nos tumores foi correlacionada com menor expressão das quimiocinas CCL2 e CCL5, menor densidade vascular e menor infiltrado de TAM's (WU *et al*., 2008). Savino *et al*. (2014) também demonstraram propriedades antitumorais de ACKR2 em Sarcomas de Kaposi (SK), onde o receptor inibiu a formação de tumores *in vivo*. Além disso, lesões de SK induzidas em camundongos imunossuprimidos por células transfectadas com o gene ACKR2 mostraram menor quantidade de linfócitos CD45, macrófagos tipo M2 e diminuição da expressão de CCL2, CCL3, CCL5 e de VEGFa e CD31 em relação aos tumores SK^{-} (SAVINO *et al*., 2014).

1.4 – O sistema quimiocinas/receptores x CCEB

Nos últimos 16 anos, algumas evidências provenientes de estudos *in vitro* e *in vivo* confirmaram a participação de quimiocinas e seus receptores na carcinogênese bucal (ALBERT *et al*., 2012; ALMOFTI *et al*., 2004; CHUANG, *et al*., 2009; FERREIRA *et al*., 2008; ISHIKAWA *et al*., 2006; JUNG *et al*., 2010; LEE *et al*., 2015; LI *et al*., 2014; LIU *et al*., 2015; MENG *et al*., 2010; OLIVEIRA-NETO *et al*., 2008; QUIAN *et al*., 2014; SHINTANI *et al*., 2004; SILVA *et al*., 2007; TSUZUKI *et al*., 2006; UCHIDA *et al*., 2003, 2004, 2007; 2011). Mais recentemente, nosso grupo revisou e discutiu os principais achados com relação a estas moléculas e o CCEB (SILVA *et al*., 2016) (ANEXO A).

Uma primeira evidência da relação entre quimiocinas e CCEB é suportada por estudos que demonstraram expressão aumentada de quimiocinas/receptores em amostras de tumores primários (ALBERT *et al*., 2012; ALMOFTI *et al*., 2004; FERREIRA *et al*., 2008; ISHIKAWA *et al*., 2006; LIU *et al*., 2015; MENG *et al*., 2010; OLIVEIRA-NETO *et al*., 2008; QUIAN *et al*., 2014; SHINTANI *et al*., 2004; SILVA *et al*., 2007; TSUZUKI *et al*., 2006; UCHIDA *et al*., 2004). Merece destaque o fato que uma correlação positiva entre a expressão de CCL2, CCL3 ou CXCL12 nessas amostras e um pior estadiamento clínico-microscópico (tamanho, profundidade de invasão, estágio, gradação histológica, presença de invasão perineural e metástases linfonodais) foi observada nestes trabalhos (ALBERT *et al*., 2012; ALMOFTI *et al*., 2004; FERREIRA *et al*., 2008; ISHIKAWA *et al*., 2006; LIU *et al*., 2015; MENG *et al*., 2010; OLIVEIRA-NETO *et al*., 2008; QUIAN *et al*., 2014; SHINTANI *et al*., 2004; SILVA *et al*., 2007; TSUZUKI *et al*., 2006; UCHIDA *et al*., 2004).

Além disso, evidências desses trabalhos utilizando técnica imuno-histoquímica e de estudos *in vitro* revelaram as células neoplásicas como uma das fontes principais da

expressão de quimiocinas, seguidas pelas células estromais (CHUANG, *et al*., 2009; FERREIRA *et al*., 2008; LEE *et al*., 2015; OLIVEIRA-NETO *et al*., 2008; SILVA *et al*., 2007; UCHIDA *et al*., 2003). Em parte, a expressão aberrante dessas moléculas nos tumores pode ser fundamentada por estudos de polimorfismos específicos, os quais sugerem que mutações em genes que codificam quimiocinas/receptores podem levar a um aumento na transcrição e consequentemente dos níveis proteicos dessas moléculas (BEKTAS-KAYHAN *et al*., 2012; SHIMIZU *et al*., 2008; TANYEL *et al*., 2013; TENG *et al*., 2009; VAIRAKTARIS *et al*., 2008; WENG *et al*., 2010). De modo semelhante, mutações em genes supressores de tumor também podem levar a um aumento da expressão de quimiocinas/receptores (YEUDALL *et al*., 2011). Outro ponto que merece destaque é que a própria sinalização desencadeada pelo sistema quimiocina/receptor nas células transformadas e células do estroma resulta na maior produção e liberação mediadores inflamatórios/quimiotáticos, levando a uma resposta redundante/ amplificada (JUNG *et al*., 2010; LI *et al*., 2014).

A segunda evidência da participação do sistema quimiocinas/receptores na patogênese do CCEB é a observação de que células neoplásicas provocam efeitos diretos sobre elas próprias (autócrinos) e/ou sobre células vizinhas (parácrinos) (JUNG *et al*., 2010; LI *et al*., 2014; ONOUE *et al*., 2006; PANDRUVADA *et al*., 2010). Estudando comparativamente fatores produzidos por monoculturas de células de CCEB (SCC-9) e CAF's, Li *et al*. (2014) verificaram que quando estas células foram colocadas em cocultivo, níveis significativamente aumentados de CCL2 foram detectados em comparação às células em monocultivo. Além disso, verificou-se um aumento na produção de espécies reativas de oxigênio pelas CAF's juntamente com ativação de NFkB e STAT3, que levariam ao aumento da transcrição de CCL2. CCL2, por sua vez, induziu aumento na proliferação e invasão de células SCC-9 (LI *et al*., 2014). Jung *et al*.

(2010), também demonstraram efeitos diretos nas células neoplásicas pela liberação de CCL7 por CAF's em coculturas. Além disso, CCL7 induziu modificações no citoesqueleto das células neoplásicas, afetando sua capacidade de invasividade (JUNG *et al*., 2010). De modo semelhante, a estimulação de células neoplásicas com CCL5 estimulou sua atividade migratória e aumentou a produção de MMP-9 *in vitro* (CHUANG *et al*., 2009).

Considerando-se todas as quimiocinas/receptores já investigados no CCEB, o eixo CXCL12/CXCR4 apresenta maior nível de evidência até o momento, especialmente com relação ao seu envolvimento no desenvolvimento de metástases em linfonodos (SILVA *et al*., 2016). Assim como demonstrado em outros tumores humanos como mama e em glioblastomas (GUO *et al*., 2014, YAO *et al*., 2015), os resultados em CCEB indicaram que a sinalização de CXCL12 via CXCR4 induziu um aumento na motilidade das células tumorais, que por sua vez influenciou a migração e invasividade dessas células (ONOUE *et al*., 2006; REHMAN *et al*., 2009, UCHIDA *et al*., 2003, 2004). De fato, a ligação CXCL12/CXCR4 foi capaz de ativar vias de sinalização como ERK1/2, AKT/PKB e NF-kB que induziu mudanças estruturais no citoesqueleto das células neoplásicas, influenciando o seu ganho de motilidade e invasividade (ONOUE *et al*., 2006; REHMAN *et al*., 2009; UCHIDA *et al*., 2003-2004). Além disso, CXCL12/CXCR4 induziu o fenômeno de EMT, previamente descrito, no qual células epiteliais malignas perderam a expressão de E-caderinas e citoqueratinas e passaram a expressar Vimentina e SNAIL, moléculas importantes na aquisição do fenótipo mesenquimal (ONOUE *et al*., 2006, TAKI *et al*., 2008). O aumento da expressão de CXCR4 foi correlacionado com um aumento na produção de MMP-9 em amostras de CCEB e em linhagens de células tumorais *in vitro* (LEE *et al*.,2009; SAMARA *et al*., 2004).

Uma terceira linha de evidência que aponta o envolvimento do sistema quimiocina/receptor na patogênese do CCEB é suportada pelos efeitos do seu antagonismo na diminuição da expressão de MMP's, redução da proliferação, invasividade e de metástases (JUNG *et al*., 2010; CHANG *et al*., 2011; UCHIDA *et al*., 2007, 2011). O efeito do bloqueio de CXCR4 utilizando-se RNA de interferência (shRNA) em linhagens de células de CCEB, por exemplo, demonstrou ser eficiente para impedir o desenvolvimento de metástases *in vivo* (UCHIDA *et al*., 2011). Neste último estudo, células de CCEB transfectadas com o RNA de interferência foram injetadas ortotopicamente no músculo masseter de camundongos imunossuprimidos, e verificou-se uma diminuição de metástases linfonodais e do volume dos tumores em relação ao grupo injetado com as células controle (UCHIDA *et al*., 2011).

1.4.1 – O eixo CCL3/CCR1/CCR5 e o receptor atípico ACKR2 no câncer de boca

O papel de CCL3 e seus receptores no câncer de boca foi pouco explorado até o momento. A primeira evidência da participação de CCL3 no CCEB foi observada por Silva *et al*. (2007). Neste estudo, uma expressão significativamente aumentada de CCL3 e CCR1, mas não de CCR5, foi encontrada em tumores primários e em linfonodos metastáticos. Uma alta expressão de CCL3 pelas células do estroma correlacionou-se diretamente com uma sobrevida média aumentada. Contudo, quando analisando a alta expressão da quimiocina pelas células tumorais (parênquima), uma pior sobrevida média foi observada (SILVA *et al*., 2007). Tentando investigar o significado clínico da expressão de CCL3 no soro de pacientes saudáveis (controles) comparativamente ao de pacientes com leucoplasias orais (LO) e com CCEB, Ding *et al*. (2014) observaram um aumento significativo de CCL3 nos pacientes com LO em relação aos controles ou ao grupo CCEB. Além disso, uma similaridade na expressão de CCL3 no soro do grupo

controle e CCEB foi observada. Entretanto, dicotomizando a expressão de CCL3 no grupo CCEB em alta e baixa observou-se correlação positiva entre o maior tamanho dos tumores e aumento dos níveis séricos de CCL3 (DING *et al*., 2014).

Considerando-se o potencial envolvimento das quimiocinas CC na patogênese do CCEB (SILVA *et al*., 2016), uma questão ganha enfoque: o da possível participação do receptor atípico de quimiocina ACKR2. Como descrito previamente este receptor atua reduzindo a biodisponibilidade de quimiocinas –CC em modelos de inflamação e em tumores induzidos quimicamente (BORDON *et al*., 2009; JAMIESON *et al*., 2005; NIBBS *et al*., 2007; SAVINO *et al*., 2014; SCHNEIDER *et al*., 2012; VETRANO *et al*., 2010). Entretanto, até o presente momento, não há relatos na literatura da participação de ACKR2 na carcinogênese oral.

Coletivamente, os dados da literatura apontam para a hipótese do envolvimento de CCL3 na carcinogênese ativando vias inflamatórias e influenciando a proliferação, angiogênese, migração e invasão das células tumorais. Considerando-se o potencial biológico do receptor ACKR2 como protetor na tumorigênese em vários modelos - promovendo a captura de quimiocinas –CC, incluindo CCL3 e outros ligantes de CCR1 e CCR5, seria razoável especular que estes sistemas podem estar conectados no CCEB. Dessa forma, o presente estudo propôs investigar os efeitos da deleção do eixo CCL3/CCR1/CCR5, bem como do receptor ACKR2 em modelo murino de carcinogênese oral quimicamente induzida.

2 – JUSTIFICATIVA

O presente estudo foi delineado a partir de dados prévios do nosso grupo de pesquisa (SILVA *et al*., 2007) demonstrando uma expressão aumentada de CCL3 e CCR1, mas não CCR5 em sítios de primários de CCEB, e em linfonodos metastáticos em humanos. As evidências da literatura descritas a seguir suportam o desenvolvimento deste trabalho:

- O papel de CCL3 e dos receptores CCR1 e CCR5 descrito em diferentes tipos de tumores (mama, hepático, leucemias) (BABA *et al*., 2013; LIDA *et al*., 2008; LU *et al*., 2003; NATH *et al*., 2006, SIVINA *et al*., 2011; YANG *et al*., 2006);
- A possível associação entre a expressão de CCL3 e dos seus receptores CCR1 e CCR5 no surgimento de metástases regionais em pacientes com CCEB (SILVA *et al*., 2007);
- A propriedade do receptor ACKR2 de reduzir a disponibilidade de quimiocinas -CC demonstrada em tumores e que lhe confere um papel protetor em diferentes modelos de tumorigênese (NIBBS *et al*., 2007; SAVINO *et al*., 2014; SCHNEIDER *et al*., 2012; VETRANO *et al*., 2010).

Desta forma, é possível hipotetizar a participação do eixo CCL3CCR1/CCR5, bem como o receptor ACKR2 na carcinogênese oral. Os resultados deste trabalho podem contribuir na busca de alvos terapêuticos para o CCEB.

3 – OBJETIVOS

3.1 – Geral

Investigar o papel da quimiocina CCL3, dos seus receptores convencionais CCR1 e CCR5 e do receptor atípico ACKR2 na carcinogênese bucal induzida por carcinógeno químico.

3.2 – Específicos

- Avaliar os aspectos macroscópicos, microscópicos e moleculares das lesões de CCEB induzidas nos animais selvagens e deficientes de CCL3, CCR1 e CCR5;
- Avaliar os aspectos macroscópicos, microscópicos e moleculares das lesões de CCEB induzidas nos animais deficientes do receptor ACKR2;
- Avaliar o efeito *in vitro* de CCL3 na proliferação e invasividade das células neoplásicas, bem como o efeito do seu bloqueio na invasividade dessas células *in vitro*.

4 – MATERIAL E MÉTODOS

4.1 – Animais

Foram utilizados camundongos machos C57BL/6 (selvagens – *wild type* – WT), provenientes do Centro de Bioterismo do Instituto de Ciências Biológicas (CEBIO) da Universidade Federal de Minas Gerais, Brasil. Os camundongos deficientes para a quimiocina CCL3 ($CCL3^{-/-}$), foram obtidos do Centro de Pesquisas René Rachou (Fiocruz Minas Gerais) e os para o receptor CCR5 ($CCR5^{-/-}$) foram gerados como previamente descrito (KUZIEL *et al*., 2003). Animais CCR1 deficientes ($CCR1^{-/-}$) foram obtidos da Taconic Farms (Hudson, New York, USA) e os animais ACKR2 ($ACKR2^{-/-}$) foram cedidos pelo Laboratório de Imunologia Pulmonar e Mecânica, Departamento de Fisiologia e Biofísica do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais. Os animais foram mantidos sob condições controladas de iluminação e temperatura, além de dieta padrão para camundongo e água *ad libitum*. Os animais foram pesados e a quantidade de ingestão de água foi avaliada semanalmente. Todos os procedimentos experimentais descritos a seguir foram executados após aprovação pelo Comitê de Ética em Experimentação Animal da Universidade Federal de Minas Gerais (ANEXO B).

4.2 – Indução de CCEB em língua de camundongos

A indução de tumores na língua dos camundongos foi realizada como previamente descrito (TANG *et al*., 2004; LI *et al*., 2013). Brevemente, uma alíquota de 1 g de 4-nitroquinolina-1-óxido (4NQO) (Sigma Aldrich, St. Louis, MO, USA) foi dissolvida em 200 mL de etilenoglicol (Sigma Aldrich) e estocada a 4°C. Semanalmente, novas alíquotas foram diluídas na água de beber dos animais na

concentração final de 50 µg/mL. Além disso, foi realizado também o tratamento com o 4NQO na concentração de 200 µg/ml, como uma proposta de estabelecer um modelo de metástase em linfonodos, conforme descrito por Li *et al*. (2013). A solução foi mantida em garrafas de polivinil protegidas da luz ambiente e os camundongos do grupo experimental (4NQO) na dose de 50 µg/mL receberam o tratamento com o carcinógeno diariamente durante 28 semanas. O grupo experimental com o 4NQO mais concentrado recebeu o tratamento somente até a vigésima semana, onde então o carcinógeno foi substituído por água filtrada e os animais mantidos nessas condições até a vigésima oitava semana. Os animais do grupo controle receberam água filtrada pelo mesmo período. Após o término de período experimental, os animais foram anestesiados com solução de quetamina e xilazina (180 mg/Kg e 24 mg/Kg, respectivamente) por via intraperitoneal para a coleta dos seguintes órgãos/tecidos: sangue, língua, linfonodos cervicais, fígado, pulmões, estômago, duodeno, jejuno, íleo e intestino grosso para análise microscópica. As línguas foram identificadas e fotografadas para posterior análise macroscópica. Os órgãos/tecidos foram coletados para verificação da ocorrência ou não de metástases à distância ou qualquer outra alteração associada ao tratamento com o 4NQO.

4.3 - Análise microscópica

4.3.1 – Amostras murinas

Todos os tecidos coletados descritos previamente, com exceção do sangue, foram fixados em solução tamponada de formaldeído 10%, pH 7.0, e processados por técnicas histológicas de rotina. As línguas foram cortadas no sentido anteroposterior, e as metades incluídas segundo a orientação da secção desejada. Cortes de 3 µm de

espessura das línguas foram obtidos para coloração com hematoxilina e eosina (H&E) e ensaios imuno-histoquímicos.

Critérios de avaliação das alterações cito-arquiteturais foram adotados a fim de classificar a gravidade das displasias epiteliais encontradas nas lesões linguais. Tais critérios levaram em consideração os itens descritos na tabela a seguir (TAB. 2).

Tabela 2 *- Critérios utilizados para o diagnóstico de displasia epitelial.*

Arquitetura	**Citomorfologia**
Estratificação epitelial irregular	Variação anormal no tamanho nuclear
Perda de polarização das células basais	Variação anormal na forma nuclear
Cristas epiteliais em forma de gota	Variação anormal no tamanho e forma da célula
Aumento do número de figuras de mitose	Aumento na proporção núcleo –citoplasma
Mitoses superficiais atípicas	Figuras de mitose atípicas
Queratinização prematura de células	Aumento do número e tamanho dos nucléolos
Pérolas de queratina	Hipercromatismo

Fonte: Organização Mundial de Saúde – OMS (2005) – Barnes et al.(2005).

A análise histopatológica foi realizada por dois examinadores (JMS e TAS), cegos diante do status dos grupos a qual foi realizada de forma consensual. Como método de avaliar a confiabilidade intra e interexaminadores, foi realizado o teste de Coeficiente de Correlação Intraclasse. As lesões linguais foram classificadas empregando-se escores histopatológicos, de acordo com critérios adaptados da OMS

(2005) (BARNES *et al*., 2005), a qual classifica as lesões como “displasia leve”, “displasia moderada”, “displasia severa” e “carcinoma *in situ*” (TAB. 3).

Tabela 3 *– Escores utilizados para graduação das displasias epiteliais.*

Escore	Características
0	Displasia ausente
1	Displasia leve (alterações limitadas ao terço basal do epitélio)
2	Displasia moderada (alterações representando dois terços do epitélio)
3	Displasia grave (alterações representando mais de dois terços do epitélio)
4	Carcinoma *in situ* (alterações em toda espessura epitelial, porém sem rompimento da membrana basal)
5	Carcinoma invasivo (ilhas carcinomatosas dentro do tecido conjuntivo)

Fonte: Barnes et al. (2005), adaptado.

A análise consistiu na observação de dez campos consecutivos, levando em consideração para estabelecimento da média do escore final, o campo representivo de maior gravidade, avaliando-se pelo menos dois cortes de cada animal.

4.3.2 – Amostras humanas

Foram incluídos neste estudo 33 casos de CCEB primário, 39 casos de desordem potencialmente maligna, diagnosticadas como Leucoplasias Orais (LO), e 16 fragmentos de mucosa oral clinicamente saudável (controles), obtidos durante cirurgia de extração de terceiros molares inferiores inclusos. Os casos de CCEB e LO foram

obtidos do Laboratório de Patologia Bucal da Faculdade de Odontologia da Universidade Federal de Goiás e Hospital Araújo Jorge, Associação do Combate ao Câncer em Goiás, Goiânia, Goiás, Brasil. A aprovação para utilização das amostras foi obtida do Comitê de ética em Pesquisa da Universidade Federal de Goiás (ANEXO C). As lesões diagnosticadas como LO foram graduadas quanto a presença de displasia epitelial (BARNES *et al*., 2005, adaptado). Das 39 LO selecionadas, 20 casos foram classificados como apresentando displasia epitelial leve, 14 como moderada e 5 casos, grave. Todos os casos de CCEB foram analisados seguindo os critérios cito-arquiteturais previamente descritos na Tabela 2 (TAB. 2).

4.4 – Imuno-histoquímica e análise quantitativa

4.4.1 - Amostras murinas

Nesta seção foram descritos os procedimentos para realização do método imuno-histoquímico (estreptavidina-biotina-peroxidase) nas línguas para o marcador de proliferação celular Ki67 (clone MM1, Novocastra, Newcastle, UK). Secções de aproximadamente 3 µm foram desparafinizadas em xilol, reidratadas e finalmente lavadas em água destilada. A recuperação antigênica foi realizada a 96º C em solução de ácido cítrico 10 mM, pH=6, durante 30 minutos, seguido de incubação com solução de H_2O_2 a 3% e o kit Avidin Biotin Blocking System (Dako, Carpinteria, CA, USA) segundo as instruções do fabricante. Após a incubação com o anticorpo primário, diluição 1:50 *overnight*, os cortes foram incubados com o anticorpo secundário Multi-Link (sistema LSAB/HRP, Dako, Carpinteria, CA, USA) segundo as instruções do fabricante. A reação foi revelada pela incubação com solução de 3,3-diaminobenzidina (DAB) (Dako, Carpinteria, CA, USA), durante 1-2 minutos. Finalmente, os cortes

foram contracorados com hematoxilina de Mayer, desidratados em soluções crescentes de alcoóis, diafanizados em xilol e montados com Permount (Fisher Scientific, Fair Lawn, NJ, USA). Controles negativos foram obtidos omitindo-se o anticorpo primário, o qual foi substituído por PBS-BSA a 1%.

As células imunomarcadas foram analisadas em microscópio de luz (Axioskop 40 ZEISS, Carl Zeiss, Gotingen, Germany), com aumento original de 1.000 vezes. Foram avaliados 20 campos consecutivos e pelo menos 2 cortes de cada animal. Os resultados foram expressos como o número total de células $Ki67^{+}$ em todas as camadas epiteliais, por campo.

4.4.2 – Amostras humanas

Nesta seção, foram descritos os procedimentos para imunomarcação e quantificação de CCL3 nos tecidos humanos. A expressão de CCL3 foi realizada por imuno-histoquímica, utilizando o anti-CCL3 (Clone FL-2, Santa Cruz Biotechnology, TX, USA), concentração 1:50, nas mesmas condições previamente descritas.

A imunorreatividade foi analisada por microscopia de luz (Axioskop 40 ZEISS; Carl Zeiss, Gotingen, Germany), no aumento original de 400 vezes, utilizando retículo de integração acoplado (Carl Zeiss – Axiostar 1122-100). Foram avaliados 20 campos consecutivos em pelo menos 2 cortes por amostra. Os resultados foram expressos como a porcentagem de células CCL3 positivas em relação à população total de células no epitélio e no tecido conjuntivo, por amostra.

4.5 - ELISA - Enzyme-linked Immunosorbent Assay

Fragmentos de lesões de CCEB clinicamente detectáveis e fragmentos de língua clinicamente normal (controle) foram pesados e homogeneizados em solução salina fosfato [(0.4 mM de cloreto de sódio - NaCl e 10 mM de fosfato de sódio - $NaPO_4$, pH 4.7)] contendo inibidores de protease [(0.1 mM fluoreto de fenilmetil sulfonil - PMSF, 0.1 mM cloreto de benzetônio, 10 mM ácido etilenodiamino tetra-acético - EDTA, e 0.01 mg/mL aprotinina A) e 0.05% Tween-20] na concentração final de 100 mg/mL. O homogenato foi centrifugado (10.000 r.p.m) a 4 ºC por 10 minutos. O sobrenadante foi coletado e estocado a -70 °C para análise posterior. A concentração de CCL3 foi mensurada utilizando-se kits disponíveis no mercado, de acordo com as instruções do fabricante (R&D Systems, Minneapolis, MN, USA). Os resultados foram expressos em picogramas de citocinas (± Desvio Padrão – SD) normalizadas por 100 mg de tecido.

4.6 - Reação em cadeia da polimerase (PCR array)

Fragmentos de CCEB experimentalmente induzido e de língua clinicamente normal (controle) foram coletados para realização da avaliação da expressão molecular de mediadores envolvidos com angiogênese, proliferação tumoral, invasão e metástase. As reações de PCR array foram realizadas conforme descrito por Vieira *et al*. (2015) (VIEIRA *et al*., 2015). Brevemente, foi realizada a extração do RNA total das amostras utilizando-se o kit RNeasyFFPE (Qiagen Inc, Valencia, CA) de acordo com as instruções do fabricante. Em seguida, a integridade do RNA extraído foi verificada a partir de 1 mg de RNA total pelo 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Aproximadamente 3 µg de RNA total foi utilizado para síntese da fita de DNA complementar – cDNA, através da reação da transcriptase reversa (Superscript III,

Invitrogen Corporation, Carlsbad, CA). A reação de PCR array foi realizada no equipamento Viia7 (LifeTechnologies, Carlsbad, CA) utilizando-se um painel contendo os alvos "Wound Healing" (PAMM-121) e "Inflammatory cytokines and receptors" (PAMM-011) (SABiosciences, Frederick, MD). Os dados foram analisados pelo software RT2 Profiler PCR Array Data Analysis, online (SABiosciences), no qual foi realizada normalização dos dados através da média geométrica inicial de três genes constitutivos (GAPDH, ACTB, Hprt1) e subsequentemente normalizados pelo grupo controle. Os resultados foram expressos como "*fold increase"* em relação ao grupo controle.

4.7 – Ensaios in vitro

4.7.1 - Cultura e ensaio de viabilidade celular – MTS

Para realização dos experimentos *in vitro* foram selecionadas duas linhagens humanas de células de carcinoma de células escamosas de cabeça e pescoço (HN12, e HN13 – do inglês: *Head and Neck*), descritas previamente (CARDINALI *et al*., 1995) e detalhadas na tabela a seguir (TAB. 4).

Tabela 4 *– Características das linhagens celulares utilizadas nos experimentos in vitro.*

Nome	Característica	Origem/localização
HN12	Metastática	Proveniente de linfonodo metastático de paciente diagnosticado com CCEB em língua
HN13	Não metastática	Proveniente de CCEB primário localizado em língua

Fonte: Cardinali et al. (1995).

As células foram utilizadas para analisar os efeitos diretos da quimiocina CCL3 na proliferação celular, invasão e produção e liberação de mediadores inflamatórios.

Aproximadamente $1x10^5$ células foram plaqueadas e cultivadas em meio Dulbecco's modificado (DMEM) (Sigma-Aldrich, St. Louis, MO, USA), suplementado com soro fetal bovino (SFB) a 10% (Gibco, Carlsbad, CA), e solução antibiótica/antimicótica (Cat. A5955, Sigma-Aldrich) em estufa com atmosfera de 5% de CO^2 a 37º C. Após alcançar a confluência, ou seja, quando aproximadamente 70-80% da superfície de cultivo foi ocupada, as células foram tripsinizadas em solução de Tripsina-EDTA (cat. T4174, Sigma-Aldrich) e ressuspendidas em DMEM novo para utilização nos experimentos subsequentes. Para determinação da viabilidade celular foi utilizado o CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) seguindo as instruções do fabricante. Todos os experimentos foram executados em triplicata, e três experimentos independentes foram considerados. As células ($1x10^5$) foram plaqueadas em placas de 96 poços (Corning Inc., Corning, NY), estimuladas com 3 diferentes concentrações de CCL3 (5, 10 ou 20 ng/mL), e então, acompanhadas por 24, 48 e 72 horas. Em seguida, foi adicionada solução de *Tetrazolium Compound* e *Phenazine Methosulfate* (MTS) (Promega). As células foram incubadas por 2 horas e a absorbância foi avaliada a 490 nm através de um leitor de microplacas (Mod. 680 XR, Bio-Rad, Hercules, CA).

O sobrenadante foi coletado nos tempos de 24, 48 e 72 horas para subsequentes ensaios de ELISA, nos quais foram utilizados os kits para detecção de CCL3, CCL5, IL-6 e TNF-α (R&D Systems, Minneapolis, MN, USA), como foi descrito previamente.

4.7.2 – Ensaio de proliferação celular

Para o ensaio de proliferação celular, as células ($1x10^5$) foram plaqueadas em triplicata em placas de 24 poços (Corning) por 48 horas em meio DMEM livre de soro

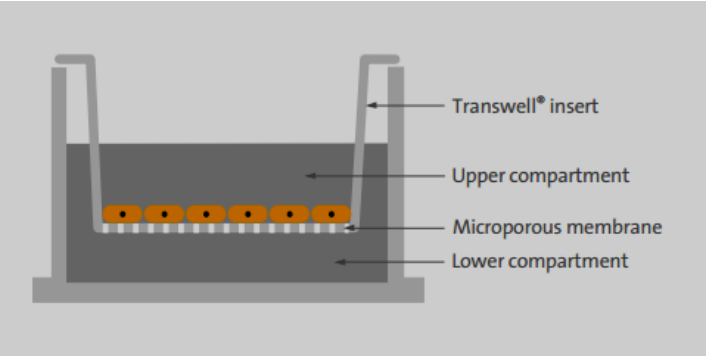
(controle), ou em DMEM contendo CCL3 (10 ng/mL) (Cat. DY270, R&D Systems, Minneapolis, MN, USA). Após 48 horas, o sobrenadante foi removido e as células aderidas na superfície das lamínulas posicionadas no fundo de cada poço foram fixadas com solução de formaldeído a 10%, por 15 minutos. Em seguida, foi realizada a técnica imuno-citoquímica, conforme descrito anteriormente, utilizando-se o anticorpo anti-Ki67 (Clone ab15580, Abcam, Cambridge, England). As lamínulas foram fotografadas usando o software AxioVision Rel. 4.8.2 (Carl Zeiss). As células Ki67 positivas foram contadas com o auxílio do software Image J e os resultados foram apresentados como a porcentagem média de células positivas, por campo.

4.7.3 – Zimograma

3×10^5 células foram plaqueadas em triplicata em placas de 6 poços. Após um período de 24 horas, o meio foi removido e substituído por 1 mL de DMEM livre de soro. Em seguida, as células foram tratadas ou não (controle) com CCL3 (10 ng/mL) e mantidas por um período adicional de 24 e 72 horas. O sobrenadante foi então coletado para análise. Aproximadamente 20 µL do sobrenadante foi dissolvido em solução de 12% SDS-PAGE (Cat. SM1841, Fermentas, Kent, UK) contendo 1 mg/mL de gelatina. O gel foi lavado com Triton X-100 a 2%, por 40 minutos e incubado com solução contendo 10 mM Tris–HCl e 5 mM CaCl2 por 16 horas a 37 °C. Em seguida, o gel foi corado com *Coomassie blue* 0,25% (Sigma-Aldrich) e subsequentemente lavado. A atividade das metaloproteinases de matriz MMP-2, MMP-8 e MMP-9 foi representada pela presença negativa das respectivas bandas. A análise semiquantitativa foi realizada utilizando-se o software Image J. Os resultados foram representados como a expressão de MMP-2, MMP-8 e MMP-9, calculados considerando a área sob cada pico.

4.7.4 – Ensaio de invasão celular

O ensaio de invasão celular foi realizado com o uso de câmaras bipartidas. Para tanto, foi utilizada placa de 24 poços (Corning), nos quais foram colocados insertos (Boyden modificado) que os dividiam em compartimentos superior e inferior, conforme mostra a figura a seguir (FIG. 1).



Fonte: http://www.corning.com/media/worldwide/cls/documents/CLS-CC-027REV6.pdf

Figura 1 *– Desenho esquemático ilustrando o ensaio de invasão celular.*

Uma membrana de policarbonato contendo poros de 8 μm de diâmetro estava presente no fundo de cada inserto. Além disso, foi utilizada matrigel (Cat.35423C, BD Biosciences, Bedford, MA) diluída em DMEM livre de soro, na proporção 1:1 para cobrir a superfície de cada membrana. Em seguida, as células ($2x10^5$) receberam um pré-tratamento com CCL3 (Cat. DY270, R&D Systems) na concentração de 10 ng/mL, ou com anticorpo anti-CCL3 (clone FL-92; Santa Cruz Biotechnology) (20 μg/mL) ou com Evasina-1 (AS9001965, 10^{-7} M) (Serono Pharmaceutical Research Institute SA, Geneva, Switzerland), um antagonista de quimiocina que impede a ação de CCL3 (FRAUENSCHUH *et al.*, 2007). Após 45 minutos, as células foram lavadas e ressuspendidas em 250 μL de DMEM livre de soro e então plaqueadas no

compartimento superior do inserto. Foi utilizado DMEM contendo CCL3 (10 ng/mL) no compartimento inferior, para a estimulação. As células foram mantidas nessa condição por 72 horas, quando então seguiu-se a remoção do meio do compartimento superior para fixação das membranas em solução de formaldeído a 10% por 15 minutos. As células aderidas na superfície do compartimento inferior foram então coradas com Giemsa (Sigma-Aldrich). Cinco campos de cada poço foram fotografados no aumento original de 200 vezes utilizando um microscópio invertido (Zeiss Axiovert 40) e as imagens analisadas pelo software AxioVision Rel. 4.8.2 software (Carl Zeiss). As células que foram capazes de transmigrar pelos poros das membranas foram então contadas usando Image J e os resultados expressos como a porcentagem média de células, por campo.

Ao final deste manuscrito é possível encontrar um fluxograma que esquematiza todos os experimentos descritos nesta seção de "Material e Métodos" (ANEXO D).

4.8 – Análise estatística

A análise estatística foi realizada utilizando o software GraphPad Prism, versão 5.0. Após testar a distribuição de normalidade dos dados, o teste *t de Student* ou a *Análise de Variância (ANOVA)* foram utilizados para comparar dois ou mais grupos experimentais, respectivamente. No caso de observação de significância estatística no teste *One-way ANOVA*, foi realizado o pós-teste *Student-Newman-Keuls*. Para amostras humanas foi realizada análise comparativa com *Kruskall-Wallis*, seguido do pós-teste de *Dunn's*. Os resultados foram expressos como médias ± Erro Padrão da Média (E.P.M) e o nível de significância estatística foi estabelecido em $p<0.05$. Os resultados do PCR

array foram analisados pelo teste *Mann-Whitney*, e os valores corrigidos pelo procedimento de *Benjamini – Hochberg*.

5 – RESULTADOS

Os resultados deste trabalho serão apresentados na forma de dois artigos científicos:

Artigo 1 *"CCL3/CCR5 is critical for oral carcinogenesis", submetido ao periódico Oral Oncology.*

Artigo 2 *"Deletion of the atypical CC chemokine receptor ACKR2 does not modify experimental squamous cell carcinogenesis" em fase de preparação para submissão.*

Elsevier Editorial System(tm) for Oral Oncology

Manuscript Draft

Manuscript Number: OO-D-16-292

Title: CCL3/CCR5 is critical for oral carcinogenesis

Article Type: Original Research Article

Section/Category: Basic

Keywords: Chemokines, CCL3, CCR1, CCR5 and OSCC

Corresponding Author: Prof. Tarcilia Silva, PhD

Corresponding Author's Institution: Universidade Federal de Minas Gerais

First Author: Janine M Silva, MSc

Order of Authors: Janine M Silva, MSc; Tâlita P Moreira dos Santos, MSc; Lays M Sobral, PhD; Celso M Queiroz-Junior, PhD; Milene A Rachid, PhD; Amanda E Proudfoot, PhD; Gustavo P Garlet, PhD; Aline C Batista, PhD; Mauro M Teixeira, PhD; Andréia M Leopoldino, PhD; Remo C Russo, PhD; Tarcilia Silva, PhD

Abstract: The chemokine CCL3 is a small chemotactic cytokine crucial for inflammatory cell recruitment in homeostatic and pathological conditions. CCL3 might affect cancer progression by stimulating leukocytes accumulation, angiogenesis and tumor growth. Objectives: The expression of CCL3 and its receptors (CCR1 and CCR5) was demonstrated in oral squamous cells carcinoma (OSCC), but their functions were not defined yet. Materials and Methods: Here, we assessed the function of CCL3 using a model of chemically-induced tongue carcinogenesis. In vitro experiments using lineages of OSCC were performed to analyze the effects of CCL3 on proliferation and invasion. Results: Results showed that chemically-induced lesions exhibited expression of CCL3. Mice deficient of CCL3 (CCL3-/-) and CCR5 (CCR5-/-) treated with chemical carcinogen presented reduced incidence of tongue tumors compared to wild-type (WT) and mice deficient for CCR1 (CCR1-/-). Consistently, microscopic analysis demonstrated attenuated cytomorphological atypia and reduced cell proliferation in lesions from CCL3-/- and CCR5-/--treated mice. We further demonstrate that OSCC from CCL3-/- mice exhibited reduced expression of angiogenic factors (Egf, Tgf-β1), adhesion molecules (Itga-4, Vtn) and matrix metalloproteinases (Mmp-1a, Mmp-2 and Mmp-9). In vitro, CCL3 induced invasion and release of CCL5, IL-6, and MMP's 2, 8 and 9 by neoplastic cells. Pharmacologic blockage of CCL3 impaired tumor cell invasion. Conclusion: Herein, we provide evidence of CCL3/CCR5 axis relevance for oral carcinogenesis. CCL3 is able to affect invasion ability of neoplastic cells and modify tumor microenvironment. These data reveal potential protective effects of CCL3 blockade in oral cancer.

Title: CCL3/CCR5 is critical for oral carcinogenesis

Keywords: *Chemokines, CCL3, CCR1, CCR5 and OSCC*

Janine Mayra da Silva[1,2], Tálita Pollyanna Moreira dos Santos[1,2], Lays Martin Sobral[3], Celso Martins Queiroz-Junior[2], Milene Alvarenga Rachid[4], Amanda EI Proudfoot[5], Gustavo Pompermaier Garlet[6], Aline Carvalho Batista[7], Mauro Martins Teixeira[8], Andréia Machado Leopoldino[3], Remo Castro Russo[9], Tarcília Aparecida da Silva[1]

[1] Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

[2]Department of Morphology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

[3]Department of Clinical Analysis, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil.

[4]Department of General Pathology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

[5]Merck Serono Geneva Research Centre, Geneva, Switzerland.

[6]Department of Biological Sciences, School of Dentistry, Universidade de São Paulo, Bauru, Brazil.

[7]Department of Stomatology, School of Dentistry, Universidade Federal de Goiás, Goiânia, Brazil.

[8]Laboratory of Immunopharmacology, Department of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

[9]Laboratory of Pulmonary Immunology and Mechanics, Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

Word count:3675

[1] Reprint requests to: Tarcília Aparecida da Silva Mailing address: Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31.270-901, Belo Horizonte, Minas Gerais, Brazil. Phone: 55 31 3409-2478 (voice); 55 31 3499-2430 (Fax). E-mail: tarcilia@ufmg.br

Abbreviations: CCL3 – CC chemokine ligand 3, CCR5 – CC chemokine receptor 5, CCR1 – CC chemokine receptor 1, OSCC – Oral squamous cell carcinoma,WT – Wild type, 4NQO – 4-Nitroquinoline-1-oxide, OLK - Oral leucoplakia, HNSCC - Head and neck squamous cell carcinoma, MMP – Matrix metalloproteinases, SCC – Squamous cell carcinoma, ECM – Extracellular matrix.

ABSTRACT

The chemokine CCL3 is a small chemotactic cytokine crucial for inflammatory cell recruitment in homeostatic and pathological conditions. CCL3 might affect cancer progression by stimulating leukocytes accumulation, angiogenesis and tumor growth. ***Objectives*****:** The expression of CCL3 and its receptors (CCR1 and CCR5) was demonstrated in oral squamous cells carcinoma (OSCC), but their functions were not defined yet. ***Materials and Methods*****:** Here, we assessed the function of CCL3 using a model of chemically-induced tongue carcinogenesis. In vitro experiments using lineages of OSCC were performed to analyze the effects of CCL3 on proliferation and invasion. ***Results*****:** Results showed that chemically-induced lesions exhibited expression of CCL3. Mice deficient of CCL3 ($CCL3^{-/-}$) and CCR5 ($CCR5^{-/-}$) treated with chemical carcinogen presented reduced incidence of tongue tumors compared to wild-type (WT) and mice deficient for CCR1 ($CCR1^{-/-}$). Consistently, microscopic analysis demonstrated attenuated cytomorphological atypia and reduced cell proliferation in lesions from $CCL3^{-/-}$ and $CCR5^{-/-}$-treated mice. We further demonstrate that OSCC from $CCL3^{-/-}$ mice exhibited reduced expression of angiogenic factors (*Egf*, *Tgf-β1*), adhesion molecules (*Itga-4*, *Vtn*) and matrix metalloproteinases (*Mmp-1a*, *Mmp-2* and *Mmp-9*). In vitro, CCL3 induced invasion and release of CCL5, IL-6, and MMP's 2, 8 and 9 by neoplastic cells. Pharmacologic blockage of CCL3 impaired tumor cell invasion. ***Conclusion*****:** Herein, we provide evidence of CCL3/CCR5 axis relevance for oral carcinogenesis. CCL3 is able to affect invasion ability of neoplastic cells and modify tumor microenvironment. These data reveal potential protective effects of CCL3 blockade in oral cancer.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy of oral cavity worldwide [1,2]. OSCC is considered highly infiltrative, locally aggressive and frequently metastasizing to cervical lymph nodes [1,3].

Tumor behavior depends on malignant cell capabilities and tumor microenvironment, which directly affects tumor growth, invasion and metastasis [4]. The interaction of tumor cells and their supporting stroma is based on production of angiogenic factors, integrins, proteases, cytokines and chemokines [4,5]. Accumulating evidence suggest that chemokines might exert pro-tumorigenic effects in different types of tumors [6-10] including OSCC [11-15]. The mechanisms by which chemokines affect tumorigenesis may include regulation of inflammatory cell trafficking to tumor microenvironment, neovascularization, extracellular matrix remodeling, neoplastic cell motility and proliferation contributing to tumor growth and spreading [16-19]. In particular, the effects of CC chemokine CCL3 are little explored in cancer. A few studies have demonstrated increased expression of CCL3 in hepatomas, multiple myeloma and chronic lymphocytic leukemia [16, 20, 21]. Functional studies demonstrated that mice deficient for CCL3 and its receptor CCR1 were significantly protected from carcinogen-induced hepatocarcinogenesis [16]. Additionally, CCL3 induced hepatoma cells to produce pseudopodia and migrate in vitro [22]. In a model of renal cell carcinoma, decreased incidence of metastasis was observed in mice deficient for CCL3 and CCR5 [18]. In the context of OSCC, one study previously showed the expression of CCL3 in tumor samples and metastatic lymph nodes and correlated it with poor cumulative survival [23]. Herein, we employed a model of chemically-induced

OSCC to investigate the role of CCL3/CCR1/CCR5 axis in oral carcinogenesis. We also used in vitro approaches to dissect the effects of CCL3 in OSCC cells.

MATERIALS AND METHODS

Animals

C57BL/6 (wild type - WT) 6-8 weeks male mice were obtained from Centro de Bioterismo, Instituto de Ciências Biológicas (CEBIO), Universidade Federal de Minas Gerais, Brazil. $CCL3^{-/-}$ deficient mice were bred and obtained from Centro de Pesquisas René Rachou (Fiocruz Minas Gerais, Brazil). $CCR5^{-/-}$ were generated as previously described [24]. $CCR1^{-/-}$ deficient mice were obtained from Taconic Farms (New York, USA). Mice were maintained under standard conditions with a 12 h light/dark cycle, controlled temperature (24 ± 2 °C) and free access to commercial chow and water ad libidum, Ethics Committee in Animal Experimentation (protocol 12/2011).

OSCC induction

The induction of oral carcinogenesis was performed as previously described [25, 26]. Briefly, 4-nitroquinoline-1-oxide (4NQO) (Sigma-Aldrich) was dissolved in ethylene glycol (Sigma-Aldrich) at 50 µg/mL or 200 µg/mL and stored at 4 °C. Experimental mice received 4NQO daily during 28 weeks and control mice (n=5, per group) received drinking water, only. After 28 weeks, mice were euthanized and tongue, cervical lymph nodes, liver, lungs, stomach, duodenum, jejunum, ileum and large intestine were collected for microscopic analysis.

Histopathological analysis

Tongues and organs of 11 WT, 11 CCL3$^{-/-}$, 6 CCR1$^{-/-}$ and 8 CCR5$^{-/-}$ treated mice were fixed in 10% buffered formalin, embedded in paraffin wax, longitudinally cut (3 µm sections) and stained with H&E. Tongue tumors were classified using scores (adapted from [27]): 0 – normal epithelial architecture, 1 - mild dysplasia (changes limited at basal third of lining epithelium), 2 - moderate (changes in two-thirds of lining epithelium), 3 - severe (more than two-thirds), 4 - carcinoma in situ (full thickness of the lining epithelium, without invasion of connective tissue) and 5 - invasive carcinoma (carcinomatous islands into connective tissue). Twenty consecutive fields were evaluated by two examiners (J.M.S and T.A.S) blinded from group status. The Intraclass Correlation Coefficient test was performed (ICC = 0.83) to validate the reliability of inter- and intra-examiner evaluations.

Immunohistochemistry and cell counting

Immunohistochemistry was performed in tongue sections using the streptavidin-biotin method [28]. Briefly, slides were deparaffinized, dehydrated and rinsed in distilled water, followed by incubation with 0.3% hydrogen peroxide and Avidin/Biotin blocking system (Dako). Sections were then incubated with monoclonal mouse anti-human Ki-67 (MM1, Novocastra), 4 ºC overnight. Negative controls were obtained by omission of primary antibody, which were substituted by 1% PBS-BSA. Cells were analyzed by light microscope (Axioskop 40 Zeiss) at 1000x original magnification, counted in 20 consecutive fields in two sections.

Human samples analysis

Thirty three cases of OSCC, 39 cases of oral potentially malignant disorder diagnosed as Oral Leucoplakia (OLK) and 16 cases of clinically oral health mucosa (controls) were obtained from Laboratório de Patologia Bucal, Faculdade de Odontologia, Universidade Federal de Goiás and Hospital Araújo Jorge, Associação do Combate ao Câncer em Goiás, Goiânia (Ethics Committee approval 013/2010). OLK samples were graded (adapted from [27]) for epithelial dysplasia (20 light, 14 moderate and 5 high). CCL3 expression was evaluated by immunohistochemistry as described above using rabbit polyclonal anti-human CCL3 (FL-92, Santa Cruz) at 1:50. CCL3 expression was quantified in epithelium and connective tissue by light microscopy in 10 consecutive fields at 400x original magnification.

Enzyme-Linked Immunosorbent Assay

SCC tongue lesions (4NQO, n=3) and clinically normal tongue samples (control, n=3) were weighed and homogenized as described previously [28]. The homogenate was centrifuged (8 946 x g) at 4 ºC for 10 min and supernatant stored at -70 °C until analysis. The concentration of CCL3 was measured using commercially available kit (R&D Systems). The results are expressed as picograms of cytokines normalized for 100 mg tissue ± standard deviation (SD).

Real Time PCR array

Real Time PCR array was performed as previously described [29]. Total RNA was extracted with the RNeasyFFPE kit (Qiagen Inc) according to the manufacturer instructions. Real Time PCR array was performed in a Viia7 instrument (LifeTechnologies) using custom panels "Wound Healing" (PAMM-121) and "Inflammatory cytokines and receptors" (PAMM-011) (SABiosciences). Data were analyzed by the RT2 profiler PCR Array Data Analysis online software (SABiosciences) for normalizing the initial geometric mean of three constitutive genes (GAPDH, ACTB, Hprt1), normalized by the control group and expressed as fold change relative to the control group.

Cell Culture and MTS assay

Head and Neck Squamous Cell Carcinoma (HNSCC) cell lines (HN12 – metastatic obtained from metastatic lymph node) and (HN13 – non metastatic from primary squamous cell carcinoma of the tongue) [30] cultured in Dulbecco's modified medium - DMEM (Sigma-Aldrich), supplemented with 10% FBS (Gibco), antibiotics and antimycotics (Cat. A5955, Sigma-Aldrich) in 5% CO^2 at 37 °C. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used to determine cell viability according to the manufacturer's instructions. Cells ($1x10^5$) were plated on 96-well plates (Corning Inc.) for 24, 48 and 72 hours before the addition of the MTS solution (tetrazolium compound and phenazine methosulfate) (Promega).

Culture supernatants were collected at 24, 48 and 72 hours for ELISA using commercial available kits for detection of CCL3, CCL5, IL-6 and TNF-α (R&D Systems) as previously described.

Proliferation assay

Cells ($1x10^5$) were plated in 24-well plates (Corning) for 48 hours in serum-free DMEM (control) or in presence of CCL3 (10 ng/mL). After incubation, supernatant was removed and immunohistochemistry was performed as described above using anti-Ki67 (ab15580, ABcam). Ki67 positive cells were counted using the Image J software.

Zymogram assay

Cells ($3×10^5$) were plated in a 6-well plate. After 24 h, medium was changed to 1 ml of serum-free DMEM, then, cells were treated or not (control) with CCL3 (10 ng/mL) and maintained for additional 24 and 72 hours. Twenty microliters of supernatant were resolved by 12% SDS-PAGE (Cat. SM1841, Fermentas) containing 1 mg/mL gelatin. The gel was washed with 2% Triton X-100, incubated in 10 mM Tris–HCl and 5 mM CaCl2 for 16 hours, 37 °C and then, stained with 0.25% Coomassie blue (Sigma-Aldrich).

Invasion assay

The cell invasion assay was performed in 24-well plates using modified Boyden chamber inserts with a polycarbonate filter membrane containing 8 μm pores. Matrigel (Cat.35423C, BD Biosciences) was diluted 1:1 with serum-free medium and used to coat the filter membranes. Cells ($2×10^5$) were pre-treated with CCL3 (Cat. DY270, R&D Systems) (10 ng/mL), antibody anti-CCL3 (FL-92; Santa Cruz Biotechnology) (20 μg/mL) or Evasin-1 (AS9001965, 10^{-7} M) (Serono Pharmaceutical Research Institute SA), a chemokine-bind protein that depletes CCL3 [31]. After 45 minutes, cells were re-suspended in 250 μL serum-free DMEM and seeded onto the upper compartment. DMEM containing CCL3 (10 ng/mL) was used in the lower chamber for

stimulation. After 72 h, filters were fixed in 10% formalin for 15 min. Cells on the lower surface were stained with Giemsa (Sigma-Aldrich). Five fields were photographed at 200x original magnification using a Zeiss Axiovert 40 inverted microscope and processed using the AxioVision Rel. 4.8.2 software (Carl Zeiss).

Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5.0 (GraphPad Software Inc.). The Student t Test was performed after checking for data normality. Comparative analysis was performed by the Kruskall-Wallis, followed by Dunn's multiple comparison post test for human specimens. The results are reported as the means ± SD. P values <0.05 are considered to be statistically significant.

RESULTS

CCL3 production during experimentally-induced and human SCC

We first evaluated CCL3 expression in chemically-induced SCC lesions and the concentration of CCL3 was significantly increased after 4NQO treatment when compared to non-treated controls (P<0.05) (Fig. 1).

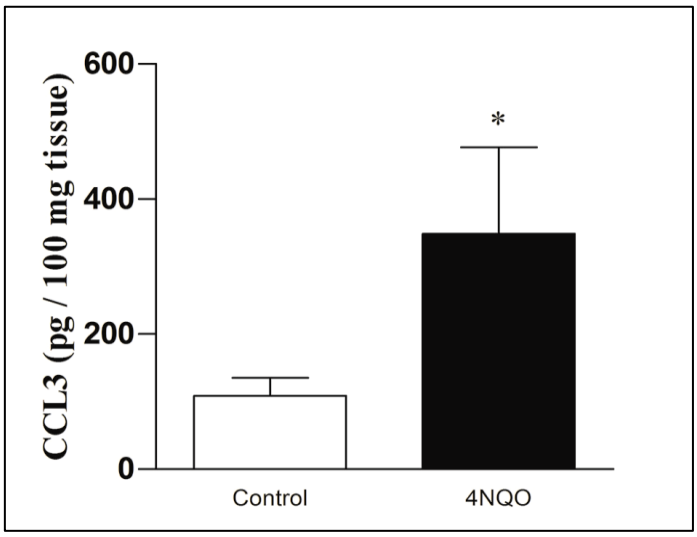


FIGURE 1 – CCL3 concentration in SCC tongue lesions of C57BL/6 mice treated with chemical carcinogen 4NQO for 28 weeks. Control mice received drink water along the experimental period. n=3, per group. * P< 0.05 in relation to control**.**

Next, we analyzed the CCL3 expression in human samples of healthy oral mucosa, Oral Leukoplakia (OLK) with different grades of epithelial dysplasia, and primary OSCC samples. CCL3 expression was markedly up-regulated in epithelial cells of OSCC (parenchyma) (Fig. 2C and 2D) when compared with healthy oral mucosa (Fig. 2A and 2D) and OLK (Fig. 2B and 2D). Additionaly, increased CCL3 expression by epithelial cells in healthy oral mucosa in relation to OLK was seen. CCL3 was also detected in connective tissue (stroma) and results showed a significant increase in oral healthy mucosa compared to OLK with different grades of dysplasia. On the other hand, no difference between CCL3 expression in stromal cells of samples of oral healthy mucosa and OSCC was seen (Fig. 2D).

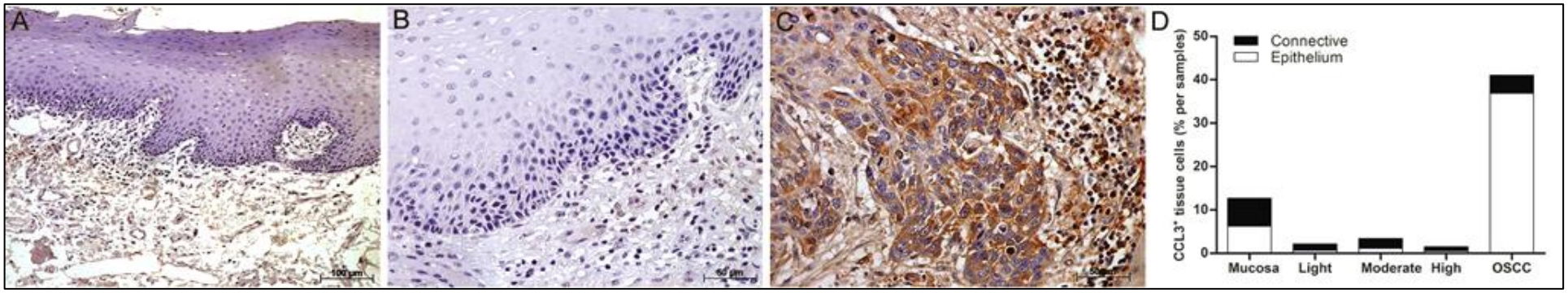


FIGURE 2 – Immunohistochemical expression of CCL3 in samples of human oral healthy mucosa (A), oral Leucoplakia (OLK) (B) and OSCC primary tumors (C). (D) The results are presented as the mean percentage of $CCL3^{+}$ cells in epithelium (parenchyma) and connective tissue (stroma).

SCC tumor formation is reduced in CCL3 and CCR5 deficient mice

Our next approach consisted of inducing oral carcinogenesis in mice lacking CCL3 or its receptors CCR1 and CCR5. SCC was induced by 28 weeks of treatment with 4NQO, as previously reported [25,32]. Clinically, SCC occurred as exophytic, papillomatous, white and base-attached tongue lesions (Fig. 3C). Comparatively, SCC tongue tumors formation was more prominent in WT (Fig. 3C) than $CCL3^{-/-}$-treated mice (Fig. 3D). No changes in tongue surface were observed in control groups (Fig. 3A and 3B).

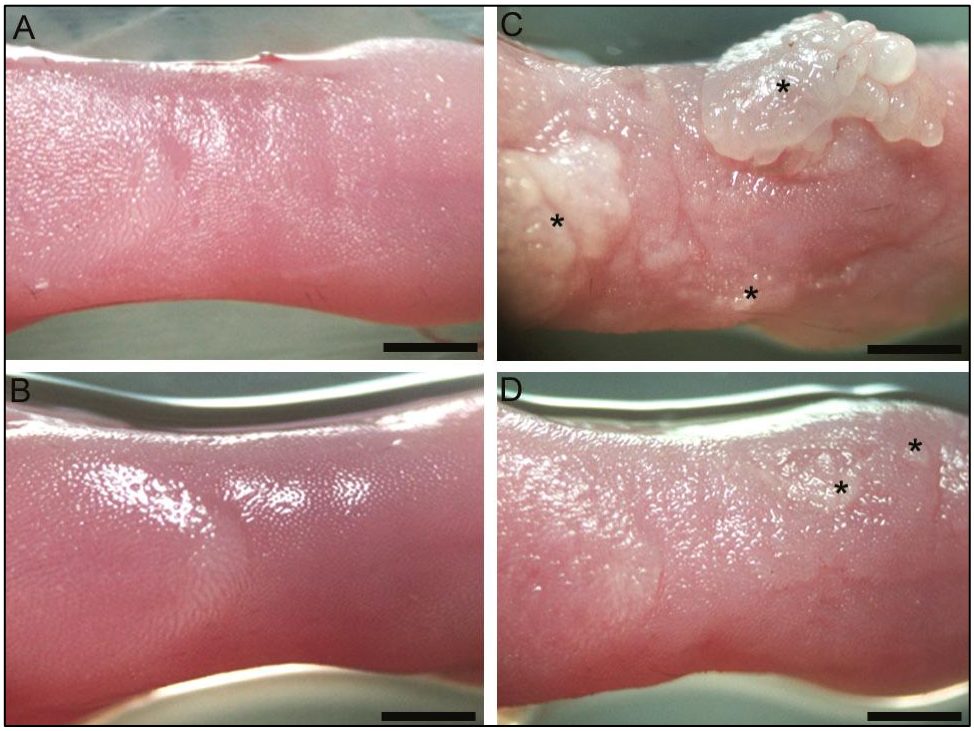


FIGURE 3 – Clinical findings in tongue of WT and $CCL3^{-/-}$ mice after 4NQO treatment (n=6 and n=7, respectively). Macroscopic appearance of WT (A) and $CCL3^{-/-}$ (B) control mice. (C) and (D) represent characteristics of SCC induced lesions in WT and $CCL3^{-/-}$ treated mice, respectively. Asterisks indicate clinical lesions (Barr = 0.1 cm).

Microscopic analysis was consistent with clinical findings and showed pronounced cytomorphological atypia ($P<0.05$), with 100% of lesions graded as carcinoma in situ and invasive carcinoma (scores 4 and 5, respectively) in WT-treated mice (Fig. 4C and 4G). In contrast, $CCL3^{-/-}$-treated mice presented lesions with lower grade of cellular atypia and severity, with 57% of the lesions graded as moderate and severe dysplasia (scores 2 and 3, respectively) (Fig. 4D and 4G) and 43% classified as carcinoma in situ. Accordingly, $CCL3^{-/-}$-treated group had significantly reduced Ki67 immunopositivity, when compared with WT-treated mice (Fig. 4F, 4E and 4H). No changes were seen in epithelium architecture in control groups (Fig. 4A, 4B and 4G).

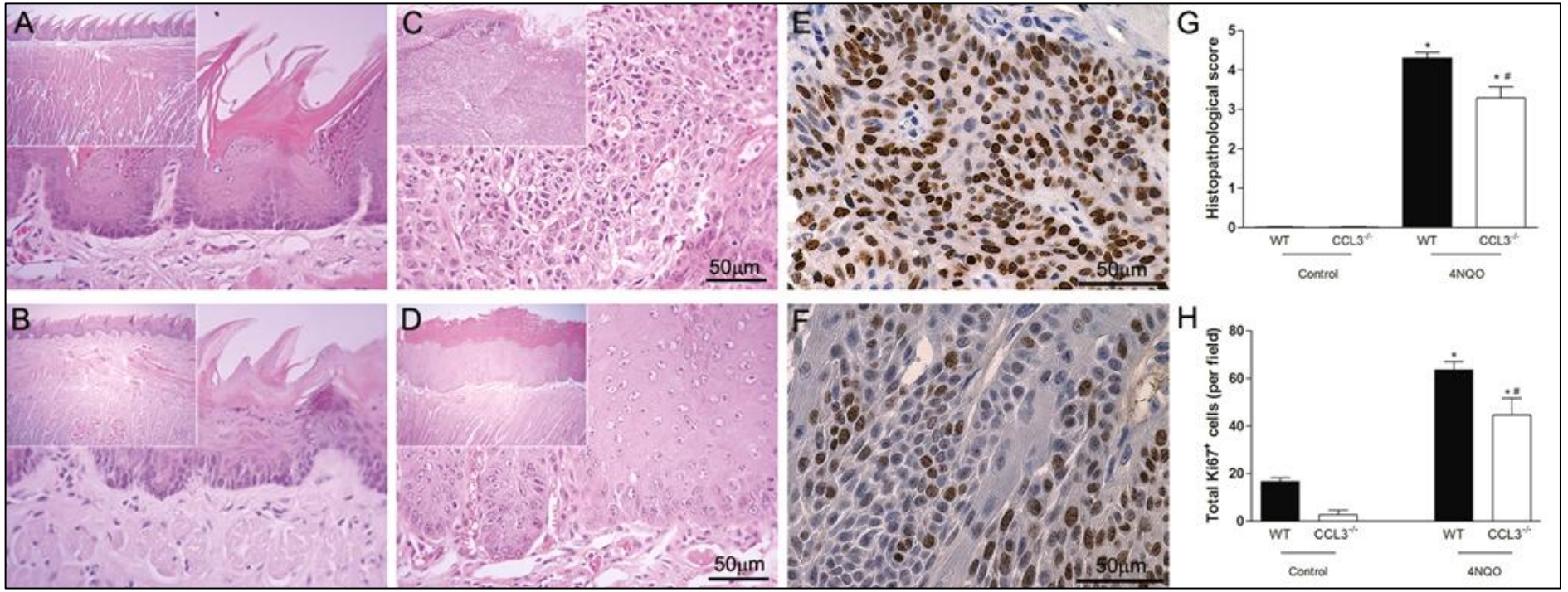


FIGURE 4 – Microscopic findings of SCC-induced lesions in WT and CCL3$^{-/-}$ mice after 4NQO treatment (n=6 and n=7, respectively). Histological characteristics of (A) WT and (B) CCL3$^{-/-}$ control mice. Histopathological characteristics of SCC induced lesions of (C) WT and *carcinoma in situ* in (D) CCL3$^{-/-}$ treated mice. Ki67 immunoexpression in tongue of (E) WT and (F) CCL3$^{-/-}$ treated mice. G – Histopathological score of tongue lesions. H – Quantification of total Ki67^{+} cells in lesions from WT and CCL3$^{-/-}$ control and treated groups. *P<0.05 in relation to respective control, #P<0.05 when comparing 4NQO- treated groups.

To confirm the relative protection of CCL3 deletion in oral carcinogenesis, a more "aggressive" OSCC model using high dose of 4NQO (200 µg/mL) was used [26]. Macroscopic and microscopic analysis revealed a similar incidence and clinical features of SCC lesions in tongue of WT mice treated with 50 or 200 µg/mL of 4NQO. Again, WT-treated mice exhibited increased tumor formation when compared with $CCL3^{-/-}$-treated mice (Fig. 5A, 5C and 5B, 5D respectively). Noteworthy, while WT-treated group exhibited pronounced cytological atypia and dysplasia (100% of lesions were graded as invasive carcinoma – score 5), $CCL3^{-/-}$-treated mice did not present any score of "invasive carcinoma" (data not shown).

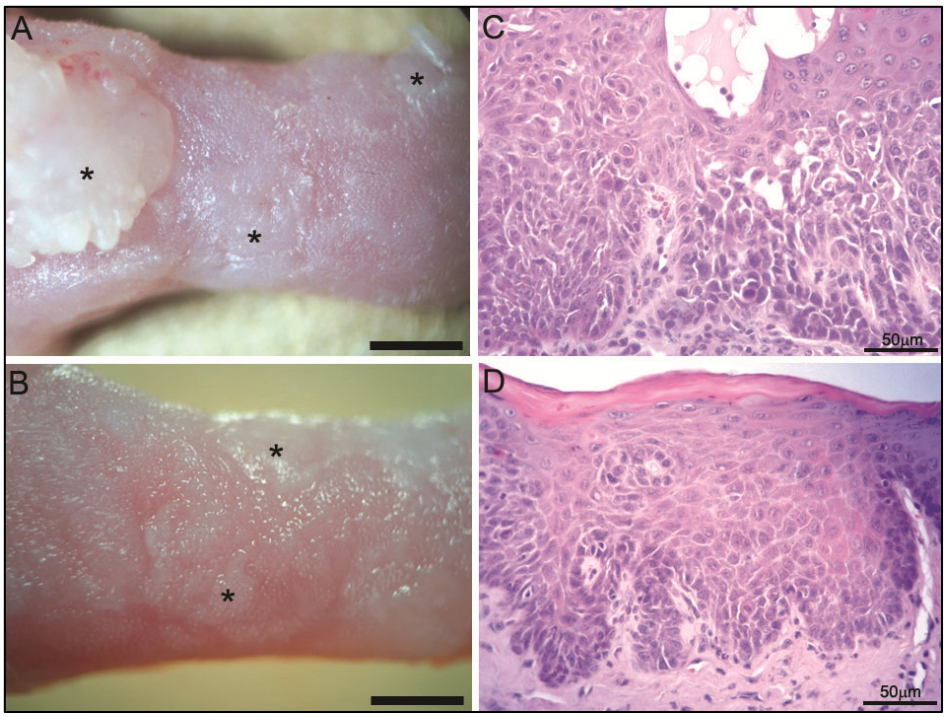


FIGURE 5 – Clinical and microscopic findings in WT and $CCL3^{-/-}$ mice after 4NQO treatment at 200 µg/mL (n=7 and n=4, respectively). Macroscopic tongue appearance of (A) WT and (B) $CCL3^{-/-}$ -treated mice. Asterisks indicate clinical lesions (Barr = 0.1 cm). (C) and (D) represent respectively, the histopathological findings in WT and $CCL3^{-/-}$-treated mice.

CCR1$^{-/-}$-treated mice presented similar production of tumors (Fig. 6A) and histopathological score (Fig. 6C and 6G) in comparison with those WT-treated mice (Fig. 6G). In contrast, CCR5$^{-/-}$-treated mice exhibited reduced incidence of tumor formation (Fig. 6B) and lower histopathological scores (Fig. 6D, 6G) that was similar to those CCL3-treated mice (Fig. 6G). Moreover, proliferative activity was decreased in lesions from CCR5$^{-/-}$-treated mice compared with CCR1$^{-/-}$-treated mice (Fig. 6F, 6E and 6H). CCR1$^{-/-}$ and CCR5$^{-/-}$ control groups did not present any changes in tongue surface (data not shown) or abnormalities in epithelium architecture (Fig. 6G).

The histopathological analysis of cervical lymph nodes and organs revealed no occurrence of metastasis after treatment with different doses of 4NQO. However, liver of treated mice presented variable degrees of hepatocytes tumefaction, steatosis and hemorrhage. The stomach and intestine also presented variable degrees of hyperqueratosis, hyperplasia and inflammation. These findings were similar for all groups of treated-mice (data not shown).

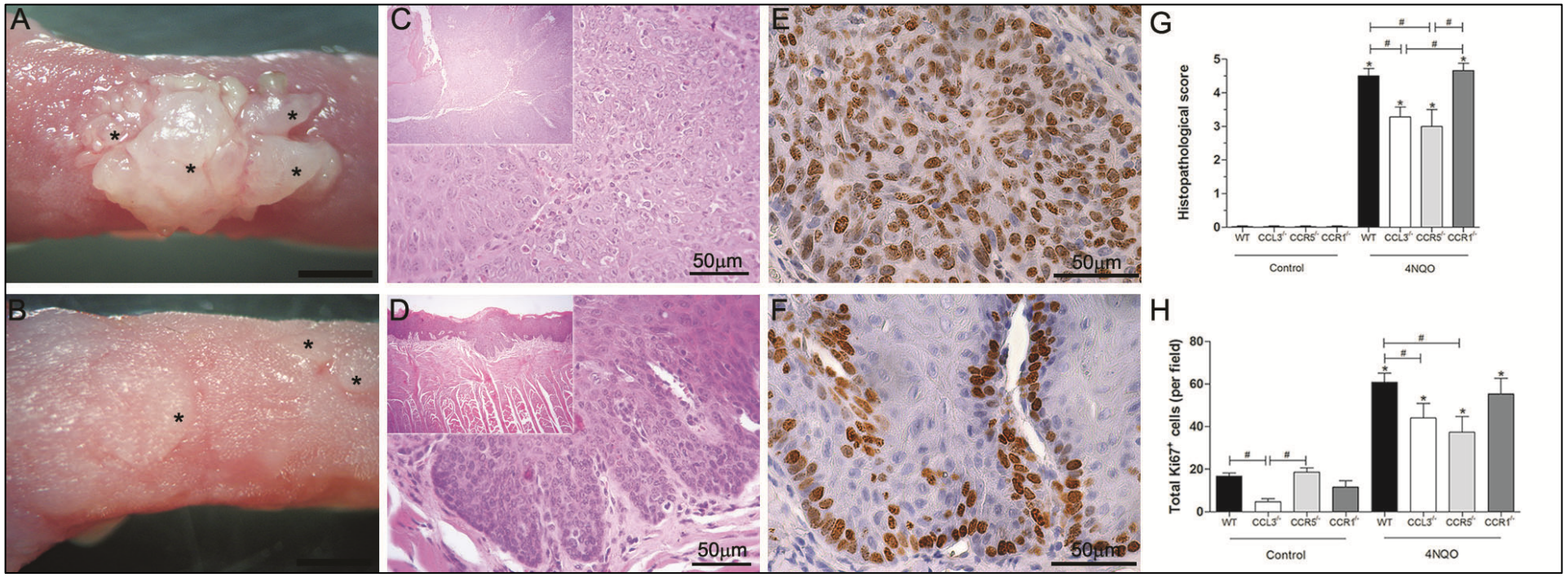


FIGURE 6 – Clinical and microscopic findings in tongue lesions of $CCR1^{-/-}$ and $CCR5^{-/-}$ mice after 4NQO treatment (n=6 and n=8, respectively). Macroscopic tongue appearance of $CCR1^{-/-}$ (A) and $CCR5^{-/-}$ (B) treated mice. Asterisks indicate clinical lesions (Barr = 0.1 cm). C-D and E-F represent respectively, the histopathological findings and Ki67 immunoexpression in tongue of $CCR1^{-/-}$ (C and E) and $CCR5^{-/-}$ (D and F) treated mice. G – Histopathological score of tongue lesions. H – Quantification of total $Ki67^{+}$ cells in WT, $CCL3^{-/-}$, $CCR1^{-/-}$ and $CCR5^{-/-}$ control and treated groups *P<0.05 in relation with respective control, #P<0.05 when comparing 4NQO- treated groups.

Mice lacking CCL3 have reduced expression of angiogenic factors and ECM components in SCC lesions

Since we have observed reduced SCC formation in CCL3 deficient mice, we next evaluated if expression of angiogenic factors, cytokines and ECM components would be consistently affected at the tumor milieu by absence of CCL3. WT-treated mice presented significantly increased expression of proliferative and angiogenic factors *Egf* , *Fgf1, Tgfβ1,Vegfa* and *Vegfb* - and inflammatory cytokines - *Il-6* and *Tnf-α*. Additionaly, there was increased expression of matrix components - *Col1a1*, *Mmp-1a*, *Mmp-2*, *Mmp-9* - and adhesion molecules - *Itga4* and *Vtn* in WT-treated mice in relation to non-treated group (Fig. 7A). On the other hand, $CCL3^{-/-}$-treated mice had significantly decreased expression of angiogenic factors *Egf, Fgf1, Tgfβ1, Vegfa, Vegfb,* inflammatory cytokines *Il-6* and *Tnf-α,* matrix components, *Mmp-1a*, *Mmp-2*, *Mmp-9*, and adhesion molecules *Itga4, Vtn* (Fig. 7B).

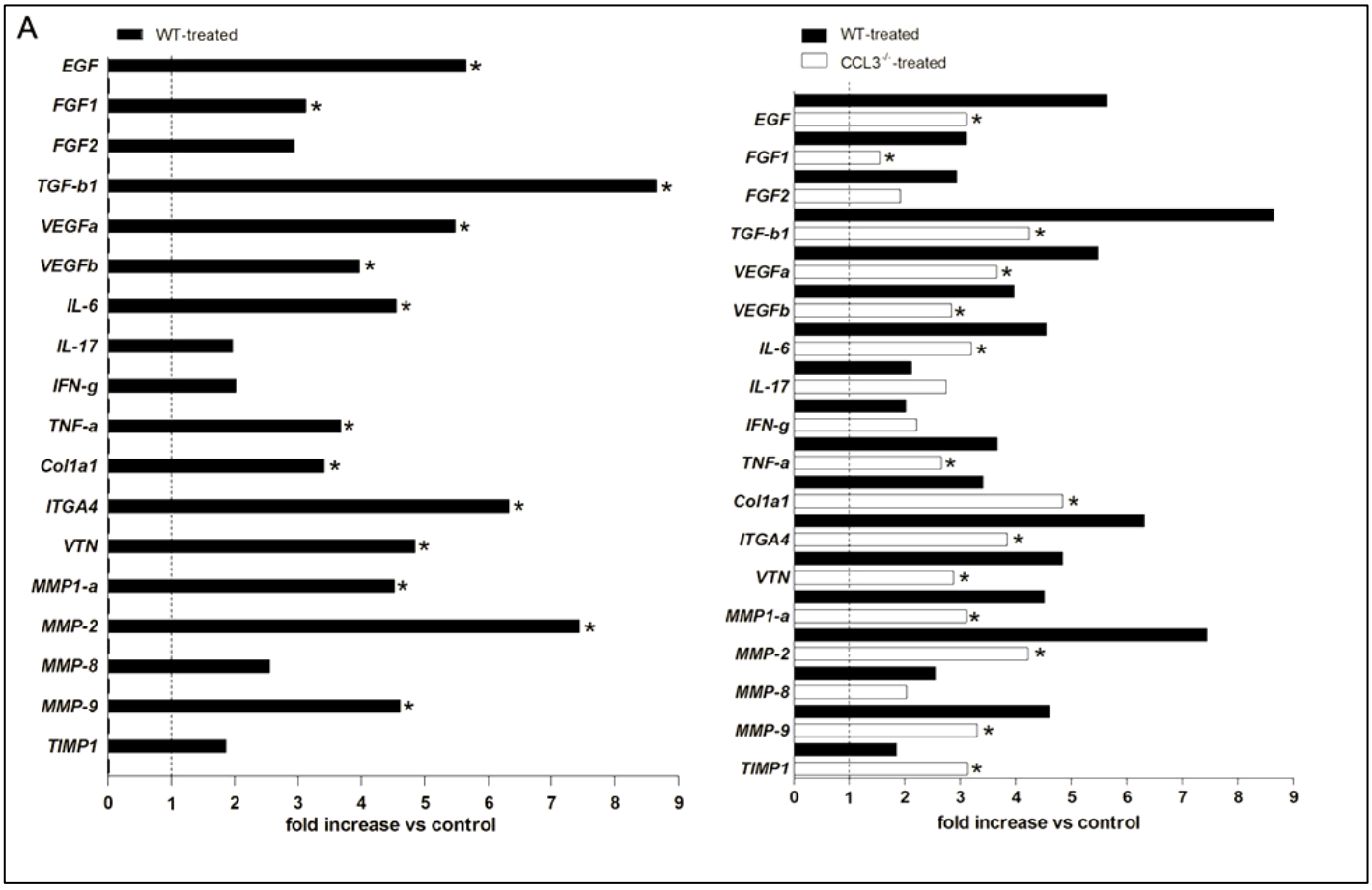


FIGURE 7 – Pattern of expression of angiogenic, pro-inflammatory cytokines, adhesion molecules and extracellular matrix components in SCC-induced lesions by PCR array in WT and CCL3$^{-/-}$ mice (n=5 per group). (A) Results after analysis comparing WT control and WT treated mice. (B) WT treated compared with CCL3$^{-/-}$ treated mice. Results were expressed as fold change relative to the control group after being normalized as the initial geometric mean of three constitutive genes (GAPDH, ACTB, Hprt1). * P<0.05.

CCL3 stimulated cell proliferation and induced expression of cytokines and MMP's

The evidence obtained in our previous experiments suggested that lack of CCL3 turned down pathways associated with invasiveness and proliferation in SCC. Next we sought to investigate if CCL3 directly affects neoplastic cell. Initially, we assessed CCL3 production by metastatic (HN12) and non-metastatic (HN13) lineages under steady state conditions, and observed that the metastatic lineage produced increased levels of CCL3 in relation to the non-metastatic (35.69±1.55 and 13.97±8.35, respectively). HN12 was then selected for subsequent experiment due to its metastatic behavior. We then determined the effect of different CCL3 concentrations (5, 10 or 20 ng/mL) on cellular viability. CCL3 at 10 ng/mL did not affect cell integrity and this concentration was used in subsequent experiments. Results showed that under CCL3 stimuli, HN12 cells produced significantly increased levels of CCL5 and IL-6 as compared with control (Fig. 8A and 8B). Levels of TNF-α did not increase significantly after CCL3 stimulation (Fig. 8C). As extracellular matrix components degradation is an essential step to tumor invasion [33] we analyzed if CCL3 would induce the expression of matrix metalloproteinases (MMPs). Indeed, there was a significant increase in production of MMP-2, MMP-8 and MMP-9 after CCL3 treatment (Fig. 8D, 8E and 8F). On the other hand, HN12 cultures stimulated with CCL3 had similar proliferation, as measured by expression of Ki67, in comparison with control (non-stimulated HN12 cells in DMEM-serum free media) (Fig. 8G).

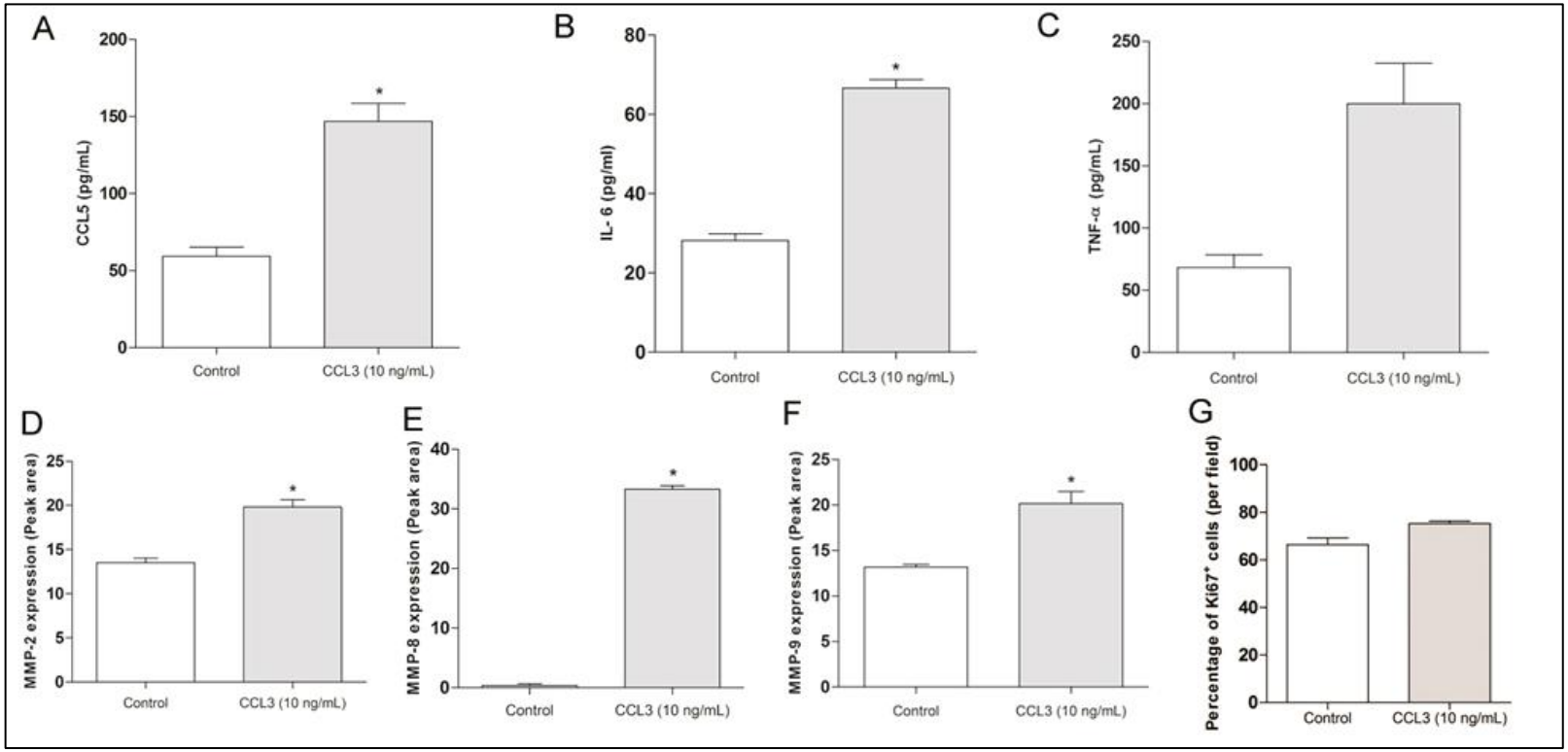


FIGURE 8 – Production of CCL5 (A), IL-6 (B) and TNF-α (C) by HN12 cultured cells after stimulation or not (control) with CCL3. Zymogram analysis of secreted MMP-2 (D), MMP-8 (E) and MMP-9 (F) by HN12 cells stimulated or not with CCL3. (G) Percentage of total HN12 $Ki67^{+}$ cells stimulated with CCL3. Experiments were performed in triplicate and results are representative of three independent experiments. *$P<0.05$ in relation with control.

Blockade of CCL3 significantly impaired invasion of HN12 cells in vitro

Previous results showed that CCL3 affects cell proliferation and ECM remodeling, then, we tested if CCL3 induces invasion of neoplastic cells in vitro. Results showed that after treatment with CCL3 (10 ng/mL), a significant increase of cells that invaded through membranes was seen in comparison with control group ($P<0.05$; Fig. 9). We further analyzed if therapies blocking CCL3 could prevent CCL3-mediated invasion. Treatment with anti-CCL3 antibody significantly impaired invasion of HN12 cells in relation to cells treated with CCL3. Evasin-1 also decreased the number of invaded cells (Fig. 9). Our results provide definitive evidence that CCL3 is relevant for oral carcinogenesis, regulating tumor cell interactions and neoplastic cells behavior.

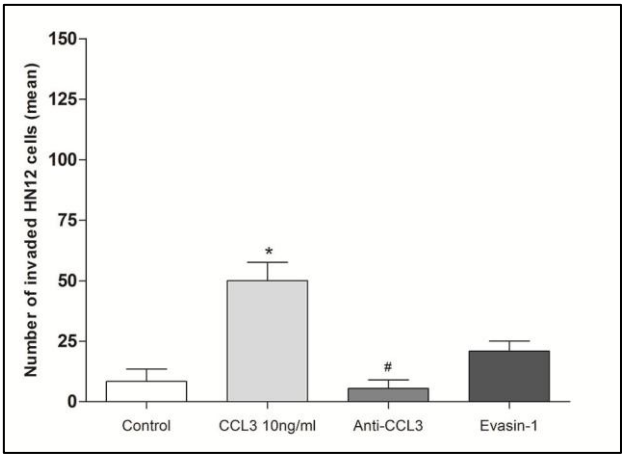


FIGURE 9 – Effect of pharmacological blockage of CCL3 on invasion of HN12 (metastatic) cells. Cells were first pre-treated with anti-CCL3 antibody (20 µg/mL) or Evasin-1 (10^{-7} M) and then, stimulated with CCL3 (10 ng/mL). Non-stimulated HN12 cells comprised the control group. Y axis represents the quantification of total number of invaded HN12 cells. Experiments were performed in triplicate and results from three independent experiments were considered. *$P<0.05$ in relation to control, #$P<0.05$ when comparing with CCL3-treated group.

DISCUSSION

The main findings of this study can be summarized as follows: 1) Enhanced expression of CCL3 was detected in experimentally-induced and human carcinogenesis; 2) Absence of CCL3 and CCR5, but not CCR1, reduced chemically-induced SCC formation as confirmed by clinical presentation, decreased histopathological scores and lower proliferative activity of lesions; 3) Oral carcinogenesis in the absence of CCL3 is associated with attenuated expression of angiogenic factors, cytokines and matrix metalloproteinases; 4) In vitro, CCL3 directly stimulates tumor cells to invade and produce cytokines and matrix metalloproteinases; 5) Specific effects of CCL3 on tumor cells were proved by using CCL3 antagonists. Finally, our results clearly demonstrated the relevance of CCL3 acting via CCR5 in oral carcinogenesis. This is the first study that provides mechanistic data linking CCL3 to oral cancer.

We first observed an increased CCL3 expression in human OSCC and experimentally-induced tongue carcinomas. Accordingly, CCL3 levels were shown to be increased in different types of tumors as hepatomas [16], multiple myeloma [20] and chronic lymphocytic leukemia [21]. We also verified the contribution of epithelial cells expressing CCL3 in human OSCC samples, which was higher compared with oral health mucosa and potentially malignant disorder OLK. OLK with different grades of epithelial dysplasia exhibited similar CCL3 expression what may suggest that this chemokine does not affect early stages of oral carcinogenesis. However, this topic needs to be further explored once malignant transformation rates in OLK could not be clearly defined [34]. Different from OSCC, in which CCL3 is significantly expressed in epithelium, in oral health mucosa there is a balanced expression in epithelium and

connective tissue. These findings might account for a role of CCL3 also in oral mucosa homeostasis as described in other processes [35].

Previous data from our group showed that CCL3 expression in primary OSCC was related with poor cumulative survival rates [23]. These results indicated a possible functional role for CCL3 in oral tumorigenesis and prompted us to explore it. Because of intrinsic limitations as the variability of tumors, stage and parameters analyzed in studies with human samples we herein used an acceptable model of oral carcinogenesis by administration of the water-soluble chemical carcinogen 4NQO. In our conditions, 4NQO treatment was efficient to produce tongue tumors as described elsewhere [25,32]. However, our data are not similar to a previous one [26], since no metastasis in cervical lymph nodes and other organs was detected using a high dose of carcinogen.

Treatment with 4NQO resulted in squamous cell carcinoma lesions formation in tongue of mice. Most of lesions analyzed were exophytic, papillomatous, white and base-attached. However, in the absence of CCL3 and CCR5, but not of CCR1, SCC formation was significantly reduced. Accordingly, microscopic analysis revealed decreased histopathological scores and decreased proliferative activity of lesions in mice lacking CCL3/CCR5. To date, this is the first demonstration that CCL3 preferably binding to CCR5 influences oral carcinogenesis. Other two studies found similar results where deficiency of CCL3/CCR5 system resulted in significantly reduced tumor formation and reduced lung metastasis [18,19]. On the other hand, a study demonstrated that absence of CCL3/CCR1 but not CCL3/CCR5 resulted in decreased incidence of hepatocellular carcinoma [17]. Such discrepancy could be explained due to differences in mice models and tumors in these studies.

Previous data from our group and others have highlighted the participation of other chemokines and receptors in OSCC development [11-15]. Some of these

molecules are capable of influencing pivotal events in tumorigenesis as regulation of inflammatory cell trafficking to tumor microenvironment and neovascularization [36,37]. In attempt to dissect if CCL3 affects tumor milieu we evaluated the expression of molecules involved with tumor proliferation (EGF, TGF-β and TNF-α) [38,39] and angiogenesis (Tgf-β1, Vegfa, Vegfb) [40-42]. We demonstrated that in the absence of CCL3, the expression of markers *Egf*, *Tgf-β1*, *Vegfa*, V*egfb*, *Il-6*, *Tnf-α*, *Itga4*, *Vtn* was significantly reduced. Previous studies corroborate these findings where reduced expression of angiogenic markers were observed in tumors from CCL3 deficient mice [17,18]. The decreased expression of adhesion molecules such as *Itga4* and *Vtn* in SCC lesions from CCL3 deficient mice may be indicative that CCL3 also interferes with neoplastic cells adhesion and motility within tumor microenvironment as described for other tumor types [43].

In vitro experiments account for a direct effect of CCL3 in neoplastic cells. First, we have observed abundant basal CCL3 production in vitro by HNSCC cell line with metastatic potential, as previously reported [44]. Although we observed in vivo the decreased Ki67 immunopositivity in mice lacking CCL3/CCR5, CCL3 stimulated HN12 cells proliferation similarly when compared with non-stimulated cells. Collectively, results indicate that CCL3 is not essential to induce cell proliferation at tumor sites because in vivo data probably resulted from reduced incidence of tumor formation in CCL3 and CCR5 deficient mice. On the other hand, HN12 cells were found to produce CCL5, IL-6 and TNF-α under CCL3 stimuli, consistently with reduced expression of these molecules in tongue tumors of CCL3 deficient mice, notably regarding the IL-6 and TNF-α. Accordingly, previous studies demonstrated the pro-inflammatory and pro-tumorigenic effects of IL-6, influencing tumor angiogenesis

for example [45]. From these results, we can hypothesize that CCL3 produced by neoplastic cells have autocrine and paracrine effects at tumor sites.

Tumor microenvironment remodeling is a critical step for invasion and metastasis [33]. MMP's production in OSCC tissues are chief in this processes [46,47]. In vitro, we verified that HN12 cells showed increased MMP's production and invasiveness when stimulated with CCL3. Interesting, SCC samples from CCL3 deficient mice exhibited diminished expression of *Mmp-1a*, *Mmp-2* and *Mmp-9*. Additionally, we observed that *Timp1* and *Col1a1* were consistently up-regulated in absence of CCL3. These results are similar to previous findings pointing CCL3 as stimuli for MMP-9 expression in metastatic murine lung tumors [18]. Confirming the specificity of CCL3 effect, a significant reduction in cellular invasion was observed by CCL3 antagonism. Similarly, previous data showed CCL3-induced pseudopodia formation in hepatoma cells [22]. The use of specific drugs to inhibit CCL3/CCR5 activity could represent potential strategies in context of oral cancer. In this regard, the use of Evasin-1, a binding-CCL3 protein [48-50], significantly reduced CCL3-mediated invasion by HNSCC cell line.

In conclusion, current findings provide definitive evidence of CCL3-CCR5 axis contribution in experimental oral carcinogenesis. Collectively, results suggest that CCL3 endogenously produced display direct regulatory effects in tumor cells and probably in neighboring cells at tumor sites. Moreover, CCL3 signaling affects pathways involved in tumor angiogenesis and tumor invasiveness through induction of extracellular matrix remodeling and gain of mobility of neoplastic cells.

ACKNOWLWDGMENTS

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - **Grant Support** (474106/2012-8 and 455334/2014-5), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

REFERENCES

1. Feller L, Lemmer J. Oral squamous cell carcinoma: epidemiology, clinical presentation and treatment. J Canc Ther 2012;3:263-8. doi:10.4236/jct.2012.34037.

2. Bagan J, Sarrion G, Jimenes Y. Oral cancer: clinical features. Oral Oncol 2010;46:414-7. doi: 10.1016/j.oraloncology.2010.03.009.

3. Massano J, Regateiro FS, Januario G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:67-6. doi: http://dx.doi.org/10.1016/j.tripleo.2005.07.038.

4. Hanaham D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Canc Cell 2012;21:309-22. doi: 10.1016/j.ccr.2012.02.022.

5. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454:436-44. doi: 10.1038/nature07205.

6. Soria G, Ben-Baruch A. The inflammatory chemokines CCL2 and CCL5 in breast cancer. Canc Lett 2008;267:271-85. doi: 10.1016/j.canlet.2008.03.018.

7. Levina V, Nolen BM, Marrangoni AM, Cheng P, Marks JR, Szczepanski MJ, et al. Role of eotaxin-1 signaling in ovarian cancer. Clin Cancer Res 2009;15:2647–56. doi: 10.1158/1078-0432.CCR-08-2024.

8. Rehman AO, Wang C. CXCL12/SDF-1 alpha activates NF-kappaB and promotes oral cancer invasion through the Carma3/Bcl10/Malt1 complex. Int J Oral Sci 2009;1:105–18. doi: 10.4248/IJOS.09059.

9. Hwang TL, Lee LY, Wang CC, Liang Y, Huang SF, Wu CM. CCL7 and CCL21 overexpression in gastric cancer is associated with lymph node metastasis and poor prognosis. World J Gastroenterol 2012;18:1249–56. doi: 10.3748/wjg.v18.i11.1249.

10. Cho YB, Lee WY, Choi SJ, Kim J, Hong HK, Kim SH, et al. CC chemokine ligand 7 expression in liver metastasis of colorectal cancer. Oncol Rep 2012;28:689–94. doi: 10.3892/or.2012.1815.

11. Uchida D, Begum N-M, Tomizuka Y, Bando T, Almofti A, Yoshida H, et al. Acquisition of lymph node, but not distant metastatic potentials, by the overexpression of CXCR4 in human oral squamous cell carcinoma. Lab Invest 2004;84:1538–46. doi:10.1038/labinvest.3700190.

12. Onoue T, Uchida D, Begum NM, Tomizuka Y, Yoshida H, Sato M. Epithelial-mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells. Int J Oncol 2006;29:1133–38. doi:10.3892/ijo.29.5.1133.

13. Uchida D, Onoue T, Tomizuka Y, Begum NM, Miwa Y, Yoshida H, et al. Involvement of an autocrine stromal cell derived factor-1/CXCR4 system on the distant metastasis of human oral squamous cell carcinoma. Mol Canc Res 2007;5:685–94. doi:10.1158/1541-7786.MCR-06-0368.

14. Chuang JY, Yang WH, Chen HT, Huang CY, Tan TW, Lin YT, et al. CCL5/CCR5 axis promotes the motility of human oral cancer cells. J Cell Physiol 2009;220:418–26. doi: 10.1002/jcp.21783.

15. Jung DW, Che ZM, Kim J, Kim K, Kim KY, Williams D, et al. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. Int J Canc 2010;127:332–44. doi: 10.1002/ijc.25060.

16. Lu P, Nakamoto Y, Nemoto-Sasaki Y, Fujii C, Wang H, Hashii M, et al. Potential interaction between CCR1 and its ligand, CCL3, induced by endogenously produced interleukin-1 in human hepatomas. Am J Pathol 2003;162:1249-58. doi:http://dx.doi.org/10.1016/S0002-9440(10)63921-1.

17. Yang X, Lu P, Fujii C, Nakamoto Y, Gao JL, Kaneko S, et al. Essential contribution of a chemokine, CCL3, and its receptor, CCR1, to hepatocellular carcinoma progression. Int J Canc 2006;118:1869–76. doi:10.1002/ijc.21596.

18. Wu Y, Li YY, Matsushima K, Baba T, Mukaida N. CCL3-CCR5 axis regulates intratumoral accumulation of leukocytes and fibroblasts and promotes angiogenesis in murine lung metastasis process. J Immunol 2008;181:6384-93. doi:10.4049/jimmunol.181.9.6384.

19. Sasaki S, Baba T, Shinagawa K, Matsushima K, Mukaida N. Crucial involvement of the CCL3/CCR5 axis-mediated fibroblast accumulation in colitis-associated carcinogenesis in mice. Int J Canc 2014;135:1207-306. doi: 10.1002/ijc.28779.

20. Vallet S, Pozzi S, Patel K, Vaghela N, Fulciniti MT, Veiby P, et al. A novel role for CCL3 (MIP-1α) in myeloma-induced bone disease via osteocalcin downregulation and inhibition of osteoblast function. Leukemia 2011;25:1174-81. doi: 10.1038/leu.2011.43.

21. Sivina M, Hartmann E, Kipps TJ, Rassenti L, Krupnik D, Lerner S, et al. CCL3 (MIP-1α) plasma levels and the risk for disease progression in chronic lymphocytic leukemia. Blood 2011;117:1662-69. doi: 10.1182/blood-2010-09-307249.

22. Yuan Y, Liu J, Liu Z, He Y, Zhang Z, Jiang C, et al. Chemokine CCL3 facilitates the migration of hepatoma cells by changing the concentration intracellular Ca. Hepatol Res 2010;40:424-31. doi: 10.1111/j.1872-034X.2009.00619.x.

23. Silva TA, Ribeiro FLL, Oliveira-Neto HH de, Watanabe S, Alencar R de CG, Fukada SY, et al. Dual role of CCL3/CCR1 in oral squamous cell carcinoma: implications in tumor metastasis and local host defense. Oncol Rep 2007;18:1107–13. doi:10.3892/or.18.5.1107.

24. Kuziel WA, Dawson TC, Quinones M, Garavito E, Chenaux G, Ahuja SS, et al. CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. Atherosclerosis 2003;167:25-32. doi: http://dx.doi.org/10.1016/S0021-9150(02)00382-9.

25. Tang XH, Knudsen B, Bemis D, Tickoo S, Gudas LJ. Oral Cavity and esophageal carcinogenesis modeled in carcinogen treated mice. Clin Canc Res 2004;10:301-13. doi:10.1158/1078-0432.CCR-0999-3.

26. Li J, Liang F, Yu D, Qing H, Yang Y. Development of a 4-nitroquinoline-1-oxide model of lymph node metastasis in oral squamous cell carcinoma. Oral Oncol 2013;49:299-305. doi: 10.1016/j.oraloncology.2012.10.013.

27. Barnes L, Eveson JW, Reichart PA, Sidransky D. World Health Organization classification of tumours. Pathology and genetics. Head and neck tumours. In: Slootweg

PJ, Eveson JW. Tumours of the oral cavity and oropharynx: Introduction. Lyon, France: IARC, 2005:166-75.

28. da Silva JM, Queiroz-Junior CM, Batista AC, Rachid MA, Teixeira MM, da Silva TA. Eosinophil depletion protects mice from tongue squamous cell carcinoma induced by 4-nitroquinoline-1-oxide. Histol Histopathol 2014;29:387–96. doi: 10.14670/HH-29.387.

29. Vieira AE, Repeke CE, Ferreira Junior S de B, Colavite PM, Biguetti CC, Oliveira RC, et al. Intramembranous bone healing process subsequent to tooth extraction in mice: micro-computed tomography, histomorphometric and molecular characterization. PLoS One 2015;10:e0128021. doi: 10.1371/journal.pone.0128021.

30. Cardinali M, Pietraszkiewicz H, Ensley JF, Robbins KC. Tyrosine phosphorylation as a marker for aberrantly regulated growth-promoting pathways in cell lines derived from head and neck malignancies. Int J Canc 1995;61:98-103. doi:10.1002/ijc.2910610117.

31. Frauenschuh A, Power CA, Deruaz M, Ferreira BR, Silva JS, Teixeira MM, et al. Molecular cloning and characterization of a highly selective chemokine-binding protein from the tick rhipicephalus sanguineus. J Biol Chem 2007;282:27250–58. doi:10.1074/jbc.M704706200.

32. Kanojia D, Vaidya MM. 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol 2006;42:655-667. doi:http://dx.doi.org/10.1016/j.oraloncology.2005.10.013.

33. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell 2011;147:275-92. doi: 10.1016/j.cell.2011.09.024.

34. van der Waal I. Oral potentially malignant disorders: is malignant transformation predictable and preventable? Med Oral Patol Oral Cir Bucal 2014;19:386-90. doi:10.4317/medoral.20205.

35. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 2014;32:659–702. doi: 10.1146/annurev-immunol-032713-120145.

36. Conti I, Rollins BJ. CCL2 (monocyte chemoattractant protein-1) and cancer. Semin Canc Biol 2004;14:149–54. doi:10.1016/j.semcancer.2003.10.009.

37. Azenshtein E, Luboshits G, Shina S, Neumark E, Shahbazian D, Weil M, et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Canc Res 2002;62:1093–1102.

38. Lee CH, Syu SH, Liu KJ, Chu PY, Yang WC, Lin P, et al. Interleukin-1 beta transactivates epidermal growth factor receptor via the CXCL1-CXCR2 axis in oral cancer. Oncotarget 2015;6:38866-80. doi: 10.18632/oncotarget.5640.

39. Bachman KE, Park BH. Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. Curr Opin Oncol 2005;17:49-54.

40. Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P. Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. J Cell Physiol 2009;219:449-58. doi: 10.1002/jcp.21706.

41. Chai ZT, Kong J, Zhu XD, Zhang YY, Lu L, Zhou JM, et al. MicroRNA-26a inhibits angiogenesis by down-regulating VEGFA through the PIK3C2α/Akt/HIF-1α pathway in hepatocellular carcinoma. PLoS One 2013;8:e77957. doi: 10.1371/journal.pone.0077957.

42. Liu G, Xu S, Jiao F, Ren T, Li Q. Vascular endothelial growth factor B coordinates metastasis of non-small cell lung cancer. Tumour Biol 2015;36:2185-91. doi: 10.1007/s13277-014-2829-5.

43. Ma YR, Ma YH. MIP-1α enhances Jurkat cell transendothelial migration by up-regulating endothelial adhesion molecules VCAM-1 and ICAM-1. Leuk Res 2014;38:1327-31. doi: 10.1016/j.leukres.2014.08.019.

44. Wolff HA, Rolke D, Rave-Fränk M, Schirmer M, Eicheler W, Doerfler A, et al. Analysis of chemokine and chemokine receptor expression in squamous cell carcinoma of the head and neck (SCCHN) cell lines. Radiat Environ Biophys 2011;50:145-54. doi: 10.1007/s00411-010-0341-x.

45. Shinriki S, Jono H, Ota K, Ueda M, Kudo M, Ota T, et al. Humanized anti-interleukin-6 receptor antibody suppresses tumor angiogenesis and in vivo growth of human oral squamous cell carcinoma. Clin Cancer Res 2009;15:5426-34. doi: 10.1158/1078-0432.CCR-09-0287.

46. Takayama S, Hatori M, Kurihara Y, Kinugasa Y, Shirota T, Shintani S. Inhibition of TGF-beta1 suppresses motility and invasiveness of oral squamous cell carcinoma cell lines via modulation of integrins and down-regulation of matrix-metalloproteinases. Oncol Rep 2009;21:205-10. doi: 10.3892/or_00000209.

47. Elahi M, Rakhshan V, Ghasemian NT, Moshref M. Prognostic value of transforming growth factor beta 1 [TGF-β1] and matrix metalloproteinase 9 [MMP-9] in oral squamous cell carcinoma. Biomarkers 2012;17:21-27. doi: 10.3109/1354750X.2011.635804.

48. Dias JM, Losberger C, De´ruaz M, Power CA, Proudfoot AEI, Shaw JP. Structural basis of chemokine sequestration by a tick chemokine binding protein: the crystal structure of the complex between Evasin-1 and CCL3. PLoS One 2009;4:e8514. doi: 10.1371/journal.pone.0008514.

49. Russo RC, Alessandri AL, Garcia CC, Cordeiro BF, Pinho V, Cassali GD, et al. Therapeutic effects of evasin-1, a chemokine binding protein, in bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 2011;45:72–80. doi: 10.1165/rcmb.2009-0406OC.

50. Castor MG, Rezende B, Resende CB, Alessandri AL, Fagundes CT, Sousa LP, et al. The CCL3/macrophage inflammatory protein-1alpha-binding protein Evasin-1 protects from graft-versus-host disease but does not modify graft-versus-leukemia in mice. J Immunol 2010;184:2646-54. doi: 10.4049/jimmunol.0902614.

Deletion of the atypical CC chemokine receptor ACKR2 does not modify experimental squamous cell carcinogenesis

Key words: *ACKR2, D6, Chemokines, OSCC*

Janine Mayra da Silva[1,2], Tálita Pollyanna Moreira dos Santos[3], Remo Castro Russo[4], Adriana Machado Saraiva[1], Gustavo Pompermaier Garlet[5], Mauro Martins Teixeira[6], and Tarcília Aparecida da Silva[1,2].

[1] Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

[2]Department of Morphology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

[3]Department of Clinical Analysis, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil.

[4]Laboratory of Pulmonary Immunology and Mechanics, Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

[5]Department of Biological Sciences, School of Dentistry, Universidade de São Paulo, Bauru, Brazil.

[6]Laboratory of Immunopharmacology, Department of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

Grant Support: This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (474106/2012-8 and 455334/2014-5).

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Reprint requests to: Tarcília Aparecida da Silva Mailing address: Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31.270-901, Belo Horizonte, Minas Gerais, Brazil. Phone: 55 31 3409-2478 (voice); 55 31 3499-2430 (Fax). E-mail: tarcilia@ufmg.br

ABSTRACT

Chemokines and chemokine receptors are considered critical in oral tumorigenesis. The atypical chemokine receptor ACKR2 is a scavenger of CC chemokines controlling the availability of these molecules. In this study, we evaluate if the deletion of ACKR2 affects oral carcinogenesis. WT and ACKR2 deficient mice ($ACKR2^{-/-}$) were treated with 4-nitroquinoline-1-oxide (4NQO) in drinking water for 28 weeks. Tongues were collected for macro and microscopic analysis and to evaluate expression of CC chemokines and its receptors, inflammatory cytokines, angiogenic factors, adhesion molecules and extracellular matrix components. Results revealed similar incidence of squamous cell carcinoma (SCC) lesions in WT and $AKCR2^{-/-}$ mice. Consistently, microscopic analysis showed similarity in histopathological parameters in both mice lineages. The mRNA expression of CC-chemokines, *CCL2*, *CCL3*, *CCL4*, *CCL5*, *CCL12* and the receptors *CCR1*, *CCR2* and *CCR5* were increased in tongue lesions of both groups in relation to controls. However, SCC lesions of $ACKR2^{-/-}$ exhibited higher expression of CCL2, IL-6 and IL-17 than WT mice. The results suggest that deletion of ACKR2 affects tumor microenvironment, but these changes are not sufficient to modify the course of chemically-induced oral carcinogenesis in mice.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common oral neoplasm worldwide and frequently presents an unfavorable prognosis (Ferlay et al., 2014; Warnakulasuriya, 2009). The locally infiltrative and metastasizing behavior of OSCC is linked to neoplastic cells and their surrounding stroma. It comprises a wide range of molecules influencing angiogenesis, tumor growth, invasion and metastasis (Koontongkaew, 2013). In this regard, chemokines display critical functions in oral tumorigenesis (reviewed by Silva et al., 2016). Some studies showed that CC chemokines e.g. CCL2, CCL5, CCL7, CCL21 and respective receptors have pro-tumorigenic effects *in vitro* and/or *in vivo* (Li, et al., 2014; Chuang et al., 2009; Shang et al., 2009; Jung et al., 2009). Moreover, an increased expression of CC chemokines was detected in OSCC (Ferreira et al., 2008; Silva et al., 2007) and in experimentally-induced oral carcinomas (Silva et al., 2014). The availability of these molecules in tumor microenvironment is regulated by atypical chemokine receptors, recently characterized, as ACKR2 (previously named D6) (Vetrano et al., 2010; Jamieson et al., 2005; Savino et al., 2014; Langenes et al., 2013). ACKR2 binds with high affinity to CC chemokines acting as "scavenger" (i.e. internalizing, but without triggering intracellular signaling function) and therefore, controls tumor-associated inflammatory response *in vivo* (Massara et al., 2016; Vetrano et al., 2010; Jamieson et al., 2005). This receptor is expressed in tissues and cell types from distinct sources, as in lymphatic cells (Hansell et al., 2015), skin (Jamieson et al., 2005) and by neoplastic cells (Langenes et al., 2013; Savino et al., 2014).

The protective effects of ACKR2 was demonstrated in Kaposi Sarcomas (Savino et al., 2014), chemically skin-induced tumors (Nibbs et al. 2007), breast cancer (Wu et

al., 2008), and colon adenocarcinomas (Langenes et al., 2013). To date, no studies have focused in ACKR2 in oral cancer. Therefore, in the current study the involvement of ACKR2 in oral tumorigenesis was investigated employing a chemically-induced model of squamous cell carcinoma in mice.

MATERIALS AND METHODS

Animals

C57BL/6 (*wild type* - WT) 6-8 weeks male mice were obtained from Centro de Bioterismo do Instituto de Ciências Biológicas (CEBIO), Universidade Federal de Minas Gerais, Brazil. ACKR2$^{-/-}$ deficient mice were bred and obtained from Laboratory of Pulmonary Immunology and Mechanics, Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Brazil. Mice were maintained in the Immunopharmacology laboratory's animal facility under standard conditions with a 12 h light/dark cycle, controlled temperature (24 ± 2°C) and free access to commercial chow and water *ad libidum*. All experimental procedures described in the current study were approved by the institutional Ethics Committee in Animal Experimentation (protocol 12/2011).

Experimental OSCC induction

The induction of oral carcinogenesis was performed as previously described (Tang et al., 2004; Li et al., 2013). The 4-nitroquinoline-1-oxide (4NQO) (Sigma-Aldrich) was obtained as a powder, dissolved in ethylene glycol (Sigma-Aldrich) and stored at 4 °C until administration. Weekly, a stock solution was prepared and diluted in mice drinking water at 50 µg/mL. Experimental mice received 4NQO daily during 28 weeks and control mice (n=5 per group) received drinking water for the same period. As a purpose of stablishment of lymph node metastasis model, mice were also submited to treatment with 4NQO at 200 µg/ml, as previously reported, (LI *et al*.,2013). Mice were treated during 20 weeks, when 4NQO was substituted by water and mice were followed until the twenty-eighth week.

No difference of mean intake of 4NQO or water was observed among groups. After 28 weeks, mice were euthanized and tongue, cervical lymph nodes, liver, lungs, stomach, duodenum, jejunum, ileum and large intestine were collected for microscopic analysis. Tongues were photographed for macroscopic analysis.

Light microscopy analysis

The tongues, cervical lymph nodes, liver, lungs, stomach, duodenum, jejunum, ileum and large intestine of 11 WT and 11 ACKR2 deficient mice were fixed in 10% buffered formalin, embedded in paraffin wax, longitudinally cut (3 µm sections) and stained with H&E. The histopathological analysis of tongue sections was performed by two examiners (J.M.S and T.A.S) that were properly blinded from the group status. The Intraclass Correlation Coefficient test was performed to validate the reliability inter- and intra-examiner. Ten representative fields were considered and the histopathological grading of tongue lesions was performed taking into account the following scores (adapted from Barnes et al., 2005): 0 – normal epithelial architecture, 1 - mild dysplasia (changes limited at basal third of lining epithelium), 2 - moderate (changes present in two-thirds of lining epithelium), 3 - severe (more than two-thirds of epithelium affected), 4 - carcinoma *in situ* (full thickness of the lining epithelium, but without involvement of the connective tissue) and 5 - invasive carcinoma (carcinomatous islands into the connective tissue). At least three sections of each organ were evaluated by a general pathologist (M.A.R) blinded of group status.

Immunohistochemistry and cell counting

The immunohistochemical staining was performed using the streptavidin-biotin method as previously reported (Silva et al., 2014). Briefly, sections of mouse tongue tumors were deparaffinized, dehydrated and rinsed in distilled water. The antigen retrieval was performed at 96 ºC for 25 minutes in citric acid buffer (pH=6.0), then, slides were incubated with 0.3% hydrogen peroxide 15 minutes twice and with Avidin/Biotin blocking system (Dako) following the manufacturer instructions. Then, sections were incubated at 4 ºC overnight with rabbit polyclonal anti-mouse Ki-67 (cat.15580, ABcam) followed by incubation with LSAB Secondary Antibody Kit (Dako). Negative controls were obtained by omission of primary antibody, which were substituted by 1% PBS-BSA. The Ki67 immunostained cells were analyzed by light microscope (Axioskop 40 Zeiss) at 1000x original magnification and counted in 20 consecutive fields in two sections. Results are expressed as the total number of $Ki67^{+}$ cells in all epithelial layers, per field.

Real Time PCR array

Real Time PCR array reactions were performed as previously described (Vieira et al., 2015). Briefly, the total RNA was obtained from gross lesions and from clinically normal tongue samples (*n=4* per group). Extraction was performed with the RNeasyFFPE kit (Qiagen Inc) according to the manufacturer instructions. The complementary DNA was synthesized by reverse transcription reaction (Superscript III, Invitrogen) from 3 µg of total RNA and Real Time PCR array was performed employing a custom panel containing the targets "Wound Healing" (PAMM-121) and "Inflammatory cytokines and receptors" (PAMM-011) (SABiosciences). Data were analyzed by the RT2 profiler PCR Array Data Analysis online software

(SABiosciences) for normalizing the initial geometric mean of the constitutive genes GAPDH, ACTB and Hprt1 and subsequently normalized by the control group, and expressed as fold change relative to the control group.

Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5.0 (GraphPad Software Inc.). The Student *t* Test was performed after checking for data normality. The results are reported as the means ± SD. *P* values <0.05 are considered to be statistically significant. For PCR array data, statistical significance was evaluated by the Mann-Whitney test, and the values tested for correction by the Benjamini - Hochberg Procedure.

RESULTS

In this study we first aimed to dissect the significance of the atypical chemokine receptor ACKR2 in chemically-induced oral squamous cell carcinoma in tongue of WT and ACKR2$^{-/-}$ deficient mice. Clinic examination of SCC-induced lesions showed apparent similarity in tumors induced by 4NQO (50 µg/mL) treatment. The lesions were mostly exophytic (protruding from tongue surface) or papillomatous, whitish and base attached (Fig. 1C and 1D). No macroscopic changes in tongue surface were observed in the control groups (Fig. 1A and 1B).

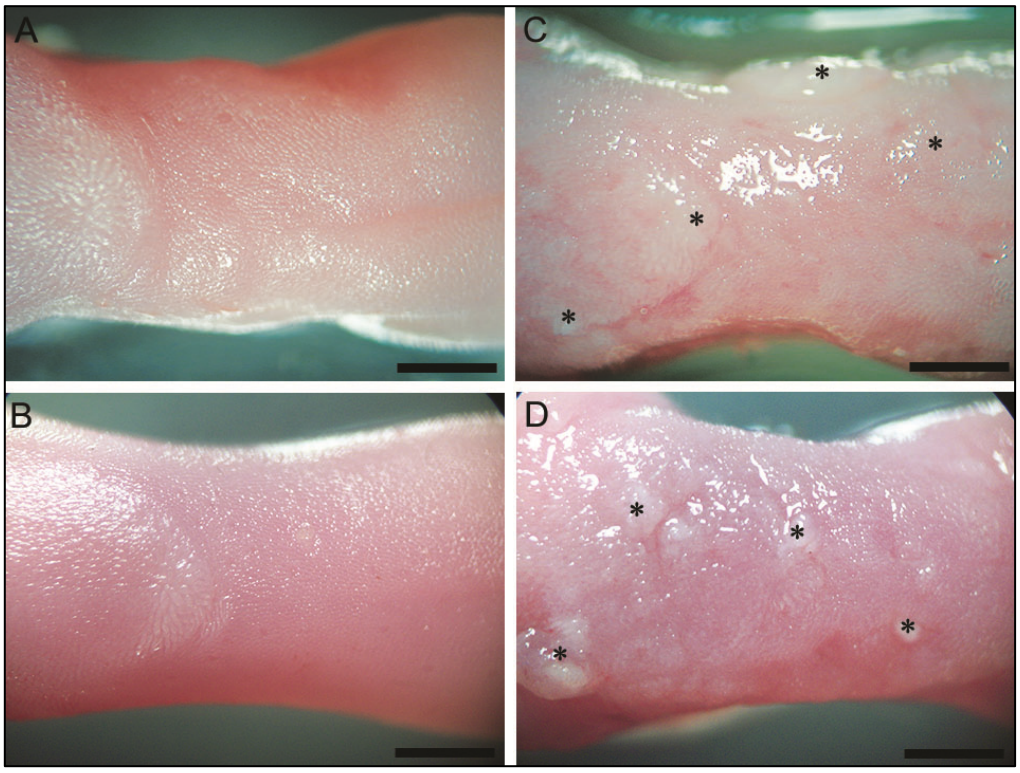


FIGURE 1 – Clinical findings of SCC-induced lesions in WT and ACKR2$^{-/-}$ mice after 4NQO treatment (*n=5*, per group). Macroscopic tongue appearance of WT (A) and ACKR2$^{-/-}$ (B) control mice (*n=5* per group). SCC gross lesions in WT (C) and ACKR2$^{-/-}$ (D) -treated mice. Asterisks indicate clinical lesions (Bar = 0.1 cm).

Microscopic analysis was consistent with clinical findings and results showed increased cytomorphological atypia and similar scores of epithelial dysplasia in WT and ACKR2$^{-/-}$-treated groups (Fig. 2C, 2D and 2G). The immunohistochemical staining revealed significantly increased percentage of Ki67^{+} keratinocytes in lining epithelium and connective tissue in both treated groups after 4NQO treatment in relation to respective control groups (Fig. 2H). However, no significant difference was seen when comparing WT and ACKR2$^{-/-}$ treated mice (Fig. 2H).

In addition, WT and ACKR2$^{-/-}$ mice were submitted to a "more aggressive" OSCC model by using four times greater concentration of 4NQO (200 µg/mL). Consistently, results showed increased cytomorphological atypia and lesions were graded with the worse epithelial dysplasia score (100% invasive carcinoma) in WT and ACKR2$^{-/-}$ treated groups, but no differences were observed between treated groups in both concentrations tested (Fig. 2E, 2F and 2G).

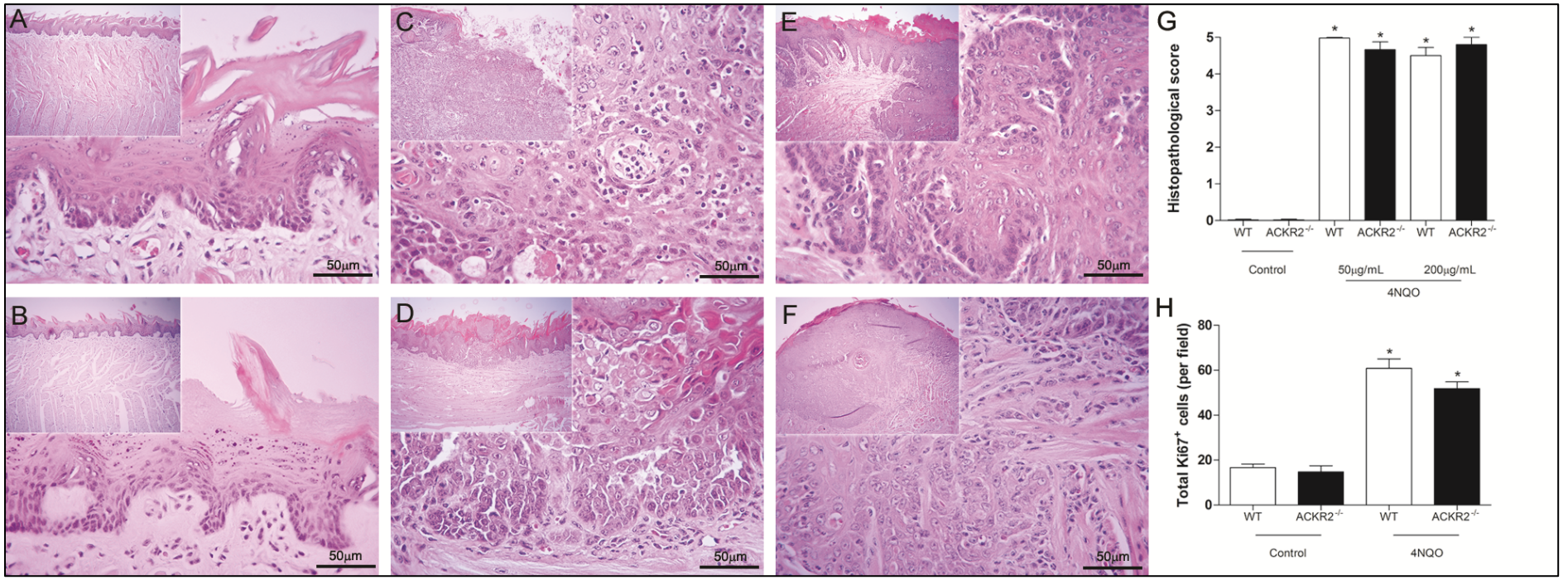


FIGURE 2 – Representative photomicrographs of histopathological findings in control and treated mice after 4NQO (*n=5*, per group). A and B – Absence of epithelial abnormality in WT and ACKR2$^{-/-}$ control mice. C and D represent the microscopic aspect of WT and ACKR2$^{-/-}$ treated with 4NQO at 50 µg/mL, and E and F WT and ACKR2$^{-/-}$ at 200 µg/mL. G – Histopathological score of tongue lesions. H - Quantitative analysis of Ki67 immunoexpression in basal and suprabasal epithelial layers in control and mice treated with 4NQO at 50 µg/mL. *p<0.05 in relation to respective control.

Taking into account the CC chemokine "scavenging" ability reported to ACKR2 in controlling tumor-associated inflammatory response, we next analyzed the expression of CC-chemokines and their receptors. We also evaluated the inflammatory cytokines *Interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-12, IL-17, Interferon gamma - IFN-γ, and Tumor Necrosis Factor-alpha – TNF-α* in tongue lesions. Interestingly, results showed important increase of chemokines *CCL3* and *CCL5* - and their receptors *CCR1, CCR2* and *CCR5*, as well as *CCL4* and *CCL12* in both treated groups when compared with controls. In addition, the mRNA expression of chemokine *CCL2* was found -*one fold increase* higher in SCC-induced lesions from ACKR2$^{-/-}$ in relation to WT (Fig. 3A). Consistently, all inflammatory cytokines analyzed showed increase in WT and ACKR2$^{-/-}$-treated groups, except for *IL-4*. The expression of *IL-6* and *IL-17* were significantly enhanced in ACKR2$^{-/-}$ -treated when compared to WT-treated mice (Fig. 3B). We further analyzed the expression of angiogenic factors, cellular adhesion molecules and extracellular matrix components. Results showed significant similar increased expression of angiogenic (*Epidermal Growth Factor – Egf, Fibroblast Growth Factor 1 – FGF1and FGF2, Vascular Endothelial Growth Factor a – Vegfa* and *Vegfb)*, the adhesion proteins (*Collagen 1a1 – Col1a1, Col1a2, Integrin A4 – Itga4*, and *Vitronectin – Vtn*) and extracellular matrix components (*Matrix Metalloproteinase 1a – Mmp-1a, MMP-8, MMP-9, Tissue inhibitor of metalloproteinase1 - Timp1 and Timp3 and Serpine*) in induced lesions of both groups after 4NQO treatment in relation to the controls (Fig. 3C). No significant differences were seen in the expression of these molecules when comparing SCC lesions from ACKR2$^{-/-}$-treated and WT-treated mice (Fig. 3C).

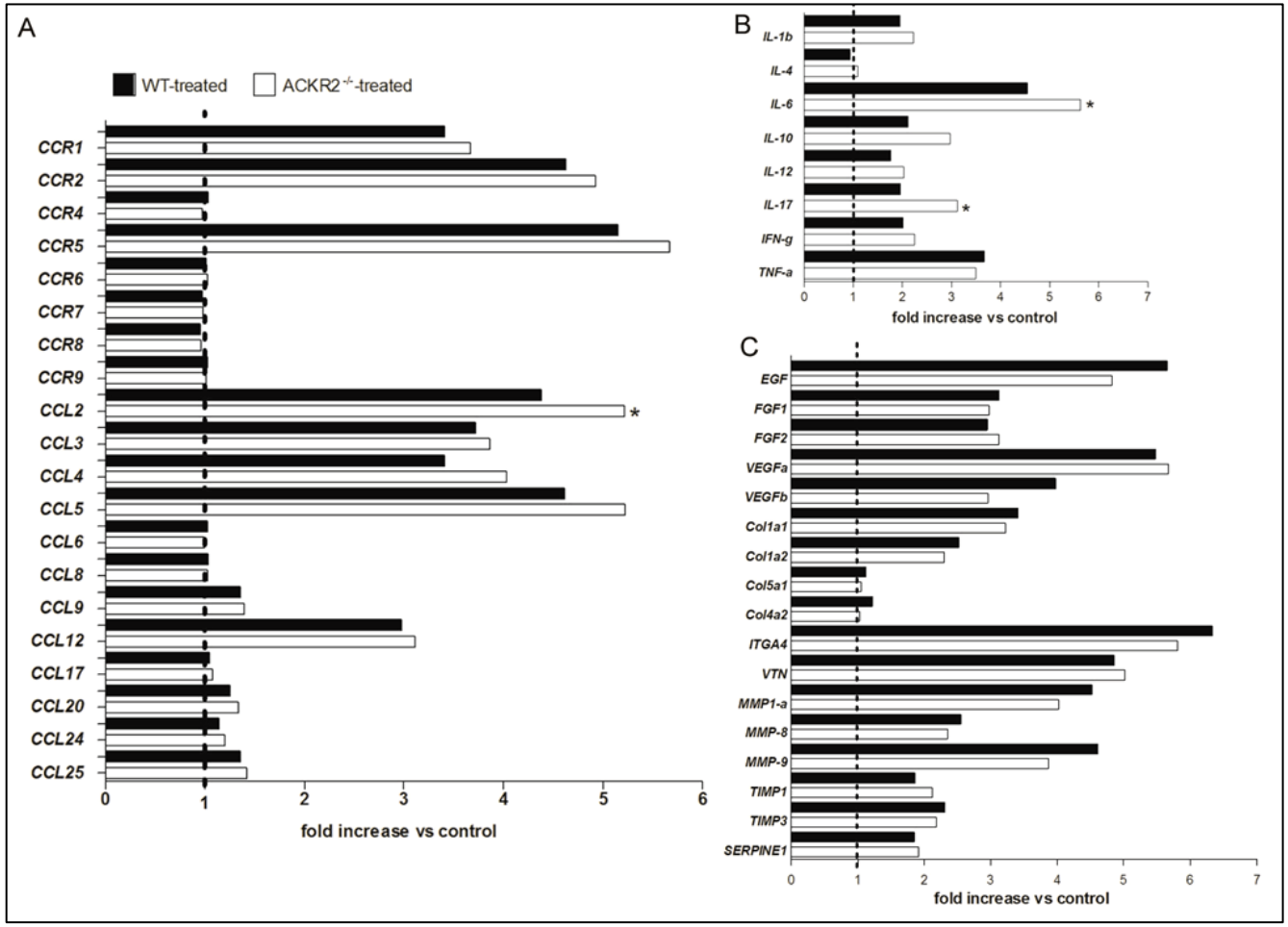


FIGURE 3 – Molecular expression profile in tongues comparing ACKR2$^{-/-}$ (*n=4*) and WT (*n=4*) mice after 4NQO treatment. A – Expression of CC chemokines and chemokine receptors, B – inflammatory cytokines expression and C – angiogenic, adhesion proteins and extracellular matrix remodeling markers. Results were expressed as fold change relative to the control group after being normalized by constitutive genes (GAPDH, ACTB, Hprt1). * $P<0.05$.

DISCUSSION

The atypical chemokine receptor ACKR2 promotes clearance of CC chemokines, controlling tumor-associated inflammatory responses (Nibbs et al., 2007; Vetrano et al., 2010, Wu et al., 2008). Previous studies highlighted the importance of CC chemokines in oral cancer (Li, et al., 2014; Chuang et al., 2009; Shang et al., 2009; Jung et al., 2010), but there are no information of ACKR2 effects in oral tumorigenesis. Our results demonstrated that ACRK2 deletion did not modify the clinical course of oral tumorigenesis in this mice model. Despite similarity of histopathological grade of SCC lesions, the expression of inflammatory molecules (*CCL2*, *IL-6* and *IL-17*) was significantly increased in lesions of ACKR2 knockout mice. These results indicate that deletion of ACRK2 impacts on SCC microenvironment, but the changes are insufficient to modify tumor progression.

Herein, both ACKR2$^{-/-}$ and WT -treated mice had similar characteristics in induced-SCC lesions, regarding macroscopic and histopathological parameters. The current findings are in accordance with previous studies showing that mice lacking ACRK2 did not differ for hepatic tumor formation when compared with WT, but these mice exhibited significant increase of CC chemokines and leucocytes (Schneider et al., 2012). On the other hand, preclinical models with constant induction of inflammation (i.e. DSS-induced colon cancer and DMBA/TPA skin-induced tumors) showed that mice lacking ACKR2 were more susceptible for tumor formation when compared to WT. Indeed, these studies have observed a predominant pro-inflammatory phenotype in absence of ACKR2 with significative accumulation of chemokines and massive infiltration of inflammatory cells (Vetrano et al., 2010; Nibbs et al., 2007). In addition, protective activities of ACKR2 were demonstrated by others, where down-regulation of

ACKR2 in colon adenocarcinomas correlated with increased expression of chemokine CCL22 and worse tumor parameters (Langenes et al., 2013).

Because we did not observe significant difference in SCC induction between WT and ACKR2$^{-/-}$, we submitted mice to a high-dose of 4NQO, as previously reported (Li et al., 2013). Nevertheless, no difference in clinical or microscopic parameters was detected between the groups. Also, high-dose of 4NQO did not induce lymph node metastasis or distant metastasis. In addition, the proliferative index of tongue lesions was consistent with our clinical and histopathological findings. An increased index of Ki67 staining were similarly seen in WT and ACKR2$^{-/-}$ -treated mice suggesting that ACKR2 does not affect cell proliferation. However, a previous study did observe a significant increase of epidermal proliferation in basal and suprabasal layers after topic application of phorbol-ester in skin of ACKR2$^{-/-}$ mice (Jamieson et al., 2005).

The protective effects of ACKR2 are attributed to its anti-inflammatory properties due to reduced availability of inflammatory mediators and infiltration of leukocytes (Wu et al., 2008; Nibbs et al., 2007; Vetrano et al., 2010, Savino et al., 2014). Interestingly, Schneider et al. (2012) showed that although DEN-induced hepatocarcinogenesis increased intrahepatic expression of CC chemokines and tumor-associated inflammation in ACKR2$^{-/-}$ mice, these changes were not sufficient to influence tumor formation and progression (Schneider et al., 2012). Regarding our findings, one important point that deserves discussion is that by 4NQO-induced SCC usually does not exhibit pronounced immune cell infiltration (Gannot et al., 2004; Silva et al., 2014). Moreover, the experimental design with one point evaluated in time (28 weeks of carcinogen treatment), precludes drawing inferences of ACKR along neoplastic events. From this, the current results do not allow us to exclude ACKR2 relevance on oral carcinogenesis. Moreover, other ACKR's were implicated in cancer

progression (Massara et al., 2016). In this regard, ACKR1 (previously called DARC), ACKR2 and ACKR4 (CCX-CKR) showed protective roles in tumors. For example, ACRK1 which is expressed by red blood cells promoted scavenging of angiogenic chemokines and suppressed blood vessel density and prostate tumor growth (Shen et al., 2006). ACKR2 inhibited leukocyte infiltration and consequently inflammation in tumors (Nibbs et al., 2007, Vetrano et al., 2010; Savino et al., 2014). Moreover, ACKR4 was demonstred to control tumor growth and metastasis because its properties of scavenging homeostatic chemokines CCL19/CCL21 in hepatocelullar carcinomas (Shi et al., 2014). On the other hand, ACKR3 (termed CXCR7) exhibited dual functions in breast cancer once its expression by cancer cells promoted tumor proliferation and angiogenesis (enhancing microvessel density and VEGFa) *in vitro*, whereas it was able to impair cell invasion in response to metastatic chemokine CXCL12 *in vivo* and *in vitro* (Hernandez et al., 2011). These findings clearly indicate that ACKR's deserve further investigation in oral cancer.

Because tumor milieu is a rich environment that comprises a wide range of molecules as chemokines, growth factors, inflammatory mediators that may interfere with tumorigenesis (Hanahan, Coussens 2012; Balkwill, 2004) and because ACKR2 was demonstrated to efficiently control inflammatory response *in vivo*, our next approach was to evaluate expression of CC-chemokines and receptors related with ACKR2 functions. Indeed, we have seen a coherent residual accumulation of β-chemokines *CCL3*, *CCL4* and *CCL5* in absence of ACKR2. Additionally, *CCL2* was significantly accumulated in tongue lesions of ACKR2$^{-/-}$ treated mice. These findings are in accordance with previous reports (Jamieson, et al., 2005; Nibbs et al., 2007; Vetrano et al., 2010; Schneider et al., 2012; Hansell et al., 2015) and corroborate the CC-chemokine scavenging abilities of ACKR2.

Moreover, expression of inflammatory cytokines showed significant two- to six fold increase of most evaluated molecules, *IL-10*, *IFN-γ* and *TNF-α*, which was similar when comparing WT and ACKR2$^{-/-}$mice. Interestingly, *IL-6* and *IL-17* were significantly increased in ACKR2$^{-/-}$ compared to WT. Consistently, increased production of TNF-α and IL-17 was seen in inflamed joints of ACKR2$^{-/-}$ arthritic mice compared to WT (Hansell et al., 2015). Additionally, ACKR2 deletion resulted in enhanced TNF-α, IFN-γ and IL-17A levels in late stages of DSS-induced colitis, whereas CC chemokines and IL-2, IL-10, IL-12 or IL-23 remained unchanged, regardless of better clinical symptoms and histological score. This paper showed IL-17^{+} γδ T cells into inflamed colon. Curiously, these protective effects observed in absence of ACKR2 were abolished after anti-IL-17 administration, indicating a regulatory effect in host inflammatory responses and concluding that impact of ACKR2 deficiency depends on the precise nature of challenge (Bordon et al., 2009).

Taking into account the increased activation of some inflammatory pathways in SCC-induced lesions in absence of ACKR2, we next evaluated the expression of mediators involved with tumor proliferation, angiogenesis and tissue remodeling. Herein, we did observe that 4NQO treatment induced coherent increase of proliferative (*EGF*), angiogenic (*FGF1* and *FGF2*, *VEGFa* and *VEGFb*) and tissue remodeling (collagen, adhesion proteins, MMP's and TIMP's) markers in SCC-induced lesions, although no significative differences were achieved when comparing ACKR2$^{-/-}$ with WT. In contrast, Savino et al. (2014) showed increase of VEGFa and CD31 in transfected Kaposi sarcoma sections that did not express the receptor ACKR2 (KS^{-}) (Savino et al., 2014). The significance of these mediators triggering cell proliferation, angiogenesis and extracellular matrix remodeling have been reported in oral tumorigenesis (Datekoa et al., 2012; Vassilakopoulou et al., 2015; Stokes et al., 2010).

Current results suggest that ACKR2 does not affect proliferation/angiogenic and matrix remodeling pathways in our conditions. In conclusion, the precise mechanism of ACKR2 deficiency in SCC-induced tumorigenesis and progression (i.e. if inflammatory mediators activate specific host immune responses, and/or possible effects of other ACKR's) could be explored in further studies. Results of the present study indicate that ACKR2 may turned up inflammatory pathways at tumor sites but these changes did not influence the course of tongue carcinogenesis in this mice model.

ACKNOWLEGMENTS

We are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

REFERENCES

Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004;4:540–50. doi:10.1038/nrc1388.

Barnes L, Eveson JW, Reichart PA, Sidransky D. World Health Organization classification of tumours. Pathology and genetics. Head and neck tumours. In: Slootweg PJ, Eveson JW. Tumours of the oral cavity and oropharynx: Introduction. Lyon, France: IARC, 2005. p.166-175.

Bordon Y, Hansell CA, Sester DP, Clarke M, Mowat AM, Nibbs RJ. The atypical chemokine receptor D6 contributes to the development of experimental colitis. J Immunol 2009;182:5032-40. doi: 10.4049/jimmunol.0802802.

Chuang JY, Yang WH, Chen HT, Huang CY, Tan TW, Lin YT, et al. CCL5/CCR5 axis promotes the motility of human oral cancer cells. J Cell Physiol 2009;220:418–26. doi:10.1002/jcp.21783.

da Silva JM, Queiroz-Junior CM, Batista AC, Rachid MA, Teixeira MM, da Silva TA. Eosinophil depletion protects mice from tongue squamous cell carcinoma induced by 4-nitroquinoline-1-oxide. Histol Histopathol 2014;29:387–96. doi: 10.14670/HH-29.387.

da Silva JM, Soave DF, Moreira Dos Santos TP, Batista AC, Russo RC, Teixeira MM, Silva TA. Significance of chemokine and chemokine receptors in head and neck squamous cell carcinoma: A critical review. Oral Oncol 2016;56:8-16. doi: 10.1016/j.oraloncology.2016.02.016.

Dateoka S, Ohnishi Y, Kakudo K. Effects of CRM197, a specific inhibitor of HB-EGF, in oral cancer. Med Mol Morphol 2012;45:91-7. doi: 10.1007/s00795-011-0543-6.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2014;136:359–86. doi:10.1002/ijc.29210.

Ferreira FO, Ribeiro FLL, Batista AC, Leles CR, de Cássia Gonçalves Alencar R, Silva TA. Association of CCL2 with lymph node metastasis and macrophage infiltration in oral cavity and lip squamous cell carcinoma. Tumour Biol 2008;29:114–21. doi:10.1159/000137669.

Gannot G, Buchner A, Keisari Y. Interaction between the immune system and tongue squamous cell carcinoma induced by 4-nitroquinoline N-oxide in mice. Oral Oncol 2004;40:287-97. doi:10.1016/j.oraloncology.2003.08.008.

Hanahan D, Coussens LM. Accessories to the Crime: Functions of cells recruited to the tumor microenvironment. Cancer Cell 2012;21:309-22. doi: 10.1016/j.ccr.2012.02.022.

Hansell CA, MacLellan LM, Oldham RS, Doonan J, Chapple KJ, Anderson EJ, et al. The atypical chemokine receptor ACKR2 suppresses Th17 responses to protein autoantigens. Immunol Cell Biol 2015;93:167-76. doi: 10.1038/icb.2014.90.
Hernandez L, Magalhaes MA, Coniglio SJ, Condeelis JS, Segall JE. Opposing roles of CXCR4 and CXCR7 in breast cancer metastasis. Breast Cancer Res 2011;13:R128. doi: 10.1186/bcr3074.

Jamieson T, Cook DN, Nibbs RJ, Rot A, Nixon C, McLean P, et al. The chemokine receptor D6 limits the inflammatory response in vivo. Nat Immunol 2005;6:403-11. doi:10.1038/ni1182.

Jung DW, Che ZM, Kim J, Kim K, Kim KY, Williams D, et al. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. Int J Cancer 2010;127:332–44. doi:10.1002/ijc.25060.

Koontongkaew S. The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. J Cancer 2013;4:66-83. doi: 10.7150/jca.5112.

Langenes V, Svensson H, Börjesson L, Gustavsson B, Bemark M, Sjöling A, et al. Expression of the chemokine decoy receptor D6 is decreased in colon adenocarcinomas. Cancer Immunol Immunother 2013;62:1687-95. doi: 10.1007/s00262-013-1472-0.

Li J, Liang F, Yu D, Qing H, Yang Y. Development of a 4-nitroquinoline-1-oxide model of lymph node metastasis in oral squamous cell carcinoma. Oral Oncol 2013;49:299-305. doi: 10.1016/j.oraloncology.2012.10.013.

Li X, Xu Q, Wu Y, Li J, Tang D, Han L et al. A CCL2/ROS autoregulation loop is critical for cancer-associated fibroblasts-enhanced tumor growth of oral squamous cell carcinoma. Carcinogenesis 2014;35:1362-70. doi: 10.1093/carcin/bgu046.

Massara M, Bonavita O, Mantovani A, Locati M, Bonecchi R. Atypical chemokine re ceptors in cancer: friends or foes? J Leukoc Biol 2016;99:1-7. [Epub ahead of print]. doi:10.1189/jlb.3MR0915-431RR.

Nibbs RJ, Gilchrist DS, King V, Ferra A, Forrow S, Hunter KD, et al. The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. J Clin Invest 2007;117:1884-92. doi:10.1172/JCI30068.

Onoue T, Uchida D, Begum NM, Tomizuka Y, Yoshida H, Sato M. Epithelial-mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells. Int J Oncol 2006;29:1133–8. doi:10.3892/ijo.29.5.1133.

Savino B, Caronni N, Anselmo A, Pasqualini F, Borroni EM, Basso G, et al.
ERK-dependent downregulation of the atypical chemokine receptor D6 drives tumor aggressiveness in Kaposi sarcoma. Cancer Immunol Res 2014;2:679-89. doi: 10.1158/2326-6066.CIR-13-0202.

Schneider C, Teufel A, Yevsa T, Staib F, Hohmeyer A, Walenda G, et al. Adaptive immunity suppresses formation and progression of diethylnitrosamine-induced liver cancer. Gut 2012;61:1733–1743. doi: 10.1136/gutjnl-2011-301116.

Shang ZJ, Liu K, Shao Z. Expression of chemokine receptor CCR7 is associated with cervical lymph node metastasis of oral squamous cell carcinoma. Oral Oncol 2009;45:480–5. doi:10.1016/j.oraloncology.2008.06.005.

Shen H, Schuster R, Stringer KF, Waltz SE, Lentsch AB. The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. FASEB J 2006;20:59-64.

Shi JY, Yang LX, Wang ZC, Wang LY, Zhou J, Wang XY, Shi GM, Ding ZB, Ke AW, Dai Z, Qiu SJ, Tang QQ, Gao Q, Fan J. CC chemokine receptor-like 1 functions as a tumour suppressor by impairing CCR7-related chemotaxis in hepatocellular carcinoma. J Pathol 2015;235:546-58. doi: 10.1002/path.4450.

Silva TA, Ribeiro FLL, Oliveira-Neto HH de, Watanabe S, Alencar R de CG, Fukada SY, et al. Dual role of CCL3/CCR1 in oral squamous cell carcinoma: implications in tumor metastasis and local host defense. Oncol Rep 2007;18:1107–13. doi:10.3892/or.18.5.1107.

Stokes A, Joutsa J, Ala-Aho R, Pitchers M, Pennington CJ, Martin C, Premachandra DJ, Okada Y, Peltonen J, Grénman R, James HA, Edwards DR, Kähäri VM. Expression profiles and clinical correlations of degradome components in the tumor microenvironment of head and neck squamous cell carcinoma. Clin Cancer Res 2010;16:2022-35. doi: 10.1158/1078-0432.CCR-09-2525.

Tang XH, Knudsen B, Bemis D, Tickoo S, Gudas LJ. Oral Cavity and esophageal carcinogenesis modeled in carcinogen treated mice. Clin Canc Res 2004;10:301-313.

Uchida D, Begum NM, Almofti A, Nakashiro K, Kawamata H, Tateishi Y, et al. Possible role of stromal-cell-derived factor-1/CXCR4 signaling on lymph node metastasis of oral squamous cell carcinoma. Exp Cell Res 2003;290:289–302. doi:10.1016/S0014-4827(03)00344-6.

Uchida D, Begum N-M, Tomizuka Y, Bando T, Almofti A, Yoshida H, et al. Acquisition of lymph node, but not distant metastatic potentials, by the overexpression of CXCR4 in human oral squamous cell carcinoma. Lab Invest 2004;84:1538–46. doi:10.1038/labinvest.3700190.

Uchida D, Onoue T, Tomizuka Y, Begum NM, Miwa Y, Yoshida H, et al. Involvement of an autocrine stromal cell derived factor-1/CXCR4 system on the distant metastasis of human oral squamous cell carcinoma. Mol Cancer Res 2007;5:685–94. doi:10.1158/1541-7786.MCR-06-0368.

Vassilakopoulou M, Psyrri A, Argiris A. Targeting angiogenesis in head and neck cancer. Oral Oncol 2015;51:409-15. doi: 10.1016/j.oraloncology.2015.01.006.

Vieira AE, Repeke CE, Ferreira Junior S de B, Colavite PM, Biguetti CC, Oliveira RC, et al. Intramembranous bone healing process subsequent to tooth extraction in mice: micro-computed tomography, histomorphometric and molecular characterization. PLoS One 2015;10:1-22. doi: 10.1371/journal.pone.0128021.

Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, et al. The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through thechemokine decoy receptor D6. Gut 2010;59:197-206. doi: 10.1136/gut.2009.183772.

Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009;45:309-16. doi: 10.1016/j.oraloncology.2008.06.002.

Wu FY, Ou ZL, Feng LY, Luo JM, Wang LP,Shen ZZ, et al. Chemokine decoy receptor d6 plays a negative role in human breast cancer. Mol Cancer Res. 2008 Aug;6(8):1276-88. doi: 10.1158/1541-7786.MCR-07-2108.

6 – DISCUSSÃO

No presente estudo foi proposta a utilização de um modelo de carcinogênese induzida quimicamente pelo 4NQO para estudar o eixo CCL3/CCR1/CCR5 *in vivo* como uma alternativa às limitações inerentes ao se realizar estudos em humanos permitindo um melhor controle de variáveis experimentais. Além das vantagens como solubilidade e facilidade na administração, o modelo com o 4NQO também permite estudar todas as etapas envolvidas no processo de carcinogênese. Entretanto, o longo tempo necessário para o desenvolvimento das lesões, a toxicidade utilizando-se altas concentrações, e a ausência da ocorrência de metástases linfonodais foram algumas das limitações observadas neste estudo. Por isso, neste trabalho avaliações *in vitro* também foram empregadas para o melhor entendimento dos mecanismos pelos quais CCL3 afeta a carcinogênese oral.

Um número crescente de evidências tem destacado a participação de quimiocinas e seus receptores na patogênese do CCEB, conforme recentemente revisado por SILVA *et al*. (2016). Tais evidências mostraram que quimiocinas produzidas tanto pelas células do tumor quanto pela heterogênea população de células que compõem seu estroma podem modular direta e indiretamente vias que controlam a proliferação, angiogênese e o ganho de motilidade e invasividade das células tumorais (ALBERT *et al*., 2012; ALMOFTI *et al*., 2004; CHUANG, *et al*., 2009; FERREIRA *et al*., 2008; ISHIKAWA *et al*., 2006; JUNG *et al*., 2010; LEE *et al*., 2015; LI *et al*., 2014; LIU *et al*., 2015; MENG *et al*., 2010; OLIVEIRA-NETO *et al*., 2008; QUIAN *et al*., 2014; SHINTANI *et al*., 2004; SILVA *et al*., 2007; TSUZUKI *et al*., 2006; UCHIDA *et al*., 2003, 2004, 2007; 2011). Em conjunto, estes dados colocam o sistema

quimiocinas/receptores como alvos em potencial em terapias para diferentes tipos de neoplasias, incluindo-se o CCEB.

Dentre a grande família das quimiocinas, a quimiocina CCL3 foi estudada previamente pelo nosso grupo, que demonstrou um aumento na sua expressão em amostras de CCEB primário e em linfonodos metastáticos em humanos (SILVA *et al*., 2007). Entretanto, existem algumas limitações neste tipo de estudo, principalmente devido a grande variabilidade de amostras com relação às características clínicas, demográficas, genotípicas e histopatológicas. Diante desses aspectos, utilizamos neste trabalho um modelo de indução de carcinogênese oral bem estabelecido na literatura (KANOJIA; VAIDYA, 2006; *LI et* al., 2013; TANG *et al*., 2004), utilizando a administração prolongada do carcinógeno químico 4NQO. A utilização desse modelo permitiu minimizar as variabilidades inerentes ao uso de amostras humanas, além de possibilitar realizar interferências funcionais na carcinogênese, como o estudo do processo em animais deficientes da quimiocina CCL3 e receptores de quimiocinas.

Inicialmente, foi observado um aumento significativo na expressão de CCL3 nas lesões linguais induzidas quimicamente por 4NQO. Consistente com nossos achados, outros trabalhos na literatura também mostraram um aumento de CCL3 em diferentes neoplasias como hepatomas (LU *et al*., 2003), leucemias (BABA *et al*., 2013; SIVINA *et al*., 2011), mieloma múltiplo (LENTZSCH *et al*., 2003; VALLET *et al*., 2011), em modelo de hepatocarcinogênese induzida (YANG *et al*., 2006), carcinogênese intestinal associada à colite (SASAKI *et al*., 2014) e em modelo murino de metástase pulmonar induzida por células de carcinoma renal (WU *et al*., 2008).

O modelo com 4NQO foi eficaz na indução dos tumores na língua dos camundongos como descrito previamente (KANOJIA; VAIDYA, 2006; TANG *et al*., 2004). Entretanto, diferentemente dos achados descritos em outro estudo (LI *et al*.,

20130) não pudemos verificar a ocorrência de metástases linfonodais ou em outros órgãos quando da utilização do 4NQO na concentração de 200 µg/mL. Esses autores utilizaram linhagem de camundongos BALB/c, enquanto no presente estudo utilizamos camundongos C57/BL6. Além disto, o período de acompanhamento no estudo de Li *et al*. (2013) foi maior, sendo que os animais tratados tiveram 100% de incidência de carcinoma e 100% de metástases em linfonodos submandibulares entre a trigésima terceira e a quadragésima semana experimental (LI *et al*., 2013). Neste sentido, o fator tempo é uma das principais limitações da utilização do modelo de indução de carcinogênese com o 4NQO em camundongos. Em nossas condições experimentais, o 4NQO na dose de 50 µg/mL na água de beber mostrou ser bem tolerado pelos animais, contudo somando-se a idade dos animais no início do tratamento (entre 6-8 semanas) mais o período de tratamento durante 28 semanas torna-se dificultoso prolongar o prazo de experimentação com objetivos de estratégias terapêuticas. Além disso, em nossas condições observou-se que a dosagem maior de 4NQO apresentou maior toxicidade o que elevou os índices de letalidade.

O tratamento com 4NQO induziu a formação de lesões exofíticas (protuberantes) ou papilomatosas, de base ampla e colocação esbranquiçada, por vezes exibindo ulceração e com bordas elevadas, semelhante ao descrito em estudos prévios (TANG *et al*., 2004). De modo interessante, na ausência do eixo CCL3/CCR5, mas não de CCR1 a formação de tumores na língua dos camundongos foi significativamente atenuada. Consistentemente, a análise microscópica mostrou também escores histopatológicos mais brandos, e reduzida atividade proliferativa nos tumores de animais deficientes para CCL3 e CCR5. Dessa forma, nosso estudo demonstrou que a participação de CCL3 na carcinogênese oral envolve principalmente a sua ligação ao receptor CCR5. Outros dois trabalhos também mostraram redução na formação de

tumores e metástases em animais deficientes para CCL3 e CCR5 em modelo de hepatocarcinogênese induzida e metástase pulmonar induzida por células de carcinoma renal (SASAKI *et al*., 2014; WU *et al*., 2008). Por outro lado, o estudo de Yang *et al*. (2006) revelou a contribuição de CCL3 e seu receptor CCR1 na hepatocarcinogênese induzida, onde animais CCL3/CCR1 deficientes exibiram incidência reduzida de tumores no fígado em relação aos WT (YANG *et al*., 2006). Contudo, a interpretação dessas discrepâncias deve ser feita com cautela, uma vez que modelos biológicos e tumores diferentes foram utilizados nesses estudos.

Embora uma reduzida atividade proliferativa foi detectada nas lesões de animais $CCL3^{-/-}$ e $CCR5^{-/-}$, os resultados do ensaio de proliferação *in vitro* revelando uma aparente similaridade na proliferação de HN12 sob estímulo ou não com CCL3 sugeriram que essa quimiocina não afeta a proliferação tumoral em nossas condições experimentais. Logo, a reduzida atividade proliferativa observada *in vivo* resulta da menor formação de tumores nesses animais.

Conforme descrito previamente, quimiocinas e receptores podem influenciar a carcinogênese oral regulando o recrutamento de células inflamatórias para o microambiente tumoral e favorecendo angiogênese, proliferação celular, motilidade e invasividade das células neoplásicas (CHUANG *et al*., 2009; LI *et al*., 2014; SHANG *et al*., 2009; UCHIDA *et al*., 2003, 2004, 2007; 2011). Neste sentido, a reduzida formação de tumores na ausência de CCL3/CCR5 poderia indicar que a quimiocina afeta o microambiente tumoral. De fato, observou-se significativa redução na expressão de EGF, TGF-β1, VEGFa e VEGFb nas lesões de animais $CCL3^{-/-}$. Reforçando esses achados, outros trabalhos também encontraram expressão reduzida de marcadores de angiogênese (ex. CD31, HGF e CD34) em tumores de animais $CCL3^{-/-}$ (YANG *et al*., 2006; WU *et al*., 2008). Além disto, estes animais apresentaram ainda reduzida

expressão de integrinas (ITGA4 e VTN). Estudo prévio demonstrou que CCL3 regula a expressão de moléculas de adesão em células leucêmicas, influenciando sua transmigração (MA; MA, 2014).

Utilizando uma linhagem de CCEB metastática, nós mostramos que o tratamento das células tumorais HN12 com CCL3 resultou na produção aumentada das citocinas pró-inflamatórias IL-6 e TNF-α. Esses resultados corroboram os achados prévios da expressão reduzida de IL-6 e TNF-α encontradas nas lesões de animais deficientes de CCL3. De fato, o bloqueio de IL-6 com anticorpo humanizado anti-IL-6 (Tocilizumab®) mostrou reduzir angiogênese e crescimento tumoral *in vivo* no CCEB (SHINRIK *et al*., 2009). Além disso, CCL3 induziu a expressão aumentada de outra quimiocina, CCL5 pela linhagem de células metastáticas HN12. Estudos prévios demonstraram um papel de CCL5 e o receptor CCR5 como faclitadores na tumorigênese no câncer de mama (SORIA; BEN-BARUCH, 2008). No contexto do CCEB um estudo demonstrou que CCL5 via CCR5 foi capaz de estimular a motilidade e invasividade de linhagem de células de CCEB, e induziu a produção de MMP-9 por essas células (CHUANG *et al*., 2009). Embora não exploramos o papel de CCL5 via CCR5 em nossas condições experimentais, a participação desse eixo na carcinogênese oral pode ser invertigada em estudos futuros. O aumento da expressão de CCL5, IL-6 e TNF-α induzida por estímulo com CCL3 reforçam a ideia de que CCL3 pode atuar como modulador do microambiente tumoral.

Os resultados da reduzida expressão de MMP1-a, -2 e -9 na ausência de CCL3 nas lesões *in vivo*, juntamente com a detecção aumentada de MMP-2, -8 e -9 no sobrenadante de células HN12 estimuladas com a quimiocina, sugerem sua possível participação no remodelamento da matriz extracelular. Nesse sentido, o remodelamento do estroma tumoral com a participação de proteinases no carcinoma de células

escamosas de cabeça e pescoço tem sido correlacionado com invasão, metástases e pior prognóstico para os pacientes (STOKES *et al*., 2010). Stokes *et al*. (2010) conduziram um estudo de tipo coorte no qual a expressão aumentada de proteinases das classes de MMP's, "*A disintegrin and metalloproteinase"* (ADAMs), "*ADAMs with thrombospondin motif"* (ADAMTSs) e "*tissue inhibitor of metalloproteinases"* (TIMP's) foi encontrada em amostras de pacientes com CCEB. De modo interessante, a expressão aumentada de diversas destas proteases mostrou forte correlação entre parâmetros clínicos dos tumores, como o sítio primário e o grau de profundidade de invasão (STOKES *et al*., 2010). Interessantemente, nós observamos aumento na expressão de TIMP1 e Col1a1 na ausência de CCL3, sugerindo mais uma vez que CCL3 regula o remodelamento da matriz tecidual durante a carcinogênese.

Além disso, observamos efeitos diretos de CCL3 sobre a invasividade das células metastáticas HN12, uma vez que a administração de anticorpo anti-CCL3 e Evasina-1 (uma proteína que se liga a CCL3, impedindo seus efeitos) (FRAUENSCHUH *et al*., 2007) reduziu o número de células transmigradas. Em concordância com nossos dados, CCL3 afetou a motilidade e invasividade de células de câncer hepático, onde um aumento intracelular no influxo de íons cálcio levou a reorganização do citoesqueleto e formação de pseudópodes nessas células (YUAN *et al*., 2010). Em conjunto, os presentes achados indicam que CCL3 influencia na tumorigênese oral exercendo efeitos regulatórios diretos nas células tumorais e modificando o microambiente tumoral.

Paralelamente, nós estudamos os efeitos do receptor atípico ACKR2 na carcinogênese oral, considerando seu papel na modulação da resposta inflamatória em diferentes situações. Sua potencial habilidade em capturar quimiocinas –CC reduzindo a biodisponibilidade em tumores de animais ACKR2$^{-/-}$ descrita por alguns autores

(NIBBS *et al*., 2007; SAVINO *et al*., 2014; VETRANO *et al*., 2010) motivaram essa investigação, juntamente com os dados descritos acima acerca do envolvimento de CCL3 na carcinogênese bucal. Além disso, até o presente momento não existem dados na literatura associando o receptor ACKR2 e câncer de boca. Nossos resultados demonstraram que a deleção de ACKR2 não resultou em modificação da carcinogênese, uma vez que as lesões observadas nos animais ACKR2$^{-/-}$ e WT tratados exibiram características clinico histopatológicas e atividade proliferativa similares nas duas doses testadas. De forma semelhante aos nossos achados, o estudo de Schneider *et al*. (2012) mostrou similaridade microscópica entre os grupos ACKR2$^{-/-}$ e WT na análise comparativa entre tumores hepáticos induzidos (SCHNEIDER *et al*., 2012). Por outro lado, outros trabalhos mostraram maior suscetibilidade na formação de tumores em animais deficientes do receptor ACKR2 (NIBBS *et al*., 2007; SAVINO *et al*., 2014; VETRANO *et al*., 2010). Esses autores observaram um aumento significativo na produção de quimiocinas CC, como CCL2, CCL3, CCL5 e o acúmulo de leucócitos nos tumores induzidos (NIBBS *et al*., 2007; SAVINO *et al*., 2014; VETRANO *et al*., 2010). Interessantemente, nós não notamos um aumentosignificativo na produção de CCL3, CCL4, CCL5 e CCL12 na ausência de ACKR2 em relação aos animais selvagens tratados. Contudo, das quimiocinas analisadas somente CCL2 foi significativamente aumentada nos tumores de animais ACKR2$^{-/-}$.

Os efeitos protetores de ACKR2 em tumores são, em parte, atribuídos às suas propriedades anti-inflamatórias, reduzindo a liberação de mediadores inflamatórios e o recrutamento de células do sistema imune (NIBBS *et al*., 2007; SAVINO *et al*., 2014; VETRANO *et al*., 2010; WU *et al*., 2008). No nosso estudo, observamos um aumento de citocinas inflamatórias nos tumores induzidos em ambos os grupos. Contudo, especialmente para IL-6 e IL-17 foi detectado aumento significativo na produção nas

lesões de animais deficientes de ACKR2. Estes resultados indicam que a deleção ACKR2 de algum modo afeta a produção destas citocinas inflamatórias durante o processo de carcinogênese oral, entretanto, pode-se considerar que as mudanças causadas pela deleção deste receptor são insuficientes para modificar a progressão tumoral. O aumento de mediadores inflamatórios em tumores de animais deficientes para ACKR2 foi associado com o acúmulo de leucócitos nas lesões na maioria dos modelos estudados (NIBBS *et al*., 2007; SAVINO *et al*., 2014; VETRANO *et al*., 2010). Vetrano *et al*. (2010) demonstrou um aumento de CCL2, CCL3, CCL5, CXCL1 e CXCL2 e aumento de células $CD3^+$, $CD68^+$, $CD11c^+$ e $CD45^+$ recrutados na ausência de ACKR2 em modelo de carcinogênese intestinal associada à colite (VETRANO *et al*., 2010). Por outro lado, o aumento de linfócitos T $CD8^+$ e das quimiocinas CCL2, CCL5 e CXCL9 em tumores hepáticos induzidos quimicamente não foram suficientes para mudar o curso da carcinogênese em animais $ACKR2^{-/-}$ em relação aos selvagens, conforme o estudo de Schneider *et al*. (2012) onde uma similaridade na formação de tumores hepáticos foi observada entre em animais $ACKR2^{-/-}$ e selvagens (SCHNEIDER *et al*., 2012). Nesse sentido, pode-se admitir que os resultados obtidos sejam explicados pelo tipo de modelo empregado, o qual induz formação de lesões que não exibem um infiltrado inflamatório expressivo (GANNOT *et al*., 2004; SILVA *et al*., 2014). Além disso, avaliou-se a indução de tumores em apenas um tempo experimental, o que nos impede de inferir sobre o papel de ACKR2 em estágios anteriores na carcinogênese.

Outro ponto que merece atenção é que além de ACKR2, outros receptores atípicos de quimiocinas também foram descritos e implicados na carcinogênese (revisado por MASSARA *et al*., 2016). Os resultados mostram papéis duais para ACKR's em tumores, onde ACKR1, ACKR2 e ACKR4 tiveram efeitos antitumorais (NIBBS *et al*., 2007; SAVINO *et al*., 2014; SHEN *et al*., 2006; SHI *et al*., 2014

VETRANO *et al*., 2010) e ACKR3 pró-tumorais (HERNANDEZ *et al*., 2011). Não há dados disponíveis relacionando estes receptores na carcinogênese oral, portanto, o papel dos ACKR's precisa ser explorado em estudos subsequentes.

Finalmente, os resultados da expressão molecular dos mediadores de proliferação (EGF), angiogênese (FGF1 e FGF2, VEGFa e VEGFb) e de remodelamento da matriz extracelular (colágeno, integrinas, MMP's e TIMP's) revelou um aumento coerente nas lesões induzidas de ambos os grupos tratados, entretanto como não houve diferença na produção destes mediadores entre os grupos sugere-se que ACKR2 não afeta a proliferação celular, nem o remodelamento da matriz em nossas condições. Coletivamente, nossos resultados indicam que ACKR2 influencia na ativação de vias inflamatórias nas lesões estudadas, mas que estas mudanças não influenciaram o curso da carcinogênese induzida por 4NQO.

Nos últimos 16 anos, uma série de trabalhos na literatura vem demonstrando o potencial de várias quimiocinas e seus receptores incluindo CCL2/CCR2, CCL5/CCR5, e CXCL12/CXCR4 como moduladores do microambiente tumoral no CCEB, promovendo motilidade, invasão e metástases para linfonodos. Evidências da participação dessas moléculas também em outros tumores reforçam a ideia de que o desenvolvimento de terapias anti-quimiocinas/receptores como adjuvantes no tratamento do câncer pode ser útil. Em um estudo, a utilização de um inibidor de CXCR4 – *1,10-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride* – AMD3100 – por exemplo, reduziu significativamente a ocorrência de metástases *in vivo*, indicando que o bloqueio de CXCR4 poderia ser utilizado como agente terapêutico em pacientes com CCEB (UCHIDA *et al*., 2011). Entretanto, a utilização de AMD3100 ou outros inibidores de CXCR4/CXCL12 (Plerixafor®, Mozobil®, BKT 140, MDX1338 - Medarex®) na quimioterapia adjuvante para

tratamento de metástase em pacientes com câncer de laringe e hipofaringe ainda estão em fase de recrutamento de pacientes e não há dados disponíveis da sua eficácia em pacientes com CCEB (SILVA *et al*., 2016). Outro medicamento, o CNTO888 (Centocor®), um anticorpo monoclonal anti-CCL2 foi testado em um estudo clínico de fase 1 e 2 em pacientes com tumores sólidos (colorretal, próstata, ovário, útero), incluindo um paciente com câncer de esôfago e mostrou ser bem tolerado pelos pacientes, contudo, sua atividade antitumoral não pode ser confirmada (SANDHU *et al*., 2013). Outro trabalho recente incluiu um grupo pequeno de pacientes com câncer de cabeça e pescoço (*n=4*), sendo um caso de CCEB, onde apesar de Carlumab® não ter causado efeitos colaterais significativos, também não foram apresentadas evidências de atividade antitumoral (BRANA *et al*., 2015). O Maraviroc (Maracon®), um medicamento inibidor do receptor CCR5 inicialmente aprovado pela Food and Drug Administration (FDA) para o tratamento de pacientes com HIV, tem sido testado também em pacientes com câncer colorretal em um estudo clínico de fase I (revisado por SILVA *et al*., 2016). Entretanto, ainda não há dados disponíveis sobre sua eficácia nesses estudos em pacientes, mas um estudo recente demonstrou que Maraviroc administrado por via oral na dose de 30 mg/Kg duas vezes ao dia por até um periodo de 15 dias foi capaz de reduzir o tamanho de tumores intestinais murinos induzidos por injeção ortotópica de células tumorais de linhagem murina e humana (TANABE *et al*., 2016).

Apesar dos resultados preliminares, a utilização desses inibidores no tratamento de tumores colocam os sistemas CCL2/CCR2, CCL5/CCR5 e CXCL12/CXCR4 como possíveis alvos terapêuticos para intervenção no CCEB. Os resultados do presente trabalho apontam para a participação de CCL3 e seu receptor CCR5 na carcinogênese

oral. Os efeitos protetores resultantes do bloqueio de CCL3 com anti-CCL3 ou Evasina-1 neste estudo incluem essa molécula no *rol* de candidatos em terapias futuras.

7 – CONCLUSÃO

Os resultados do presente estudo permitem concluir que:

- O aumento da expressão de CCL3 em lesões de CCEB quimicamente induzidas por 4NQO podem indicar a participação desta quimiocina na carcinogênese bucal.
- A reduzida formação de CCEB em animais deficientes de CCL3 e CCR5 indica que o eixo CCL3/CCR5 regula vias “pro-tumorigênicas” no CCEB.
- Os efeitos de CCL3 nas células de CCEB *in vitro* sugerem que esta quimiocina influencia diretamente a capacidade de invasão e a liberação de marcadores inflamatórios por estas células.
- A atividade antitumorigênica postulada para ACKR2 não foi observada em nossas condições experimentais.

REFERÊNCIAS

ALBERT, S. *et al*. Prognostic value of the chemokine receptor CXCR4 and epithelial-to-mesenchymal transition in patients with squamous cell carcinoma of the mobile tongue. *Oral Oncol*., v. 48, n.12, p. 1263–71, Dec. 2012.

ALLEN, S. J.; CROWN, S. E.; HANDEL, T. M. Chemokine: receptor structure, interactions, and antagonism. *Annu. Rev. Immunol*., v.25, p. 787-820, 2007.

ALMOFTI, A. *et al*. The clinicopathological significance of the expression of CXCR4 protein in oral squamous cell carcinoma. *Int. J. Oncol*., v. 25, n. 1, p. 65-71, Jul. 2004.

ARIMA, Y. *et al*. 4-Nitroquinoline 1-oxide forms 8-hydroxydeoxyguanosine in human fibroblasts through reactive oxygen species. *Toxicol. Sci*., v. 91, n. 2, p. 382-92, Jun. 2006.

BABA, T. *et al*. MIP-1α/CCL3-mediated maintenance of leukemia-initiating cells in the initiation process of chronic myeloid leukemia. *J. Exp. Med*., v. 210, n. 12, p. 2661-73, Nov. 2013.

BACHELERIE, F. *et al*. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol. Rev*., v. 66, n. 1, p.1-79, Nov. 2013.

BAGAN, J.; SARRION, G.; JIMENES, Y. Oral cancer: clinical features. *Oral Oncol*., v. 46, n. 6, p. 414–417, Jun. 2010.

BAGGIOLINI, M.; WALZ, A.; KUNKEL, S. L. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest*., v. 84, n. 4, p. 1045-9, Oct. 1989.

BALBO, S. *et al*. Carcinogenicity and DNA adduct formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and enantiomers of its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in F-344 rats. *Carcinogenesis*, v.35, n. 12, p. 2798-806, Dec. 2014.

BALKWILL, F. Cancer and the chemokine network. *Nature Rev*., v.4, n. 7, p. 540-549, Jul. 2004.

BALKWILL, F. The chemokine system and cancer. *J. Pathol*., v. 226, n. 2, p. 148-157, Jan. 2012.

BARNES, L. *et al*. World Health Organization classification of tumours. Pathology and genetics. Head and neck tumours. *In*: Slootweg, P. J.; Eveson, J.W. Tumours of the oral cavity and oropharynx: Introduction. Lyon, France: IARC, 2005: p. 166-75.

BEKTAS-KAYHAN, K. *et al*. MCP-1 and CCR2 gene variants in oral squamous cell carcinoma. *Oral Dis*., v. 18, n. 1, p. 55–9, Jan. 2012.

BORDON, Y. *et al*. The atypical chemokine receptor D6 contributes to the development of experimental colitis. *J. Immunol*., v. 182, n. 8, p. 5032-40, Apr. 2009.

BOYD, N. M.; READE, P.C. Mechanisms of carcinogenesis with particular reference to the oral mucosa. *J. Oral Pathol*., v. 17, n. 5, p. 193-201, May 1988.

BRANA, I. *et al*. Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody, in combination with four chemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study. *Target Oncol*., v. 10, n. 1, p. 111-23, Mar. 2015.

BRASIL. MINISTÉRIO DA SAÚDE. INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA. Coordenação de Prevenção e Vigilância Estimativa 2016: incidência de câncer no Brasil / Instituto Nacional de Câncer José Alencar Gomes da Silva – Rio de Janeiro: INCA, 122 p. 2015.

BROUWER, A. F.; EISENBERG, M. C.; MEZA, R. Age Effects and Temporal Trends in HPV-Related and HPV-Unrelated Oral Cancer in the United States: A Multistage Carcinogenesis Modeling Analysis. *PLoS One*, v.11, n. 3, e0151098, Mar. 2016.

CARDINALI, M. *et al*. Tyrosine phosphorylation as a marker for aberrantly regulated growth-promoting pathways in cell lines derived from head and neck malignancies. *Int. J. Canc*., v. 61, n. 1, p. 98-103, Mar. 1995.

CHANG, K. P. *et al*. Overexpression of macrophage inflammatory protein-3α in oral cavity squamous cell carcinoma is associated with nodal metastasis. *Oral Oncol*., v. 47, n. 2, p. 108–13, Feb. 2011.

CHOW, M. T.; LUSTER, A. D. Chemokines in cancer. *Cancer Immunol. Res*., v. 2, n. 12, p.1125-31, Dec. 2014.

CHUANG, J. Y. *et al*. CCL5/CCR5 axis promotes the motility of human oral cancer cells. *J. Cell Physiol*., v. 220, n.2, p. 418–26, Aug. 2009.

CONTALDO, M. *et al*. Prognostic implications of node metastatic features in OSCC: a retrospective study on 121 neck dissections. *Oncol. Rep.*, v. 30, n. 6, p. 2697-704, Dec. 2013.

DERKA, S. *et al*. Cell proliferation and apoptosis culminate in early stages of oral oncogenesis. *Oral Oncol*., v. 42, n. 5, p. 540-50, May 2006.

DING, L. *et al*. Serum CCL2 and CCL3 as potential biomarkers for the diagnosis of oral squamous cell carcinoma. *Tumor Biol*., v. 35, n. 10, p. 10539-46, Oct. 2014.

FELLER, L.; LEMMER, J. Oral squamous cell carcinoma: epidemiology, clinical presentation and treatment. *J. Canc. Ther*., v. 3, p. 263-268, Aug. 2012.

FERLAY, J. *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer*, v. 136, p. 359–86, Sep. 2014.

FERREIRA, F. O. *et al*. Association of CCL2 with lymph node metastasis and macrophage infiltration in oral cavity and lip squamous cell carcinoma. *Tumor Biol*., v. 29, n. 2, p. 114–121, 2008.

FRA, A. M. *et al*. Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J. Immunol*., v. 170, n. 5, p. 2279-82, Mar. 2003.

FRAUENSCHUH, A. *et al*. Molecular cloning and characterization of a highly selective chemokine-binding protein from the tick rhipicephalus sanguineus. *J. Biol. Chem*., v. 282, n. 37, p. 27250–58, Sep. 2007.

GANNOT, G.; BUCHNER, A.; KEISARI, Y. Interaction between the immune system and tongue squamous cell carcinoma induced by 4-nitroquinoline N-oxide in mice. *Oral Oncol*., v. 40, n.3, p. 287-97, Mar. 2004.

GRIFFITH, J. W.; SOKOL, C. L.; LUSTER, A. D. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol*., v. 32, p. 659-702, 2014.

GRIVENNIKOV, S. I.; KARIN, M. Inflammation and oncogenesis: a vicious connection. *Curr. Opin. Genet. Dev*.; v. 20, n. 1, p. 65-71, Feb. 2010.

GUO, Q. *et al*. Axis Promotes Proliferation, Migration, Invasion, and Metastasis of Ovarian Cancer. *Oncol Res*., v.22, n. 5-6, p. 247-58, 2014.

HANAHAM, D.; COUSSENS, L. M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Canc. Cell*, v. 21, n.3, p. 309-22, Mar. 2012.

HANSELL, C. A. *et al*. The atypical chemokine receptor ACKR2 suppresses Th17 responses to protein autoantigens. *Immunol. Cell Biol*., v. 93, n. 2, p. 167-76, Feb. 2015.

HASHIBE, M. *et al*. Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiol. Biomarkers Prev*., v. 18, n. 2, p. 541-50, Feb. 2009.

HERNANDEZ, L. *et al*. Opposing roles of CXCR4 and CXCR7 in breast cancer metastasis. *Breast Cancer Res*., v. 13, n. 6, p. R128, 2011.

HONG, Y. *et al*. p16(CDKN2A) expression during rat tongue carcinogenesis induced by 4-nitroquinoline-1-oxide. *Oral Oncol*., v. 45, n. 7, p. 640-4, Jul. 2009.

IIDA, N. *et al*. Tumor cell apoptosis induces tumor-specific immunity in a CC chemokine receptor 1- and 5-dependent manner in mice. *J. Leukoc. Biol*., v. 84, n. 4, p. 1001-10, Oct. 2008.

ISHIKAWA, T. *et al*. CXCR4 expression is associated with lymph-node metastasis of oral squamous cell carcinoma. *Int. J. Oncol*., v. 28, n. 1, p. 61–6, Jan. 2006.

JAMIESON, T. *et al*. The chemokine receptor D6 limits the inflammatory response in vivo. *Nat. Immunol*., v. 6, n.4, p. 403-11, Apr. 2005.

JUNG, D. W. *et al*. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. *Int. J. Canc*., v. 127, n. 2, p. 332–44, Jul. 2010.

KANOJIA, D.; VAIDYA, M. M. 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. *Oral Oncol*., v. 42, p. 655-667, Aug. 2006.

KIM, M. M. *et al*. Head and neck cancer cell lines exhibit differential mitochondrial repair deficiency in response to 4NQO. *Oral Oncol*., v. 42, n. 2, p. 201-7. Feb. 2006.

KO, H. H. *et al*. Factors influencing the incidence and prognosis of second primary tumors in patients with oral squamous cell carcinoma. *Head Neck*, [Epub ahead of print], Apr. 2016.

KUZIEL, W. A. *et al*. CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. *Atherosclerosis*, v. 167, v. 1, p. 25-32, Mar. 2013.

LANGENES, V. *et al*. Expression of the chemokine decoy receptor D6 is decreased in colon adenocarcinomas. *Cancer Immunol. Immunother*., v. 62, n. 11, p. 1687-95, Nov. 2013.

LEE, C. C. *et al*. MCP1-induced epithelial-mesenchymal transition in head and neck cancer by AKT activation. *Anticancer Res*., v. 35, n. 6, p. 3299-306, Jun. 2015.

LEE, J. I. *et al*. Prognostic significance of CXCR-4 expression in oral squamous cell carcinoma. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*., v. 107, n. 5, p. 678–84, May 2009.

LENTZSCH, S. *et al*. Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. *Blood*, v. 101, n. 9, p. 3568-73, May 2003.

LI, J. *et al*. Development of a 4-nitroquinoline-1-oxide model of lymph node metastasis in oral squamous cell carcinoma. *Oral Oncol*., v. 49, n.4, p. 299-305, Apr. 2013.

LI, X. *et al*. A CCL2/ROS autoregulation loop is critical for cancer-associated fibroblasts-enhanced tumor growth of oral squamous cell carcinoma. *Carcinogenesis*, v. 35, n. 6, p. 1362-70, Jun. 2014.

LIU, F. Y. *et al*. CCR7 regulates cell migration and invasion through MAPKs in metastatic squamous cell carcinoma of head and neck. *Int. J. Oncol*., v. 45, n. 6, p. 2502-10, Dec. 2014.

LU, P. *et al*. Potential Interaction between CCR1 and its ligand, CCL3, induced by endogenously produced interleukin-1 in human hepatomas. *Am. J. Pathol*., v. 162, n. 4, p. 1249-1258, Apr. 2003.

LU, S. L.; HERRINGTON, H.; WANG, X. J. Mouse models for human head and neck squamous cell carcinomas. *Head Neck*., v. 28, n. 10, p. 945-54, Oct. 2006.

MA, Y. R.; MA, Y. H. MIP-1α enhances Jurkat cell transendothelial migration by up-regulating endothelial adhesion molecules VCAM-1 and ICAM-1. *Leuk. Res*., v. 38, n. 11, p. 1327-31, Nov. 2014.

MANTOVANI, A. *et al.* Cancer-related inflammation. *Nature*, v. 454, n. 7203, p. 436-444, Jul. 2008.

MASSANO, J. *et al*. Oral squamous cell carcinoma: Review of prognostic and predictive factors. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*., v. 102, n. 1, p. 67-76, Jan. 2006.

MASSARA, M. *et al*. Atypical chemokine re ceptors in cancer: friends or foes? *J. Leukoc. Biol*., v. 99, p. 1-7, 2016.

MATSUO, Y. *et al*. CXC-chemokine/CXCR2 biological axis promotes angiogenesis in vitro and in vivo in pancreatic cancer. *Int. J. Cancer*, v.125, n. 5, p. 1027-37, Sep. 2009.

MAURER, M.; VON STEBUT, E. Macrophage inflammatory protein-1. *Int. J. Biochem. Cell Biol*., v. 36, n. 10, p. 1882-6, Oct. 2004.

MELCHERS, L. J. *et al*. Detection of HPV-associated oropharyngeal tumours in a 16-year cohort: more than meets the eye. *Br. J. Cancer*, v. 112, n. 8, p. 1349-57, Apr. 2015.

MENG, X. *et al*. Expression of CXCR4 in oral squamous cell carcinoma: correlations with clinicopathology and pivotal role of proliferation. *J. Oral Pathol. Med*., v. 39, n.1, p. 63–8, Jan. 2010.

MENTEN, P.; WUYTS, A.; VAN DAMME, J. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev*., v. 13, n. 6, p. 455-81, Dec. 2002.

MINICUCCI, E. M. *et al*. The role of the TP53 gene during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. *Exp.Toxicol. Pathol*., v. 63, n. 5, p. 483-489, Jul. 2011.

NAGINI, S. *et al*. Of humans and hamsters: a comparative evaluation of carcinogen activation, DNA damage, cell proliferation, apoptosis, invasion, and angiogenesis in oral cancer patients and hamster buccal pouch carcinomas. *Oral Oncol*., v.45, n. 6, p. 31-7, Jun. 2009.

NAKAHARA, W.; FUKUOKA, F.; SUGIMURA, T. Carcinogenic action of 4-nitroquinoline-N-oxide. *Gan*., v.48, n. 2, p. 129-37, Jun. 1957.

NATH, A. *et al*. Macrophage inflammatory protein (MIP) 1α and MIP1β differentially regulate release of inflammatory cytokines and generation of tumoricidal monocytes in malignancy. *Canc. Immunol. Immunother*., v. 55, p. 1534-1541, Dec. 2006.

NAUTA, J. M.; ROODENBURG, J. L.; NIKKELS, P. G. Epithelial dysplasia and squamous cell carcinoma of the Wistar rat palatal mucosa: 4NQO model. *Head Neck*, v. 18, n. 5, p. 441-449, Sep-Oct. 1996.

NIBBS, R. J. *et al*. The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am. J. Pathol*., v. 158, n. 3, p. 867-77, Mar. 2001.

NIBBS, R. J. *et al*. The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J. Clin. Invest*., v. 117, n. 7, p. 1884-92, Jul. 2007.

NUNOSHIBA, T.; DEMPLE, B. Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. *Cancer Res*., v. 53, n. 14, p. 3250-2, Jul. 1993.

OKAZAKI, Y. *et al*. Investigation of environmental factors for diagnosing malignant potential in oral epithelial dysplasia. *Oral Oncol*., v. 38, n. 6, p. 562-73, Sep. 2002.

OLIVEIRA NETO, H. H. *et al*. Involvement of CXCL12 and CXCR4 in lymph node metastases and development of oral squamous cell carcinomas. *Tumor Biol*., v. 29, n. 4, p. 262–71, 2008.

ONOUE, T. *et al*. Epithelial-mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells. *Int. J. Oncol*., v. 29, n. 5, p. 1133–8, Nov. 2006.

PANDRUVADA, S. N. M. *et al*. Role of CXC chemokine ligand 13 in oral squamous cell carcinoma associated osteolysis in athymic mice. *Int. J. Cancer*, v. 126, n. 10, p. 2319–29, May 2010.

QIAN, Y. *et al*. The chemokine receptor-CXCR2 plays a critical role in the invasion and metastases of oral squamous cell carcinoma in vitro and in vivo. *J. Oral Pathol. Med*., v. 43, n. 9, p. 658-66, Oct. 2014.

REHMAN, A. O.; WANG, C. CXCL12/SDF-1 alpha activates NF-kappaB and promotes oral cancer invasion through the Carma3/Bcl10/Malt1 complex. *Int. J. Oral Sci*., v. 1, n. 3, p. 105–18, Sep. 2009.

RIBEIRO, D. A. *et al*. Genomic instability in non-neoplastic oral mucosa cells can predict risk during 4-nitroquinoline 1 –oxide-induced rat tongue carcinogenesis. *Oral Oncol*., v. 40, p. 910-915, Oct. 2004.

ROTHENBERG, S. M.; ELLISEN, L.W. The molecular pathogenesis of head and neck squamous cell carcinoma. *J. Clin. Invest.*, v. 122, n. 6, p. 1951-7, Jun. 2012.

RUSSO, R. C.; GARCIA, C. C.; TEIXEIRA, M. M. Anti-inflammatory drug development: Broad or specific chemokine receptor antagonists? *Curr. Opin. Drug Discov. Devel*., v. 13, n. 4, p. 414-7, Jul. 2010.

SAKAKI, T. *et al*. Changing expression of E- and P-cadherin during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. *J. Oral Pathol. Med*., v. 32, n. 9, p. 530-7, Oct. 2003.

SAMARA, G. J. *et al*. CXCR4-mediated adhesion and MMP-9 secretion in head and neck squamous cell carcinoma. *Cancer Lett*., v. 214, n. 2, p. 231-41, Oct. 2004.

SANDHU, S. K. *et al*. A first-in-human, first-in-class, phase I study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 in patients with solid tumors. *Cancer Chemother. Pharmacol*., v. 71, n. 4, p. 1041-50, Apr. 2013.

SASAKI, S. *et al*. Crucial involvement of the CCL3/CCR5 axis-mediated fibroblast accumulation in colitis-associated carcinogenesis in mice. Int. J. Canc., v. 135, n.6, p. 1207-306, Sep. 2014.

SAVINO, B. *et al*. ERK-dependent down regulation of the atypical chemokine receptor D6 drives tumor aggressiveness in Kaposi sarcoma. *Cancer Immunol. Res*., v. 2, n. 7, p. 679-89, Jul. 2014.

SCHNEIDER, C. *et al*. Adaptive immunity suppresses formation and progression of diethyl nitrosamine-induced liver cancer. *Gut*, v. 61, n. 12, p. 733–1743, Dec. 2012.

SCHOOP, R. A. L.; NOTEBORN, M. H. M.; JONG, R. J. B. A mouse model for oral squamous cell carcinoma. *J. Mol. Hist*., v. 40, p. 177-181, Jun. 2009.

SCULLY, C.; BAGAN, J. Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. *Oral Dis*., v. 15, n. 6, p. 388-99, Sep. 2009.

SHAH, J. P.; GIL, Z. Current concepts in management of oral cancer-surgery. *Oral Oncol*., v. 45, n. 4-5, p. 394-401, Apr-May 2009.

SHANG, Z. J.; LIU, K.; SHAO, Z. Expression of chemokine receptor CCR7 is associated with cervical lymph node metastasis of oral squamous cell carcinoma. *Oral Oncol*., v. 45, p. 480-485, 2009.

SHEN, H. *et al*. The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. *FASEB J*., v. 20, n. 1, p. 59-64, Jan. 2006.

SHI, J. Y. *et al*. CC chemokine receptor-like 1 functions as a tumour suppressor by impairing CCR7-related chemotaxis in hepatocellular carcinoma. *J. Pathol.*, v. 235, n. 4, p. 546-58, Mar. 2015.

SHIMIZU, Y. *et al*. A single nucleotide polymorphism in the matrix metalloproteinase-1 and interleukin-8 gene promoter predicts poor prognosis in tongue cancer. *Auris. Nasus Larynx*, v. 35, n. 3, p. 381–9, Sep. 2008.

SHINRIKI, S. *et al*. Humanized anti-interleukin-6 receptor antibody suppresses tumor angiogenesis and in vivo growth of human oral squamous cell carcinoma. *Clin. Cancer Res*., v. 15, n. 17, p. 5426-34, Sep. 2009.

SHINTANI, S. *et al*. Growth-regulated oncogene-1 expression is associated with angiogenesis and lymph node metastasis in human oral cancer. *Oncology*, v. 66, n. 4, p. 316–22, 2004.

SICA, A.; ALLAVENA, P.; MANTOVANI, A. Cancer related inflammation: the macrophage connection. *Cancer Lett*., v. 267, n. 2, p. 204-15, Aug. 2008.

SILVA, J. M. *et al*. Eosinophil depletion protects mice from tongue squamous cell carcinoma induced by 4-nitroquinoline-1-oxide. *Histol. Histopathol*., v. 29, n. 3, p. 387–96, Mar. 2014.

SILVA, J. M. *et al*. Significance of chemokine and chemokine receptors in head and neck squamous cell carcinoma: A critical review. *Oral Oncol*., v.56, p. 8-16, May 2016.

SILVA, T. A. *et al*. Dual role of CCL3/CCR1 in oral squamous cell carcinoma: implications in tumor metastasis and local host defense. *Oncol. Rep*., v. 18, n. 5, p. 1107-1113, Nov. 2007.

SIVINA, M. *et al*. CCL3 (MIP-1α) plasma levels and the risk for disease progression in chronic lymphocytic leukemia. *Blood*, v. 117, n. 5, p. 1662-1669, Feb. 2011.

SORIA, G.; BEN-BARUCH, A. The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Canc. Lett*., v. 267, n. 2, p. 271-85, Aug. 2008.

SOUDA, M. *et al*. Gene expression profiling during rat mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene. *Int. J. Cancer*., v. 125, n. 6, p. 1285-97, Sep. 2009.

STOKES, A. *et al.* Expression profiles and clinical correlations of degradome components in the tumor microenvironment of head and neck squamous cell carcinoma. *Clin. Cancer Res*., v. 16, n. 7, p. 2022-35, Apr. 2010.

SUZUKI, T. *et al*. Target organ and time-course in the mutagenicity of five carcinogens in Muta Mouse: a summary report of the second collaborative study of the transgenic mouse mutation assay by JEMS/MMS. *Mutat. Res*., v. 444, n. 2, p. 259-68, Aug. 1999.

TAKE, Y. *et al*. Lymph node metastases in hamster tongue cancer induced with 9,10-dimethyl-1,2-benzanthracene: association between histological findings and the incidence of neck metastases, and the clinical implications for patients with tongue cancer. *Br. J. Oral Maxillofac. Surg*., v 37, n. 1, p. 29-36, Feb. 1999.

TAKI, M. *et al*. Up-regulation of stromal cell-derived factor-1alpha and its receptor CXCR4 expression accompanied with epithelial-mesenchymal transition in human oral squamous cell carcinoma. *Oncol. Rep*., v. 19, n. 4, p. 993-8, Apr. 2008.

TANABE, Y. *et al*. Blockade of the chemokine receptor, CCR5, reduces the growth of orthotopically injected colon cancer cells via limiting cancerassociated fibroblast accumulation. *Oncotarget*, v. 7, p. 48335-48345, Jun. 2016.

TANG, X. H. *et al*. Oral Cavity and esophageal carcinogenesis modeled in carcinogen treated mice. *Clin. Canc. Res*., v. 10, p. 301-313, Jan. 2004.

TANYEL, C. R. *et al*. Effects of genetic variants of CCR5 chemokine receptors on oral squamous cell carcinoma. *Genet. Mol. Res*., v. 12, n. 4, p. 5714–20, Nov. 2013.

TENG, Y. H. *et al*. Contribution of genetic polymorphisms of stromal cell-derived factor-1 and its receptor, CXCR4, to the susceptibility and clinicopathologic development of oral cancer. *Head Neck*, v. 31, n. 10, p, 1282–8, Oct. 2009.

TSUZUKI, H. *et al*. Oral and oropharyngeal squamous cell carcinomas expressing CCR7 have poor prognoses. *Auris. Nasus Larynx*, v. 33, n. 1, p. 37–42, Mar. 2006.

UCHIDA, D. *et al*. Acquisition of lymph node, but not distant metastatic potentials, by the overexpression of CXCR4 in human oral squamous cell carcinoma. *Lab. Invest*., v. 84, n. 12, p. 1538–46, Dec. 2004.

UCHIDA, D. *et al*. Blockade of CXCR4 in oral squamous cell carcinoma inhibits lymph node metastases. *Eur. J. Cancer*, v. 47, n. 3, p. 452-9, Feb. 2011.

UCHIDA, D. *et al*. Involvement of an autocrine stromal cell derived factor-1/CXCR4 system on the distant metastasis of human oral squamous cell carcinoma. *Mol. Canc. Res*., v. 5, n. 7, p. 685–94, Jul. 2007.

UCHIDA, D. *et al*. Possible role of stromal-cell-derived factor-1/CXCR4 signaling on lymph node metastasis of oral squamous cell carcinoma. *Exp. Cell Res*., v. 290, n. 2, p. 289–302, Nov. 2003.

UENO, T. *et al*. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin. Cancer Res*., v.6, n. 8, p. 3282-9, Aug. 2000.

VAIRAKTARIS, E. *et al*. The hamster model of sequential oral oncogenesis. *Oral Oncol*., v. 44, n. 4, p. 315-24, Apr. 2008.

VAIRAKTARIS, E. *et al*. The interleukin-18 -607A/C polymorphism is not associated with risk for oral cancer. *Anticancer Res*., v. 27, n. 6B, p. 4011–4, Nov-Dec. 2007.

VALLET, S. *et al*. A novel role for CCL3 (MIP-1α) in myeloma-induced bone disease via osteocalcin downregulation and inhibition of osteoblast function. *Leukemia*, v. 25, n. 7, p. 1174-1181, Jul. 2011.

VERED, M. *et al*. Stromal myofibroblasts and malignant transformation in a 4NQO rat tongue carcinogenesis model. *Oral Oncol*., v. 43, n. 10, p. 999-1006, Nov. 2007.

VETRANO, S. *et al*. The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. *Gut*, v. 59, n.2, p. 197-206, Feb. 2010.

VIEIRA, A. E. *et al*. Intramembranous bone healing process subsequent to tooth extraction in mice: micro-computed tomography, histomorphometric and molecular characterization. *PLoS One*, v. 10, n. 5, May 2015.

WARNAKULASURIYA, S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol.*, v. 45, n. 4-5, p. 309-16, Sep. 2009.

WENG, C. J. *et al*. Effect of CC chemokine ligand 5 and CC chemokine receptor 5 genes polymorphisms on the risk and clinicopathological development of oral cancer. *Oral Oncol*., v. 46, n. 10, p. 767–72, Oct. 2010.

WOLPE, S. D. *et al*. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J. Exp. Med*., v.167, n. 2, p. 570-81, Feb. 1988.

WU, F. Y. *et al*. Chemokine decoy receptor d6 plays a negative role in human breast cancer. *Mol. Cancer Res*., v. 6, n. 8, p. 1276-88, Aug. 2008.

WU, Y. *et al*. CCL3-CCR5 axis regulates intratumoral accumulation of leukocytes and fibroblasts and promotes angiogenesis in murine lung metastasis process. *J. Immunol*., v. 181, p. 6384-6393, Nov. 2008.

YANG, X. *et al*. Essential contribution of a chemokine, CCL3, and its receptor, CCR1, to hepatocellular carcinoma progression. *Int. J. Canc*., v. 118, p. 1869–1876, Apr. 2006.

YAO, C. *et al*. CXCL12/CXCR4 Axis Upregulates Twist to Induce EMT in Human Glioblastoma. *Mol. Neurobiol*., [Epub ahead of print], Jul. 2015.

YEUDALL, W. A. *et al*. Gain-of-function mutant p53 upregulates CXC chemokines and enhances cell migration. *Carcinogenesis*, v. 33, n. 2, p. 442-51, Feb. 2012.

YUAN, Y. *et al*. Chemokine CCL3 facilitates the migration of hepatoma cells by changing the concentration intracellular Ca. *Hepatol. Res*., v. 40, n. 4, p. 424-31, Apr. 2010.

ZOU, Y. R. *et al*. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*, v. 393, n. 6685, p. 595-9, Jun. 1998.

ANEXOS

ANEXO A

Oral Oncology 56 (2016) 8–16

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Review

Significance of chemokine and chemokine receptors in head and neck squamous cell carcinoma: A critical review

Janine Mayra da Silva [a], Danilo Figueiredo Soave [b], Tálita Pollyanna Moreira dos Santos [a], Aline Carvalho Batista [b], Remo Castro Russo [c], Mauro Martins Teixeira [d], Tarcília Aparecida da Silva [a,*]

[a] Department of Oral Surgery and Pathology, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[b] Department of Stomatology (Oral Pathology), Faculdade de Odontologia, Universidade Federal de Goiás, Goiânia, Brazil
[c] Department of Physiology and Biophysics, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[d] Department of Biochemistry and Immunology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form 14 January 2016
Accepted 29 February 2016

Keywords:
Head and neck squamous cell carcinoma
Chemokines
Inflammation

SUMMARY

Chemokines are small chemotactic proteins that coordinate circulation of immune/inflammatory cells throughout body compartments. Because of this property chemokines and their cell surface receptors are implicated in several physiological and pathological conditions, including cancer. These molecules are expressed by neoplastic or stromal cells and have effects at tumor primary site (e.g. stimulating angiogenesis and tumor cells motility) and lymph nodes (creating a gradient to direct migration of neoplastic cells). In this article we review the current knowledge about the function(s) of chemokines and receptors in squamous cell carcinoma from the oral cavity and head and neck region. Accumulating evidence suggests some chemokine(s) and receptor(s) as potential targets in adjuvant therapies for these malignancies.

Introduction

Squamous cell carcinoma (SCC) is the most common malignancy of head and neck region arising from mucosal linings of the upper aerodigestive tract, comprising (1) nasal cavity and paranasal sinuses, (2) nasopharynx, (3) hypopharynx, larynx, and trachea, and (4) oral cavity and oropharynx [1,2]. Head and neck squamous cell carcinomas (HNSCC) are characterized by high morbidity and mortality rates and strong tendency to regional and distant metastasis [1–4]. These tumor characteristics depend on individual properties of neoplastic cells and tumor microenvironment, which is comprised by diverse inflammatory/immune cells population; stromal cells; cancer stem cells; nutrients, growth factors, and a network of cytokines and chemokines [5–7]. It is widely accepted that cancer progression and prognosis is affected by immune/inflammatory cell infiltration into the tumor [6,8], a process tighly regulated by chemokines [9,10].

Chemokines are chemotactic cytokines able to control cell trafficking and positioning throughout the body compartments [11]. Chemokines have a defined chemical structure and comprise four subfamilies – CC, CXC, C and CX3C – distinguished according to the position of the first two cysteines adjacent to the amino terminus region. In chemokine nomenclature [12], the letter "L" followed by a number denotes a specific chemokine (e.g. CCL2 or CXCL8) [9,12]. The receptors are labelled by the letter R followed by the number (e.g. CCR2 or CXCR1). These molecules have key roles in tissue homeostasis, adaptive and acute immune responses [11]. In cancer, chemokines are implicated in progression by affecting tumor microenvironment and organ-specific spread of different tumor cells (e.g. breast, ovarian and colorectal) [13–15]. The biological and clinical relevance of these molecules is achieved by successful results from studies blocking chemokines system in some cancers [16–18].

Abbreviations: SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma; CAF's, carcinoma associated fibroblasts; MMP-9, matrix metalloproteinase-9; PI3K, phosphoinositide 3-kinase; AKT/PKB, protein kinase b; MAPK'S, mitogen activated protein kinases; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenquimal transition; SNP'S, single nucleotide polymorphisms; OLK, oral leukoplakia; ERK1/2, extracellular signal-regulated kinase; NF-KB, nuclear factor kappa-light-chain-enhancer of activated b cells; FDA, food and drug administration.

* Corresponding author at: Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31.270-901 Belo Horizonte, Minas Gerais, Brazil. Tel.: +55 31 3409 2478; fax: +55 31 3499 2430.
E-mail address: tarcilia@ufmg.br (T.A.d. Silva).

http://dx.doi.org/10.1016/j.oraloncology.2016.02.016
1368-8375/

Concerning SCC of oral cavity and head and neck region, a considerable number of studies were published, particularly in the last ten years, and had contributed to delineate the relevance of chemokines in this malignancy [19–70]. Nevertheless, despite accumulated knowledge, clinical trials targeting chemokines as part of chemotherapy regimen are incipient and included a small numbers of patients [16,18]. Herein, we review the most important studies on SCC of oral cavity and head and neck region and chemokines. In Table 1, we summarize the main findings of in vivo and in vitro studies highlighting the function and clinical relevance of these molecules in oral cancer. We also provide a critical perspective of exploiting the chemokine system as future chemotherapeutic opportunities for SCC.

CC chemokines

CCL2

A significant number of studies highlighted the CCL2 importance in different cancer types [71,72] and striking preclinical antitumor activity was verified blocking CCL2 [16–18] (Table 2). In addition, studies have shown expression of CCL2 in SCC from oral cavity (OSCC) and HNSCC [19–22,73,74]. One study did observed an increase of CCL2 expression in OSCC and in metastatic lymph nodes [73]. Similarly, CCL2 expression in HNSCC was associated with tumor invasion [20]. A functional evidence of CCL2 in OSCC was demonstrated by observation that proliferation, invasion and tumor growth induced by oral carcinoma-associated fibroblasts (CAF's) are mediated by CCL2 [19]. Therefore, inhibition of CCL2 resulted in significant reduction of tumor burden in vivo [19].

Genetic polymorphisms of CCL2 and CCR2 genes and increased risk of developing OSCC were described [74]. In this study patients with G allele and GG genotype of CCL2 and 64I allele and wt/64I genotype of CCR2 had significative increased risk for OSCC. The underlying proposed mechanism is that polymorphism results in increase of chemokine transcription and consequently augment of its biological activity [74].

CCL3 and CCL5

The expression of CCL3 and CCL5 was observed in different types of malignancies as hematological disorders, colitis-associated carcinogenesis, breast and prostate carcinomas [75–79]. Although there are no clinical trial targeting these molecules, one ongoing study is testing an approved agent for inhibition of CCL3 and CCL5 receptor, CCR5 (Maraviroc), to treat metastatic colorectal cancer (Table 2). In the context of oral cancer, just one report showed an augmented expression of CCL3 and its receptor CCR1 in OSCC and in metastatic lymph nodes [80].

Regarding CCL5, Chuang et al. [24] did observe its expression in OSCC cells lines. Stimulation of these cells with CCL5 induced directed migration and production of matrix metalloproteinase-9 (MMP-9). Consistently, blockade of MMP-9 using small interfering RNA inhibited the CCL5-induced cell motility providing evidence of CCL5/CCR5 axis involvement in invasive and metastatic phenotype of oral cancer cells [24]. However, it remains unclear the specific receptor (s) involved in the CCL5 effects in HNSCC.

CCL5 and CCR5 gene polymorphisms were correlated with increased risk for OSCC development in two case-control studies [81,82]. It was observed a positive association of CCR5 gene variants, delta32 and 59029 A/G, and of genotypes CCL5-28 CG, CCL5-28 CG or GG, CCL5-28 CG/-403 CT and CCL5-28 CG/-403 TT with increased risk for OSCC development [81,82].

CCL7

CCL7 exhibited marked up regulation in colorectal and gastric tumors and were correlated with metastasis [15,83]. Recently, CCL7 has been identified as a key regulator of OSCC cell invasion and migration in vitro and in vivo [25]. Jung et al. [25] demonstrated that CCL7 stimulated cytoskeleton changes in oral cancer cells enhancing invasion and migration. Accordingly, the invasiveness was inhibited by treatment with neutralizing antibodies against CCL7 or it receptors CCR1 and CCR3 [25]. These findings might put CCL7 as a candidate for therapeutic intervention in oral cancer, but clearly, questions regarding another functions of CCL7 and its receptors needs to be answered.

CCL19 and CCL21

Some studies showed significant correlation between CCL19/CCL21/CCR7 expression, lymph node metastasis and poor prognosis of OSCC [27,84] and HNSCC [29]. In these studies, increased expression of CCR7 in primary tumors and lymph nodes was correlated with tumor size, clinical stage, recurrence, lymph node metastasis and poor survival rates [27,29,84]. Additionally, using heterotopic transplantation of HNSCC cells in mouse, it was observed an increase of CCR7-expressing cells in the lymph nodes of mice [29]. In contrast, Oliveira et al. [85] did not find any association between CCL19/CCL21/CCR7 expression with microscopic and clinical parameters of OSCC.

Data from in vitro experiments demonstrated that CCR7-positive SCC cells had increased capacity to adhere to lymph nodes, which was inhibited when these cells were treated with anti-CCR7 antibody [27]. Additionally, CCL21 is a potent stimuli for SCC migration [27]. Accordingly, CCL21 receptor – CCR7 – induces cytoskeleton modifications which in turn stimulate tumor cell migration, invasion, adhesion and survival of HNSCC cells via activation of PI3K/AKT and MAPK's and MMP-9 release [30,32–34].

CCL20

Abiko et al. [86] were first to demonstrate CCL20 expression in OSCC samples and in six different oral SCC lines. They observed CCL20 upregulation after bacterial or inflammatory stimuli of OSCC cell cultures. A recent study found an association of CCL20 expression with nodal metastasis and worse prognosis of OSCC [35]. In line with these findings, the suppression of CCL20 in SCC cell line using interfering RNA's reduced migration and invasion [35].

CXC chemokines

CXCL1 and CXCL2

CXCL1/CXCR2 axis was found to mediate angiogenesis, and promote tumor progression in different types of cancer, (e.g. gastric, colorectal and pancreatic) [87–90]. Shintani et al. [37] observed an increased CXCL1 expression in different SCC cell lines and tumor specimens and correlated it with microvessel density, leukocyte infiltration and lymph node metastasis. Moreover, one additional study demonstrated that CXCL1 production is essential to activate Epidermal Growth Factor Receptor (EGFR) signaling and to increase proliferation of human dysplastic oral keratinocytes [38].

The expression of CXCL2 was also demonstrated in human samples and SCC cell lines. This molecule seems to be involved in cancer-induced bone destruction and invasion once CXCL2-expressing OSCC cells injected into calvaria of athymic mice induced bone resorption via activation of pro-osteoclastogenic pathways [39]. Therefore, a significant reduction of osteoclast formation was observed after blocking CXCL2 [39].

CXCL8

In human cancers, it is well established that CXCL8/CXCR1 and CXCL8/CXCR2 axis can induce angiogenesis, tumor growth, motility of neoplastic cells and Epithelial–mesenquimal transition (EMT) [89–92]. In the context of oral cancer, it was previously shown that expression of CXCL8, CXCL6 (a CXCL8 homologue) and CXCR2 in tumor samples correlates with lymph node

Table 1
Current knowledge of chemokine/chemokine receptor system expression in studies of Head and Neck Squamous Cell Carcinoma.

Chemokine	Receptor(s)	Key target(s)	Sample type	Main findings(s)	References
CC chemokines					
CCL2 (MCP-1)	CCR2	Monocytes, lymphocytes and NK	OSCC	↑ CCL2 ↑ lymph node metastasis	[73]
			OSCC, OSCC cell line and xenografts	↑ CCL2 ↑ ROS ↑ proliferation, ↑ invasion ↑ tumor growth	[19]
			HNSCC	↑ invasion, ↑ macrophage infiltration ↑ angiogenesis	[20]
			HNSCC and OSCC cell lines	↑ CCL2 ↓ patient survival ↑ CCL2 ↑ ERK/AKT/STAT3 phosphorilation ↑ survival in vitro	[21]
				↑ CCL2 ↑ EMT via AKT	[22]
CCL3 (MIP1-α)	CCR1, CCR5	Macrophages, monocytes, basophils and NK	OSCC	↑ CCL3 ↑ lymph node metastasis	[80]
CCL4 (MIP1-β)	CCR5, CCR8		HNSCC and HNSCC cell lines	↑ CCL4 ↑ T lymphocytes infiltration ↑ survival	[23]
CCL5 (RANTES)	CCR1, CCR3, CCR5		OSCC cell line	↑ CCL5 ↑ cell motility ↑ migration ↑ MMP-9	[24]
CCL7 (MCP-3)	CCR1, CCR2, CCR3, CCR5	Monocytes, neutrophils	OSCC and OSCC cell line	CCR1/CCR3 inhibition ↓ cell invasion	[25]
CCL11 (Eotaxin)	CCR2, CCR3, CCR5	Eosinophil and basophils	OSCC	↑ Eosinophil infiltration	[109]
CCL17 (TARC)	CCR4, CCR8	T lymphocytes	HNSCC and HNSCC cell lines	↑ CCL17/CCL22 ↑ Foxp3$^+$ and Tregs tumor infiltration	[26]
CCL19 (ELC, Exodus-3 and MIP3-β)/CCL21 (SLC, Exodus-2 and TCA-4)	CCR7	T and B lymphocytes and DC	OSCC	↑ CCR7 ↑ lymph node metastasis ↑ tumor size ↑ clinical stage and local recurrence	[84]
			OSCC, and OSCC cell line	↑ CCR7 ↑ lymph node metastasis ↑ stage ↑ tumor size↑ CCL21/CCR7 ↑ invasion ↑ adhesion to lymph nodes in vitro	[27]
			HNSCC, HNSCC cell lines and mice	↑ CCL19 ↑ lymph node metastasis CCR7 blockade ↓ AKT ↓ BCL-2 ↓ tumor growth	[28]
			HNSCC and HNSCC cell lines	↑ CCR7 ↑ lymph node metastasis ↑ adhesive ability	[29]
			HNSCC and HNSCC cell lines	↑ CCR7 ↑ lymph node metastasis	[30]
				↑ CCR7 ↑ migration ↑ adhesion and cytoskeleton reorganization	
				↑ CCR7/CCL19 ↑ PKCα ↑ NF-KB	[31]
				↑ CCR7 ↑ PKCα ↑ stage ↑ lymph node metastasis	
				↑ CCR7/CCL19 ↑ PI3K ↑ CDC42 and actin polymerization	[32]
				↑ CCR7 ↑ migration ↑ MMP-9 ↑ β1 integrin ↑ cytoskeleton reorganization	[33]
				↑ CCR7/CCL19 ↑ ERK/JNK ↓ E-cadherin ↑ vimentin ↑ invasion	[34]
CCL20 (LARC, Exodus-1 and MIP3-α)	CCR6	Macrophages, lymphocytes and monocytes	OSCC and OSCC cell line	↑ CCL20 ↑ lymph node metastasis CCL20 blockade ↓ cell invasion	[23,35]
CCL22 (MDC)	CCR4	Macrophages, DC	HNSCC and HNSCC cell lines	↑ CCR4/CCL22 ↑ lymph node metastasis ↑ lymphatic invasion	[36]
				↑ CCR4/CCL22 ↑ migration ↑ M2-like macrophages	
CXCL1(Gro-α, GRO1)	CXCR2	Neutrophils	OSCC and OSCC cell lines	↑ CXCL1 lymph node metastasis	[37]
				↑ CXCL1 ↑ leukocyte differentiation and ↑ microvessel density	
			OSCC cell lines	↑ IL-1β ↑ CXCL1 ↑ EGFR	[38]
CXCL2 (Gro-β, GRO2 and MIP2-α)	CXCR2		OSCC, OSCC cell line and xenografts	↑ CXCL2 in OSCC cell line ↑ osteoclast differentiation ↑ bone resorption	[39]
CXCL5 (ENA-78)	CXCR1, CXCR2		HNSCC and HNSCC cell lines	↑ CXCL5 ↑ proliferation ↑ invasion in vitro and in vivo	[40]
CXCL8 (IL-8)	CXCR1; CXCR2		OSCC cell lines	↑ CXCL8 ↑ invasion ↑ MMP-7	[41]
				↑ CXCL8 ↑ proliferation ↑ invasion	[42]
				↑ CXCL8 and CXCL1 ↑ ERK1/2 phosphorylation ↑ proliferation ↑ invasion and ↑ MMP-7 and 9	[43]
			OSCC, OSCC cell line and xenografts	↑ CXCR2 ↑ lymph node metastasis	[44]
				↑ CXCR2 ↑ migration ↑ invasion and cytoskeleton reorganization	
CXCL9 (MIG)	CXCR3	Lymphocytes and NK	OSCC and OSCC cell line	↑ CXCL9 OSCC ↑ tumor burden ↑ aggressiveness CXCL9 inhibition ↓ cell proliferation, migration and invasion	[45,46]
CXCL10 (IP-10)			OSCC	↑ CXCL10 ↓ response to radiotherapy ↓ overall survival	[47]
CXCL11 (I-TAC, IP-9)			OSCC	↑ CXCL11/CXCR7/CXCL12 ↑ OLK and OSCC	[48]
CXCL12 (SDF-1)	CXCR4/CXCR7	Lymphocytes, monocytes	OSCC and OSCC cell line	CXCL12 activated ↑ ERK1/2 and ↑ AKT phosphorilation in vitro	[49]
			OSCC cell line and mice	OSCC cell line transfected with CXCR4 ↑ calcium influx ↑ ERK1/2 ↑ AKT/PKB and ↑ metastasis in vivo	[50]
			OSCC, OSCC cell line and mice	↑ CXCL12/CXCR4 in metastatic lymph nodes ↓ survival CXCR4 antagonism ↓ lung metastasis ↑ survival in mice CXCL12 ↑ cell motility and ↑ anchorage-independent growth	[51]

Table 1 (*continued*)

Chemokine	Receptor(s)	Key target(s)	Sample type	Main findings(s)	References
			OSCC	↑ CXCL12/CXCR4 ↑ lymph node metastasis	[52–54]
			HNSCC	↑ CXCL12 ↑ lymph node metastasis	[55]
			OSCC and OSCC cell line	↑ CXCR4 ↑ lymph node metastasis ↑ tumor size ↑ stage and grade CXCL12 ↑ proliferation	[56]
			OSCC	↑ CXCR4/CXCL12 ↑ tumor grade ↑ lymph node metastasis ↑ perineural invasion ↓ survival ↑ CXCR4/Vimentin ↓ survival	[57]
			OSCC and OSCC cell line	↑ CXCR4 ↑ lymph node metastasis ↑ invasion ↑ recurrence ↑ CXCR4 ↑ invasiveness and scattering in vitro	[58]
			OSCC	↑ CXCR4 ↑ MMP-9 ↑ proliferation	[59]
			OSCC cell line	↑ CXCR4 ↑ NF-κB by CBM-complex ↑ invasion	[60]
			OSCC cell line	↑ CXCL12 ↑ vimentin and ↓ E-cadherin by PI3 K-AKT	[61]
			OSCC cell line	Forced EMT ↑ CXCL12 ↑ migration	[62]
			HNSCC cell line	↑ CXCR4 ↑ adhesion ↑ MMP-9	[63]
			HNSCC cell line	CXCR4 blockade ↓ proliferation	[64]
			OSCC cell line	LPS ↑ CXCR4 ↑ invasiveness	[65]
CXCL13 (BCA-1, BLC)	CXCR5	T and B lymphocytes	OSCC, OSCC cell line and xenografts	↑ CXCL13/CXCR5 ↑ bone invasion	[66–68]
CXCL14 (BRAK)	Unknown	B lymphocytes, monocytes	OSCC cell lines and xenografts	CXCL14^{+} cells ↓ proliferation in vitro ↓ tumor growth in vivo	[69]
XCL1 (Lymphotactin α)	XCR1	Lymphocytes and NK	OSCC and OSCC cell lines	↑ XCL1 ↑ lymph node metastasis XCL1/XCR1 ↑ ERK1/2 phosphorylation ↑ proliferation ↑ invasion ↑ adhesion and ↑ MMP-7	[70]

Abbreviations: MCP-1 (monocyte chemoattractant protein-1); OSCC (oral squamous cell carcinoma); NK (natural killer); ROS (reactive oxygen species); HNSCC (head and neck squamous cell carcinoma); ERK (extracellular signal-regulated kinase); AKT/PKB (protein kinase b); STAT3 (signal transducer and activator of transcription 3); EMT (epithelial–mesenquimal transition); MIP1-α (macrophage inflammatory protein 1-α); MIP1-β (macrophage inflammatory protein-1β); RANTES (regulated upon activation normal T-cell expressed and secreted); MMP-9 (matrix metalloproteinase-9); MCP-3 (monocyte chemoattractant protein-3); MIP-3β (macrophage inflammatory-3β); SLC (secondary lymphoid tissue chemokine); DC (dendritic cells); BCL-2 (membrane proteins encoded by the BCL-2 genes); PKC (protein kinase c); NF-KB (nuclear factor kappa-light-chain-enhancer of activated b cells); PI3K (phosphoinositide 3-kinase); CDC42 (cell division cycle 42); JNK (c-Jun N-terminal kinase JNK); MIP3-α (macrophage protein-3α); MDC (macrophage derived chemokine); Tregs (regulatory T cells); GRO-1α (growth-regulated oncogene-1α); IL-1β (interleukin-1β); EGFR (epidermal growth factor receptor); GRO-β (growth-related gene product β or MIP-2α (macrophage inflammatory protein-2α); IL-8 (interleukin-8); MMP-7 (matrix metalloproteinase-7); MIG (monokine induced by gamma-interferon); IP-10 (interferon-induced protein-10); SDF-1α (stromal cell-derived factor-1α); CMB (carma3, bcl10 and malt1 complex); LPS (lipopolysaccharide); BLC/BCA-1 (B-lymphocyte chemoattractant/B-cell attracting chemokine-1); RANKL (receptor activator of nuclear factor kappa-b ligand); C-JUN (protein encoded by JUN gene); NFATC3 (nuclear factor of activated t cells, cytoplasmic 3); C-MYC (cellular dna-binding proteins encoded by the c-myc gene); BM (bone marrow); ENA (epithelial-derived neutrophil-activating peptide 78); IP-9 (interferon-induced protein-9); BRAK (breast and kidney expressed chemokine); TARC (thymus- and activation-regulated chemokine); OLK (oral leukoplakia).

metastasis and worse prognosis [44,93]. Moreover, in vitro studies verified that CXCL8 and CXCR2 promoted migration and invasion of OSCC cells [42–44,93]. Consistently, a significant inhibition was seen when knockdown CXCL8 interfering RNA or receptor blocking antibodies were employed [42,43].

Analysis of genetic polymorphisms revealed that a mutant allele (A-251) in the CXCL8 promoter was significantly associated with increased risk of OSCC and a positive correlation between this mutation and poor prognosis [94]. In contrast, Vairaktaris et al. [95] and Liu et al. [96] did not find association of CXCL-8 single nucleotide polymorphisms (SNP's) with oral cancer susceptibility. However, the latter study observed a strong correlation between OSCC risk and tobacco or betel quid chewing consumption and OSCC risk, suggesting that environmental carcinogens associated with CXCL8 polymorphisms might increase the risk of OSCC development [96]. A meta analysis evaluating six different studies did not reach positive correlation between CXCL8 gene polymorphysm and oral cancer susceptibility [97].

CXCL9 and CXCL10

The expression of CXCL9, CXCL10 and CXCR3 was previously detected in cancer tissues (i.e. nasopharyngeal and pancreatic) [46,98]. A recent study reported augmented expression of CXCL9 in serum of patients with OSCC compared to healthy individuals which was positively correlated with poor clinical parameters [46]. Moreover, it was observed that knockdown of CXCL9 in a SCC cell lineage using interfering RNA resulted in significant reduction of cell proliferation, migration and invasiveness [46]. CXCL9/CXCR3 was also shown to be expressed by CAF's [99]. In the latter study, CAF supernatants stimulated tongue carcinoma cells (Tca8113) to increase levels of CXCR3 and Bcl-2. This effect was significantly attenuated after neutralization with anti-CXCR3 and CXCL9 antibodies [99].

CXCL10 was also associated with response to radiotherapy and poor overall survival in tongue squamous cell carcinomas [47]. In this study, CXCL10 was upregulated (16-fold increase) and its expression was correlated with worse response to radiotherapy [47].

CXCL11, CXCL12, CXCR4 and CXCR7

The axis CXCL11/CXCL12/CXCR4/CXCR7 is the most studied chemokine system in oral tumorigenesis [48–65,100–103]. Noteworthy, substantial progress was made targeting CXCR4/CXCL12 in other cancer types including the use of an approved chemotherapeutic based on antagonism of CXCR4 (Table 2). Studies have shown increased expression of CXCL12 and CXCR4 in Oral Leukoplakia (OLK), OSCC, HNSCC and metastatic lymph nodes [48,51–58]. In vitro, CXCL12/CXCR4 induces cytoskeleton changes that facilitate migration of OSCC cells via activation of ERK1/2, AKT/PKB and NF-KB [49,50,60,61] (Fig. 1).

The influence of CXCL12/CXCR4 axis in EMT was assessed in vitro, once EMT is critical for tumor microenvironment remodeling. Some studies did obtain that CXCL12/CXCR4 promotes down-regulation of epithelial markers (i.e. Cytokeratin, E-cadherin and β-catenin), and up-regulation of mesenchymal components (i.e. Vimentin and Snail), suggesting that this system contributes to lymph node metastasis via EMT activation [61,62].

Table 2
Current clinical trial studies focusing in chemokine/chemokine receptors targeting in malignancies.

Agent	Target	Class	Study type/tumor type/status/	Reference
Carlumab	CCL2	Anti-CCL2 monoclonal antibody	Phase I and Phase II prostate cancer (completed)	[17] NCT00992186[a]
CNTO888 Centocor			Phase I – advanced tumors (completed)	[16][b] [18][b] NCT01204996[a] NCT00537368[a]
KW-0761	CCR4	Neutralizing monoclonal CCR4 antibody	Phase II – adult T-cell Leukemia-lymphoma (completed)	[110] NCT00920790[a]
Maraviroc (Maracon)	CCR5	Antagonist	Phase I – metastatic colorectal cancer (completed)	NCT01736813[a]
NI	CXCR2	NI	Study type unknown – pancreatic cancer (completed)	NCT00851955[a]
Plerixafor (Trade name: Mozobil) (AMD3100)	CXCR4	Antagonist	Phase I – advanced pancreas, ovarian, colorrectal cancer (recruiting) Multiple Myeloma, Non-Hodgkin Lymphoma, Leukemia	NCT02179970[a] NCT00512252[a] NCT00241358[a] NCT00694590[a] NCT00914849[a] NCT00322842[a] NCT00665314[a]
BKT 140	CXCR4	Antagonist	Phase I and Phase II – Multiple myeloma (completed)	[111] NCT01010880[a]
MDX1338 (Medarex)	CXCR4	Neutralizing monoclonal CXCR4 antibody	Phase I – Multiple myeloma (completed)	NCT01120457[a]
NOX-A12 (Noxxon)	CXCL12	Oligonucleotide	Phase II – Multiple myeloma, Chronic lymphocytic leukemia (ongoing)	NCT01486797[a] NCT01521533[a]
NI	CXCL12/ CXCR4	NI	Study type unknown – metastasis of laryngeal and hypopharyngeal SCC (recruiting)	NCT00174096[a]

Informations included in this table were adapted from Ref. [112,113].
NI (not informed).
[a] ClinicalTrials.gov Identifier.
[b] Includes: SCC and HNSCC.

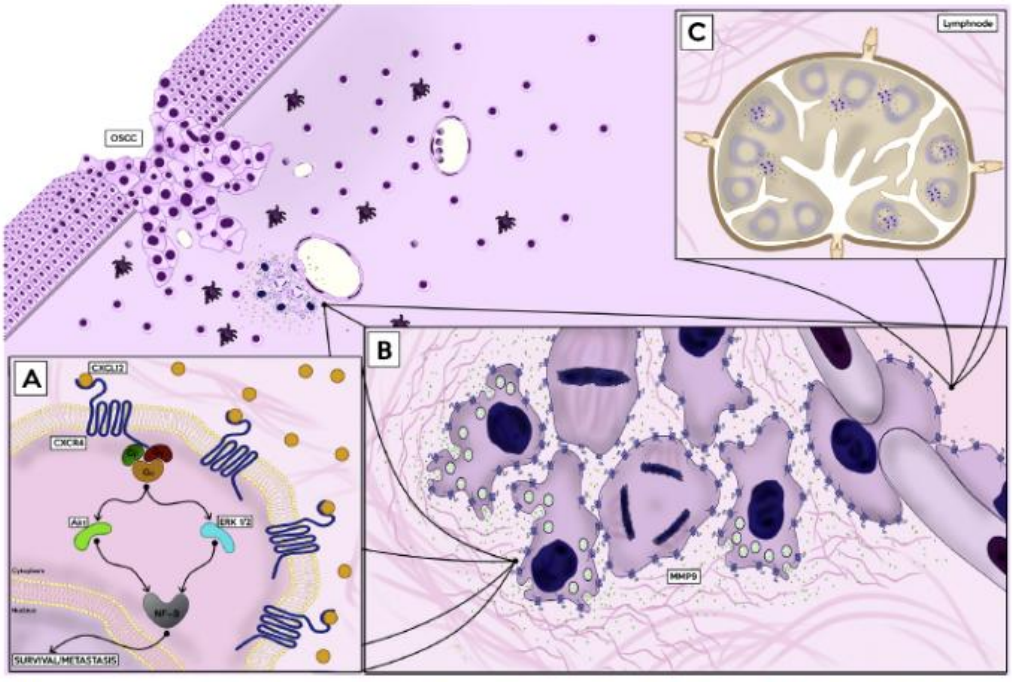


Fig. 1. CXCL12/CXCR4 in OSCC. (A) CXCL12 engagement in CXCR4 induces ERK1/2 and Akt/PKB activation, thus influencing cell survival and spreading of neoplastic cells, (B) neoplastic cells expressing CXCR4 co-express MMP-9, which might in turn stimulate tissue remodeling and migration of these cells. The CXCR4 positivity was also associated with increase of tumor proliferative index, (C) CXCL12/CXCR4 expression in OSCC was positively correlated with neoplastic cell migration to lymph nodes.

Genetic polymorphisms of CXCL12 and CXCR4 have been associated with increase of OSCC risk [102,103]. Vairaktaris et al. [102] studied the CXCL12 G801A gene polymorphism in Europeans and found that allele A was associated with advanced stages of oral cancer, especially among alcohol abusers [102]. Another study evaluated the same polymorphism in the CXCL12 gene and CXCR4 in Taiwanese patients [103]. This study obtained that individuals with at least 1 mutated allele A in CXCL12 gene, had increased risk to develop OSCC when compared with those with G/G genotype. No significant difference was observed in allele distribution for CXCR4 gene [103].

XCL1

Little is known about XCL1 effects in oral cancer. Khurram et al. [70] observed, for the first time, the expression of XCR1 by normal oral keratinocytes and OSCC cell lines. In this study, XCR1/XCL1 interaction induced phosphorylation of ERK1/2 and stimulated migration, invasion and proliferation of OSCC cells. Additionally, XCR1/XCL1 stimulated production of MMP-2, MMP-7 and MMP-9 by SCC cells in vitro [70].

Future directions

Despite the advances in therapeutic procedures (surgery, chemo-radiotherapy) over the last years, the prognosis of patients with HNSCC has remained largely unchanged. The high potential of this type of cancer for local invasion, the elevated rates of cervical metastasis, the tumor size and stage at the time of diagnosis have been considered the most important prognostic factors [1,2].

In the last ten years considerable progress was obtained on chemokines effects in HNSCC (Table 1). Furthermore, lessons taken from other cancer types based on targeting chemokine system brought up successful outcomes (Table 2). Therefore, the use of anti-chemokine antibodies, chemokine-binding proteins, chemokine receptor antagonists or inhibitors interfering with the signaling emerged as adjuvant chemotherapy alternative in HNSCC's. Clearly, data discussed in the current review demonstrate that CCL5/CCR5, CCL19/CCR7, CXCL12/CXCR4 and CCL2/CCR2 axis are strong candidates for intervention. To date, only two clinical studies, with four patients included, have tested a CCL2 antibody, CNTO 888 (Carlumab), in HNSCC patients. Nevertheless, no long term significant clinical effects were reported until now [16,18].

Proof of concept from the therapeutic CXCL12/CXCR4 blockade in tumors has been demonstrated by Porvasnik et al. [104] A CXCR4 antagonist (CTCE-9908) was able to reduce tumor cell proliferation and to induced modifications in tumor stroma as inhibition of angiogenesis and lymphangiogenesis and induction of apoptosis in prostate tumors [104]. Another CXCR4 blocker, AMD 3100 (Plerixafor), approved by the Food and Drug Administration (FDA), exhibited antitumoral activity via induction of brain tumor cells apoptosis [105]. In contrast, there are some studies reporting that Plerixafor alone was ineffective in tumor reduction of glioblastomas and mouse mammary carcinomas [106,107]. In a clinical trials data base (https://clinicaltrials.gov), there were more than eighty studies using Plerixafor in anti-neoplastic therapies, mainly for hematopoietic tumors. Currently, Plerixafor use is accepted in clinical routine to treat multiple myeloma or non-Hodgkin lymphoma [108] (Table 2).

The anti-CCL2 antagonist, Carlumab, has been well tolerated as adjuvant therapy to treat solid malignancies (e.g. prostate, breast, ovarian, colorectal, and esophageal SCC) [16–18]. However, preliminary results showed only transient blocking CCL2/CCR2 in serum without effects in anti-tumoral activity [17,18].

Maraviroc, a CCR5 blocker, was the first chemokine-based therapy approved by FDA for treatment of HIV. Currently, there is one clinical trial testing this agent to treat metastatic colorectal cancer (Table 2), but no information regarding preliminary results are available.

As summarized in Table 2, except for CXCL12/CXCR4-based therapy for hematologic malignancies, there are a few clinical studies targeting chemokines or chemokine receptors in solid tumors. Thus, a vast field for discovery is existing, in particular for two approved drugs (Maraviroc and Plerixaflor) and unexplored, but potentially important targets in HNSCC: CCL5/CCR5, CCL19/CCR7, CXCL12/CXCR4 and CCL2/CCR2.

Concluding remarks

Squamous cell carcinoma of the head and neck region is a severe disease and, in most cases, fatal. A better understanding of molecular mechanisms associated with these tumors might help to find efficient adjuvant therapies. Available studies revealed that cells from HNSCC express chemokine receptors and also release different types of chemokines. These molecules orchestrate critical functions in oral tumorigenesis by favoring angiogenesis, tumor growth and metastasis. These results put these molecules as targets for therapies to these malignancies. Most studies have focused on the CXCL12/CXCR4 axis. In vitro and in vivo data clearly demonstrate the significance of this axis in metastasis through increasing motility and proliferation of neoplastic cells and induction of EMT. Furthermore, data concerning CCL5/CCR5, CCL19/CCR7 and CCL2/CCR2 support the notion that therapies directed to these chemokines would improve HNSCC outcomes. Currently, a wide variety of compounds targeting chemokines and chemokine receptors in malignancies are in clinical trial and two approved drugs are available. It should encourage clinical strategies to test these molecules as adjuvant therapies in HNSCC.

Conflict of interest statement

None declared.

Acknowledgements

We are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

References

[1] Rothenberg SM, Ellisen LW. The molecular pathogenesis of head and neck squamous cell carcinoma. J Clin Invest 2012;122:1951–7. http://dx.doi.org/10.1172/JCI59889.
[2] Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Canc 2011;11:9–22. http://dx.doi.org/10.1038/nrc2982.
[3] Feller L, Lemmer J. Oral squamous cell carcinoma: epidemiology, clinical presentation and treatment. J Cancer Ther 2012;03:263–8. http://dx.doi.org/10.4236/jct.2012.34037.
[4] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2014;136:E359–86. http://dx.doi.org/10.1002/ijc.29210.
[5] Hanahan D, Coussens LM. Accessories to the Crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012;21:309–22. http://dx.doi.org/10.1016/j.ccr.2012.02.022.
[6] Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454:436–44. http://dx.doi.org/10.1038/nature07205.
[7] Ritchie KE, Nör JE. Perivascular stem cell niche in head and neck cancer. Cancer Lett 2013;338:41–6. http://dx.doi.org/10.1016/j.canlet.2012.07.025.
[8] Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883–99. http://dx.doi.org/10.1016/j.cell.2010.01.025.
[9] Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004;4:540–50. http://dx.doi.org/10.1038/nrc1388.

[10] Chow MT, Luster AD. Chemokines in cancer. Canc Immunol Res 2014;2:1125–31. http://dx.doi.org/10.1158/2326-6066.CIR-14-0160.

[11] Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 2014;32:659–702. http://dx.doi.org/10.1146/annurev-immunol-032713-120145.

[12] Chemokine/Chemokine Receptor Nomeclature. IUIS/WHO Subcommittee on Chemokine Nomenclature. J Interferon Cytok Res 2002; 22: 1067–1068.

[13] Soria G, Ben-Baruch A. The inflammatory chemokines CCL2 and CCL5 in breast cancer. Cancer Lett 2008;267:271–85. http://dx.doi.org/10.1016/j.canlet.2008.03.018.

[14] Levina V, Nolen BM, Marrangoni AM, Cheng P, Marks JR, Szczepanski MJ, et al. Role of eotaxin-1 signaling in ovarian cancer. Clin Cancer Res 2009;15:2647–56. http://dx.doi.org/10.1158/1078-0432.CCR-08-2024.

[15] Cho YB, Lee WY, Choi SJ, Kim J, Hong HK, Kim SH, et al. CC chemokine ligand 7 expression in liver metastasis of colorectal cancer. Oncol Rep 2012;28:689–94. http://dx.doi.org/10.3892/or.2012.1815.

[16] Sandhu SK, Papadopoulos K, Fong PC, Patnaik A, Messiou C, Olmos D, et al. A first-in-human, first-in-class, phase I study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 in patients with solid tumors. Canc Chemother Pharmacol 2013;71:1041–50. http://dx.doi.org/10.1007/s00280-013-2099-8.

[17] Pienta KJ, Machiels JP, Schrijvers D, Alekseev B, Shkolnik M, Crabb SJ, et al. Phase 2 study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 (CCL2), in metastatic castration-resistant prostate cancer. Invest New Drugs 2013;31:760–8. http://dx.doi.org/10.1007/s10637-012-9869-8.

[18] Brana I, Calles A, LoRusso PM, Yee LK, Puchalski TA, Seetharam S. Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody, in combination with four hemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study. Target Oncol 2015;10:111–23. http://dx.doi.org/10.1007/s11523-014-0320-2.

[19] Li X, Xu Q, Wu Y, Li J, Tang D, Han L, et al. A CCL2/ROS autoregulation loop is critical for cancer-associated fibroblasts-enhanced tumor growth of oral squamous cell carcinoma. Carcinogenesis 2014;35:1362–70. http://dx.doi.org/10.1093/carcin/bgu046.

[20] Ohta M, Kitadai Y, Tanaka S, Yoshihara M, Yasui W, Mukaida N, et al. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. Int J Canc 2002;102:220–4. http://dx.doi.org/10.1002/ijc.10705.

[21] Ji WT, Chen HR, Lin CH, Lee JW, Lee CC. Monocyte chemotactic protein 1 (MCP-1) modulates pro-survival signaling to promote progression of head and neck squamous cell carcinoma. PLoS ONE 2014;9:e88952. http://dx.doi.org/10.1371/journal.pone.0088952.

[22] Lee CC, Ho HC, Su YC, Lee MS, Hung SK, Lin CH. MCP1-induced epithelial–mesenchymal transition in head and neck cancer by AKT activation. Anticancer Res 2015;35:3299–306.

[23] Liu JY, Li F, Wang LP, Chen XF, Wang D, Cao L, et al. CTL- vs Treg lymphocyte-attracting chemokines, CCL4 and CCL20, are strong reciprocal predictive markers for survival of patients with oesophageal squamous cell carcinoma. Br J Canc 2015;113:747–55. http://dx.doi.org/10.1038/bjc.2015.290.

[24] Chuang JY, Yang WH, Chen HT, Huang CY, Tan TW, Lin YT, et al. CCL5/CCR5 axis promotes the motility of human oral cancer cells. J Cell Physiol 2009;220:418–26. http://dx.doi.org/10.1002/jcp.21783.

[25] Jung DW, Che ZM, Kim J, Kim K, Kim KY, Williams D, et al. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. Int J Cancer 2010;127:332–44. http://dx.doi.org/10.1002/ijc.25060.

[26] Maruyama T, Kono K, Izawa S, Mizukami Y, Kawaguchi Y, Mimura K, et al. CCL17 and CCL22 chemokines within tumor microenvironment are related to infiltration of regulatory T cells in esophageal squamous cell carcinoma. Dis Esoph 2010;23:422–9. http://dx.doi.org/10.1111/j.1442-2050.2009.01029.x.

[27] Shang ZJ, Liu K, Shao Z. Expression of chemokine receptor CCR7 is associated with cervical lymph node metastasis of oral squamous cell carcinoma. Oral Oncol 2009;45:480–5. http://dx.doi.org/10.1016/j.oraloncology.2008.06.005.

[28] Wang J, Seethala RR, Zhang Q, Gooding W, van Waes C, Hasegawa H, et al. Autocrine and paracrine chemokine receptor 7 activation in head and neck cancer: implications for therapy. J Natl Cancer Inst 2008;100:502–12. http://dx.doi.org/10.1093/jnci/djn059.

[29] Irino T, Takeuchi H, Matsuda S, Saikawa Y, Kawakubo H, Wada N, et al. CC-Chemokine receptor CCR7: a key molecule for lymph node metastasis in esophageal squamous cell carcinoma. BMC Canc 2014;14:291. http://dx.doi.org/10.1186/1471-2407-14-291.

[30] Li P, Zhao ZJ, Liu FY, Sun LY, Ding X, Zhang WZ, et al. The chemokine receptor 7 regulates cell adhesion and migration via beta1 integrin in metastatic squamous cell carcinoma of the head and neck. Oncol Rep 2010;24:989–95. http://dx.doi.org/10.3892/or_00000946.

[31] Zhen-jin Z, Peng L, Fa-yu L, Liyan S, Chang-fu S. PKCα take part in CCR7/NF-κB autocrine signaling loop in CCR7-positive squamous cell carcinoma of head and neck. Mol Cell Biochem 2011;357:181–7. http://dx.doi.org/10.1007/s11010-011-0888-0.

[32] Zhao ZJ, Liu FY, Li P, Ding X, Zong ZH, Sun CF. CCL19-induced chemokine receptor 7 activates the phosphoinositide-3 kinase-mediated invasive pathway through Cdc42 in metastatic squamous cell carcinoma of the head and neck. Oncol Rep 2011;25:729–37. http://dx.doi.org/10.3892/or.2010.1109.

[33] Guo N, Liu F, Yang L, Huang J, Ding X, Sun C. Chemokine receptor 7 enhances cell chemotaxis and migration of metastatic squamous cell carcinoma of head and neck through activation of matrix metalloproteinase-9. Oncol Rep 2014;32:794–800. http://dx.doi.org/10.3892/or.2014.3242.

[34] Liu FY, Safdar J, Li ZN, Fang QG, Zhang X, Xu ZF, et al. CCR7 regulates cell migration and invasion through MAPKs in metastatic squamous cell carcinoma of head and neck. Int J Oncol 2014;45:2502–10. http://dx.doi.org/10.3892/ijo.2014.2674.

[35] Chang KP, Kao HK, Yen TC, Chang YL, Liang Y, Liu SC, et al. Overexpression of macrophage inflammatory protein-3α in oral cavity squamous cell carcinoma is associated with nodal metastasis. Oral Oncol 2011;47:108–13. http://dx.doi.org/10.1016/j.oraloncology.2010.11.012.

[36] Tsujikawa T, Yaguchi T, Ohmura G, Ohta S, Kobayashi A, Kawamura N, et al. Autocrine and paracrine loops between cancer cells and macrophages promote lymph node metastasis via CCR4/CCL22 in head and neck squamous cell carcinoma. Int J Canc 2013;132:2755–66. http://dx.doi.org/10.1002/ijc.27966.

[37] Shintani S, Ishikawa T, Nonaka T, Li C, Nakashiro K-I, Wong DTW, et al. Growth-regulated oncogene-1 expression is associated with angiogenesis and lymph node metastasis in human oral cancer. Oncology 2004;66:316–22. http://dx.doi.org/10.1159/000078333.

[38] Lee CH, Syu SH, Liu KJ, Chu PY, Yang WC, Lin P, et al. Interleukin-1 beta transactivates epidermal growth factor receptor via the CXCL1-CXCR2 axis in oral cancer. Oncotarget 2015;6:38866–80. http://dx.doi.org/10.18632/oncotarget.5640.

[39] Oue E, Lee JW, Sakamoto K, Iimura T, Aoki K, Kayamori K, et al. CXCL2 synthesized by oral squamous cell carcinoma is involved in cancer-associated bone destruction. Biochem Biophys Res Commun 2012;424:456–61. http://dx.doi.org/10.1016/j.bbrc.2012.06.132.

[40] Miyazaki H, Patel V, Wang H, Edmunds RK, Gutkind JS, Yeudall WA. Down-regulation of CXCL5 inhibits squamous carcinogenesis. Canc Res 2006;66:4279–84. http://dx.doi.org/10.1158/0008-5472.CAN-05-4398.

[41] Watanabe H, Iwase M, Ohashi M, Nagumo M. Role of interleukin-8 secreted from human oral squamous cell carcinoma cell lines. Oral Oncol 2002;38:670–9.

[42] Christofakis EP, Miyazaki H, Rubink DS, Yeudall WA. Roles of CXCL8 in squamous cell carcinoma proliferation and migration. Oral Oncol 2008;44:920–6. http://dx.doi.org/10.1016/j.oraloncology.2007.12.002.

[43] Khurram SA, Bingle L, McCabe BM, Farthing PM, Whawell SA. The chemokine receptors CXCR1 and CXCR2 regulate oral cancer cell behaviour. J Oral Pathol Med 2014;43:667–74. http://dx.doi.org/10.1111/jop.12191.

[44] Qian Y, Wang Y, Li DS, Zhu YX, Lu ZW, Ji QH, et al. The chemokine receptor-CXCR2 plays a critical role in the invasion and metastases of oral squamous cell carcinoma in vitro and in vivo. J Oral Pathol Med 2014;43:658–66. http://dx.doi.org/10.1111/jop.12189.

[45] Hsin LJ, Kao HK, Chen IH, Tsang NM, Hsu CL, Liu SC, et al. Serum CXCL9 levels are associated with tumor progression and treatment outcome in patients with nasopharyngeal carcinoma. PLoS ONE 2013;8:e80052. http://dx.doi.org/10.1371/journal.pone.0080052.

[46] Chang KP, Wu CC, Fang KH, Tsai CY, Chang YL, Liu SC, et al. Serum levels of chemokine (C-X-C motif) ligand 9 (CXCL9) are associated with tumor progression and treatment outcome in patients with oral cavity squamous cell carcinoma. Oral Oncol 2013;49:802–7. http://dx.doi.org/10.1016/j.oraloncology.2013.05.006.

[47] Rentoft M, Coates PJ, Loljung L, Wilms T, Laurell G, Nylander K. Expression of CXCL10 is associated with response to radiotherapy and overall survival in squamous cell carcinoma of the tongue. Tumour Biol 2014;35:4191–8. http://dx.doi.org/10.1007/s13277-013-1549-6.

[48] Xia J, Chen N, Hong Y, Chen X, Tao X, Cheng B, et al. Expressions of CXCL12/CXCR4 in oral premalignant and malignant lesions. Mediators Inflamm 2012;2012:516395. http://dx.doi.org/10.1155/2012/516395.

[49] Uchida D, Begum NM, Almofti A, Nakashiro K, Kawamata H, Tateishi Y, et al. Possible role of stromal-cell-derived factor-1/CXCR4 signaling on lymph node metastasis of oral squamous cell carcinoma. Exp Cell Res 2003;290:289–302. http://dx.doi.org/10.1016/S0014-4827(03)00344-6.

[50] Uchida D, Begum N-M, Tomizuka Y, Bando T, Almofti A, Yoshida H, et al. Acquisition of lymph node, but not distant metastatic potentials, by the overexpression of CXCR4 in human oral squamous cell carcinoma. Lab Invest 2004;84:1538–46. http://dx.doi.org/10.1038/labinvest.3700190.

[51] Uchida D, Onoue T, Tomizuka Y, Begum NM, Miwa Y, Yoshida H, et al. Involvement of an autocrine stromal cell derived factor-1/CXCR4 system on the distant metastasis of human oral squamous cell carcinoma. Mol Cancer Res 2007;5:685–94. http://dx.doi.org/10.1158/1541-7786.MCR-06-0368.

[52] Oliveira-Neto HH, Silva ET, Leles CR, Mendonça EF, Alencar Rde C, Silva TA, et al. Involvement of CXCL12 and CXCR4 in lymph node metastases and development of oral squamous cell carcinomas. Tumour Biol 2008;29:262–71. http://dx.doi.org/10.1159/000152944.

[53] Delilbasi CB, Okura M, Iida S, Kogo M. Investigation of CXCR4 in squamous cell carcinoma of the tongue. Oral Oncol 2004;40:154–7. http://dx.doi.org/10.1016/S1368-8375(03)00144-1.

[54] Ishikawa T, Nakashiro KI, Hara S, Klosek SK, Li C, Shintani S, et al. CXCR4 expression is associated with lymph-node metastasis of oral squamous cell carcinoma. Int J Oncol 2006;28:61–6. http://dx.doi.org/10.3892/ijo.28.1.61.

[55] Sasaki K, Natsugoe S, Ishigami S, Matsumoto M, Okumura H, Setoyama T, et al. Expression of CXCL12 and its receptor CXCR4 correlates with lymph

node metastasis in submucosal esophageal cancer. J Surg Oncol 2008;97:433–8. http://dx.doi.org/10.1002/jso.20976.
[56] Albert S, Hourseau M, Halimi C, Serova M, Descatoire V, Barry B, et al. Prognostic value of the chemokine receptor CXCR4 and epithelial-to-mesenchymal transition in patients with squamous cell carcinoma of the mobile tongue. Oral Oncol 2012;48:1263–71. http://dx.doi.org/10.1016/j.oraloncology.2012.06.010.
[57] Meng X, Wuyi L, Yuhong X, Xinming C. Expression of CXCR4 in oral squamous cell carcinoma: correlations with clinicopathology and pivotal role of proliferation. J Oral Pathol Med 2010;39:63–8. http://dx.doi.org/10.1111/j.1600-0714.2009.00801.x.
[58] Almofti A, Uchida D, Begum NM, Tomizuka Y, Iga H, Yoshida H, et al. The clinicopathological significance of the expression of CXCR4 protein in oral squamous cell carcinoma. Int J Oncol 2004;25:65–71. http://dx.doi.org/10.3892/ijo.25.1.65.
[59] Lee JI, Jin BH, Kim MA, Yoon HJ, Hong SP, Hong SD. Prognostic significance of CXCR-4 expression in oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:678–84. http://dx.doi.org/10.1016/j.tripleo.2008.12.047.
[60] Rehman AO, Wang C. CXCL12/SDF-1 alpha activates NF-kappaB and promotes oral cancer invasion through the Carma3/Bcl10/Malt1 complex. Int J Oral Sci 2009;1:105–18. http://dx.doi.org/10.4248/IJOS.09059.
[61] Onoue T, Uchida D, Begum NM, Tomizuka Y, Yoshida H, Sato M. Epithelial–mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells. Int J Oncol 2006;29:1133–8. http://dx.doi.org/10.3892/ijo.29.5.1133.
[62] Taki M, Higashikawa K, Yoneda S, Ono S, Shigeishi H, Nagayama M, et al. Up-regulation of stromal cell-derived factor-1alpha and its receptor CXCR4 expression accompanied with epithelial–mesenchymal transition in human oral squamous cell carcinoma. Oncol Rep 2008;19:993–8.
[63] Samara GJ, Lawrence DM, Chiarelli CJ, Valentino MD, Lyubsky S, Zucker S, et al. CXCR4-mediated adhesion and MMP-9 secretion in head and neck squamous cell carcinoma. Canc Lett 2004;214:231–41.
[64] Wang DF, Lou N, Qiu MZ, Lin YB, Liang Y. Effects of CXCR4 gene silencing by lentivirus shRNA on proliferation of the EC9706 human esophageal carcinoma cell line. Tumour Biol 2013;34:2951–9. http://dx.doi.org/10.1007/s13277-013-0858-0.
[65] Takabayashi T, Takahashi N, Okamoto M, Yagi H, Sato M, Fujieda S. Lipopolysaccharides increase the amount of CXCR4, and modulate the morphology and invasive activity of oral cancer cells in a CXCL12-dependent manner. Oral Oncol 2009;45:968–73. http://dx.doi.org/10.1016/j.oraloncology.2009.06.006.
[66] Yuvaraj S, Griffin AC, Sundaram K, Kirkwood KL, Norris JS, Reddy SV. A novel function of CXCL13 to stimulate RANK ligand expression in oral squamous cell carcinoma cells. Mol Cancer Res 2009;7:1399–407. http://dx.doi.org/10.1158/1541-7786.MCR-08-0589.
[67] Pandruvada SNM, Yuvaraj S, Liu X, Sundaram K, Shanmugarajan S, Ries WL, et al. Role of CXC chemokine ligand 13 in oral squamous cell carcinoma associated osteolysis in athymic mice. Int J Cancer 2010;126:2319–29. http://dx.doi.org/10.1002/ijc.24920.
[68] Sambandam Y, Sundaram K, Liu A, Kirkwood KL, Ries WL, Reddy SV. CXCL13 activation of c-Myc induces RANK ligand expression in stromal/preosteoblast cells in the oral squamous cell carcinoma tumor-bone microenvironment. Oncogene 2013;32:97–105. http://dx.doi.org/10.1038/onc.2012.24.
[69] Ito S, Ozawa S, Ikoma T, Yajima N, Kiyono T, Hata R. Expression of a chemokine BRAK/CXCL14 in oral floor carcinoma cells reduces the settlement rate of the cells and suppresses their proliferation in vivo. Biomed Res 2010;31:199–206. http://dx.doi.org/10.2220/biomedres.31.199.
[70] Khurram SA, Whawell SA, Bingle L, Murdoch C, McCabe BM, Farthing PM. Functional expression of the chemokine receptor XCR1 on oral epithelial cells. J Pathol 2010;221:153–63. http://dx.doi.org/10.1002/path.2695.
[71] Zhang J, Lu Y, Pienta KJ. Multiple roles of chemokine (C-C motif) ligand 2 in promoting prostate cancer growth. J Natl Cancer Inst 2010;102:522–8. http://dx.doi.org/10.1093/jnci/djq044.
[72] Gazzaniga S, Bravo AI, Guglielmotti A, van Rooijen N, Maschi F, Vecchi A, et al. Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. J Invest Dermatol 2007;127:2031–41. http://dx.doi.org/10.1038/sj.jid.5700827.
[73] Ferreira FO, Ribeiro FLL, Batista AC, Leles CR, de Cássia Gonçalves Alencar R, Silva TA. Association of CCL2 with lymph node metastasis and macrophage infiltration in oral cavity and lip squamous cell carcinoma. Tumour Biol 2008;29:114–21. http://dx.doi.org/10.1159/000137669.
[74] Bektas-Kayhan K, Unur M, Boy-Metin Z, Cakmakoglu B. MCP-1 and CCR2 gene variants in oral squamous cell carcinoma. Oral Dis 2012;18:55–9. http://dx.doi.org/10.1111/j.1601-0825.2011.01843.x.
[75] Vallet S, Anderson KC. CCR1 as a target for multiple myeloma. Expert Opin Ther Targets 2011;15:1037–47. http://dx.doi.org/10.1517/14728222.2011.586634.
[76] Sivina M, Hartmann E, Kipps TJ, Rassenti L, Krupnik D, Lerner S, et al. CCL3 (MIP-1α) plasma levels and the risk for disease progression in chronic lymphocytic leukemia. Blood 2011;117:1662–9. http://dx.doi.org/10.1182/blood-2010-09-307249.
[77] Sasaki S, Baba T, Shinagawa K, Matsushima K, Mukaida N. Crucial involvement of the CCL3-CCR5 axis-mediated fibroblast accumulation in colitis-associated carcinogenesis in mice. Int J Cancer 2014;135:1297–306. http://dx.doi.org/10.1002/ijc.28779.
[78] Azenshtein E, Luboshits G, Shina S, Neumark E, Shahbazian D, Weil M, et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res 2002;62:1093–102.
[79] Vaday GG, Peehl DM, Kadam PA, Lawrence DM. Expression of CCL5 (RANTES) and CCR5 in prostate cancer. Prostate 2006;66:124–34. http://dx.doi.org/10.1002/pros.20306.
[80] Silva TA, Leite Ribeiro FL, Oliveira-Neto HH, Watanabe S, Alencar Rde C, Fukada SY. Dual role of CCL3/CCR1 in oral squamous cell carcinoma: implications in tumor metastasis and local host defense. Oncol Rep 2007;18:1107–13. http://dx.doi.org/10.3892/or.18.5.1107.
[81] Tanyel CR, Cincin ZB, Gokcen-Rohlig B, Bektas-Kayhan K, Unur M, Cakmakoglu B. Effects of genetic variants of CCR5 chemokine receptors on oral squamous cell carcinoma. Genet Mol Res 2013;12:5714–20. http://dx.doi.org/10.4238/2013.November.18.20.
[82] Weng CJ, Chien MH, Lin CW, Chung TT, Zavras AI, Tsai CM, et al. Effect of CC chemokine ligand 5 and CC chemokine receptor 5 genes polymorphisms on the risk and clinicopathological development of oral cancer. Oral Oncol 2010;46:767–72. http://dx.doi.org/10.1016/j.oraloncology.2010.07.011.
[83] Hwang TL, Lee LY, Wang CC, Liang Y, Huang SF, Wu CM. CCL7 and CCL21 overexpression in gastric cancer is associated with lymph node metastasis and poor prognosis. World J Gastroenterol 2012;18:1249–56. http://dx.doi.org/10.3748/wjg.v18.i11.1249.
[84] Tsuzuki H, Takahashi N, Kojima A, Narita N, Sunaga H, Takabayashi T, et al. Oral and oropharyngeal squamous cell carcinomas expressing CCR7 have poor prognoses. Auris Nasus Larynx 2006;33:37–42. http://dx.doi.org/10.1016/j.anl.2005.07.019.
[85] Oliveira-Neto HH, de Souza PPC, da Silva MRB, Mendonça EF, Silva TA, Batista AC. The expression of chemokines CCL19, CCL21 and their receptor CCR7 in oral squamous cell carcinoma and its relevance to cervical lymph node metastasis. Tumour Biol 2013;34:65–70. http://dx.doi.org/10.1007/s13277-012-0511-3.
[86] Abiko Y, Nishimura M, Kusano K, Nakashima K, Okumura K, Arakawa T, et al. Expression of MIP-3alpha/CCL20, a macrophage inflammatory protein in oral squamous cell carcinoma. Arch Oral Biol 2003;48:171–5. http://dx.doi.org/10.1016/S0003-9969(02)00167-X.
[87] Wei ZW, Xia GK, Wu Y, Chen W, Xiang Z, Schwarz RE, et al. CXCL1 promotes tumor growth through VEGF pathway activation and is associated with inferior survival in gastric cancer. Canc Lett 2015;359:335–43. http://dx.doi.org/10.1016/j.canlet.2015.01.033.
[88] Wang D, Wang H, Brown J, Daikoku T, Ning W, Shi Q, et al. CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. J Exp Med 2006;203:941–51. http://dx.doi.org/10.1084/jem.20052124.
[89] Matsuo Y, Raimondo M, Woodward TA, Wallace MB, Gill KR, Tong Z, et al. CXC-chemokine/CXCR2 biological axis promotes angiogenesis in vitro and in vivo in pancreatic cancer. Int J Canc 2009;125:1027–37. http://dx.doi.org/10.1002/ijc.24383.
[90] Campbell LM, Maxwell PJ, Waugh DJ. Rationale and means to target pro-inflammatory interleukin-8 (CXCL8) signaling in cancer. Pharmaceuticals (Basel) 2013;6:929–59. http://dx.doi.org/10.3390/ph6080929.
[91] Cheng XS, Li YF, Tan J, Sun B, Xiao YC, Fang XB, et al. CCL20 and CXCL8 synergize to promote progression and poor survival outcome in patients with colorectal cancer by collaborative induction of the epithelial–mesenchymal transition. Cancer Lett 2014;348:77–87. http://dx.doi.org/10.1016/j.canlet.2014.03.008.
[92] Li XJ, Peng LX, Shao JY, Lu WH, Zhang JX, Chen S, et al. As an independent unfavorable prognostic factor, IL-8 promotes metastasis of nasopharyngeal carcinoma through induction of epithelial–mesenchymal transition and activation of AKT signaling. Carcinogenesis 2012;33:1302–9. http://dx.doi.org/10.1093/carcin/bgs181.
[93] Wang N, Feng Y, Wang Q, Liu S, Xiang L, Sun M, et al. Neutrophils infiltration in the tongue squamous cell carcinoma and its correlation with CEACAM1 expression on tumor cells. PLoS ONE 2014;9:e89991. http://dx.doi.org/10.1371/journal.pone.0089991.
[94] Shimizu Y, Kondo S, Shirai A, Furukawa M, Yoshizaki T. A single nucleotide polymorphism in the matrix metalloproteinase-1 and interleukin-8 gene promoter predicts poor prognosis in tongue cancer. Auris Nasus Larynx 2008;35:381–9. http://dx.doi.org/10.1016/j.anl.2007.12.002.
[95] Vairaktaris E, Serefoglou ZC, Yapijakis C, Agapi C, Vassiliou S, Nkenke E, et al. The interleukin-18 -607A/C polymorphism is not associated with risk for oral cancer. Anticancer Res 2007;27:4011–4.
[96] Liu CM, Yeh CJ, Yu CC, Chou MY, Lin CH, Wei LH, et al. Impact of interleukin-8 gene polymorphisms and environmental factors on oral cancer susceptibility in Taiwan. Oral Dis 2012;18:307–14. http://dx.doi.org/10.1111/j.1601-0825.2011.01882.x.
[97] Yang L, Zhu X, Liang X, Ling Z, Li R. Association of IL-8-251A>T polymorphisms with oral cancer risk: evidences from a meta-analysis. Tumour Biol 2014;35:9211–8. http://dx.doi.org/10.1007/s13277-014-2193-5.
[98] Lunardi S, Jamieson NB, Lim SY, Griffiths KL, Carvalho-Gaspar M, Al-Assar O, et al. IP-10/CXCL10 induction in human pancreatic cancer stroma influences lymphocytes recruitment and correlates with poor survival. Oncotarget 2014;5:11064–80. http://dx.doi.org/10.18632/oncotarget.2519.
[99] Bian L, Sun X, Jin K, He Y. Oral cancer-associated fibroblasts inhibit heat-induced apoptosis in Tca8113 cells through upregulated expression of Bcl-2

through the Mig/CXCR3 axis. Oncol Rep 2012;28:2063–8. http://dx.doi.org/10.3892/or.2012.2019.
[100] Xia J, Wang J, Chen N, Dai Y, Hong Y, Chen X, et al. Expressions of CXCR7/ligands may be involved in oral carcinogenesis. J Mol Histol 2011;42:175–80. http://dx.doi.org/10.1007/s10735-011-9322-x.
[101] Clatot F, Picquenot JM, Choussy O, Gouérant S, Moldovan C, Schultheis D, et al. Intratumoural level of SDF-1 correlates with survival in head and neck squamous cell carcinoma. Oral Oncol 2011;47:1062–8. http://dx.doi.org/10.1016/j.oraloncology.2011.07.021.
[102] Vairaktaris E, Vylliotis A, Spyridonodou S, Derka S, Vassiliou S, Nkenke E, et al. A DNA polymorphism of stromal-derived factor-1 is associated with advanced stages of oral cancer. Anticancer Res 2008;28:271–5.
[103] Teng YH, Liu TH, Tseng HC, Chung TT, Yeh CM, Li YC, et al. Contribution of genetic polymorphisms of stromal cell-derived factor-1 and its receptor, CXCR4, to the susceptibility and clinicopathologic development of oral cancer. Head Neck 2009;31:1282–8. http://dx.doi.org/10.1002/hed.21094.
[104] Porvasnik S, Sakamoto N, Kusmartsev S, Eruslanov E, Kim W-J, Cao W, et al. Effects of CXCR4 antagonist CTCE-9908 on prostate tumor growth. Prostate 2009;69:1460–9. http://dx.doi.org/10.1002/pros.21008.
[105] Rubin JB, Kung AL, Klein RS, Chan JA, Sun Y, Schmidt K, et al. A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. Proc Natl Acad Sci USA 2003;100:13513–8. http://dx.doi.org/10.1073/pnas.2235846100.
[106] Kioi M, Vogel H, Schultz G, Hoffman RM, Harsh GR, Brown JM. Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. J Clin Invest 2010;120:694–705. http://dx.doi.org/10.1172/JCI40283.
[107] Kozin SV, Kamoun WS, Huang Y, Dawson MR, Jain RK, Duda DG. Recruitment of myeloid but not endothelial precursor cells facilitates tumor regrowth after local irradiation. Cancer Res 2010;70:5679–85. http://dx.doi.org/10.1158/0008-5472.CAN-09-4446.
[108] DiPersio JF, Micallef IN, Stiff PJ, Bolwell BJ, Maziarz RT, Jacobsen E, et al. Phase III prospective randomized double-blind placebocontrolled trial of plerixafor plus granulocyte colonystimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-hodgkin's lymphoma. J Clin Oncol 2009;27:4767–73. http://dx.doi.org/10.1200/JCO.2008.20.7209.
[109] Lorena SCM, Oliveira DT, Dorta RG, Landman G, Kowalski LP. Eotaxin expression in oral squamous cell carcinomas with and without tumour associated tissue eosinophilia. Oral Dis 2003;9:279–83. http://dx.doi.org/10.1034/j.1601-0825.2003.00958.x.
[110] Ishida T, Joh T, Uike N, Yamamoto K, Utsunomiya A, Yoshida S, et al. Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study. J Clin Oncol 2012;30:837–42. http://dx.doi.org/10.1200/JCO.2011.37.3472.
[111] Abraham M, Biyder K, Begin M, Wald H, Weiss ID, Galun E, et al. Enhanced unique pattern of hematopoietic cell mobilization induced by the CXCR4 antagonist 4F-benzoyl-TN14003. Stem Cells 2007;25:2158–66. http://dx.doi.org/10.1634/stemcells.2007-0161.
[112] Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, et al. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypicalchemokine receptors. Pharmacol Rev 2013;66:1–79. http://dx.doi.org/10.1124/pr.113.007724.
[113] Crusz SM, Balkwill FR. Inflammation and cancer: advances and new agents. Nat Rev Clin Oncol 2015;12:584–96. http://dx.doi.org/10.1038/nrclinonc.2015.105.

ANEXO A

Histol Histopathol (2014) 29: 387-396

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Eosinophil depletion protects mice from tongue squamous cell carcinoma induced by 4-nitroquinoline-1-oxide

Janine Mayra da Silva[1], Celso Martins Queiroz-Junior[1], Aline Carvalho Batista[2], Milene Alvarenga Rachid[3], Mauro Martins Teixeira[4] and Tarcília Aparecida da Silva[1]

[1]Department of Oral Surgery and Pathology, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, [2]Department of Stomatology (Oral Pathology), Faculdade de Odontologia, Universidade Federal de Goiás, Goiânia, Brazil, [3]Department of General Pathology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil and [4]Laboratory of Immunopharmacology, Department of Biochemistry and Immunology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Summary. Aims: Tumor-associated tissue eosinophilia (TATE) has been correlated with prognosis in oral squamous cell carcinoma (OSCC). This study aimed to investigate whether eosinophils depletion affects experimental oral carcinogenesis.

Methods and Results: BALB/c (wild type - WT) and eosinophil-deficient (*Δdb*/GATA-1) mice were treated with the carcinogen 4-nitroquinoline-1-oxide (4NQO) in drinking water for 28 weeks. Tongues were collected for histopathological and immunohistochemical analysis, as well as for the evaluation of cytokines/chemokines by ELISA. The tongue SCC induced by 4NQO was associated with a rise in eosinophil numbers. WT-treated group showed a significantly increased incidence of SCC, with higher cytological atypia, in comparison with *Δdb*/GATA-1 mice. Consistently, the proliferative index was higher in WT compared to the *Δdb*/GATA-1/GATA-1-treated group. No significant changes in the concentration of CCL3, CCL11 and TNF-α were detected for both groups after 4NQO treatment. Conclusions: These results suggest that eosinophils might be responsible for the deleterious outcome of experimental tongue carcinogenesis, given that their ablation protects mice from OSCC.

Key words: Eosinophil, Squamous cell carcinoma, Tonge, GATA, 4NQO

Offprint requests: Tarcília Aparecida da Silva, Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31.270-901, Belo Horizonte, Minas Gerais, Brazil. e-mail: tarcilia@ufmg.br

Introduction

Eosinophils are considered multifunctional leucocytes that participate in innate and adaptive immune responses (Hothenberg and Hogan, 2006; Gatault et al., 2012). After different stimuli, including carcinogens, eosinophils are recruited to inflammatory sites, releasing a variety of cytotoxic proteins, interleukins (e.g. TNF-α) and chemokines (e.g. CCL5 and CCL11) (Hothenberg and Hogan, 2006; Martinelli-Kläy et al., 2009). Some of these molecules induce pro-inflammatory effects, such as upregulation of adhesion mechanisms, cellular trafficking and vascular permeability, which may promote angiogenesis and, thus, favor tumor growth (Munitz and Schaffer-Levi, 2004; Hothenberg and Hogan, 2006; Martinelli-Kläy et al., 2009).

The increased number of infiltrated eosinophils in tumors (also called Tumor-Associated Tissue Eosinophilia-TATE) has been implicated as a prognostic factor in human cancers (Munitz and Schaffer-Levi, 2004; Oliveira et al., 2011; Gatault et al., 2012). However, the exact role of eosinophils in malignant tumors is controversial (Martinelli-Kläy et al., 2009; Pereira et al., 2010; Gatault et al., 2012). While some authors suggested that TATE may be correlated with better prognosis (Goldsmith et al., 1992; Dorta et al., 2002), others reported their association with worse tumor evolution (Wong et al., 1999; Alrawi et al., 2005; Said et al., 2005; Oliveira et al., 2011) or even no effect (Oliveira et al., 2009; Tadbir et al., 2009). In part, these controversies could be attributed to some limitations associated to human studies, such as differences in

genetic background, tumor staging, and also in the criteria used to assess the density of eosinophils (Dorta et al., 2002; Alkhabuli and High, 2006; Falconieri et al., 2008).

In this regard, the use of mouse models of squamous cell carcinoma (SCC) could be an alternative to reduce some variables associated with clinical samples. The tongue SCC model induced by 4-nitroquinoline-1-oxide (4NQO) administration in drinking water is characterized by significant DNA damage and dysplastic changes in the tongue epithelium (Tang et al., 2004; Lu et al., 2006). This process culminates in neoplastic transformation, mimicking several parameters of the human condition (Tang et al., 2004; Kanojia and Vaidya, 2006; Carvalho et al., 2012; Moon et al., 2012).

In the current study the involvement of eosinophils in SCC was investigated employing an experimental model of tongue SCC induced by 4NQO in eosinophil-depleted mice (*Δdb*/GATA-1 deficient-mice).

Materials and methods

Mice

Twenty-five *Δdb*/GATA-1 (eosinophil-deficient mice) (donated by Dr. A. Humbles, Harvard, Boston, MA) (Humbles et al., 2004) and BALB/c (wild type – WT) male mice were bred in the animal facility of Universidade Federal de Minas Gerais, Brazil. *Δdb*/GATA-1 and BALB/c mice present the same genetic background (Humbles et al., 2004; Jackson laboratory, available at http://jaxmice.jax.org/strain/005653.html). Mice were maintained under standard conditions with a 12 h light/dark cycle, controlled temperature (24±2°C) and had free access to commercial chow and drinking water. All experimental procedures described in the current study were approved by the institutional Ethics Committee (CETEA/UFMG-protocol number 12/2011). The mice were weighed weekly and no changes in their weight were observed during the experimental period.

Induction of SCC by 4NQO

The mouse model of tongue carcinogenesis used in the current study was adapted from the protocol described by Tang et al. (2004). Briefly, 4-Nitroquinoline-1-oxide (4NQO), obtained as a powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethylene glycol (Sigma-Aldrich) to a final concentration of 50 μg/ml and stored at 4°C. The stock solution was prepared weekly and diluted in the drinking water of mice.

Experimental mice (9 WT and 6 *Δdb*/GATA-1) received treatment with 4NQO daily for 28 weeks. The control groups (n=5 per group) received drinking water without 4NQO during the same period. The mean intake of water was recorded, but there was no difference between the groups. After 28 weeks of carcinogen treatment, mice were euthanized and tongue, cervical lymph nodes, liver, stomach, duodenum, jejunum, ileum and large intestine were collected for microscopic analysis.

Light microscopy

Tongue, cervical lymph nodes, liver, stomach, duodenum, jejunum, ileum and large intestine tissues were fixed in 10% buffered formalin, embedded in paraffin wax and cut longitudinally (3 μm). The slides were deparaffinized, rehydrated, and stained with H&E. The tongue lesions were classified using the following score (adapted from Barnes et al., 2005): 0 - normal, 1 - mild dysplasia (changes limited at basal third of the lining epithelium), 2 - moderate (when the changes represented two-thirds of the lining epithelium), 3 - severe (more than two-thirds of the epithelium affected), 4 - carcinoma in situ (full thickness of the lining epithelium, but without involvement of the connective tissue) and 5 - invasive carcinoma (carcinomatous islands into the connective tissue). For microscopic analysis, 20 consecutive fields were evaluated by two examiners (S.J.M and S.T.A) blinded to the group status. To validate the reliability of the inter- and intra-examiner evaluations, the Intraclass Correlation Coefficient test was performed and there were significant positive correlations ($p<0.001$). The other organs were also evaluated for general histopathological analysis by a pathologist (R.M.A).

Eosinophils staining technique

For the eosinophil staining, the Sirius Red staining protocol was followed (Meyerholz et al., 2009), adapted from the original method published by Llewellyn (1970) with elimination of the sodium chloride step (Llewellyn, 1970). Slides were incubated in Harris hematoxylin (two minutes) and rinsed in tap water followed by a rinse in 100% ethanol. The slides were then immersed in an alkaline (pH 8-9) Sirius Red solution (Sigma-Aldrich, CI 35780) for two hours and rinsed in tap water. Stained eosinophils were counted in 20 consecutive fields of the epithelium lining area (including a third of lamina propria, under epithelial layer), at x 400 magnification. Results were expressed as a total number of eosinophils per sample.

Ki67 and PCNA immunohistochemistry and cell counting

Immunohistochemistry was performed using the streptavidin-biotin method. Briefly, serial sections of tongue tumors were deparaffinized, rehydrated and rinsed in distilled water. They were then incubated with 0.3% hydrogen peroxide twice for 15 min. For antigen retrieval, the slides were incubated in citric acid buffer (pH=6.0) at 96°C for 20 min. The slides were incubated at 4°C overnight with the monoclonal mouse anti-human Ki67 antibody (clone MM1; Novocastra, Newcastle, UK) at 1:50 or the monoclonal mouse anti-human PCNA

antibody (clone 124; Dako, Glostrup, Denmark) at 1:800. The immunolabeling was visualized through incubation in 3,3-diaminobenzidine (DAB) solution (Dako). Finally, the sections were stained with Mayer's hematoxylin and covered. Negative controls were obtained by omission of the primary antibody, which was substituted by 1% PBS-BSA.

The immunostained cells were analyzed by light microscopy (Axioskop 40 ZEISS; Carl Zeiss, Gottingen, Germany) at 1.000x magnification and counted in the basal and suprabasal epithelial layers in the total area of 20 consecutive fields in two sections. A proliferative index of Ki67 and PCNA positive cells was obtained by adding the results of basal and suprabasal epithelium layers.

ELISA and MPO activity

Tongue lesional samples were also collected for immunoenzimatic assays. The samples were weighed and homogenized in phosphate buffered saline (0.4 mM NaCl and 10 mM $NaPO_4$) containing protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20 at 100 mg/mL. The homogenate was centrifuged (8.946 x g) at 4°C for 10 min. The supernatant was then collected and stored at -70°C until further analysis. The concentration of CCL3, CCL5, CCL11 and TNF-α was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The results were expressed as picograms of cytokines (± S.E.M.) normalized for 100 mg tissue.

The MPO activity, a neutrophil enzyme marker, was also evaluated in homogenized tongue tissues by enzymatic reaction, as previously described (Queiroz-Junior et al., 2009). The MPO contents were expressed as relative units calculated from standard curves based on the MPO activity from 5% casein peritoneal-induced neutrophils.

Statistical analysis

Results were expressed as the mean ± standard error mean (SEM). Statistical analysis was performed using the software GraphPad Prism 5.0. The non-parametric unpaired Student t test and analysis of variance (ANOVA) followed by *Student-Newman-Keuls post hoc* analysis were performed. Results with $p<0.05$ were considered statistically significant.

Results

4NQO treatment increases the number of eosinophils in tongue lesions

The first step of the current study was to investigate whether the induction of SCC by 4NQO was associated with the presence of eosinophils in tongue lesions. Indeed, the number of Sirius Red-stained eosinophils was significantly increased in tongue samples of WT-treated mice compared to their respective non-treated controls ($p<0.05$) (Fig. 1). No eosinophils have been detected in tongue samples of *Δdb*/GATA-1 mice.

Histopathological investigation of tongue SCC and metastatization after 4NQO treatment

After detecting increased numbers of eosinophils in tongue lesions induced by 4NQO, potential differences between WT and eosinophil-deficient (*Δdb*/GATA-1) mice were evaluated. The histopathological analysis of tongue samples showed that WT mice treated with 4NQO for 28 weeks (Fig. 2D-E) presented pronounced cytological atypia (pleomorphism, atypical mitosis, hyperchromatism) and islands of squamous cells invading the connective tissue, in comparison with the control (Fig. 2B), in which no changes in epithelium architecture have been observed. *Δdb*/GATA-1 mice also presented significant changes in tongue epithelium after treatment with 4NQO as indicated by the histological score (Fig. 2A), but these alterations were less

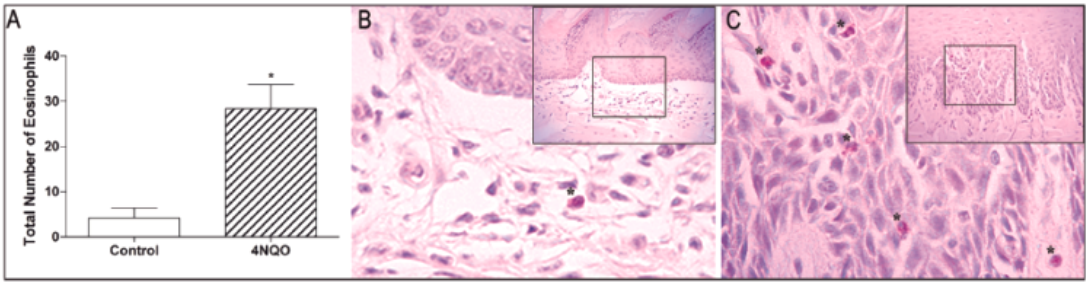


Fig. 1. Detection of eosinophils in tongue SCC induced by 4NQO. **A.** Total number of Sirius Red-stained eosinophils in tongue samples of WT control and 4NQO-treated mice. * $p<0.05$ in relation to control; unpaired Student's t test; **B and C.** Representative photomicrographs of WT control and 4NQO-treated tongue samples, respectively. Asterisks indicate Sirius Red-stained eosinophils.

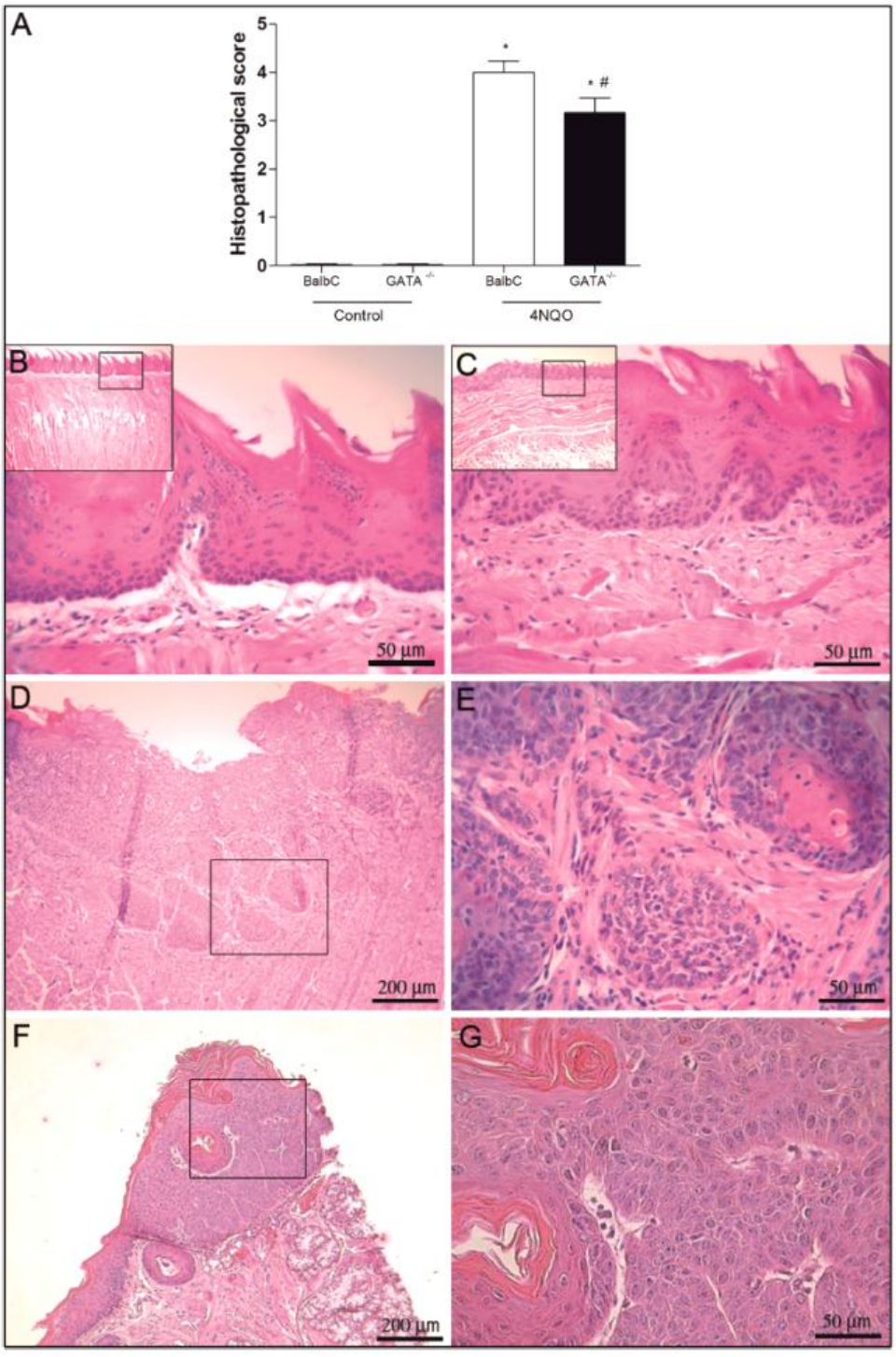


Fig. 2. Histopathological evaluation of tongue samples of control and experimental mice. **A.** Histopathological scores of tongue samples. * indicates $p<0.05$ versus the respective control group. # indicates $p<0.05$ versus WT-treated group. **B and C** show representative photomicrographs of WT and *Δdb*/GATA-1control tongue samples, respectively. **D and F.** Representative photomicrographs of WT and *Δdb*/GATA-1 tongue samples of mice treated with 4NQO for 28 weeks. **E and G** correspond to the higher increase of the squares indicated in D and F, respectively.

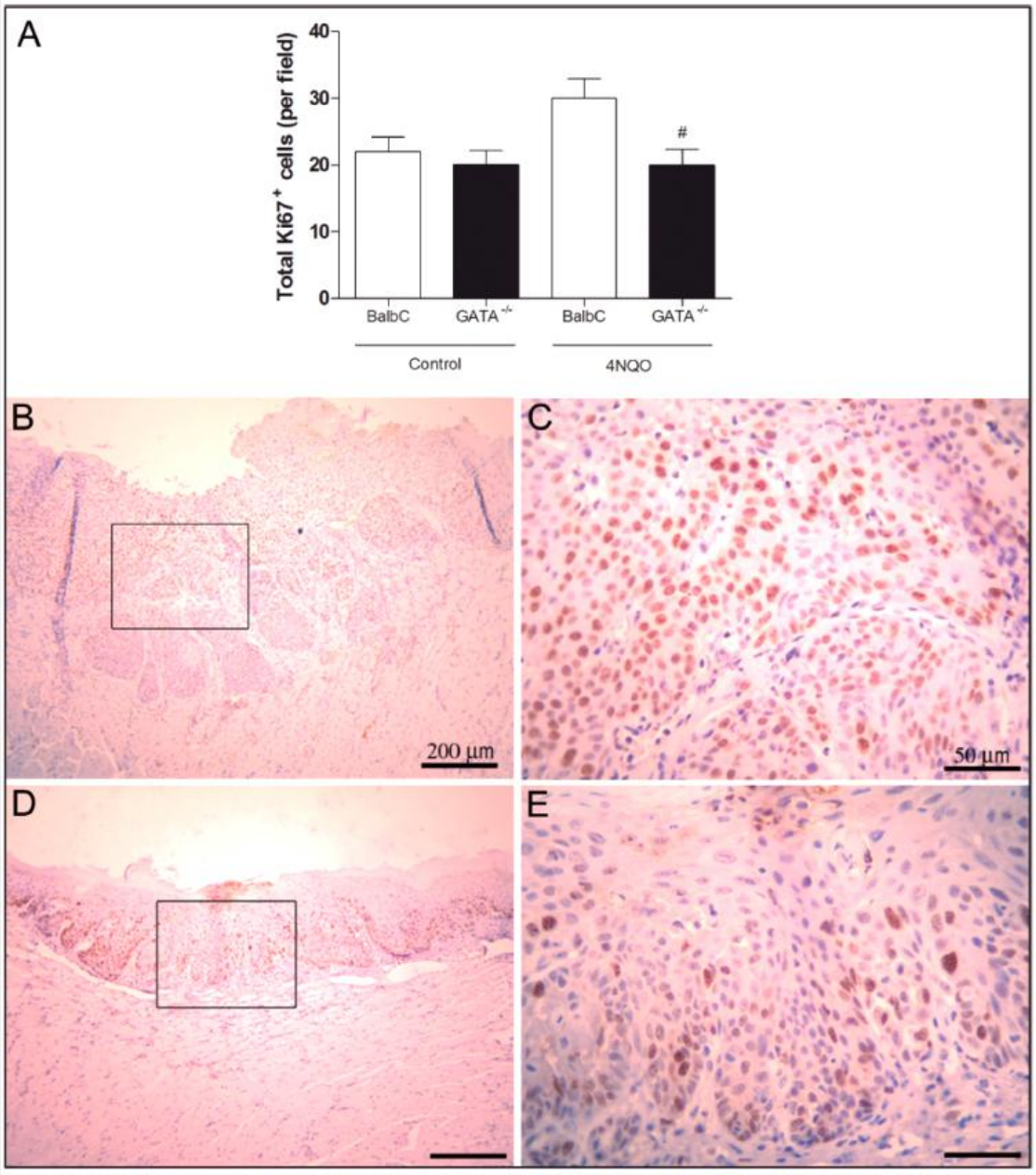


Fig. 3. Immunohistochemical expression of Ki67 in the tongue of mice. **A.** Quantification of total Ki67 positive cells in control and experimental groups. # $p<0.05$ when compared to WT-treated mice. **B and D** represent WT and *Δdb*/GATA-1 tongue samples of mice treated with 4NQO for 28 weeks. **C and E** correspond to the higher view of the squares in panels B and D, respectively.

pronounced in comparison to WT mice (Fig. 2F-G). WT-treated mice had 78% of lesions graduated with scores 4 and 5, while *Δdb*/GATA-1-treated mice presented better histopathological scores (67% scores 2 and 3). Tongue samples of WT and *Δdb*/GATA-1 treated mice were also graduated histopathologicaly until the twentieth week, but there were no differences between these groups (data not shown).

Consistently with these findings, the immuno-expression of Ki67, a proliferative marker, in the epithelial cells was significantly increased after 4NQO-treatment in WT when compared to *Δdb*/GATA-1 mice ($p<0.05$) (Fig. 3A-E). The number of PCNA immunostained cells was also significantly increased in WT-treated compared to *Δdb*/GATA-1-treated group, (Fig. 4).

The histopathological analysis of cervical lymph nodes, liver, stomach, duodenum, jejunum, ileum and large intestine of WT and *Δdb*/GATA-1 mice revealed no occurrence of metastasis. The liver samples of WT-treated mice presented variable degrees of hepatocyte tumefaction, steatosis and hemorrhage. Intestine samples presented variable degrees of inflammation in the lamina propria. These histopathological alterations were less pronounced in *Δdb*/GATA-1-treated mice (data not shown). Analysis of the stomach of WT-treated mice (n=2) showed squamous cell carcinoma in the aglandular portion of the organ (Fig. 5). In contrast, no histopathological alterations have been detected in the stomach of *Δdb*/GATA-1-treated mice (Fig. 5).

Inflammatory parameters in 4NQO-induced tongue SCC

In addition to the evaluation of microscopic aspects of the lesions induced by 4NQO, some inflammatory parameters were also investigated. The MPO activity (a neutrophil-enzyme marker) in tongue samples was marginal and there were no differences when comparing the experimental and control groups (Fig. 6).

The concentrations of the chemokines CCL3, CCL5, and CCL11 in tongue samples did not differ between WT control and WT-treated mice. Nevertheless, the levels of CCL3 and CCL11 were significantly decreased in *Δdb*/GATA-1-treated mice in comparison to the WT-treated group (Fig. 7A and B, respectively). The levels of CCL5 were significantly smaller in *Δdb*/GATA-1-treated mice than the respective control (Fig. 7C). The expression of TNF-α was also reduced in the *Δdb*/GATA-1-treated group when compared to the WT-treated mice, but this result reached no statistical significance (Fig. 7D).

Discussion

The major findings of the current study can be summarized as follows: i) the treatment of mice with 4NQO induced invasive tongue SCC, which was associated with increased numbers of eosinophils; ii) mice lacking eosinophils (*Δdb*/GATA-1) had OSCC with better histopathological scores and lower proliferative indexes than the respective WT mice.

In the current study, 4NQO, a water soluble chemical carcinogen, was used to induce SCC in the tongue of mice. The classical advantages of employing this carcinogen are its solubility and its ability to induce genetic damage (DNA adduct formation and DNA strand breaks) that culminate in neoplastic transformation similar to humans (Vered et al., 2003; Ribeiro et al., 2004; Tang et al., 2004). It also causes damage similar to tobacco (Kanojia and Vaidya, 2006; Lu et al., 2006), one of the major risk factors for SCC (Massano et al., 2006). Herein, 4NQO treatment in drinking water induced histopathological SCC features in the tongue epithelium of WT mice in a time-dependent manner, given that the histopathological scores found after 28 weeks were worse than those observed after 20 weeks of treatment (data not shown). Indeed, some authors support that chemical carcinogenesis is a multi-step process, which involves the long-term exposure to one or more chemical carcinogens (Boyd and Reade, 1988). A previous report has shown that 4NQO induces clinical signs of SCC slowly (Gannot et al., 2004). In the current study, mice did not exhibit significant clinical changes in their tongue surfaces and in the mean body weight after 4NQO treatment. Furthermore, no evidence of metastasis has been detected as previously described (Tang et al., 2004). In line with this, a recent study demonstrated lymph node metastasis in this model, but the dose used was 4 times greater than that employed in the current study (Li et al., 2012). Despite these clinical data, mice exhibited pronounced epithelial atypia and unfavorable histopathological scores, consistent with SCC. Accordingly, the immunoexpression of the

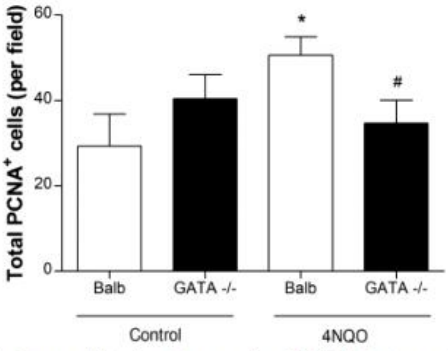


Fig. 4. Immunohistochemical expression of PCNA in the tongue of mice. Quantification of total PCNA positive cells in WT and *Δdb*/GATA-1 tongue samples of mice treated or not with 4NQO for 28 weeks. * $p<0.05$ when comparing the WT-treated mice to the respective control. # $p<0.05$ when compared to WT-treated mice.

proliferative markers Ki67 and PCNA was significantly increased in WT-treated mice. The expression of these markers is strongly related to increased cellular proliferation and neoplastic conversion during SCC development (Silva et al., 2007; Fracalossi et al., 2011).

All these histopathological parameters induced by the treatment with 4NQO were associated with increased numbers of eosinophils, when compared with the control. These findings suggested for the first time that eosinophils could be associated with the development of tongue SCC in this experimental model.

The presence of eosinophils in human cancers has been reported in different anatomical sites (Fernández-Aceñero et al., 2000; Cuschieri et al., 2002; Spiegel et al., 2002; Tadbir et al., 2009). Nevertheless, the precise role of TATE in SCC is controversial. Several studies have shown that TATE may be correlated with better (Goldsmith et al., 1992; Dorta et al., 2002), worse (Wong et al., 1999; Alrawi et al., 2005; Said et al., 2005) or even may have no influence on SCC prognosis (Oliveira et al., 2009; Tadbir et al., 2009). Here, after detecting increased numbers of eosinophils in tongue lesions induced by 4NQO, the involvement of this cell type in tumor development was explored using *Δdb*/GATA-1 mice. This mouse lineage is known to be devoid of eosinophils due to an engineered deletion of a palindromic double-enhancer binding site for GATA proteins in the gene encoding GATA-1 (Humbles et al., 2004). The data showed that the treatment of these eosinophil-deficient mice with 4NQO induced histopathological signs of SCC in their tongue, but these signs were significantly decreased when compared with

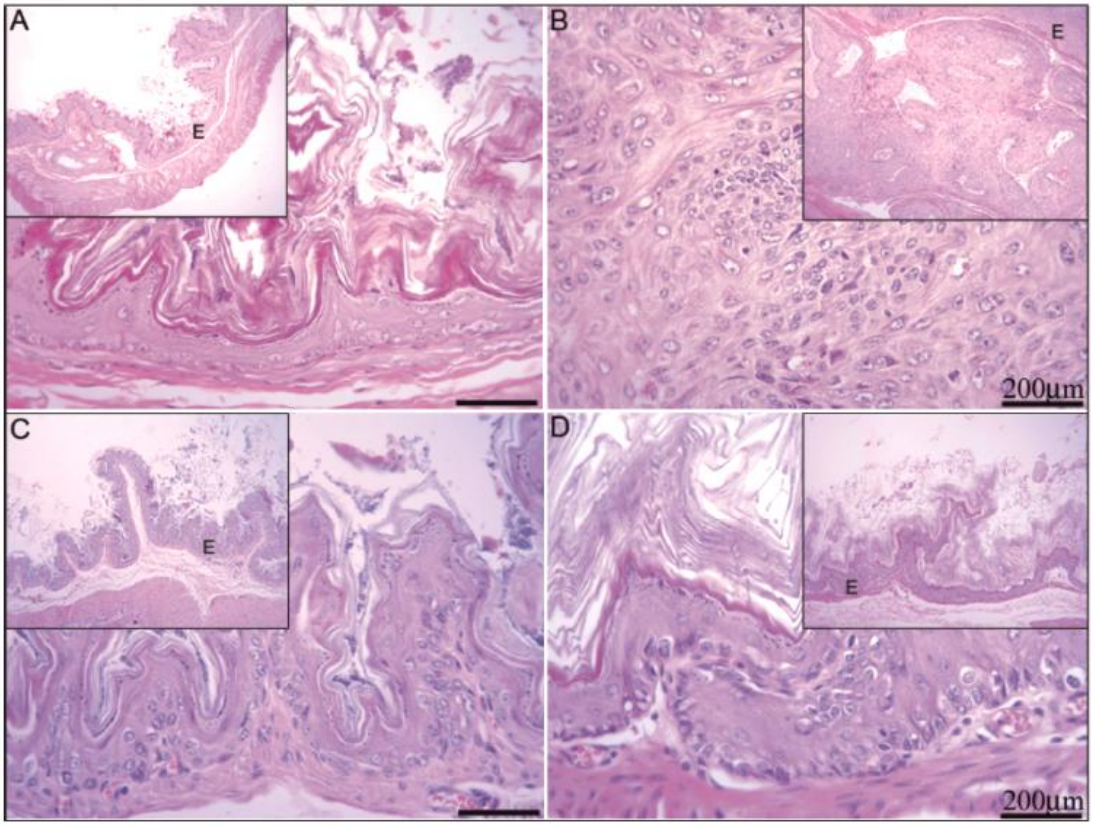


Fig. 5. Histopathological analysis of stomach of mice after 4NQO treatment. **A.** Microscopical appearance of stomach of WT. **B.** Histopathological analysis of the stomach of WT-treated mice showing squamous cell carcinoma in the aglandular portion of the organ. **C.** *Δdb*/GATA-1 stomach sample of control mice. **D.** No evidence of SCC was seen in *Δdb*/GATA-1-treated mice. E: epithelium stomach surface. x 400; insets, x 100

WT mice. The lack of eosinophils was associated with a better status of SCC after 28 weeks of 4NQO treatment. *Δdb*/GATA-1 mice also seemed to present protection against potential systemic commitment induced by 4NQO, i.e. these mice did not present alterations in other evaluated organs, except for the tongue, in contrast to WT mice, which presented malignant transformation of the stomach epithelium in some cases. These findings are in line with the study of Wong and colleagues (1999), who found that the ablation of TATE by administration of anti-interleukin-5 monoclonal antibody in hamsters induced smaller tumor burden and delayed the onset of SCC development in these animals (Wong et al., 1999).

Eosinophils are multifunctional leucocytes involved in cytotoxicity, inflammatory processes, tissue remodeling and modulation of immune responses (Gatault et al., 2012). Moreover, eosinophils are considered to be active components of peritumoral and intratumoral inflammatory infiltrate (Dorta et al., 2002; Hogan, 2007; Martinelli-Kläy et al., 2009). Under specific stimuli, eosinophils are able to promote directly or indirectly the release of several inflammatory mediators, including cytokines and chemokines (CCL2, CCL3, CCL5 and CCL11) that may recruit inflammatory cells to the tumor environment and allow the modulation of the immune responses (Hothenberg and Hogan, 2006; Gatault et al., 2012). In this regard, the induction of SCC lesions by 4NQO did not trigger an increase in the expression of CCL3, CCL5, CCL11 and TNF-α in the tongue of mice in the evaluated time-point. These data are in agreement with some evidence from the literature, indicating that the experimental model of 4NQO-induced tongue is not associated with marked expression

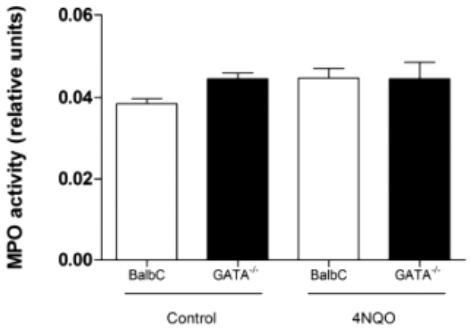


Fig. 6. Myeloperoxidase activity in tongue samples of mice. The presence of neutrophils was indirectly measured by MPO activity. The MPO expression in both experimental and control groups was marginal and there were no differences among the groups.

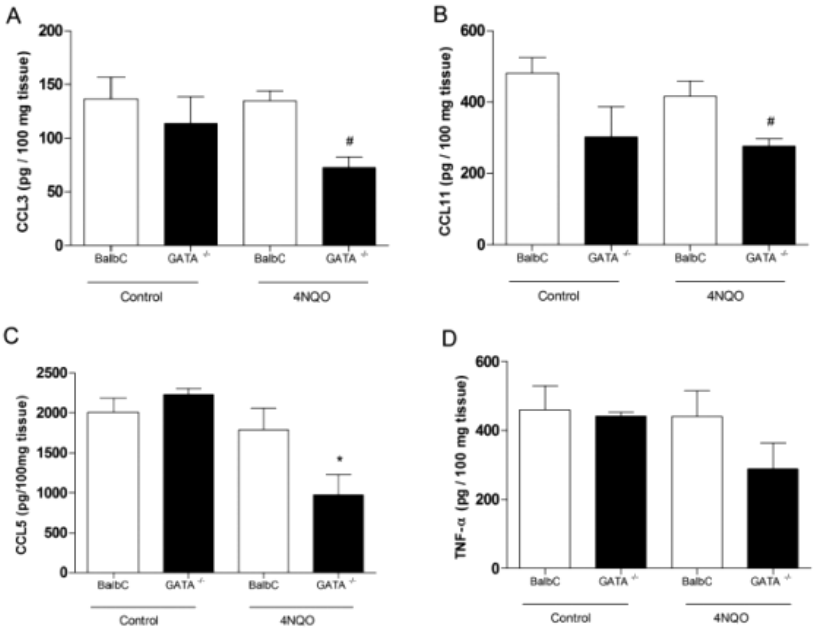


Fig. 7. Inflammatory mediators induced by 4NQO in tongue samples. **A-D.** Concentration of CCL3, CCL11, CCL5 and TNF-α in control and experimental WT and *Δdb*/GATA-1 mice, respectively. *p <0.05 when compared to the respective control; # p<0.05 when comparing WT- and *Δdb*/GATA-1-treated mice.

of inflammatory mediators in tumor sites (Gannot et al., 2004; Tang et al., 2004; Schoop et al., 2009). One hypothesis is that the expression of such mediators could be increased in the earlier stages of tumor development, although this remains to be addressed in further studies. Nevertheless, the levels of the CC chemokines 3 and 11 were significantly decreased in tongue lesional samples of *Δdb*/GATA-1-treated mice versus WT-treated mice. Moreover, the 4NQO-treatment was associated with reduced expression of CCL5 in *Δdb*/GATA-1-treated mice in relation to the respective control. These results may suggest that the recruitment of inflammatory cells may be partially impaired in *Δdb*/GATA-1-treated mice during carcinogenesis. However, no changes in MPO were observed after 4NQO treatment between the groups. In view of the non-mechanistic nature of the current data, these findings deserve further investigation.

Taken together the current results indicate that eosinophils may participate in tongue carcinogenesis, given that their ablation protected mice from SCC. Studies using mouse models can be helpful in the clarification of the roles of specific cell types in tumoral biology and can provide a better understanding about their involvement in tumor development.

Acknowledgements. We are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Grants 470516/2009-7 and 474106/2012-8); Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

References

Alkhabuli J.O. and High A.S. (2006). Significance of eosinophil counting in tumor associated tissue eosinophilia (TATE). Oral Oncol. 42, 849-850.

Alrawi S.J., Tan D., Stoler D.L., Dayton M., Anderson G.R., Mojica P., Douglas W., Hicks W. Jr, Rigual N. and Loree T. (2005). Tissue eosinophilic infiltration: a useful marker for assessing stromal invasion, survival and locoregional recurrence in head and neck squamous neoplasia. Cancer J. 11, 217-225.

Barnes L., Eveson J.W., Reichart P.A. and Sidransky D. (2005). World Health Organization classification of tumours. Pathology and genetics of Head and neck tumours. In: Tumours of the oral cavity and oropharynx. Slootweg P.J. and Everson J.W. (eds). IARC Press. Lyon. pp 168-175.

Boyd N.M. and Reade P.C. (1988). Mechanisms of carcinogenesis with particular reference to the oral mucosa. J. Oral Pathol. 17, 193-201.

Carvalho J.G., Noguti J., Silva V.H.P., Dedivitis R.A., Franco M.F. and Ribeiro D.A. (2012). Alkylation-induced genotoxicity as a predictor of DNA repair deficiency following experimental oral carcinogenesis. J. Mol. Histol. 43, 145-150.

Cuschieri A., Talbot I.C. and Weeden S. (2002). Influence of pathological tumour variables on long-term survival in resectable gastric cancer. Br. J. Cancer 86, 674-679.

Dorta R.G., Landman G., Kowalski L.P., Lauris J.R., Latorre M.R. and Oliveira D.T. (2002). Tumour-associated tissue eosinophilia as a prognostic factor in oral squamous cell carcinomas. Histopathology 41, 152-157.

Falconieri G., Luna M.A., Pizzolitto S., DeMaglio G., Angione V. and Rocco M. (2008). Eosinophil-rich squamous carcinoma of the oral cavity: a study of 13 cases and delineation of a possible new microscopy entity. Ann. Diag. Pathol. 12, 322-327.

Fernández-Aceñero M.J., Galindo-Gallego M., Sanz J. and Aljama A. (2000). Prognostic influence of tumor-associated eosinophilic infiltrate in colorectal carcinoma. Cancer 88, 1544-1548.

Fracalossi A.C.C., Comparini L., Funabashi K., Godoy C., Iwamura E.S.M., Nascimento F.D., Nader H.B., Oshima C.T.F. and Ribeiro D.A. (2011). Ras gene mutation is not related to tumour invasion during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. J. Oral Pathol. Med. 40, 325–333.

Gannot G., Buchner A. and Keisari Y. (2004). Interaction between the immune system and tongue squamous cell carcinoma induced by 4-nitroquinoline N-oxide in mice. Oral Oncol. 40, 287-297.

Gatault S., Legrand F., Delbeke M., Loiseau S. and Capron M. (2012). Involvement of eosinophils in the anti-tumor response. Cancer Immunol. Immunother. 61, 1527-1534.

Goldsmith M.M., Belchis D.A., Cresson D.H., Merritt W.D. 3rd and Askin F.B. (1992). The importance of the eosinophil in head and neck cancer. Otolaryngol. Head Neck Surg. 106, 27-33.

Hogan S.P. (2007). Recent advances in eosinophil biology. Int. Arch. Allergy Immunol. 143, 3-14.

Hothenberg M.E. and Hogan S.P. (2006). The eosinophil. Annu. Rev. Immunol. 24, 147-174.

Humbles A.A., Lloyd C.M., McMillan S.J., Friend D.S., Xanthou G., McKenna E.E., Ghiran S., Gerard N.P., Yu C., Orkin S.H. and Gerard C. (2004). A critical role for eosinophils in allergic airways remodeling. Science 305, 1776-1779.

Kanojia D. and Vaidya M.M. (2006). 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol. 42, 655-667.

Li J., Liang F., Yu D., Qing H. and Yang Y. (2012). Development of a 4-nitroquinoline-1-oxide model of lymph node metastasis in oral squamous cell carcinoma. Oral Oncol. 49, 299-305.

Llewellyn B.D. (1970). An improved Sirius red method for amyloid. J. Med. Lab. Tech. 23,308.

Lu S.L., Herrington H. and Wang X.J. (2006). Mouse models for human head and neck squamous cell carcinomas. Head Neck 28, 945-954.

Martinelli-Kläy C.P., Mendis B.R.R.N. and Lombardi T. (2009). Eosinophils and oral squamous cell carcinoma: a short review. J. Oncol. 2009, 1-6.

Massano J., Regateiro F.S., Januário G. and Ferreira A. (2006). Oral squamous cell carcinoma: Review of prognostic and predictive factors. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 102, 67-76.

Meyerholz D.K., Griffin M.A., Castilow E.M. and Varga S.M. (2009). Comparison of histochemical methods for murine eosinophil detection in an RSV vaccine-enhanced inflammation model. Toxicol. Pathol. 37, 249-255.

Moon S.M., Ahn M.Y., Kwon S.M., Kim S.A., Ahn S.E. and Yoon J.H. (2012). Homeobox C5 expression is associated with the progression of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. J. Oral Pathol. Med. 41, 470-476.

Munitz A. and Schaffer-Levi F. (2004). Eosinophils: 'new' roles for 'old' cells. Allergy 59, 268-275.

Oliveira D.T., Tjioe K.C., Assao A., Faustino S.S.E., Carvalho A.L., Landman G. and Kowalski L.P. (2009). Tissue eosinophilia and its association with tumoral invasion of oral cancer. Int. J. Surg. Pathol.

17, 244-249.

Oliveira D.T., Biassi T.P., Faustino S.E.S., Carvalho A.L., Landman G. and Kowalski L.P. (2011). Eosinophils may predict occult lymph node metastasis in early oral cancer. Clin. Oral Invest. 16, 1523-1528.

Pereira M.C., Oliveira D.T. and Kowalski L.P. (2010). The role of eosinophils and eosinophil cationic protein in oral cancer: a review. Arch. Oral Biol. 56, 353-358.

Queiroz-Junior C.M., Pacheco C.M.F., Fonseca A.H., Klein A., Caliari M.V. and Francischi J.N. (2009). Myeloperoxidase content is a marker of systemic inflammation in a chronic condition: the example given by the periodontal disease in rats. Mediators Inflamm. 1-7.

Ribeiro D.A., Salvadori D.M.F., Silva R.N., Darros B.R. and Marques M.E.A. (2004). Genomic instability in nonneoplastic oral mucosa cells can predict risk during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Oral Oncol. 40, 910-915.

Said M., Wiseman S., Yang J., Alrawi S., Douglas W., Cheney R., Hicks W., Rigual N., Loree T., Spiegel G. and Tan D. (2005). Tissue eosinophilia: a morphologic marker for assessing stromal invasion in laryngeal squamous neoplasms. BMC Clin. Pathol. 5, 1-8.

Schoop R.A.L., Noteborn M.H.M. and De Jong R.J.B. (2009). A mouse model for oral squamous cell carcinoma. J. Mol. Hist. 40, 177-181.

Silva R.N., Ribeiro D.A., Salvadori D.M.F. and Marques M.E.A. (2007). Placental glutathione S-transferase correlates with cellular proliferation during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Exp. Toxic. Pathol. 59, 61-68.

Spiegel G.W., Ashraf M. and Brooks J.J. (2002). Eosinophils as a marker for invasion in cervical squamous neoplastic lesions. Int. J. Gynecol. Pathol. 21, 117-124.

Tadbir A.A., Ashraf M.J. and Sardari Y. (2009). Prognostic significance of stromal eosinophilic infiltration in oral squamous cell carcinoma. J. Craniof. Surg. 20, 287-289.

Tang X.H., Knudsen B., Bemis D., Tickoo S. and Gudas L.J. (2004). Oral Cavity and esophageal carcinogenesis modeled in carcinogen treated mice. Clin. Canc. Res. 10, 301-313.

Vered M., Daniel N., Hirshberg A. and Dayan D. (2003). Histomorphologic and morphometric changes in minor salivary glands of the rat tongue during 4-nitroquinoline 1-oxide-induced carcinogenesis. Oral Oncol. 39, 491-496.

Wong D.T., Bowen S.M., Elovic A., Gallagher G.T. and Weller P.F. (1999). Eosinophil ablation and tumor development. Oral Oncol. 35, 496-501.

Accepted September 11, 2013

ANEXO B

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 12/2011,** relativo ao projeto intitulado "***Participação do infiltrado inflamatório peritumoral e da quimiocina MIP-1A/CCL3 na carcinogênese bucal***", que tem como responsável(is) **Tarcília Aparecida Silva** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **30/ 03/2011.**

Este certificado expira-se em **30/ 03/ 2016.**

CERTIFICATE

We hereby certify that the **Protocol nº 12/2011,** related to the project entitled "***Role of peritumoral inflammatory infiltrate and the chemokine MIP-1ª/CCL3 on oral carcinogenesis***", under the supervisiors of **Tarcília Aparecida Silva,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **March 30, 2011.**

This certificate expires in **March 30, 2016.**

Belo Horizonte, 4 de Abril de 2011.

Profª. Jacqueline Isaura Alvarez-Leite
Coordenadora do CETEA/UFMG

Universidade Federal de Minas Gerais
Avenida Antônio Carlos, 6627 – Campus Pampulha
Unidade Administrativa II – 2º Andar, Sala 2005
31270-901 - Belo Horizonte, MG - Brasil
Telefone: (31) 3499-4516
www.ufmg.br/bioetica/cetea - cetea@prpq.ufmg.br

(Mod.Cert. v1.0)

ANEXO B

Universidade Federal de Minas Gerais
Comitê de Ética em Experimentação Animal - CETEA

OF. CETEA-009/13 Belo Horizonte, 15 de abril de 2013.

Sra.
Profa. Tarcília Aparecida da Silva
Departamento de Clínica, Cirurgia e Patologia Oral
Faculdade de Odontologia
Nesta

O Comitê de Ética em Experimentação Animal (CETEA) da UFMG informa que na reunião ordinária do dia 27/03/2013, em face da solicitação apresentada por V. Sa., aprovou o pedido de adendo ao projeto de pesquisa protocolado sob o número 12/2011, titulo "*Participação do infiltrado inflamatório peritumoral e da quimiocina MIP-1A/CCL3 na carcinogênese bucal*" para a inclusão de novo experimento para a indução da carcinogênese na língua dos camundongos pelo carcinógeno químico 4-Nitroquinolina-1-óxido cujo objetivo é avaliar o papel dos receptores de CCL3(CCr1, CCr5 e D6) e da quimiocina CCL2 na carcinogênese.

Foi aprovada inclusão dos seguintes camundongos (grupos): 5 animais CCR1-/- (KO) que beberão água filtrada (controles); 5 animais CCR1-/- (KO) que receberão o 4NQO na água de beber durante 28 semanas; 5 animais CCR5-/- (KO) que beberão água filtrada (controles); 5 animais CCR5-/- (KO) que receberão o 4NQO na água de beber durante 28 semanas; ; 5 animais D6-/- (KO) que beberão água filtrada (controles); 5 animais D6-/- (KO) que receberão o 4NQO na água de beber durante 28 semanas; 5 animais CCR2-/- (KO) que beberão água filtrada (controles); 5 animais CCR2-/- (KO) que receberão o 4NQO na água de beber durante 28 semanas.

Sendo o que nos apresenta no momento, ficamos à sua inteira disposição para outros esclarecimentos que se fizerem necessários.

Atenciosamente

Profa. Jacqueline Isaura Alvarez Leite
Coordenadora do CETEA/UFMG

O CETEA tem novo site: http://www.ufmg.br/bioetica/cetea/.
E-mail : cetea@prpq.ufmg.br
Endereço: *Unidade Administrativa II, 2º. andar, sala 2005.*
Tel. 3409-4516, Fax: 3409-4592

ANEXO C

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

COMITÊ DE ÉTICA EM PESQUISA

UFG

Goiânia, 01/05/2010

PARECER CONSUBSTANCIADO REFERENTE AO PROJETO DE PESQUISA, PROTOCOLADO NESTE COMITÊ SOB O Nº013/2010

I – Identificação

– Título do projeto: Significância clínico-patológica da expressão das quimiocinas CCL19 e CCL21 e de seu receptor específico no carcinoma espinocelular de boca
– Pesquisador Responsável: Helenisa Helena de Oliveira Neto
– Orientador (quando necessário): Aline Carvalho Batista
– Pesquisadores participantes:
– Instituição onde será realizado o estudo:
– Data de apresentação ao CEP/UFG:
– Área Temática:

Comentários do relator frente à Resolução CNS 196/96 e complementares em particular sobre:

V– Parecer do CEP

Os pesquisadores atenderam as sugestões propostas e, portanto, meu parecer é favorável à aprovação do projeto, salvo melhor juízo deste Comitê.

O pesquisador responsável deverá encaminhar ao CEP/UFG, relatórios da pesquisa encerramento, conclusão (ões) e publicação (ões) de acordo com as recomendações da Resolução 196/96.

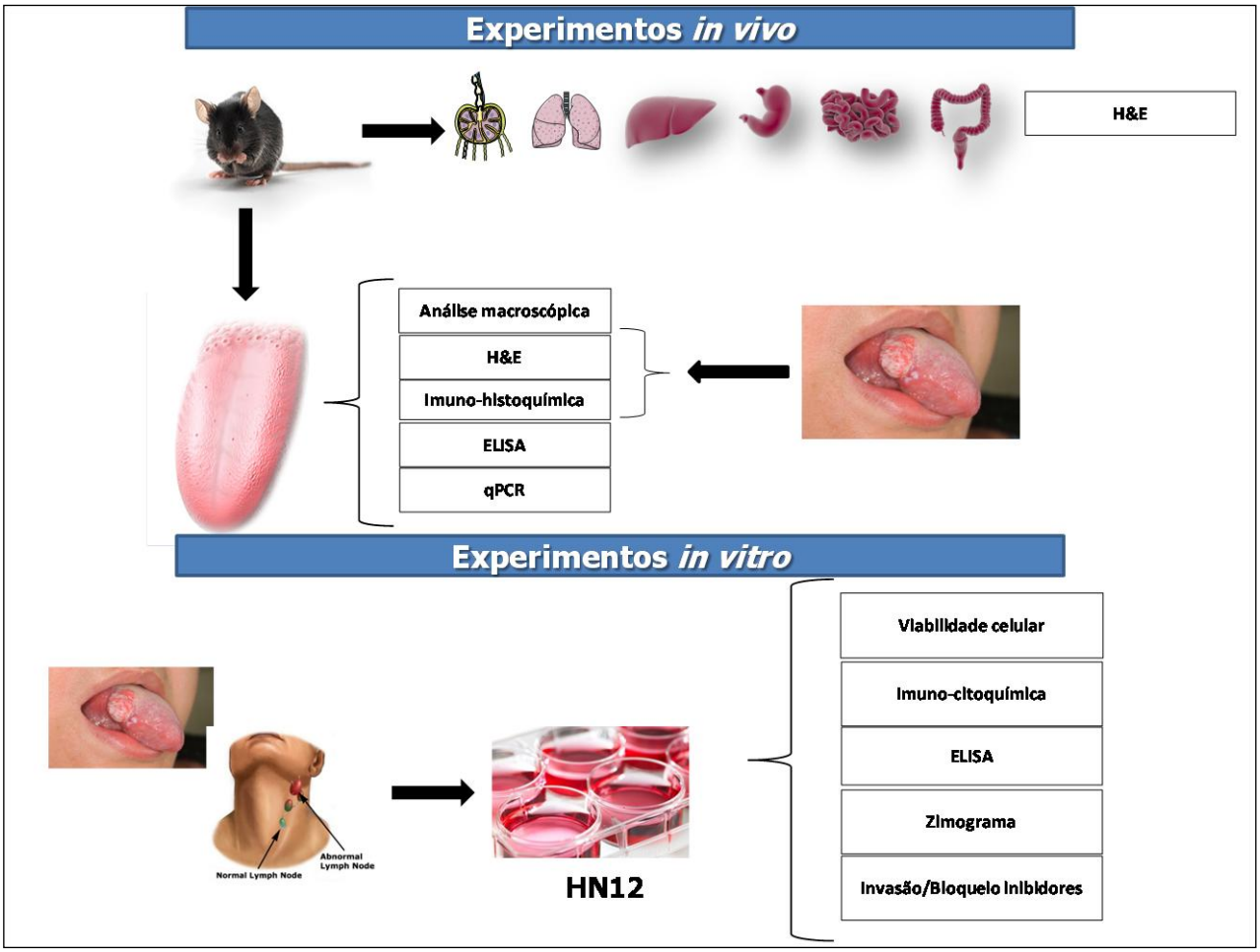
VI – Data da reunião: 03/05/2010

Assinatura do(a) relator(a):

Assinatura do(a) Coordenador(a)/ CEP/UFG:

Prof. Maurício Martins Sales
Coordenador do Comitê de Ética em Pesquisa
Pró-Reitoria de Pesquisa e Pós-Graduação/UFG

ANEXO D


Experimentos in vivo
H&E
Análise macroscópica
H&E
Imuno-histoquímica
ELISA
qPCR
Experimentos in vitro
Normal Lymph Node
Abnormal Lymph Node
HN12
Viabilidade celular
Imuno-citoquímica
ELISA
Zimograma
Invasão/Bloquelo inibidores