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CARCINOMA ESPINOCELULAR DE BOCA E INFLAMAÇÃO: PAPEL DOS MACRÓFAGOS NO PROGNÓSTICO E INFLUÊNCIA DE CITOCINAS INFLAMATÓRIASNO COMPORTAMENTO MIGRATÓRIO.

Linha de Pesquisa: Câncer Bucal

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“Há um menino, há um moleque
Morando sempre no meu coração
Toda vez que o adulto balança
Ele vem pra me dar a mão

Há um passado no meu presente
Um sol bem quente lá no meu quintal
Toda vez que a bruxa me assombra
O menino me dá a mão

E me fala de coisas bonitas
Que eu acredito
Que não deixarão de existir
Amizade, palavra, respeito
Caráter, bondade alegria e amor
Pois não posso
Não devo, não quero
Viver como toda essa gente
Insiste em viver
E não posso aceitar sossegado
Qualquer sacanagem ser coisa normal

Bola de meia, bola de gude
O solidário não quer solidão
Toda vez que a tristeza me alcança
O menino me dá a mão

Há um menino, há um moleque
Morando sempre no meu coração
Toda vez que o adulto fraqueja
Ele vem pra me dar a mão”

Bola de meia, bola de gude
Autores: Milton Nascimento e Fernando Brant

RESUMO

ALVES, Alessandro Menna. **Carcinoma espinocelular de boca e inflamação: papel dos macrófagos no prognóstico e influência de citocinas inflamatórias no comportamento migratório.** 2016. 78f. Tese (Doutorado) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2016.

O carcinoma espinocelular de boca (CEB) é a neoplasia maligna mais comum da cavidade oral, correspondendo à aproximadamente 94% dos casos dessa região. Apesar dos diversos estudos moleculares e celulares do CEB, a taxa de sobrevida dos pacientes é de aproximadamente 50%, devido principalmente ao tamanho do tumor, metástase em linfonodos regionais, grau de diferenciação das células e sítio anatômico. O microambiente tumoral do CEB, é extremamente complexo e diversificado, tendo como característica principal um estado inflamatório crônico imunossupressivo. Este microambiente é sustentado pela liberação de diferentes citocinas inflamatórias, como IL-6, TNF-α e IL-1β, as quais promovem a comunicação celular e osquestram as atividades exercidas tanto pelas células tumorais quanto pelas estromais. Dentre essas atividades, tem sido relatado na literatura que as citocinas inflamatórias são capazes de aumentar a migração e a capacidade de invasão das células tumorais. Entre as células estromais, os macrófagos são as mais abundantes e participam da manutenção do microambiente tumoral. De acordo com o estímulo, podem ser polarizados M1, com papel pró-inflamatório e anti-tumoral, e M2, com papel anti-inflamatório e pró-tumoral. O objetivo desta tese foi compreender o papel dos macrófagos no prognóstico de CEB e das citocinas inflamatórias IL-6, TNF-α e IL-1β no comportamento migratório de linhagens celulares de CEB. Para verificar o papel dos macrófagos no prognóstico, foi realizada uma revisão sistemática na qual foram incluídos apenas os estudos que utilizavam amostra de pacientes com CEB e avaliavam o prognóstico com marcadores para macrófagos. Foi observado que maiores concentrações de macrófagos CD68+ e CD163+ estavam relacionados com pior prognóstico de pacientes com CEB, embora não tenha sido possível concluir qual região tumoral a presença destas células seja mais importante

para o desfecho. Para analisar o papel das citocinas inflamatórias IL-6, TNF-α e IL-1β no comportamento migratório de células de CEB foram realizados ensaios *in vitro* utilizando duas linhagens celulares, SCC25 e Cal27, em condições promotoras de migração sob a influência dessas citocinas. Foi observado que a citocina IL-6 foi capaz de aumentar a velocidade de migração e a direcionalidade tanto da SCC25 quanto da Cal 27 e que esta melhora na capacidade migratória ocorreu através de um *crosstalk* entre a via de sinalização relacionada a IL6 (STAT3) e a via reguladora de migração celular, Rho GTPase Rac1. Estes dados reforçam o papel do microambiente tumoral no processo de progressão tumoral e sugerem potenciais alvos terapêuticos como a modulação do perfil da população de macrófagos e o papel de interleucinas no controle de invasão tecidual e metástase.

PALAVRAS-CHAVES: câncer oral; microambiente tumoral; macrófagos associados ao tumor; SCC25; Cal27; IL-6; Rac1; Rho GTPase.

ABSTRACT

ALVES, Alessandro Menna. **Oral squamous cell carcinoma and inflammation: role of macrophages in the prognosis and the influence of inflammatory cytokines on migratory behavior.** 2016. 78f. Thesis (Doctorate) – School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, 2016.

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity, corresponding to approximately 94% of the cases in this region. Despite the diverse molecular and cellular studies of OSCC, the patient survival rate is approximately 50%, mainly due to tumor size, regional lymph node metastasis, cell differentiation and anatomic site. The OSCC tumor microenvironment is extremely complex and diverse, with the main characteristic being an immunosuppressive chronic inflammatory state. This microenvironment is supported by the release of different inflammatory cytokines, such as IL-6, TNF-α and IL-1β, which promote cell communication and enhance the activities of both tumor and stromal cells. Among these activities, it has been reported in the literature that inflammatory cytokines are capable of increasing migration and invasiveness of tumor cells. Among stromal cells, macrophages are the most abundant and participate in the maintenance of the tumor microenvironment. According to the stimulus, macrophages can be polarized in M1, with pro-inflammatory and anti-tumoral role, and M2, with anti-inflammatory and pro-tumoral role. Thus, the aim of this thesis was to evaluate the role of macrophages in the prognosis of OSCC and the influence of inflammatory cytokines IL-6, TNF-α and IL-1β in the migratory behavior of OSCC cell lines. To assess the role of macrophages in the prognosis, a systematic review was conducted in which only studies using a sample of OSCC patients were evaluated and the prognosis was evaluated with macrophage markers. It was observed that higher concentrations of CD68 + and CD163 + macrophages were related to worse prognosis in patients with OSCC, although it was not possible to conclude which tumor region the presence of these cells is more important for the outcome. In order to analyze the role of the inflammatory cytokines IL-6, TNF-α and IL-1β in the migratory

behavior of OSCC cells, *in vitro* assays using two cell lines, SCC25 and Cal27, were performed in migration-promoting conditions under the influence of these cytokines. It was observed that IL-6 was able to increase the speed migration and directionality of both SCC25 and Cal 27 and that this improvement in migratory capacity occurred through a crosstalk between the IL6-related signaling pathway (STAT3) and cell migration-related pathway, RhoGTPase Rac1. These data reinforce the role of the tumor microenvironment in the tumor progression process and suggest potential therapeutic targets such as the modulation of the profile of the macrophages population and the role of interleukins in the control of tissue invasion and metastasis.

KEYWORDS: oral cancer; tumor microenvironment; tumor-associated macrophages; SCC25; Cal27; IL-6; Rac1; Rho GTPase.

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Fundamentação teórica

Artigo científico 1

LISTA DE ABREVIATURAS:

CEB – carcinoma espinocelular de boca
OMS – Organização Mundial da Saúde
INCA – Instituto Nacional do Câncer José de Alencar
MAT – Microambiente tumoral
TGF-β1 – Fator de crescimento transformante β1
TNF-α – Fator de necrose tumoral α
IL – Interleucina
IFN-γ – Interferon gama
MMP – Metaloproteinase da matriz
VEGF – Fator de crescimento endotelial vascular
PDGF – Fator de crescimento derivado de plaqueta
FGF – Fator de crescimento fibroblástico
TEM – transição epitélio-mesênquima

SUMÁRIO

1. FUNDAMENTAÇÃO TEÓRICA

1.1 Carcinoma espinocelular de boca

O câncer de boca corresponde à aproximadamente 5% de todos os tumores malignos do corpo (1), embora haja uma ampla variação geográfica na incidência deste câncer (2). O câncer bucal apresenta alta incidência em diferentes partes do globo, como Sri Lanka, Taiwan, França, Leste Europeu, Rússia, EUA, Uruguai, Brasil, sendo que na Índia é o tipo de malignidade mais comum (2, 3). Segundo a Organização Mundial da Saúde (OMS), no seu último relatório no ano de 2012, foram relatados 14,1 milhões de novos casos de câncer no mundo, sendo 339.564 casos de câncer oral (GLOBOCAN). Já no Brasil, segundo o Instituto Nacional do Câncer (INCA), o câncer oral é a sexta neoplasia mais comum, sendo previsto para o ano de 2016, 11.140 novos casos em homens e 4.350 novos casos em mulheres. O carcinoma espinocelular de boca (CEB), também chamado de carcinoma de células escamosas ou epidermóide, é o tipo mais prevalente (94% dos tumores orais malignos), e ocorre, normalmente, em indivíduos acima dos 45 anos de idade. Embora várias pesquisas tenham sido realizadas buscando o melhor entendimento do comportamento do CEB, a taxa de sobrevida ainda é menor que 50% em 5 anos, principalmente nos casos em que há a presença de metástase em linfonodos regionais (4).

1.2 Microambiente tumoral e CEB

Microambiente tumoral (MAT) é definido como um ambiente complexo formado não só pelas células tumorais, mas também pelas células estromais, como macrófagos, linfócitos, células endoteliais, pericitos e fibroblastos (5, 6), as quais são atraídas para este local e estão em constante interação entre si e com as células tumorais, através da liberação de citocinas (7-9). Somado à isso, ainda temos a presença da matriz extracelular, a qual pode interagir de diferentes maneiras com os componentes do MAT, dependendo da sua composição e das integrinas que essas células possuem (10). Além disso,

durante a progressão tumoral, essa matriz vai sendo modificada e degradada, liberando outras citocinas para o MAT.

Em 2011, Hanahan e Weinberg publicaram uma revisão de literatura intitulada “Hallmarks of Cancer: The Next Generation” (11), na qual eles mostram que para o crescimento e progressão neoplásica, o tumor adquire dez capacidades: mutação e instabilidade genômica, resistência à morte celular, desregulação do metabolismo energético, sinalização proliferativa contínua, evasão dos supressores de crescimento, fuga da destruição imune, imortalidade replicativa, inflamação promotora de tumor, ativação de invasão e metástase, e angiogênese. Já em 2012, uma outra revisão de literatura conduzida por Hanahan e Coussens, intitulada “Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment” (5), ressalta que dos dez pontos básicos para o desenvolvimento tumoral, em sete deles temos a participação das células do estroma, ressaltando a importância destes componentes celulares para o desenvolvimento do tumor (Figura 1). Alguns autores em revisões de literatura tem ressaltado que as células do estroma seriam alvos terapêuticos interessantes, uma vez que, apesar de estarem programadas para atuar a favor do tumor, não apresentam mutações e são passíveis de “reeducação”, consequentemente, impedindo/controlando o desenvolvimento do tumor (5, 6).

Desde o início da formação do tumor, a partir das primeiras células alteradas e formação de lesões potencialmente malignas, passando pelo rompimento da membrana basal e invasão do tecido conjuntivo subjacente, entrada nos vasos sanguíneos e formação dos êmbolos tumorais, até o preparo do nicho metastático e a presença de macrometástases, temos a participação das células do estroma (6) (Figuras 2 e 3). No CEB, assim como em outros tumores sólidos, no primeiro momento, as células do estroma ainda não foram “educadas” pelas células tumorais, então elas atuam impedindo o crescimento do tumor, induzindo essas células com pequenas alterações no DNA à apoptose. Entretanto, conforme as células tumorais vão adquirindo novas mutações e habilidades, as células do estroma vão sendo “educadas” e ativadas de modo favorável ao tumor. No caso específico do CEB, conforme a lesão potencialmente maligna vai evoluindo, há um aumento na atividade angiogênica, produção de metaloproteinases da matriz e uma mudança no

perfil das células inflamatórias, diminuindo a quantidade de células dendríticas, apoptose de linfócitos T CD8, e aumento de macrófagos e linfócitos T CD4 reguladores (7, 9) (Figura 4).

1.3 MAT e inflamação

Uma importante característica do MAT é o estado inflamatório imunossupressivo constante, gerado tanto pela presença das células imunes quanto pelas citocinas inflamatórias que são liberadas neste ambiente (6, 11). Este processo inflamatório constante ajuda na proliferação das células presentes no MAT, promove angiogênese, aumenta a sobrevida das células tumorais e modifica o infiltrado inflamatório do tumor (12).

Dentre as células imunes presentes no MAT, podemos destacar: os macrófagos e suas polarizações em M1 e M2; os linfócitos T (CD8, CD4 auxiliar e CD4 regulador), B e NK (*natural killer*); células supressoras de origem mielóide (MDSCs); neutrófilos e suas polarizações em N1 e N2 (6, 12-14). Já entre as citocinas inflamatórias, temos: IL-6, TNF-α, IL-1β, IL-10, IL-4, IL-12, IL-4, IL-2, TGF-β (14, 15). As funções de cada citocina inflamatória estão resumidas na tabela 1.

Vale ressaltar ainda que essas citocinas inflamatórias são produzidas por todas as células presentes no MAT, dependendo do estímulo recebido, e também são liberadas neste espaço quimiocinas, citocinas responsáveis pela quimioatração de células.

1.4 Macrófagos e o MAT

Entre as células estromais presentes no MAT, os macrófagos são as mais abundantes e que apresentam maior importância para o desenvolvimento do tumor. Estas células têm sido relacionadas à proliferação e sobrevida de células tumorais, angiogênese, invasão de tecidos adjacentes e metástase (13, 16). Além disso, estudos clínicos têm mostrado que maiores concentrações de macrófagos em amostras de tumor estão relacionados a piores prognósticos (17). Outro ponto a ser destacado em relação aos macrófagos é a sua plasticidade, podendo ser polarizados basicamente de duas maneiras,

dependendo do estímulo ao seu redor: M1, o qual é considerado pró-inflamatório e anti-tumoral, e M2, o qual é considerado imunossupressivo e pró-tumoral (13, 16, 18-20) (Figura 5). Também tem sido sugerido na literatura que a polarização dos macrófagos não seja tão dicotômica, mas sim um estado flutuante em que as células fluam em estágios entre M1 e M2 (19, 20) (Figura 5).

A polarização em M1 ocorre principalmente na presença de interferon gama (IFN-γ) ou devido à exposição à microrganismos e seus produtos, como lipopolissacarídeo (LPS) (13, 18, 19) (Figura 5). Macrófagos M1 estão associados com respostas inflamatórias do tipo Th1, atração de linfócitos Th1, atividade microbicida e tumoricida. Além disso, M1 são macrófagos capazes de realizar apresentação de antígeno (13, 21). Seus principais marcadores são CD11c, CD80 and HLA-DR (13, 17, 18, 22, 23). Além disso, produzem TNF e IL-6, e aumentam a atividade de linfócito T CD8 (20) (Figura 6).

Já a polarização M2 é estimulada por IL-4, IL-10, IL-13 ou TGFβ (16, 18, 19, 24) (Figura 5). Esta polarização é relacionada à respostas inflamatórias do tipo Th2 e atração de linfócitos T reguladores, a qual é imunossupresiva (25). M2 libera fatores de crescimento como fator de crescimento endotelial vascular (VEGF), fator de crescimento derivado de plaqueta (PDGF), fator de crescimento transformador (TGF-β), e o fator de crescimento de fibroblasto (FGF), os quais promovem angiogênese em vários tumores (21, 26), liberação de metaloproteinases da matriz (MMP-1, MMP-2, MMP-3, MMP-9 e MMP-12) e citocinas inflamatórias, como IL-6, TNF, IL-1,IL-23, IL-10. Seus principais marcadores são CD163, CD11b, CD206 and MRC1 (13, 17, 18, 22, 23). Em adição, diminuem a atividade linfócitos T CD8 e T CD4 auxiliar do tipo 1, ao passo que aumentam a de linfócitos T CD4 auxiliares do tipo 2 e T CD4 reguladores (20) (Figura 6).

Ainda é importante ressaltar que não deve ser avaliado apenas a polarização do macrófago, mas também em qual região do tumor ele está presente, se na porção tumoral, no estroma ou no fronte de invasão, devido à heterogeneidade do MAT (24, 27, 28).

1.5 Mecanismos de invasão tumoral

Os diferentes eventos que ocorrem durante a migração celular são coordenados pela família das Rho GTPases, as quais são ativadas e desativadas de maneira coordenada, permitindo que a célula migre. Dentre as proteínas presentes nesta família, as mais importantes são a Rac1, RhoA e Cdc42 (29). O primeiro evento da migração celular é a determinação da direção do movimento, a qual ocorre pela ação de Cdc42. Logo após, ocorre a formação do lamelipódio na porção frontal da célula e formação de adesões novas, as quais estabilizam o lamelipódio, sendo controlado principalmente por Rac1. Em seguida, fibras de tensão são formadas ao longo do corpo celular, causando a contração da célula na direção do movimento, coordenada por RhoA (29) (Figura 7). Vários estudos têm demonstrado que Rac1 está superexpressa em diferentes tipos de tumor, incluindo CEB (30). Além disso, esta superexpressão de Rac1 está relacionada com invasividade, metástase e prognóstico pobre (31-35). Alguns artigos têm demonstrado que os componentes do MAT são capazes de modular essas atividades migratórias (36, 37).

A invasão tecidual e o desenvolvimento do potencial metastático de tumores malignos são a maior causa de insucessos clínicos em termos de terapia e prognóstico. As invasões tumorais, sejam elas individuais ou coletiva, são caracterizadas por modificações na adesão entre as células, e na adesão das células à matriz extracelular, reorganização do citoesqueleto e mudança do formato celular (38-41). A composição e a rigidez da matriz extracelular, e as mutações e a presença de proteínas ligadas à adesão celular, irão determinar qual o tipo de migração que será adotada pelas células e o formato que irão assumir. Ainda, os fatores de crescimento e as citocinas inflamatórias presentes no MAT também influenciarão o comportamento migratório das células tumorais (40).

Durante a carcinogênese, um grande número de mutações ocorre, as quais levam à modificações no citoesqueleto e no formato celular, aumentando a motilidade celular, em um processo chamado transição epitélio-mesênquima (TEM) (42, 43). TEM é marcada pela perda de marcadores epiteliais e ganho de marcadores mesenquimais, como e-caderina e n-caderina, respectivamente

(44). Alguns autores têm sugerido que o termo “transição” da TEM deveria ser substituído por “transformação”, porque refletiria melhor o espectro de plasticidade das células durante este processo (42) (Figura 8). Além disso, TEM pode ser influenciado por diferentes citocinas inflamatórias, as quais são liberadas pelos componentes celulares do MAT (42, 43). Alguns estudos tem tem demonstrado que IL-6 é capaz de induzir TEM em células de CEB, gerando maior invasão tecidual e metástase (45, 46).

1.6 Citocinas inflamatórias, MAT e migração das células tumorais

Como foi mencionado anteriormente, as citocinas inflamatórias liberadas pelas células presentes no MAT são extremamente importantes para a manutenção da intensa rede de comunicação celular do MAT e, consequentemente, as diferentes atividades do tumor. Lippitz (15), em 2013, realizou uma revisão sistemática, na qual foi determinado a existência de um padrão de expressão dessas citocinas nos diferentes tipos de neoplasias malignas do corpo. Este estudo também mostra que as citocinas que beneficiam a atividade tumoral direta ou indiretamente, apresentam-se superexpressas, e as que teriam potencial de inibir o crescimento e atividade tumoral, estariam subexpressas (15) (Tabela 2 e 3).

No microambiente tumoral do CEB, tanto as células tumorais quanto as células do estroma liberam inúmeras citocinas inflamatórias, como IL-6, IL-1β ou TNF-α e aumentam a expressão de seus fatores de transcrição: STAT3 para a IL-6 e NF-κB para IL-1β e TNF-α (47, 48). Alguns estudos tem revelado que IL-6 induz angiogênese e linfangiogênese, TEM e resistência à quimioterapia (46, 49, 50), enquanto IL-1β promoveu *in vitro* transformação maligna de queratinócitos orais displásicos e estimulou células de CEB à secretar IL-6, IL-8 e a quimiocina CXCL1 (51, 52), e TNF-α aumentou *in vitro* a união entre as células endoteliais e células de CEB, provavelmente aumentando o potencial metastático deste tumor (53). TNF-α também está relacionado com angiogênese, crescimento e o fenótipo de células tronco tumorais (54, 55).

Pesquisas recentes têm mostrado que as células inflamatórias e suas citocinas afetam o comportamento migratório das células tumorais, levando alguns autores a acreditar que sejam as grandes responsáveis pelo início do

processo de TEM, através da ativação de diferentes fatores de transcrição, como o NF-κB, Snail e STAT-3 (56, 57). Segundo Odenthal et al. (2016) (40), os fatores de crescimento e as citocinas presentes no MAT se ligam aos seus receptores nas células tumorais e levam a ativação de fatores de transcrição que estão relacionados com os eventos migratórios das células (Figura 9). Ainda, os fatores de crescimento e as citocinas irão modular a dinâmica e o tipo de migração celular (40) (Tabela 4).

Apesar dos avanços, o entendimento do papel da resposta inflamatória sobre o prognóstico e o comportamento invasivo de células CEB ainda é limitado.

1.7 Figuras

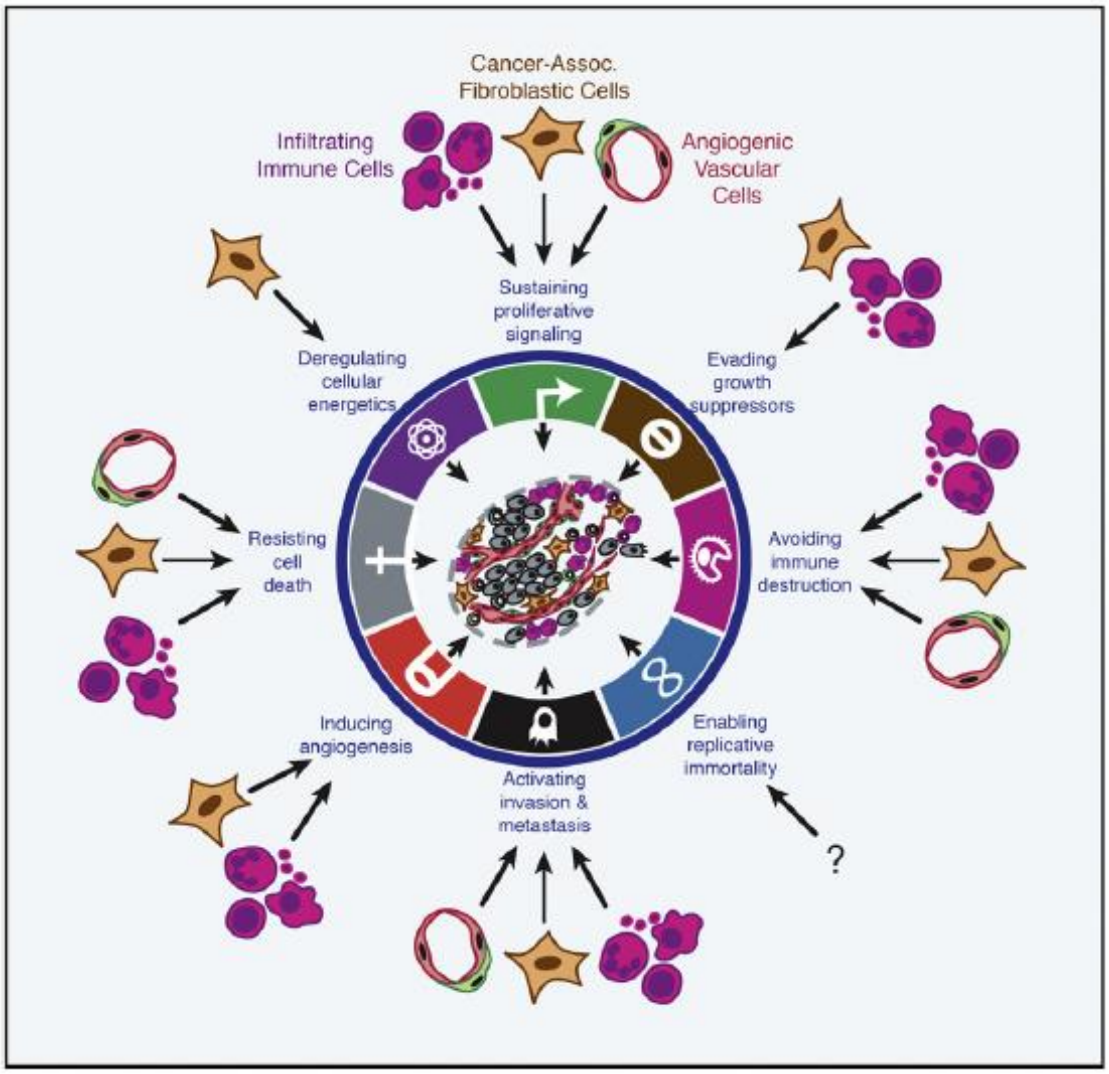

Figura 1: Contribuição multifatorial das células ativadas/recrutadas do estroma para os "Hallmarks of Cancer". Dos dez "Hallmarks of Cancer", as células do estroma atuam diretamente em sete pontos, sendo que as células imunes atuam sustentando a sinalização proliferativa, evasão dos supressores de crescimento, fuga da destruição imune, resistência à morte celular, indução à angiogênese e ativação da invasão tecidual e metástase. Fonte: Hanahan & Coussens, 2012.

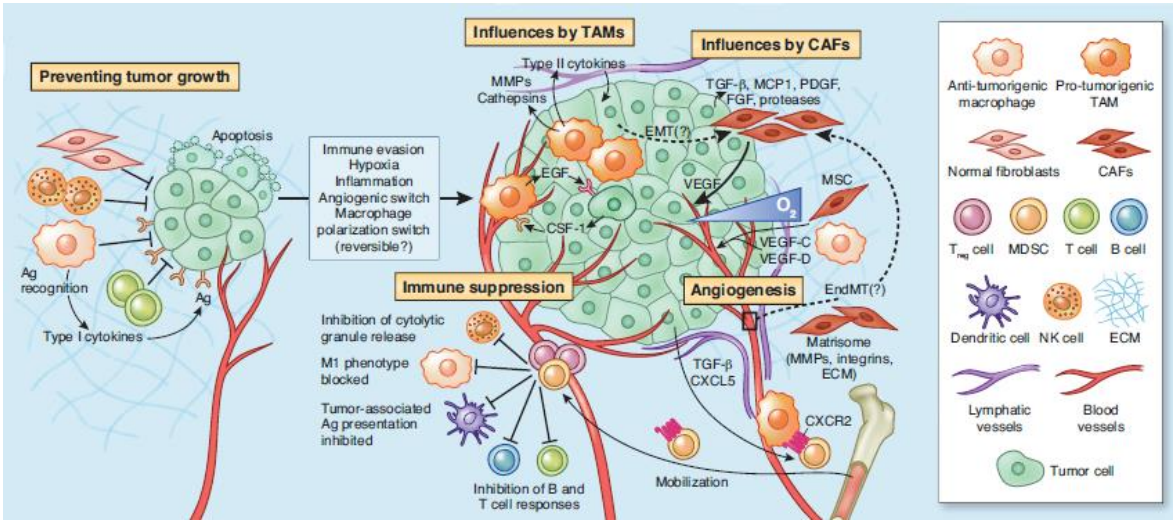

Figura 2: Múltiplas células do estroma convergem para o tumor primário dando suporte ao seu crescimento e progressão. Desde o início da formação do tumor, existe a participação das células do estroma, sendo que no início elas impedem a formação do tumor, e conforme as células neoplásicas vão adquirindo novas mutações e habilidades, estas células do estroma são “educadas” para trabalhar a favor do crescimento e progressão tumoral. Fonte: Quail & Joyce, 2013.

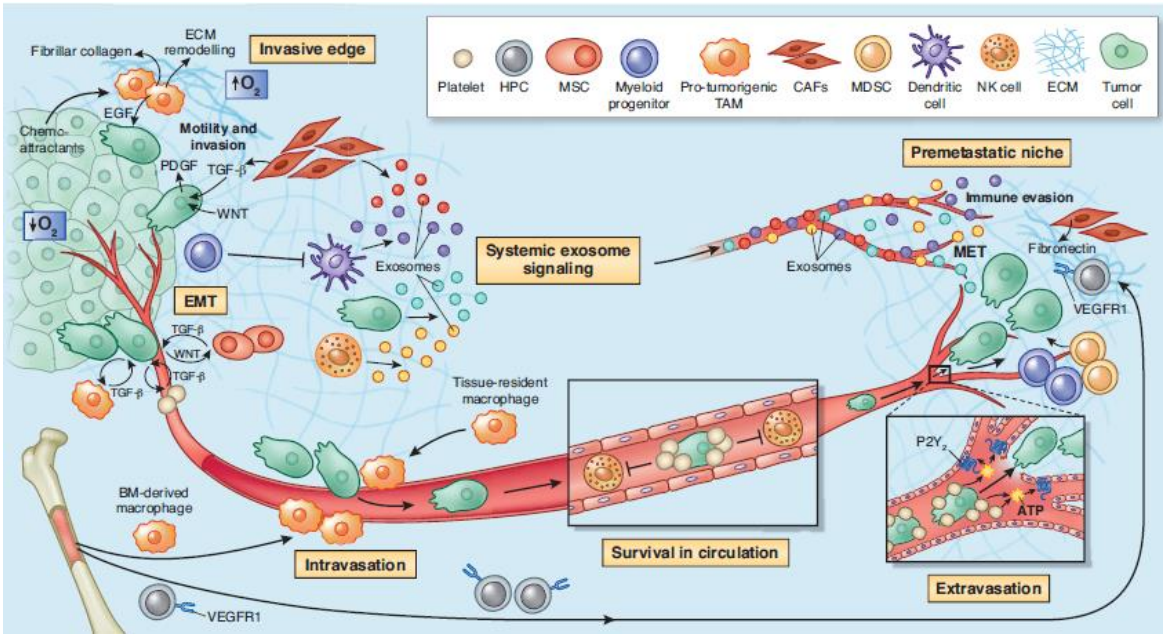

Figura 3: As células do estroma também atuam no processo metastático. As células do estroma induzem a transição epitélio-mesênquima, auxiliam as células tumorais no intravasamento, na sobrevivência dentro da corrente sanguínea e no extravasamento. Além de estar envolvidas no preparo do nicho metastático, através da liberação de inúmeras citocinas. Fonte: Quail & Joyce, 2013.

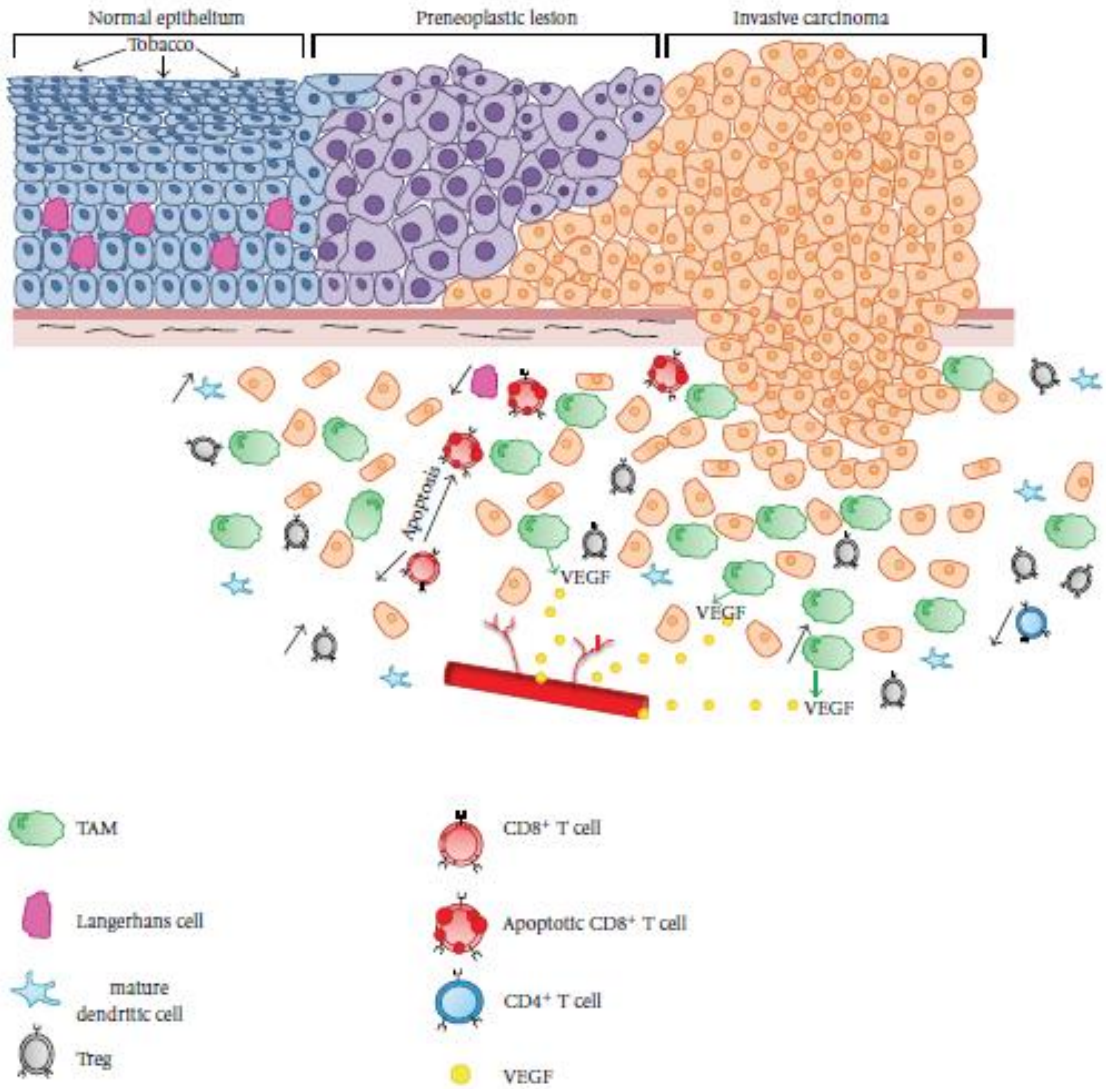

Figura 4: Evolução de uma lesão potencialmente maligna de boca em um carcinoma invasivo e as principais modificações que ocorrem no estroma tumoral. Conforme ocorre a progressão de uma lesão potencialmente maligna para uma lesão neoplásica na cavidade bucal, há uma mudança no estroma ao redor dessa lesão, caracterizada por um aumento na atividade angiogênica, diminuição no número de células dendríticas, aumento no número de macrófagos e apoptose de linfócitos T CD8. Fonte: Duray et al., 2010.

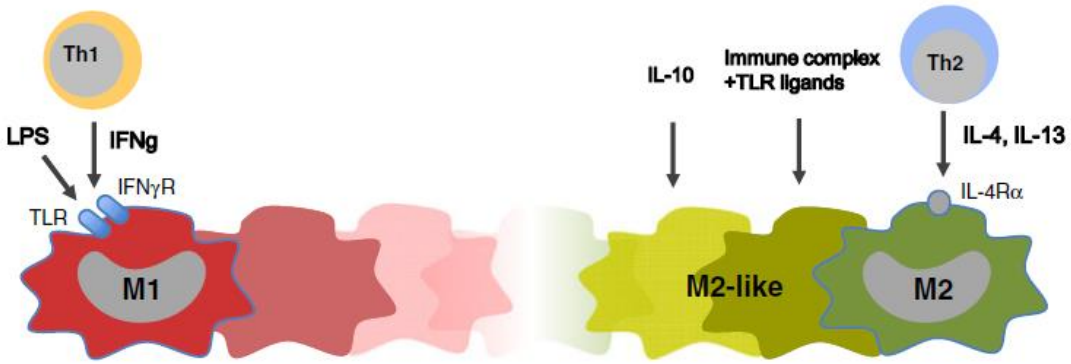

Figura 5: Espectro de ativação de polarização dos macrófagos em M1 e M2. Este esquema apresenta os fatores determinantes para a polarização dos macrófagos, seja em M1 ou M2. Também mostra o que vem sendo sugerido pela literatura, que o macrófago, na maioria das vezes, não está exclusivamente com característica de M1 ou M2, e sim oscilando em estágios intermediários entre estas duas polarizações. Fonte: Biswas et al., 2013.

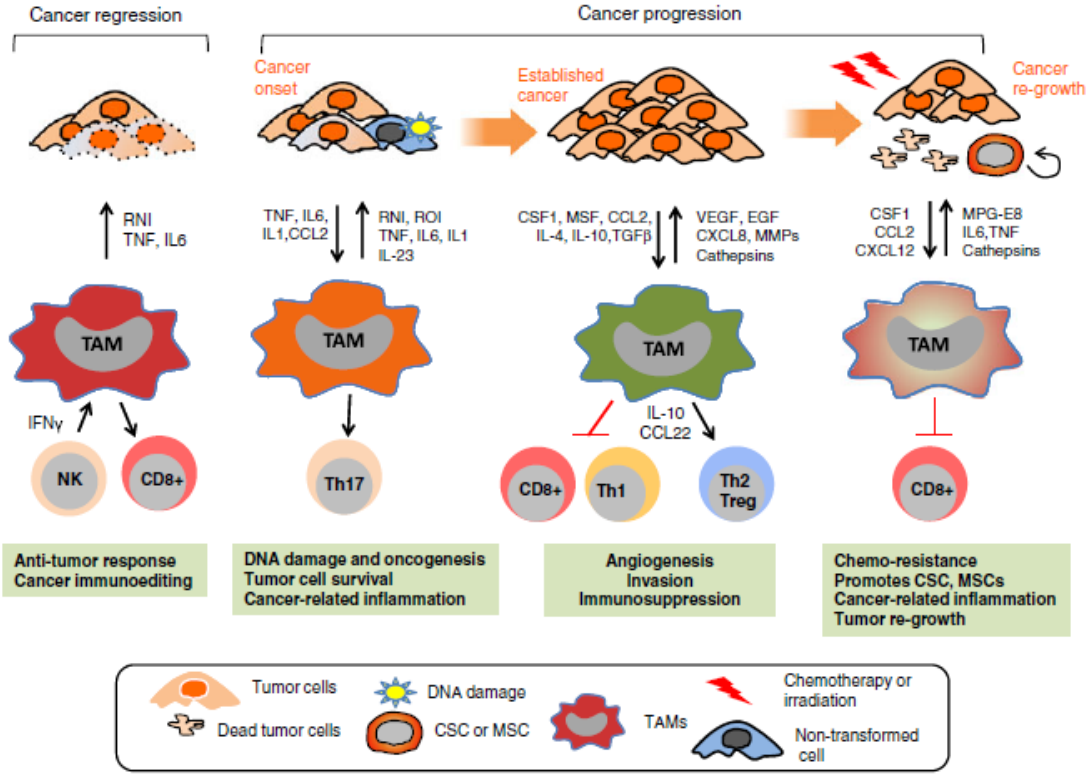

Figura 6: Diversas funções dos macrófagos associados ao tumor. A figura mostra as diversas funções que os macrófagos apresentam dentro do microambiente tumoral, citocinas liberadas e a relação com as demais células imunes deste espaço. Fonte: Biswas et al., 2013.

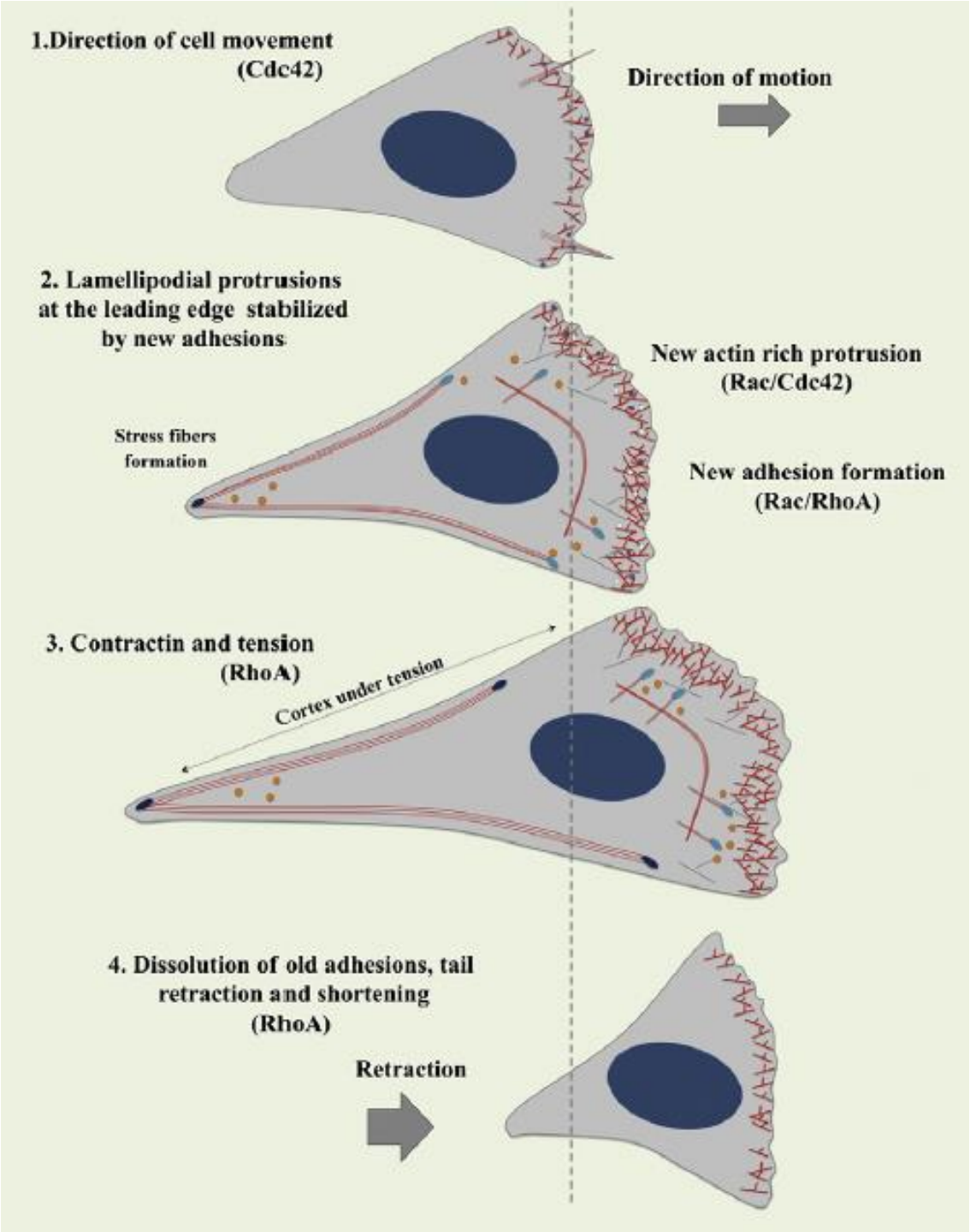

Figura 7: Rho GTPases no ciclo da migração celular. O esquema acima nos mostra a sequência de eventos e a ativação das diferentes Rho GTPases em cada fase do ciclo celular.

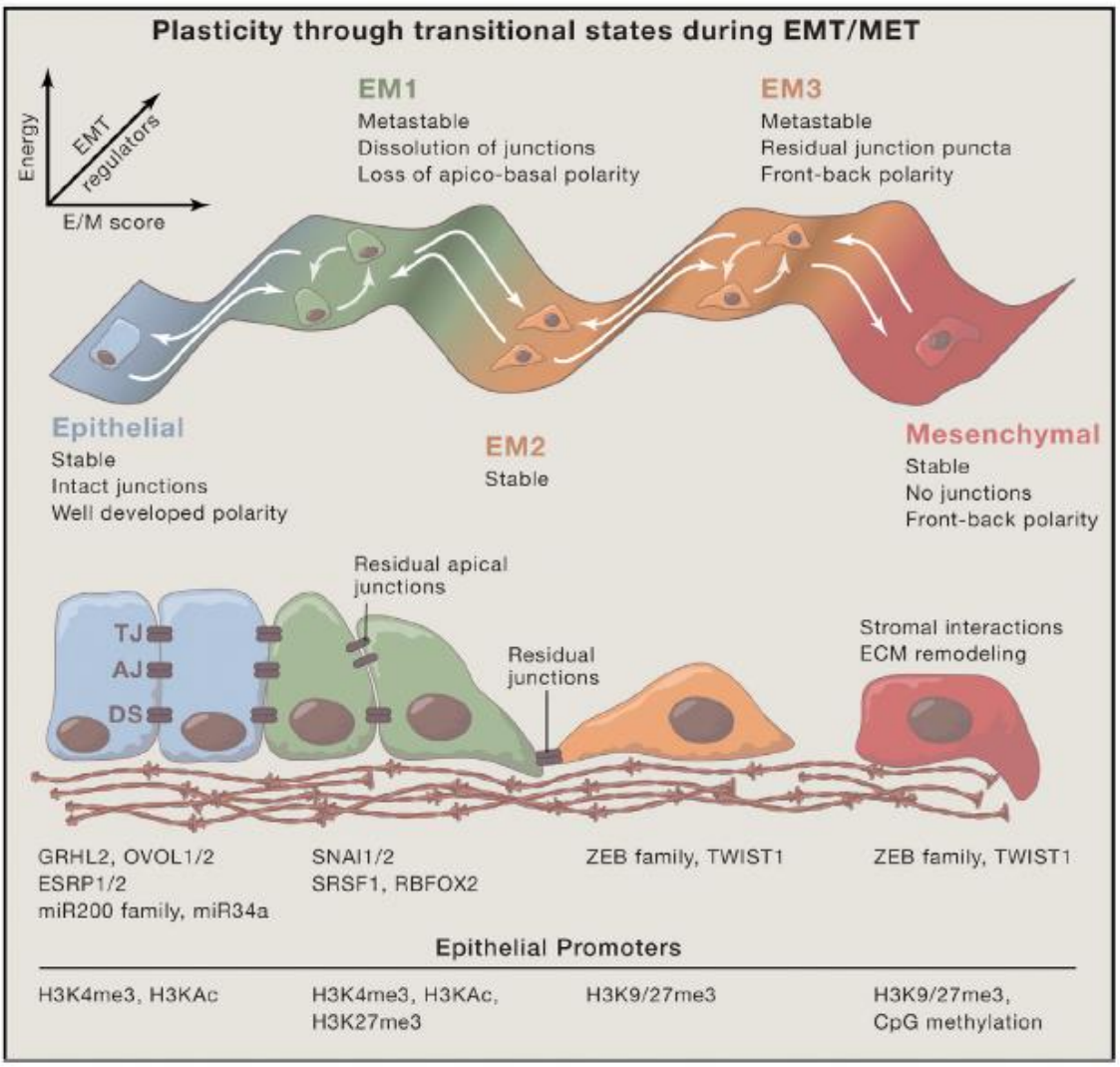

Figura 8: Transição através dos diferentes estados pertencentes ao espectro da transição epitélio-mesênquima (TEM). TEM pode ser considerado como um estado contínuo de transição, em que as células tumorais, dependendo do estímulo recebido do meio ambiente, flutuam de um fenótipo epitelial até um mesenquimal, e as formas intermediárias entre estes dois. Conforme o processo progride, as células vão perdendo sua polaridade e adesões célula-célula, e aumentando sua interação com a matriz extracelular. Fonte: Nieto et al., 2016.

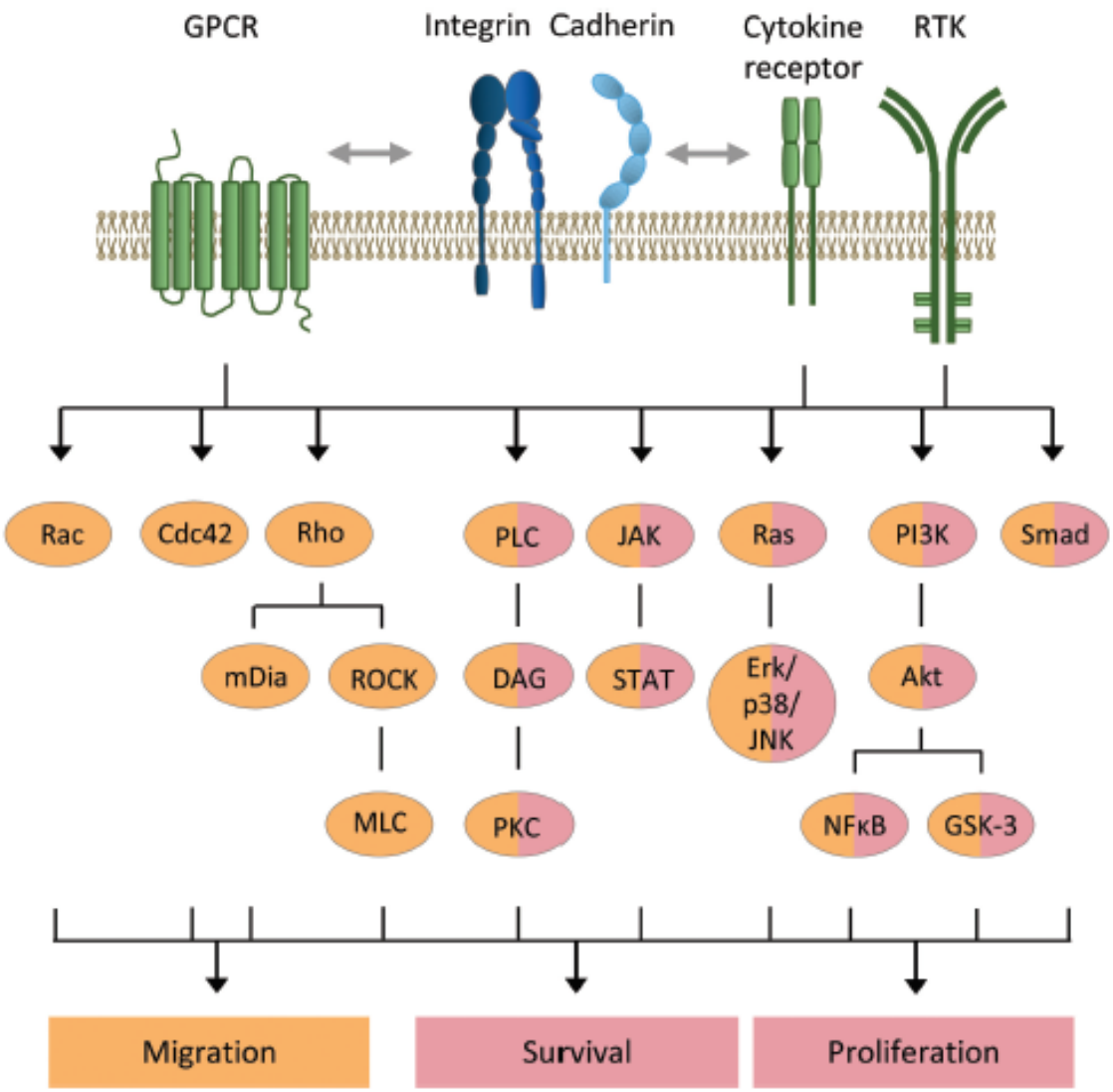

Figura 9: Vias de sinalização envolvidas no controle da migração. Os fatores de crescimento e as citocinas se ligam à receptores transmembrana acoplados à proteína G levando à ativação de fatores de transcrição associados à diferentes funções da célula tumoral, entre elas a migração. Fonte: Odenthal et al., 2016.

1.8 Tabelas

Citocina inflamatória	Pró ou anti-tumoral	Função
TGF-β1	Pró-tumoral	Diminui a expressão de IL-12 e IL-2; impede a maturação de linfócitos CD8 e NK pela inibição de IFN-γ; induz a maturação de linfócitos Treg.
IL-10	Pró-tumoral	Inibe a apresentação de antígenos e a expressão de MHC classe II, inibe a produção de IL-2 e IFN-γ; inibe a diferenciação de células dendríticas, diminui resposta imune.
IL-2	Anti-tumoral	Expansão clonal de linfócitos T; proliferação e diferenciação de linfócitos NK.
IL-12	Anti-tumoral	Estímula a produção de IFN-γ; geração e atividade de linfócitos T citotóxicos; maturação e ativação de linfócitos NK; estímula respostas inflamatórias do Thelper1
IFN-γ	Anti-tumoral	Estímula a expressão de MHC classe II; inibe TGF-β1.
TNF-α	Pró-tumoral	Estimula a ativação e recrutamento de monócitos e neutrófilos; aumenta permeabilidade vascular; induz a expressão de moléculas de adesão pelas células endoteliais.
IL-6	Pró-tumoral	Estimula a produção de metaloproteinases da matriz; diferenciação de linfócitos B; induz transição epitélio-mesênquima.

Tabela 1: Citocinas inflamatórias, suas funções e seu papel no microambiente tumoral. Lippitz, 2013.

	Pulmão	Mama	Colorretal	Gástrico	Melanoma	Pâncreas	Glioma maligno	Hepático	Renal	Cabeça e pescoço
Fator inibitório de migracão de macrófagos expressa pelo tecido tumoral	+	+	+		+	+	+	+		+
IL-8 produzida pelas células tumorais	+	+	+	+	+	+	+		+	
Concentração sérica de IL-6 elevada	+	+	+	+	+	+		+	+	+
Expressão diminuída de IL-12			+	+	+		+	+	+	+
Produção diminuída de IFN-γ pelas células imunes	+		+	+	+		+		+	+
Expressão reduzida de HLA-DR	+		+		+	+	+			+
Concentração sérica de TGF-β elevada	+	+	+	+			+	+	+	
Expressão tumoral de quimiocina CXCR4	+	+	+	+	+	+	+	+	+	
Concentração sérica elevada de IL-10	+	+	+	+	+	+	+	+		

Tabela 2: Evidências publicadas da expressão de citocinas específicas em alguns tumores. Fonte: Adaptado de Lippitz (2013).

	Pulmão	Mama	Colorretal	Gástrico	Melanoma	Esôfago	Pâncreas	Hepático	Renal	Linfoma difuso de células B
Expressão do fator inibitório de migracão de macrófagos e efeito prognóstico negativol	+	+		+		+		+		
IL-8 é associada com tamanho do tumor, profundidade de invasão, ou estágio avançado	+	+	+	+		+		+		+
Concentração sérica de IL-6 e efeito prognóstico negativo	+	+	+	+	+	+	+		+	+
Concentração sérica de IL-18 associada com estágio avançado	+	+				+		+	+	
Concentração sérica de IL-18 elevada e prognóstico negativo	+			+			+	+		+
Expressão elevada de HLA-DR e prognóstico positivo	+	+	+		+		+	+		+
Expressão tumoral de quimiocina CXCR4 associada com metástase	+	+	+	+	+	+	+	+	+	
Concentração sérica de IL-10 associada com um efeito prognóstico negativo	+		+	+	+		+	+	+	+

Tabela 3: Evidências publicadas do efeito de citocinas específicas em vários tipos de tumor. Fonte: Adaptado de Lippitz (2013).

Efeito primário	Resposta adaptativa
Modulação das dinâmicas de adesão célula-matriz extracelular	Migração aumentada, alongamento celular, capacidade migratórias em diferentes substratos
Modulação das adesões célula-célula	Migração coletiva x migração individual
Aumento das dinâmicas de citoesqueleto, polaridade e contratilidade	Alongamento e contração celular, perda das junções célula-célula, migração individual no formato amebóide ou mesenquimal
Mudança na composição da matriz extracelular	Migração facilitada, estimulação parácrina de migração, direcionalidade

Tabela 4: Plasticidade induzida pelos fatores de crescimento e pelas citocinas na migração de células tumorais. Os fatores de crescimento e as citocinas têm efeitos primários sobre as células tumorais e estromais influenciand as adesões célula-matriz e célula-célula, dinâmica do citoesqueleto e composição e estrutura da matriz extracelular. Isto leva, dependendo do tipo de célula e do contexto local, a uma resposta plástica na migração de células tumorais, podendo ser de maneira coletiva, ou individual (formato mesenquimal ou ameboide). Fonte: Odenthal et al., 2016.

2. OBJETIVOS

2.1 Objetivos gerais

O objetivo desta tese foi avaliar o papel da inflamação no prognóstico e no comportamento migratório do carcinoma espinocelular de boca.

2.2 Objetivos específicos

2.2.1 Papel dos macrófagos no prognóstico de pacientes com carcinoma espinocelular de boca

Foi realizada uma revisão sistemática utilizando a base de dados Pubmed. Foram selecionados artigos que utilizaram apenas amostras de pacientes e avaliaram a presença de macrófagos nos tumores e sua relação com o prognóstico. Também observados os tipos de marcadores utilizados e as áreas do tumor avaliadas.

2.2.2) Papel de interleucinas inflamatórias no comportamento migratório de linhagens celulares de carcinoma espinocelular de boca

Foram realizados ensaios *in vitro* nas linhagens tumorais de carcinoma espinocelular de boca SCC25 e Cal27 em condições promotoras de migração (fibronectina para SCC25 e laminina para Cal27). Os diferentes ensaios foram realizados sob a influência das citocinas inflamatórias IL-6, TNF-α e IL-1β. Além disso, foi utilizado Stattic®, um inibidor de STAT3. Os parâmetros avaliados foram: responsividade das células às diferentes interleucinas, velocidade de migração, direcionalidade do movimento e ativação de Rac1.

A presente tese será apresentada sob a forma de artigos, sendo dois no total.

3. ARTIGO CIENTÍFICO 1

O artigo intitulado “Macrophages and prognosis of oral squamous cell carcinoma: a systematic review” foi formatado de acordo com as normas da Revista Oral Oncology, a qual tem fator de impacto 4,286 (2015).

Macrophages and prognosis of oral squamous cell carcinoma: a systematic review.

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ABSTRACT: The most prevalent cancer in the oral cavity is oral squamous cell carcinoma (OSCC). Like other tumors, macrophages are the most numerous cells in the stroma of the OSCC tumor microenvironment (TME) and can be polarized in two ways, depending on the stimulus of the TME: M1, which shows a pro-inflammatory and anti-tumoral activity, and M2, with a anti-inflammatory and pro-tumoral activity. Also, macrophages collaborate with some events in the TME, such as angiogenesis, extracellular matrix degradation, proliferation, escape from immune destruction, tissue invasion and metastasis. In the literature, several studies have shown that macrophages are important to the prognosis of patients in different types of cancer. Thus, the aim of the present study was to conduct a systematic review to evaluate the role of macrophages in the prognosis of OSCC patients. A search in the Pubmed database was performed and were included only studies that evaluate the importance of macrophages in the prognosis of OSCC patients. From initial 133 articles, 13 fully attended the inclusion criteria. The most articles evaluated only CD68, a panmacrophage marker, or CD163, M2 marker. Only one evaluated M1 marker, CD11c. Besides, 5 articles analyzed the presence of macrophages in different areas of the tumor. CD68 and CD163 higher concentrations were associated with worse survival. In conclusion, macrophages are important to OSCC patients' prognosis, however it is not possible to conclude which region is more important to the outcome.

KEYWORS: tumor associated macrophages; CD68; CD163; M1; M2; oral cancer; head and neck cancer; disease-free survival; overall survival; outcome.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common cancer in the oral cavity and represents more than 90% of all head and neck cancers [1]. Despite advances in the understanding of the biology of OSCC, the patient survival rate in general is around 50%, mainly due to the presence of regional lymph node metastasis [2]. Like other cancer types, OSCC have a complex tumor microenvironment (TME) with the presence of several stromal cells, which are attracted to this local and are related to an intense release of growth

factors, chemokines and interleukins that allow tumor progression, invasion and metastasis [3-5]. Some authors have reported that resistance and failure to antineoplastic treatment are often associated to the stromal elements of TME [4, 6].

TME is an emerging field in cancer therapy and it is defined as the complex environment that support cancer progression, proliferation of neoplastic cells and invasion of adjacent tissues. It is composed by cancer cells and stromal cells, such as fibroblasts, endothelial cells, pericytes, and immune cells [7, 8]. These cells produce a multitude of molecules with the potential to influence the tumorigenesis process. For instance, cytokines from tumor microenvironment recruit cells from normal adjacent tissues, which are reprogrammed to produce various growth factors and other cytokines, and then contributes to tumor cell growth, tissue invasion, metastasis and, consequently, poor prognosis [9]. Since these cells are linked to the majority of hallmarks of cancer, researchers have focused on new therapeutics modalities targeted to modulated the behavior of the cellular components of TME, Furthermore, stroma cells do not have mutations like tumor cells and the changes on cell behavior are modulated by several cytokines expressed in the TME [7, 10].

Among immune cells, tumor-associated macrophages (TAMs) are the most abundant and important stromal cells in the TME. TAMs are considered important players in tumor progression and are related to proliferation and survival of tumor cells, angiogenesis, invasion of surrounding tissues and metastasis [11, 12]. Furthermore, TAMs density are related to worse prognosis in several types of cancer [13]. According to stimuli in TME, TAMs can be polarized in two main groups: M1, which is considered pro-inflammatory and anti-tumoral, and M2, which is considered immunosuppressive and pro-tumoral [11, 12, 14].

M1 macrophage polarization occurs mainly in the presence of interferon gamma (IFN-γ) or via exposure to microorganisms or their products such as lipopolysaccharide (LPS) [11, 14, 15]. Then, M1 secretes several pro-inflammatory cytokines such as IL-1, IL-6, INF-γ, which are associated with activation of Th1 response and Th1 lymphocytes attraction. Moreover, M1 function as antigen presenting cells, which is considered to have a potential antitumor role [11, 16].

M2 macrophage polarization is stimulated in the presence of IL-4, IL-10, IL-13 or TGFβ [12, 14, 15, 17]. Some authors suggest that this type of polarization may be divided in three groups: M2a, M2b and M2c [18]. This classification is related to the immunosuppressive response and the attraction of regulatory T cells (Treg) and Th2 lymphocytes. M2 releases growth factors such as vascular endothelial growth factor (VEGF), growth factor platelet derived (PDGF), transforming growth factor (TGF-β), and fibroblast growth factor (FGF) that can promote angiogenesis in various tumors [16, 19] and release matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9 and MMP-12) and plasminogen activator urokinase-type (uPA) [20].

Herein, we conducted a systematic review to evaluate the influence of macrophages on the prognosis of patients with OSCC. We observed that higher concentrations of TAMs, mainly CD68+ and CD163+, are related to poor prognosis.

METHODS

Search strategy

The search was performed on PUBMED database with the following terms: “head and neck neoplasms” OR “mouth neoplasms” OR “oral squamous cell carcinoma” OR “head and neck squamous cell carcinoma" OR "head and neck cancer" OR "oral cancer" AND “survival” OR “mortality” OR “prognosis” OR "disease free survival" OR "survival analysis" AND “macrophage” (the last access was realized on October 2016). Furthermore, searches were done in the references of the selected papers. The articles were reviewed by two independent authors (AMA e LFD) and the studies that generated disagreement between reviewers were reevaluated to be reached on a consensus.

Inclusion and exclusion criteria

In this paper were included exclusively studies that evaluated the importance of macrophages to the prognosis of the patients with OSCC.

Patients in treatment, *in vitro* and animal models studies, and review articles were excluded.

Data extraction

The data were extracted from the included studies by two authors independently using a standardized instrument. The following data were collected: first author, year, sample size, markers, method, median follow-up, evaluated tumor zone, and main results.

RESULTS

The search in the PUBMED database resulted in 133 articles. Of these, 106 were removed in the title analysis phase: 96 were out of research scope, 5 were written in a language different than English and another 5 were reviews. Another 11 studies were declassified by the abstract evaluation: 5 were out of research scope, 5 analyzed other aspects of macrophages related to OSCC and 1 were review. After these two steps, remained 16 works in which the full text was analyzed. At this moment, we discarded 3 papers due to the analysis other aspects of macrophages related to OSCC. At the end, 13 studies were included in this review (Fig. 1). Table 1 summarizes the results of this systematic review.

The publication date of the articles ranged from 2004 to 2016. The median sample size was 92 patients (range from 17 to 240) and the median for patient follow-up was 39 months, varying from 18 to 61.5 months.

All studies applied immunohistochemistry staining for macrophage markers and the most used markers in the selected studies were CD68 (panmacrophage antigen, n=10) and CD163 (M2 macrophage antigen, n=8). Other 3 different cell markers were also used: MRC1 (M2 macrophage antigen, n=1), CD11b (M2 macrophage antigen, n=1) and CD11c (M1 macrophage antigen, n=1).

From the 13 selected articles, only 5 reported that evaluated different areas of the tumor - the main areas assessed were tumor stroma and tumor epithelium. Two articles related that the higher density of CD68+ or CD163+ or

MRC1 macrophages in the epithelial fraction/tumor nest were related to poor outcome, such as. Other two articles related that higher density of CD68+ or CD163+ macrophages in the tumor stroma were related to poor prognosis. And one study showed that the higher density of CD163 in the invasive front of the tumor was associated with worse prognosis. Even in relation to prognosis, all articles evaluated overall survival (OS) followed by disease-free survival (DFS), and one article also evaluated local failure-free survival (LFFS), distant metastasis-free survival (DMFS), and progression-free survival (PFS). Together, the results of these studies revealed that the increased concentration of TAMs CD68+/CD163+/MRC1+, depending on the tumor region, were related to worse prognosis. Only one paper showed no association between TAMs CD68+ and tumor outcome.

DISCUSSION

Tumor microenvironment is a complex system where tumor cells reprogram stromal cells for their own benefit. From the ten hallmarks of cancer described by Hanahan and Weinberg (2011) [10], these reprogramed cells contribute at least with seven: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, deregulating cellular energetics, resisting cell death, inducing angiogenesis and activating invasion and metastasis [7]. For instance, during oral squamous cell carcinoma development, the reprograming of stromal cells already starts in potentially malignant lesions where altered non-neoplastic cells induce angiogenesis and modifications in immune cells in the adjacent connective tissue [3, 34]. When these altered non-neoplastic cells become true malignant cells and invade adjacent connective tissue shortly after basal membrane rupture, there is a switch to an immunosuppressive TME, which allows tumor development [3, 5]. In this review, it was included only studies that evaluated the role of macrophages, the main player on TME, in the prognosis of OSCC. The results of the selected studies showed that a high concentration of macrophages was related to a worse prognosis. Thus, the development of new therapeutic modalities target to TME-related cells might improve the success rate for the treatment of the cancer [35].

TAMs may be polarized into two different phenotypes: classic M1, which exerts antitumoral role; and alternative M2 - which exerts protumoral role [11]. For M1 macrophages, the most used markers are CD11c, CD80 and HLA-DR, while for M2 detection it is used CD163, CD11b, CD206 and MRC1. Moreover, it can be used CD68, which is a pan macrophage marker [11, 13, 14, 23, 30]. However, some studies have related that CD68 is not specific for macrophages, since fibroblasts and tumor cells might be detected by this marker [21, 36]. From the articles selected for this study, only one used an appropriated marker for M1 [22] and some articles used only the pan macrophage marker CD68. Due to the variability of the data and the methodology of the studies, we did not perform a meta-analysis. The failure to use specific markers may lead to unspecific findings and does not identify the true role of macrophages in tumor progression and prognosis.

Several studies have shown that the TAMs are important for the prognosis of patients in different types of solid tumors [13]. In human gastric cancer, it was observed that the higher density of CD68+ macrophage was associated with worse overall survival [37]. In addition, CD68 expression was related to a higher expression of vimentin and low expression of e-cadherin, important markers of epithelial-mesenchymal transition, suggesting that macrophages can induce EMT [37]. Other study conducted in patients with pancreatic cancer, the presence of M2 macrophages was associated with worse prognosis [38]. The articles selected in this review, except for one [33], showed association among macrophages, CD68+, CD163+ or MRC1+, and worse prognosis. These findings indicate that the TAMs are important for the prognosis of patients with OSCC.

Although it may be concluded that macrophages - CD68 +, CD163 + or MRC1+ - are important for the outcome of OSCC patients, it has not been possible to conclude in which region of the tumor, whether epithelial part, stroma, or invasive front of the tumor, TAMs have more influence on the prognosis. The organization of TME might vary according to the tumor region due to anatomical and biological challenges to the recruitment of stromal cells [6, 17, 39]. In lung cancer, a meta-analysis showed that the infiltration of M2 into the tumor stroma was associated with poor prognosis, while the presence M1 TAMs into the tumor islands was related to good prognosis [40]. In breast

cancer samples, the infiltration of CD68+ macrophages in intratumoral compartment [41] or the presence of CD163+ macrophages in the tumor stroma [42] were related with poor prognosis. In the present review, the high density of macrophages CD68+, CD163 or MRC1+ in the epithelial part [21, 22] or the high density of macrophages CD68+ or CD163+ in tumor stroma 23, 26] were related with worse prognosis. In addition, one study [25] showed that high density of macrophages CD163+ in invasive front of the OSCC were related with worse prognosis. These data show the relevance of assessing not only the polarization of the macrophages, but the zone of the tumor where it is present.

One of the main causes of decreased survival in cancer patients is related to the development of metastasis. Besides the modulation of the tumor cell migration performance [43], components of TME might contribute to the preparation of the pre-metastatic niche (PMN) [8, 44]. It was demonstrated in cancer that TAMs contribute to the formation of PMN by releasing molecules that promote angiogenesis as well as the chemoattraction of tumor cells and naive macrophages to the local of metastasis [44]. In an *in vitro* study with triple negative breast cancer, it was demonstrated that the metastatic suppressors reduce the macrophage infiltration in the tumor, and consequently decreased metastasis [45]. For OSCC, patients with metastasis in lymph nodes exhibited high infiltration of M2 macrophages both in the tumor and in the lymph node [46]. Based on these results, is possible that TAMs participate not only in the invasive process of tumor cells, but also modify the PMN to receive the metastatic cells.

The present review showed that macrophages are important components of OSCC TME and M2 TAMs are related with worse OS and DFS. Nevertheless, it was not possible to identify in which tumor zone the presence of these cells are more relevant to prognosis, being necessary other studies that evaluate the characteristics of TAMs, whether M1 or M2, and in which region of the TME is more relevant to the outcome.

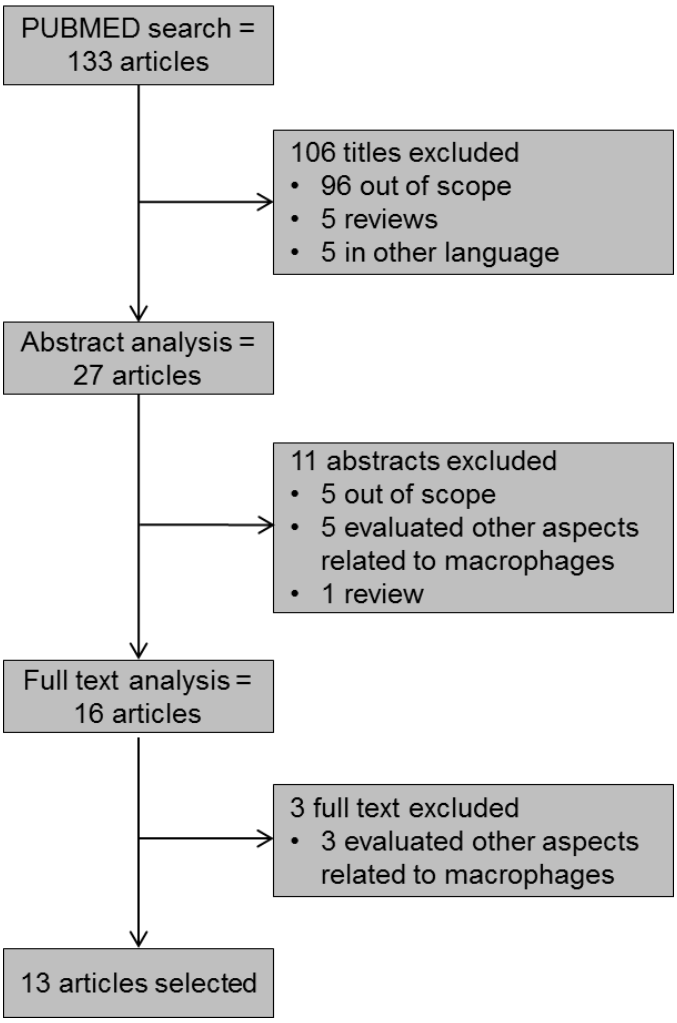

Figure 1: Flowchart showing the different steps for the selection of articles included in the review.

Table 1: Characteristics of the selected studies in the systematic review.

Authors/Year	Sample	Median follow-up	Markers	Methods	Evaluated tumor zone	Main results
Hu et al., 2016 [21]	127	39 months	CD68, CD163	IHC	Tumor nest and tumor stroma	Higher expression of CD68 and CD163 in tumoral nest were associated with worse OS.
Weber et al., 2016 [22]	17	Minimun of 36 months	CD68, CD11c, CD163 e MRC1	IHC	Epithelial and stroma fraction	Higher expression of MRC1 in epitelial fraction was associated with poor outcome*.
Ni et al., 2015 [23]	91	NR	CD68	IHC	Normal tissue, tumor nest and tumor stroma	Higher expression of CD68 in tumor stroma was associated with worse OS and worse DFS.
Matsuoka et al., 2015 [24]	60	NR	CD163	IHC	NR	Higher expression of CD163 was associated with worse OS and worse DFS.
Fujita et al., 2014 [25]	50	NR	CD163	IHC	Intratumoral zone and tumor invasive front	Higher expression of CD163 in the tumor invasive front was associated with worse OS and worse DFS.
Balermpas et al., 2014 [26]	106	40 months	CD68, CD163, CD11b	IHC	Tumor intraepithelial zone, tumor stroma and tumor periphery	Higher expression of CD68 and CD163 in tumor stroma were associated with worse OS, worse LFFS, worse DMFS and worse PFS.
He et al., 2014 [27]	43	24 months	CD68, CD163	IHC	NR	Higher expression of CD163 was associated with worse OS.
Wang et al., 2014 [28]	240	61.5 months	CD163	IHC	NR	Higher expression of CD163 was associated with worse OS.
Costa et al., 2013 [29]	45	Minimun of 18 months	CD68	IHC	NR	Higher expression of CD68 was associated with worse OS.
Fuji et al., 2012 [30]	108	NR	CD68, CD163	IHC	NR	Higher expression of CD163 was associated with worse OS.
Lu et al., 2010 [31]	92	NR	CD68	IHC	NR	Higher expression of CD68 was associated with worse OS and worse DFS.
Liu et al., 2008 [32]	112	NR	CD68	IHC	NR	Higher expression of CD68 was associated with worse OS.
Marcus et al., 2004 [33]	102	41.1 months	CD68	IHC	NR	Association was note found.

*poor outcome = ocurrence of local recurrence, a second oral tumor, lymph node metastasis or distant metastasis during the follow-up period.

OS = overall survival; DFS = disease free survival; LFFS = local failure-free survival; DMFS = distant metastasis-free survival; PFS = progression-free survival; IHC = immunohistochemistry; NR = not reported

CD68=panmacrophage marker; CD163, CD11b and MRC1=M2 macrophage; CD11c=M1 macrophage.

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4. ARTIGO CIENTÍFICO 2

O artigo intitulado “Interleukin-6 increases Rac1 activity and enhances the migratory behavior of oral squamous cell carcinoma tumor cells” foi formatado de acordo com as normas da Revista Oral Oncology, a qual tem fator de impacto 4,286 (2015).

Interleukin-6 increases Rac1 activity and enhances the migratory behavior of oral squamous cell carcinoma tumor cells

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ABSTRACT: During cancer progression, tumor cells develop different skills, as the ability to migrate in the host tissue, an important step for invasion and metastasis. This capability can be modulated for several factors present in the tumor microenvironment, such as interleukins (ILs). In the current literature there are few evidences about the role of ILs in the migratory behavior of oral squamous cell carcinoma (OSCC) tumor cells. The aim of this study was to evaluate the effect of different ILs in the migratory behavior of OSCC tumor cell lines. Cells (SCC25 and Cal27) were stimulated with IL-6 (10 or 100ng/ml), IL1B (1 or 10ng/ml) or TNF-α (5 or 25ng/ml) for 30min and we observed phosphorylation of STAT3 (IL6 stimuli) and NFkB (IL-1β or TNF-α stimuli), indicating responsiveness of OSCC tumor cells to cytokines. Then cells were plated on migration promoting condition in the presence of the selected ILs, imaged for 20h and migratory cells were tracked. Among the cytokines tested, only IL-6 increased migration speed and directionality for both cell lines, and the inhibition of STAT3 phosphorylation blocked this phenotype. Cell migration is a multistep process coordinated by RhoGTPases. We observed the IL6-mediated STAT3 phosphorylation correlated with and increase in the Rac1 RhoGTPase activation levels. Therefore, we concluded that OSCC are responsive to cytokine stimuli, and that IL-6 enhances migratory ability of OSCC tumor cells by a mechanism that involves STAT3 phosphorylation and Rac1 activation.

KEYWORDS: oral cancer; head and neck cancer; tumor cell migration; Rho GTPases; fibronectin; laminin; SCC25; Cal27, interleukins.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) corresponds to 95% of the malignant tumors of the oral cavity and, due to the late diagnosis and the metastatic behavior of the tumor, the 5 years patient survival rate is only 50% [1-3]. Like other cancers, the OSCC microenvironment is composed not only of tumor cells, but also of stromal cells - immune cells, fibroblasts, endothelial cells - which support tumor growth and is characterized by intense cellular communication through releasing of inflammatory cytokines, such as IL-6, TNF-α and IL-1β [4-6]. This complex network of interactions present in TME

represents a challenge for the treatment of OSCC patients and a better understanding of these relationships can lead to the development of more effective therapies [7].

Tumor development is an event that involves multiple steps in which tumor cells develop different skills such as the ability to invade surrounding tissue and generate metastases [8]. During carcinogenesis, a number of mutations occur that, in addition to uncontrolled cell proliferation, result in modifications in the cytoskeleton and cell shape, increasing cellular motility, a process called epithelial-mesenchymal transition (EMT) [9, 10]. EMT is also marked by the loss of epithelial markers and gain of mesenchymal markers, such as e-cadherin and n-cadherin, respectively [11]. Besides, EMT may be influenced by different inflammatory cytokines, which are released by other cellular components of the tumor microenvironment [9, 10]. Some studies have demonstrated that IL-6 is capable of inducing EMT in OSCC cells, generating more tissue invasion and metastasis [12, 13].

The tissue invasion and development of metastatic potential of malignant tumors are the major cause of clinical failure in terms of therapy and prognosis [11]. These processes also evolve the cell capability to migrate individually or collectively in the surrounding tissue [14]. For cell migration, the first step taken by the cell is to determine the direction of movement. After this, occurs the formation of lamelipodium in the leading edge of the cell and formation of new adhesions, which stabilize the lamelipodium. From this moment, tension fibers are formed along the cell body, causing the cell contraction in the direction of movement [15]. Cell migration is coordinated by a group of proteins called Rho GTPases, which are activated in a coordinated manner allowing the cell to move into the tissue. Rac1, an important Rho GTPase protein, is responsible for the formation of the lamellipodia in the leading edge of the cell and focal immature adhesions [15]. Several studies have related that Rac1 activation promotes invasion and metastasis in different types of tumor [16-21]. Some articles have demonstrated that TME components are capable to modulate these migratory activities [18, 22, 23].

In the present study, it was observed that IL-6 is the most important inflammatory cytokine to enhance the migration ability of OSCC cells,

independently of the degree of the EMT. Furthermore, it was shown that this process occurred by increasing the activation of Rac-1.

MATERIALS AND METHODS

Cell culture

OSCC cell lines used in this study were Cal27 ($ATCC_1$ CRL-2095^{TM}), cultivated in DMEM high glucose (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin (Gibco), and SCC25 ($ATCC_1$ CRL-1628^{TM}), cultivated in DMEM/F12 with 15mM HEPES and 0.5mM sodium pyruvate (Gibco) supplemented with FBS 10%, 1% streptomycin/penicillin (Gibco) and hydrocortisone (400ng/ml, Sigma), and cells were maintained in incubator (37°C, 5% CO_2). Both OSCC cell lines were obtained from the Tissue Culture Facility at School of Medicine of University of Virginia and checked for mycoplasma.

Western blotting

OSCC cells were cultured in plastic plates with regular medium overnight. Then, Cal27 and SSC25 were exposed to rhIL-6 (10 and 100ng/ml), rhTNF-α (25ng/ml) and rhIL-1β (10 ng/ml). All human recombinant proteins were purchased from PeproTech® (Rocky Hill, NJ, USA). After 30min of exposition, cells were washed with cold PBS (2x) and collected with scratches and RIPA buffer (25mMTris-HCL pH 7.6, 150mM NaCl, 1% NP- 40, 1% sodium deoxycholate, 0.1% SDS) with phosphatases (1:100, Sigma Aldrich) and proteases inhibitor (1:100, Sigma Aldrich). Cell lysates were centrifuged (12000 rpm, 4ºC, 20 minutes), the supernatants were collected, the protein contents were quantified (BCA method, Bio-Rad) and protein samples were incubated with Laemmli Buffer at 95ºC for 5min. Then, cell lysates were submitted to 4-20% SDS-PAGE gels (Biorad) and transferred to a PDVF membrane. Blocking of non-spsecific sites was performed during 45 min at room temperature with deffated milk or 5% BSA in PBS/0.5% Tween 20 during 45min. Then, membranes were incubated overnight at 4ºC with primary antibodies: anti-Stat3

(1:1000, Cell Signaling, Danvers, MA), anti-Stat3 pY705 (1:1000, Cell Signaling, Danvers, MA), anti-Nκb-65 (1:1000, Cell Signaling, Danvers, MA), anti-Nκb pS536 (1:1000, Cell Signaling, Danvers, MA), anti-e-cadherin (1:1000, Cell Signaling, Danvers, MA), and anti-n-cadherin (1:1000, Cell Signaling, Danvers, MA). After, membranes were incubated with peroxidase-conjugated secondary antibody (1:1000, Cell Signaling, Danvers, MA). Reaction was detected by chemioluminescence (Pierce, Thermo,Rockford, IL). Bands density was measured using ImageJ software (http://rsb.info.nih.gov/ij).

Microscopy and time-lapse videos

Imaging acquisition and analysis for migration assays were performed as previously described [24]. Cells were trypsinized (0,05% Tripsin), centrifuged (1000 rpm, 5 min) and suspended in CCM1 serum-free medium (HyClone, Thermo Fisher Scientific Inc.). Then, OSCC cells were plated in migration promoting conditions according to Ramos et al. (2016): SCC-25 were plated in fibronectin (2µg/ml) and CAL-27 in poly-l-lysine (1mg/ml) + laminin (2µg/ml). After 1 hour of plating, cells were incubated with rhIL-6 (10 and 100ng/ml), rhTNF-α (25ng/ml) or rhIL-1β (1 and 10 ng/ml) in the presence/absence of Stattic® (Abcam, Cambridge, MA), a STAT3 inhibitor. Then, cells were transferred to a Nikon TE300 microscope (10x 0.25 NA CFI Achro DL106 Nikon objective), equipped with a temperature controller (37ºC) and a charge coupled device camera (Orca II, Hamamatsu Photonics) and operated with Metamorph software (Molecular Devices). Images were obtained at 10 min intervals for 20 hours under low light conditions. For analysis of speed migration and directionality, the nucleus of each migratory cell was marked on “manual tracking” ImageJ software plugin. Migration speed was calculated by the ratio between the total distance traveled (distance) and the number of images (time) that cell migrated. For directionality, it was obtained the X and Y coordinates of the migratory cell in each image and normalized to start at a virtual X=0 and Y=0 position. A polar plot graph was made to demonstrate the directionality of the cells in each group [24].

Rac1 pull down assay

For analysis of Rac1 activation, pull down assays were performed as described previously [25]. SSC25 cells were suspended in CCM1 media plated in plastic dishes covered with fibronectin (2µg/ml) for 1h. Then, cells were washed with PBS (2x) and incubated in the presence/absence of IL-6 (100ng/ml) and/or Stattic (0.1 µM or 0.5µM). After 1h incubation, cells were washed (3x) with cold PBS (4º), harvested, lysed with CRIBs buffer containing 1% NP-40, 50 mM Tris pH 7.4, 10% glycerol, 100 mM NaCl, 2 mM $MgCl_2$ with protease and phosphatase inhibitors. Cell lysates were centrifuged (12000 rpm, 4ºC, 20min) and the supernatant was collected. A small fraction (40ul) was separated for determination of total Rac1 levels, while for activated Rac1 levels a large fraction (300ul) was incubated (1h, 4ºC) in the presence of GST-PAK-CRIB beads. Then, both samples were prepared for SDS-PAGE and submitted to immunoblotting for Rac1. Bands density was measured using ImageJ software (http://rsb.info.nih.gov/ij).

Statistical analysis

Statistical analysis was realized using Statistical Package for the Social Sciences 21 (SPSS Inc, Chicago, IL, EUA). Tests performed were Student-t test or One-way analysis of variance (ANOVA) followed by Tukey's post-test. Statistically significant differences were considered only when $p < 0.05$

RESULTS

OSCC tumor cells are responsive to different inflammatory cytokines

To verify if OSCC tumor cells are responsive to inflammatory cytokines, we used two OSCC cell lines with different levels of EMT markers (Figure1): Cal27 (high differentiated) and SCC25 (low differentiated). Then, both cell lines were incubated with interleukins (IL6, IL1b and TNF-a) for 30 min and we measured the phosphorylation levels of main markers of IL6 (STAT3-Y705) or IL-1β and TNF-α (Nκb65-S536) signaling pathway. It was observed that both,

highly (Cal27) and low (SCC25) differentiated OSCC cell lines were responsive for the cytokines tested (Figure 2A and Supplementary figure 1A) and it was not detected a crosstalk between both signaling pathways. This data indicated that cytokines present in the tumor microenvironment might affect the behavior of OSCC cells.

IL-6 alters the speed and the directionality of migration of OSCC cell lines

A possible effect of inflammatory cytokines on OSCC is an increase on migration properties [26]. To test this hypothesis, cells were plated on fibronectin (SCC25) or laminin (CAL27) and imaged for 24h in the presence/absence of IL-6, IL-1β or TNF-α. Migratory cells were tracked and we analyzed migration speed and directionality. The treatment with IL-1β showed no effect on migration speed for both cell lines, while TNF-α (25ng/ml) induced a slight increase on speed migration for Cal27 (21,16%), but severely impaired migration directionality for both cell lines (Figure 2 and Supplementary Figure 1). However, the treatment with IL-6 (100ng/ml) increased migration speed of both SCC25 (71,58%, $p<0.001$; Fig. 2B) and CAL27 (13,42%, $p<0.05$; Supplementary Figure 1B) when compared to control, while the lower IL-6 dose (10ng/ml) affected only the low differentiated OSCC cell line (SCC25, $p<0.05$; Fig. 2B). This IL6-mediated increase in migration speed was also accompanied by an improvement on migration directionality (Fig. 2C and Supplementary figure 1C). These results indicate that IL6 enhances the migratory behavior of OSCC cells, with more evident effects in low differentiated cell lines, suggesting a potential role for this interleukin during the invasion and metastasis process.

STAT3 inhibition avoids the IL-6 related effects on OSCC cell migration

The IL6 signaling pathway involves the phosphorylation of STAT3 protein [27]. We used Stattic®, a STAT3 inhibitor [28], in order to analyze if the IL-6 mediated effects on OSCC cell migration to confirm the involvement of the STAT3 signaling pathway. It was observed that a 1h incubation with Stattic® (0.5 µM) prior to IL6 (100ng/ml) stimuli, avoided STAT3-Y705 phosphorylation in both cell lines (Figure 3A and Supplementary figure 2A). Then, we performed

time-lapse analysis of OSCC cells stimulated with IL6 (100ng/ml) in the presence/absence of Stattic® (0.1 or 0.5 µM). It was observed that inhibition of STAT3 phosphorylation (0.5 µM) blocked the IL6-mediated increase on migration properties, for both OSCC cell lines (Figure 3C and Supplementary figure 2B). Thus, these results confirm that changes in the migratory behavior of OSCC cells induced by IL6 occur by the activation of STAT3 signaling pathway.

IL6 pathway increases Rac1 activation levels

A possible mechanism of action of the IL6-STAT3 pathway is the interaction with cell migration signaling pathways, such as the RhoGTPase Rac1. We plated the SCC25 cell line in migrating promoting conditions, stimulated with IL6 (100ng/ml) and/or Stattic® (0.1 and 0.5 µM) for 1h and quantified the activated Rac1 levels by pull down assay. It was observed that IL6 (100ng/ml) induced a 24% activation of Rac1, while the blockage of STAT3 phosphorylation by Stattic® restored Rac1 activation levels similar to the control (Figure 4). This result indicates that the improvement in the migratory behavior of OSCC cells by IL6 involves the phosphorylation of STAT3 and activation of the RhoGTPase Rac1.

DISCUSSION

Several studies have shown that inflammatory cytokines are important for different stages of tumor development, such as proliferation, escape from immune system, extracellular matrix degradation and preparation of metastatic niche [29, 30]. In this context, some interleukins, mainly IL-6, have been shown to induce EMT in tumor cells of different origins, consequently leading to an increase in tissue invasion, migration and metastasis [27, 31]. Regarding migration, some articles have demonstrated that Rac1, an important Rho GTPase implicated in lamellipodia formation and cell migration, is overexpressed in tumor cells and associated with invasiveness and metastasis [32]. In the present study, it was shown that IL-6 enhanced speed migration and directionality of OSCC cells, regardless of the degree of cell differentiation.

Furthermore, it has also been shown that IL-6 increased the activation of rac1 showing a crosstalk among these signaling pathways.

Epithelial-mesenchimal transition (EMT) is a dynamic event where tumor cells transit between a state similar to their original epithelium and a state similar to mesenchymal cells [10, 33]. Some authors have suggested that the term “transition” of EMT should be changed by “transformation”, because it would better reflect the plasticity spectrum of cells during this process [10]. In order to analyze the degree of EMT, or the degree of cell differentiation, it is consensus in the literature the use of e-cadherin and n-cadherin markers, where high e-cadherin / low n-cadherin is related to an epithelial-like state, Low e-cadherin / high n-cadherin a mesenchymal-like state [10]. The present study showed that, independently of the degree of EMT, IL-6 increased speed migration and directionality of OSCC cells.

In the OSCC microenvironment, tumor cells and stromal cells release innumerable inflammatory cytokines, such IL-6, IL-1β or TNF-α, that maintain an immunosuppressed environment and promote different tumor capabilities [4, 34]. Some studies have revealed that IL-6 induced angiogenesis and lymphangiogenesis, EMT and chemotherapeutic resistance [13, 35, 36], while IL-1β promoted *in vitro* malignant transformation of dysplastic oral keratinocytes and estimulated OSCC cells to secrete IL-6, IL-8 and chemokine CXCL1 [37, 38], and TNF-α improved *in vitro* fusion between endothelial cells and OSCC cells, probably enhancing the metastatic potential of these tumors [39]. TNF-α also is related with angiogenesis, growth and cancer stem-cell phenotype [40, 41]. The results of this study demonstrated that both Cal27 and SCC25 are responsive to these inflammatory cytokines and our hypothesis was that they can modulate the migratory behavior of these OSCC cell lines. However, only IL-6 showed this capability. Probably, IL-1β and TNF-α modulate other activities than migration.

IL-6 is an important interleukin of chronic inflammation that binds to IL-6R and result in the activation of the transcription factor STAT3 [31, 42, 43]. Several researches have demonstrated that IL-6 and activation of STAT3 promote tumor cell proliferation, apoptosis resistance, metastasis, angiogenesis, immune suppression, chemotherapeutic resistance and poor prognosis [27, 31, 42, 43]. Yadav et al. (2016) [44] demonstrated that the

blockade of IL-6 signaling by bazedoxifene reduced chemo and radioresistance, cell proliferation and migration of OSCC cells. Data from the present study, which showed that IL-6 increased the migratory capacity of OSCC cells, are similar to results found in the literature [12, 13]. Wu et al. (2016) [12] showed that IL-6 reduced e-cadherin expression and increased n-cadherin expression in low metastatic cells inducing EMT. Furthermore, it was observed that IL-6 induced migration of low metastatic cells. Similar, Yadav et al. (2011) [13] demonstrated that Cal27 and immortalized oral epithelial cells under influence of IL-6 increased migration and EMT markers expression. Our results with data from the literature prove that IL-6 improve migration of OSCC cells, even in those that express high levels of e-cadherin and are more differentiated.

Rac1 is a Rho GTPase that belong to the Ras superfamily. Together with Cdc42 and RhoA, Rac1 participates in the events of cell migration being responsible for the lamellipodia formation in the leading edge [15]. Several study have demonstrated that Rac1 is overexpressed in different types of tumor, including OSCC [32]. Moreover, this overexpression of Rac1 is related with invasiveness, metastasis and poor prognosis [32]. In the present study, IL-6 induced higher activation of Rac1 and migratory capacity of OSCC cells. However, when it was used Stattic®, a STAT3 inhibitor, even in the presence of IL-6, Rac1 activation and migratory skills of OSCC cells was reduced, suggesting a crosstalk between Rac1 and IL-6/STAT3 signaling pathways. These results are in accordance with other results of the literature. Teng et al. (2009) [22] showed that fibroblasts without STAT3 expression had a reduction in directionality and a random migration. Furthermore, they demonstrated that STAT3 binds to βPIX, a Rac1 activator, and regulates in this way Rac1 activation [22]. In hepatocellular carcinoma cells it was shown that STAT3 phosphorylation indirectly induces Rac1 activation, enhancing DOCK8 expression, a GEF for Rac1 [23].

In conclusion, IL-6 increased speed migration and directionality of both OSCC cell lines, independent of the degree of EMT of tumor cells. In addition, it was shown that this improvement in migratory behavior was induced by the greater activation of Rac1, suggesting a crosstalk between STAT3 and Rac1 signaling pathways.

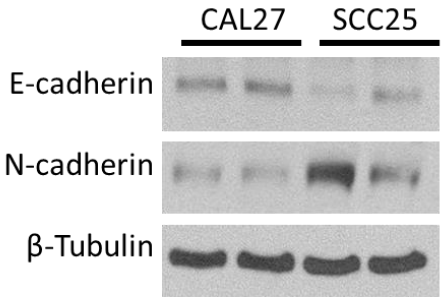


Figure 1: Western blotting analysis of e-cadherin and n-cadherin in the CAL27 and SCC25 cell lines. It was observed that while the SCC25 showed high expression of N-cadherin and low expression of E-cadherin, the CAL27 had high levels of E-cadherin and low levels of N-cadherin.

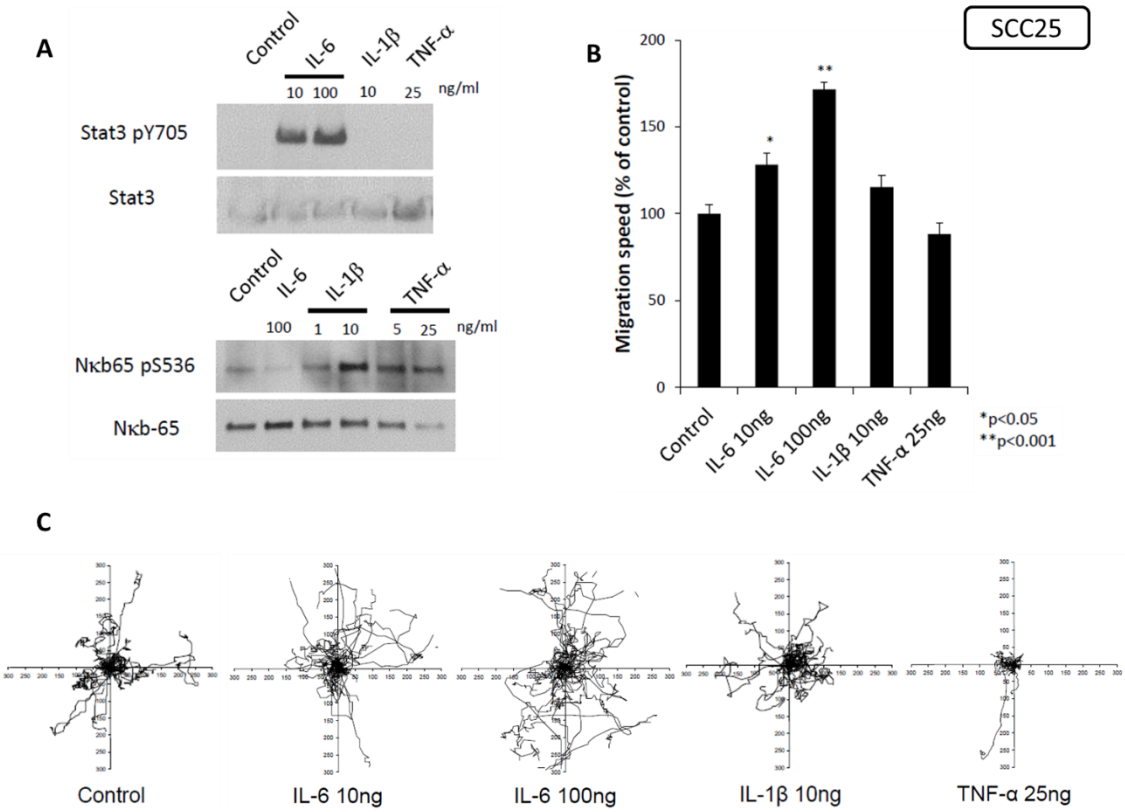
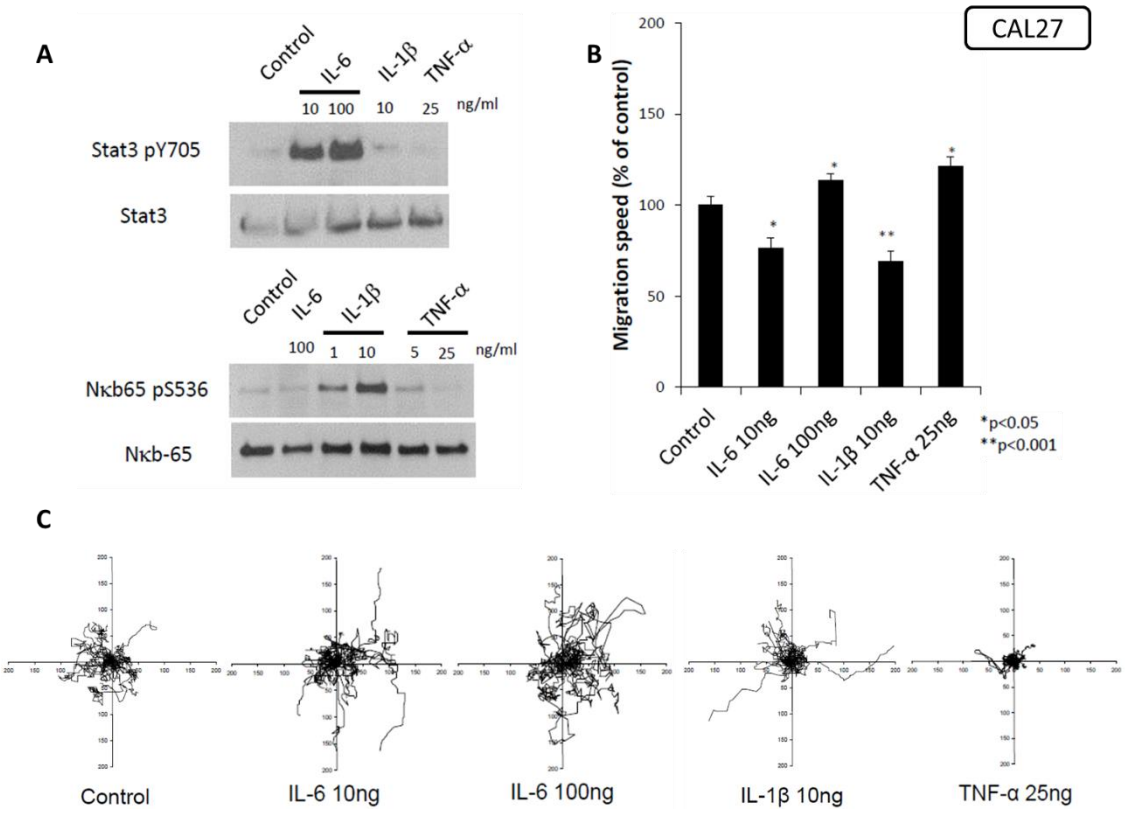


Figure 2: A) Western blotting analysis of STAT3 pY705 and Nκb65 pS536 in the SCC25 cell line. It was observed that there were phosphorylation of Stat3 and Nκb65, showing that the signaling pathway was activated. B) Analysis of migration speed of the SCC25 cell line in the presence of inflammatory cytokines. Data were expressed in percentage of control. It was observed that treatment with IL-6 10ng and 100ng increased the migration speed of tumor cells compared to control. C) Polar plot graphs of the SCC25 in the presence of inflammatory cytokines. Each line represents an only migratory cell. It was noted that the treatment with IL-6, regardless of the dose used, the directionality was increased.



Supplementary figure 1: A) Western blotting analysis of STAT3 pY705 and Nκb65 pS536 in the Cal27 cell line. It was observed that there were phosphorylation of Stat3 and Nκb65, showing that the signaling pathway was activated. B) Analysis of migration speed of the Cal27 cell line in the presence of inflammatory cytokines. Data were expressed in percentage of control. It was observed that treatment with IL-6 100ng and TNF-α25ng increased the migration speed of tumor cells compared to control. C) Polar plot graphs of the Cal27 in the presence of inflammatory cytokines. Each line represents an only migratory cell. It was noted that the treatment with IL-6, regardless of the dose used, the directionality was increased.

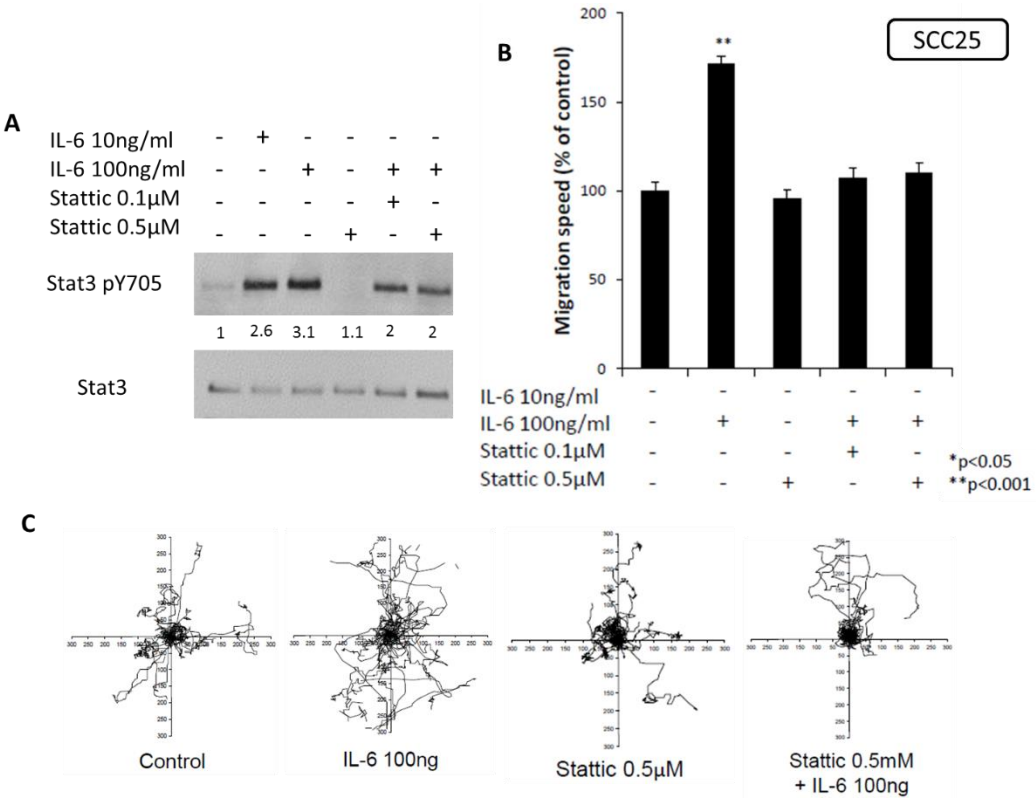
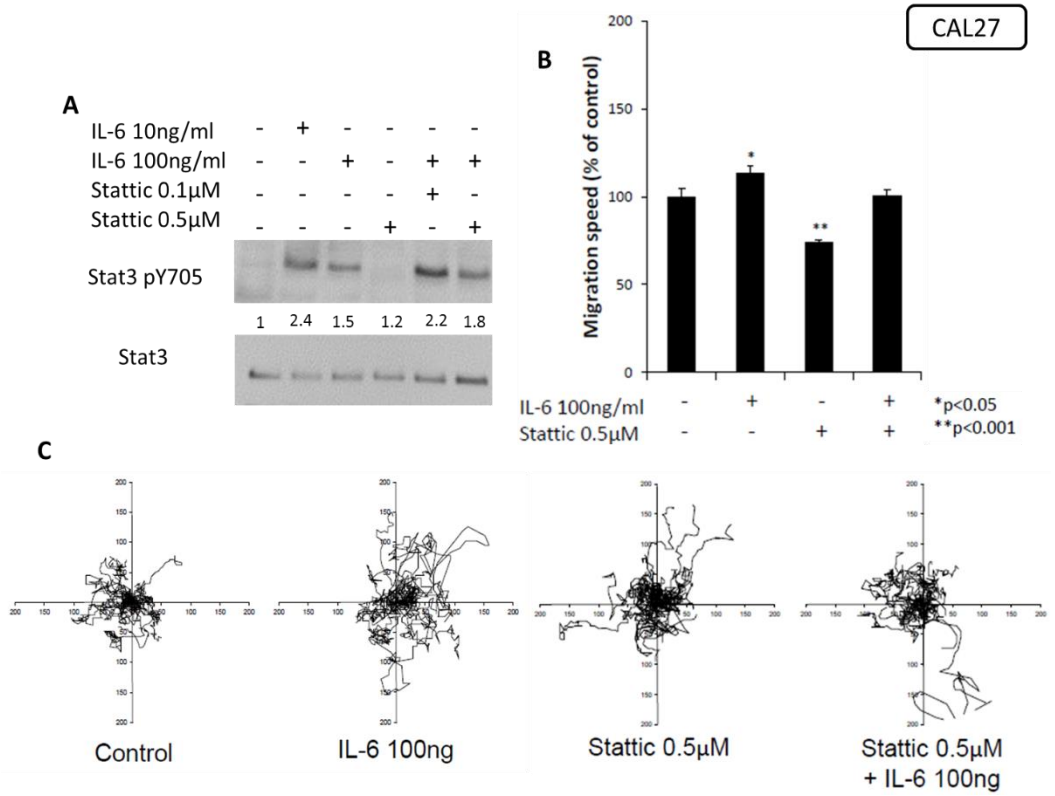


Figure 3: In the presence/absence of IL-6 and Stattic, a STAT3 inhibitor, it were performed the following analysis in the SCC25 cell line: A) Western blotting analysis of STAT3 pY705. It was observed that Stattic was capable to inhibit STAT3 phosphotilation. B) Analysis of migration speed. Data were expressed in percentage of control. It was observed that the use of a STAT3 inhibitor reduced the migration speed to levels similar to the control. C) Polar plot graphs. Each line represents only one migratory cell. The use of Stattic reduced the directionality of tumor cells, even in the presence of IL-6.



Supplementary figure 2: In the presence/absence of IL-6 and Stattic, a STAT3 inhibitor, it were performed the following analysis in the Cal27 cell line: A) Western blotting analysis of STAT3 pY705. It was observed that Stattic was capable to inhibit STAT3 phosphotilation. B) Analysis of migration speed. Data were expressed in percentage of control. It was observed that the use of a STAT3 inhibitor reduced the migration speed to levels similar to the control. C) Polar plot graphs. Each line represents only one migratory cell. The use of Stattic reduced the directionality of tumor cells, even in the presence of IL-6.

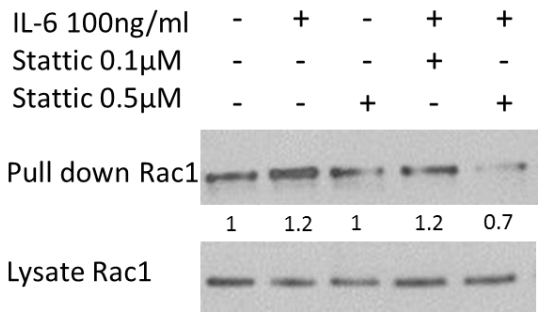


Figure 4: Pull down assay of Rac1. The inhibition of STAT3 phosphorylation led to a decrease in the levels of phosphorylated Rac1.

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5. CONSIDERAÇÕES FINAIS

O microambiente tumoral do CEB é extremamente complexo, devido à variedade de células estromais presentes e as citocinas inflamatórias liberadas tanto por este grupo de células quanto pelas células tumorais. Somado à estes componentes, ainda temos a matriz extracelular que também vai sendo modificada conforme o tumor vai evoluindo. Entendendo melhor como estes elementos se relacionam, provavelmente conseguiremos desenvolver terapias mais eficazes contra o CEB, aumentando a taxa de sobrevida, diminuindo morbidade e melhorando a qualidade de vida dos pacientes.

A presente tese mostrou que:

- Os macrófagos são importantes para o prognóstico do CEB, sendo que a alta densidade de macrófagos CD68+ e/ou CD163+ foram relacionados com prognósticos piores.
- Outros estudos são necessários utilizando mais marcadores de macrófagos e avaliando em quais zonas do tumor a presença dessas células é mais relevante para o prognóstico.
- Foi demonstrado que as citocinas inflamatórias, principalmente IL-6, são capazes de modular o comportamento migratório de linhagens celulares de CEB.
- A melhor capacidade migratória proporcionada por IL-6 se deu pela maior ativação de Rac1, importante Rho GTPase envolvida na migração celular. Isso mostra que há um *crosstalk* entre as vias de sinalização de IL-6 – STAT3 e Rac1.

Sendo assim, estes resultados mostram que os componentes inflamatórios presentes no microambiente tumoral de CEB são importantes para o seu desenvolvimento, apontando potenciais alvos terapêuticos.

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