



UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara



Laura Andrea González Maldonado

**TGF-beta derivado de células neoplásicas como mediador da modulação do
fenótipo de macrófagos e da invasão em câncer de cabeça e pescoço (HNC):
intersecção com expressão e atividade de GALR2 em células neoplásicas**

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Orientador: Carlos Rossa Junior

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González Maldonado LA. TGF-beta derivado de células neoplásicas como mediador da modulação do fenótipo de macrófagos e da invasão em câncer de cabeça e pescoço (HNC): intersecção com expressão e atividade de GALR2 em células neoplásicas [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

RESUMO

Macrófagos são as células imunes mais abundantes em carcinomas espinocelulares de cavidade bucal, podendo representar cerca de 50% da massa do tumor. A quantidade de macrófagos infiltrando a lesão tumoral é inversamente relacionada ao prognóstico e sobrevida dos pacientes. Os macrófagos presentes no microambiente tumoral (TME) são denominados macrófagos associados ao tumor (TAM) e tem seu fenótipo influenciado por moléculas presentes no TME, incluindo os produtos secretados pelas células neoplásicas. No processo de vigilância imune, os macrófagos têm a função de detectar células tumorais e ativar a imunidade adaptativa para eliminação destas, além de participar no processo de reparo tecidual / restabelecimento da homeostasia. O Fator de Transformação de Crescimento (TGF)- β e o Fator Estimulador da Formação de Colônia (CSF)-1 afetam diretamente o fenótipo de macrófagos, e sua expressão em tumores epiteliais está associada à maior agressividade / progressão. Estas proteínas favorecem a polarização de macrófagos para um fenótipo semelhante ao M2, que é associado à tumores mais agressivos. O receptor 2 de galanina (GALR2) é considerado um oncogene em HNC, favorecendo a proliferação e sobrevivência celular e também a invasão; no entanto as influências da expressão de GALR2 pelas células tumorais na modulação macrófagos e na expressão de TGF- β e CSF-1 são desconhecidas. O objetivo geral do projeto foi determinar o efeito do TGF- β secretado pelas células tumorais na biologia de macrófagos, assim como determinar o efeito da expressão/atividade de GALR2 por células tumorais no fenótipo de macrófagos e sua possível interação com a expressão de TGF- β e CSF-1 pelas células tumorais. O desenvolvimento do projeto foi baseado em três objetivos específicos e os resultados foram divididos em 3 artigos. O primeiro objetivo foi determinar a influência dos produtos secretados pelas células de linhagens neoplásicas com alta (H314, carcinoma de células escamosas de assoalho de boca) e baixa (SCC-9, carcinoma de células escamosas de língua) expressão constitutiva de TGF- β no fenótipo de macrófagos; assim como na migração, quimiotaxia e invasão de células tumorais em co-cultura com macrófagos *in vitro*. O fenótipo de macrófagos foi determinado pelo RT-qPCR e análise multiplex e indicou um perfil pró-tumoral/reparo (M2) de macrófagos estimulados com o secretoma de H314 e um perfil com tendência ao perfil pró-tumoral/inflamatório (M1) em macrófagos estimulados com o secretoma das células SCC-9. Produtos secretados por macrófagos aumentaram a quimiotaxia de ambas as linhagens neoplásicas, porém apenas o secretoma de H314 foi quimiotático para macrófagos. Macrófagos condicionados com o secretoma das células tumorais favoreceram a invasão da linhagem menos invasiva (H314) e inibirão a invasão da linhagem mais invasiva (SCC9). O segundo objetivo foi avaliar a influência específica do TGF- β secretado pelas linhagens SCC-9 e H314 no fenótipo de macrófagos pelo RT-qPCR e ELISA, assim como na quimiotaxia e invasão (*in vitro* e *in vivo*, em modelo de membrana corio-alantóica do ovo de galinha) das células neoplásicas em co-cultura com macrófagos. A inibição do efeito do TGF-

β como componente do secretoma das linhagens neoplásicas foi realizada com o tratamento dos macrófagos com inibidor bioquímico da quinase associada ao receptor de TGF- β tipo I (TGF-bRIk) previamente ao estímulo com o secretoma das linhagens neoplásicas. O bloqueio da atividade biológica de TGF- β atenuou a expressão de IL-10 (marcador M2) avaliada por RT-qPCR e ELISA. A inibição de TGF- β em macrófagos também reduziu a invasão de células H314 in vivo e in vitro e reduziu a quimiotaxia de macrófagos para esta linhagem. Por outro lado, a inibição de TGF-bRIk aumentou a quimiotaxia recíproca entre macrófagos e a linhagem SCC-9. O terceiro objetivo foi determinar a influência da expressão/atividade de GALR2 nas linhagens neoplásicas sobre o fenótipo de macrófagos e a possível interação de GALR2 com a expressão de TGF- β e CSF-1 como mediadores importantes para macrófagos. Utilizamos quatro linhagens celulares transgênicas derivadas das mesmas linhagens neoplásicas parentais (SCC-9 e H314), incluindo duas linhagens com alta expressão de GALR2 e as respectivas linhagens-controle (transfectadas com o mesmo vetor plasmidial de expressão - pcDNA3.1 - 'vazio'). A expressão constitutiva de TGF- β e CSF-1 e a meia-vida dos mRNAs foram determinados por ELISA e RT-qPCR, respectivamente. O perfil fenotípico de macrófagos foi determinado usando RT-qPCR e análise multiplex; e a invasão de células tumorais em co-cultura com macrófagos foi avaliada in vitro. A superexpressão de GALR2 reduziu a produção de TGF- β e CSF-1, bem como a meia-vida do RNAm tanto na linhagem H314 quanto SCC-9. A maior expressão de GALR2 nas células neoplásicas influenciou o perfil fenotípico dos macrófagos regulando a expressão de genes-alvo de forma dependente da linhagem celular neoplásica. Em geral, a maior expressão de GALR2 nas linhagens neoplásicas H314 e SCC-9 tendeu a acentuar o perfil pró-tumoral dos macrófagos. A invasão de H314 com alta expressão de GALR2 na presença de macrófagos foi discretamente acentuada, enquanto a expressão de GALR2 reduziu a invasão da linhagem SCC-9 na presença e ausência de macrófagos. Como conclusão geral do trabalho, os dados suportam que o secretoma de células de carcinoma oral de células escamosas (OSCC) influencia o fenótipo de macrófagos. A modulação do fenótipo de macrófagos pode afetar positiva ou negativamente diversos processos da biologia tumoral, como quimiotaxia e invasão, de maneira dependente da linhagem celular neoplásica específica. A inibição de TGF- β como um componente do secretoma das linhagens H314 e SCC-9 tem efeito atenuador no fenótipo M2 de macrófagos e pode favorecer ou inibir a quimiotaxia e invasão de diferentes linhagens celulares tumorais. A expressão de GALR2 em células OSCC influencia a expressão gênica e a produção de proteínas pelos macrófagos de uma maneira específica da linhagem celular. O aumento de GALR2 diminui a invasão de células tumorais.

Palavras chave: Neoplasias bucais. Carcinoma de Células Escamosas. Macrófagos.

González Maldonado LA. Tumor-cell derived TGF-beta as a mediator macrophage phenotype and Head and Neck Cancer (HNC) tumor cell invasion: intersection with increased expression and activity of GALR2 in neoplastic cells [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

ABSTRACT

Macrophages are the most abundant immune cell type in the tumor microenvironment (TME) of head and neck squamous cell carcinomas (HNSCC), representing up to 50% of the tumor mass. The intensity of macrophage infiltration is inversely proportional to prognosis and disease survival. In the TME, macrophages are called tumor-associated macrophages (TAM) and their phenotype is determined by molecules in this microenvironment, including neoplastic cell-secreted products. As innate immune antigen-presenting cells, macrophages have an important role in immune surveillance, detecting neoplastic cells and activating the adaptive immune response for their elimination, besides participating in the repair process after treatment. Transforming growth factor (TGF)-beta has marked effects on both macrophage and neoplastic cell phenotype and it can be produced by cancer cells. Increased expression of TGF- β is associated with worse prognosis of epithelial cancers and drive an M2-like phenotype in macrophages, which is associated with more aggressive tumors. Galanin receptor 2 (GALR2) is an oncogene in HNC, as it is associated with increased cancer cell proliferation, survival and invasion; however, the influences of increased GALR2 expression by cancer cells on macrophages and on the expression of TGF- β are unknown. The main objective of the project was to determine the relative influence of TGF- β as a component of neoplastic cell secretome on macrophage biology. As a secondary objective, the possible intersection between expression and activity of GALR2 in tumor cells and the influence on macrophage phenotype through regulation of TGF- β expression and production. This proposal was structured in three specific objectives, and the data organized, presented and arranged in three manuscripts. The first specific objective was to determine the influence of the secretome from neoplastic cell lines with low (SCC-9, squamous cell carcinoma, tongue) and high (H314, squamous cell carcinoma, floor of mouth) constitutive production of TGF- β on macrophage phenotype and macrophage-modulated tumor cell migration, chemotaxis and invasion. Macrophage phenotype was determined by RT-qPCR and multiplex analysis. Secretome of H314 cells enhanced the pro-tumoral profile (M2-like) of macrophages, whereas the secretome of SCC-9 cells skewed macrophages to a pro-inflammatory (M1-like) phenotype. Macrophage-secreted products were chemotactic for both neoplastic cell lines, but only H314 secretome was chemotactic for macrophages. Invasion of the less invasive H314 cells was increased in the presence of macrophages, whereas inhibited invasion of SCC9 cells. The second specific objective was to determine the specific relative influence TGF- β secreted by SCC-9 and H314 cell lines on macrophage phenotype. To block the biological influence of TGF- β as a component of the secretome of neoplastic cell lines, macrophages were treated with a TGF- β Type I Receptor Kinase (TGF- β RIK) biochemical inhibitor before stimulation with the secretome (conditioned medium - CM) from neoplastic cell lines. Macrophage phenotype was determined by RT-qPCR and ELISA. Influence of tumor cell-derived TGF- β on macrophage-modulated chemotaxis and invasion (in vitro and in vivo) was investigated. Blocking the influence of neoplastic cell-derived TGF- β in macrophages reduced IL-10 (M2 marker) by RT-qPCR and ELISA. In addition,

inhibition of TGF- β in macrophages attenuates the invasion capacity of H314 cells in vivo and in vitro and also reduced macrophage chemotaxis to the secretome of H314 cells. In contrast, blocking the effects of TGF- β enhanced reciprocal chemotaxis between macrophages and SCC-9 cells. The third objective was to determine the influence of GALR2 expression in neoplastic cells on macrophage phenotype and the possible crosstalk with expression of TGF- β and CSF-1, as important macrophage-related mediators, by neoplastic cells. We used four transgenic cell lines derived from the parental neoplastic lines (SCC-9 and H314), including two cell lines with high expression of GALR2 and their respective control cell lines (transfected with the same 'empty' plasmid expression vector - pcDNA3.1). Influence of GALR2 expression on constitutive production of TGF- β and CSF-1 and on the half-life of their mRNAs were determined by ELISA and RT-qPCR, respectively. Macrophage phenotype was determined using RT-qPCR and multiplex assay; and the invasion of tumor cells in co-culture with macrophages was determined in vitro and in vivo. Increased expression of GALR2 by neoplastic cells tended to enhance the pro-tumoral phenotype of macrophages. In H314 cells, increased GALR2 discretely increased invasion in the presence of macrophages, whereas in SCC9 cells increased GALR2 reduced invasion both in the presence and absence of macrophages. In conclusion, the secretome of oral squamous cell carcinoma (OSCC) cells influence macrophage phenotype. Changes in macrophage phenotype can either increase or decrease chemotaxis and invasion as outcomes of tumor biology, depending on the specific neoplastic cell line and the modulation on macrophage phenotype can affect several processes of tumor biology, such as chemotaxis and invasion in a neoplastic cell lineage-dependent manner. Inhibition of the biological effect of TGF- β as a component of the secretome of the H314 and SCC-9 cells attenuates the M2 / pro-tumoral phenotype of macrophages. GALR2 expression in OSCC cells influences gene expression and protein production by macrophages in a neoplastic cell lineage-specific manner. The increase in GALR2 decreases invasion of neoplastic cells.

Keywords: Mouth Neoplasms. Carcinoma, Squamous Cell. Macrophages.

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1 INTRODUÇÃO

Câncer de cabeça e pescoço (HNC) é a 6ª neoplasia mais prevalente no mundo¹, e o carcinoma espinocelular ou de células escamosas é o tipo mais prevalente, representando cerca de 90% dos casos de HNC^{2,3}. Carcinoma espinocelular de cabeça e pescoço (HNSCC, *head and neck squamous cell carcinoma*) é uma neoplasia agressiva, caracterizada por invasão loco-regional e disseminação metastática (especialmente para o sistema linfático) e elevadas taxas de recidiva pós-tratamento⁴. Atualmente, mesmo com a implementação dos tratamentos padrão recomendados, a expectativa de sobrevivência em 5 anos para casos avançados de HNSCC é de cerca de 50%, a qual é inferior à sobrevida de casos de câncer de mama (~ 70%) e melanoma (~80%)⁵.

Nos últimos anos, tem havido grande interesse no estudo da interação entre resposta imune e tumores sólidos, incluindo HNSCC, devido ao papel importante da resposta do hospedeiro no desenvolvimento (tumorigênese), progressão (invasão) e resposta ao tratamento^{6,7}. A interação entre células neoplásicas e da resposta imune ocorre no microambiente tumoral (TME, *tumor microenvironment*), o qual inclui células imunes, endoteliais, fibroblastos, células mesenquimais e componentes da matriz extracelular. O TME é, atualmente, considerado como parte do tumor^{8,9} e tem sido intensamente estudado como potencial alvo terapêutico de diversos tipos de câncer, incluindo HNSCC^{9,10}. Macrófagos são as células imunes mais prevalentes no TME de HNSCC¹¹ e representam um importante alvo terapêutico devido à sua participação na oncogênese, progressão/invasão e recidiva pós-tratamento¹². O número de macrófagos infiltrantes é diretamente relacionado à pior prognóstico em HNSCC¹³⁻¹⁵, e pode estar relacionado à diversos mecanismos biológicos, como: (i) produção aumentada de mediadores capazes de ativar STAT3 nas células tumorais e promover sua sobrevivência e proliferação; (ii) produção de mediadores angiogênicos, como fator de crescimento vascular-endotelial (VEGF); (iii) produção de mediadores capazes de degradar a matriz extracelular e facilitar a invasão, como metaloproteases de matriz (MMPs); e (iv) produção de mediadores biológicos imunomoduladores da resposta anti-tumoral, como IL-6, TNF e IL-10¹⁶⁻²⁰.

Uma característica fundamental dos macrófagos é sua responsividade e adaptação aos sinais do microambiente, denominada plasticidade fenotípica. Dois

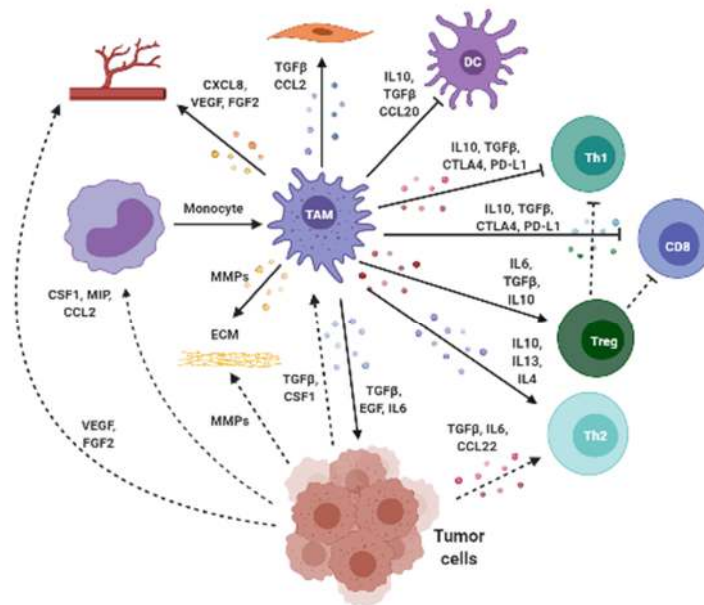
fenótipos extremos contrastantes são tradicionalmente descritos para os macrófagos: M1/clássico ou pró-inflamatório, associado à elevada produção de IL-1, IL-12, TNF e ROS; e M2/alternativo ou pró-reparativo, associado à elevada produção de IL-10, TGF e IL-1ra.

O fenótipo M2 é subclassificado em M2a (induzido por IL4), M2b (induzido por ligantes IgG / TLR) e M2c (induzido por IL10, glucocorticóide, CSF-1)²¹, refletindo a influência de diferentes pistas extracelulares nos macrófagos. Curiosamente, a polarização fenotípica dos macrófagos é transitória e dinâmica, pois pode ser revertida de acordo com os sinais do microambiente²².

Considera-se que o fenótipo dos macrófagos transita em um espectro entre os extremos 'M1' e 'M2', dependendo da natureza e relativa abundância de estímulos externos do microambiente^{21,23}. Além das diferenças entre macrófagos M1 e M2 no perfil de produção de mediadores biológicos secretados, incluindo citocinas, existem proteínas de membrana características de cada perfil, consideradas marcadores fenotípicos, como CD80 (M1) e CD163/CD206 (M2)²⁴.

No TME, os macrófagos assumem um fenótipo típico, sendo denominados 'macrófagos associados ao tumor' (TAM, tumor associated macrophages) o qual é, tradicionalmente, considerado como 'semelhante à M2' (M2-like), de perfil reparador/imunossupressivo. No entanto, em tecidos de casos de HNSCC podem ser encontrados tanto marcadores do perfil M1 quanto do perfil M2²⁵ sugerindo a participação de macrófagos associados aos dois extremos do espectro fenotípico e/ou que TAMs apresentam um fenótipo híbrido e não simplesmente um fenótipo do perfil M2.

Figure 1 – Macrófagos associados a tumor



Created in BioRender.com 

TAMs são macrófagos com fenótipo M2 que respondem a moléculas secretadas pelo tumor (por ex., TGF- β e CSF-1) e secretam mediadores biologicamente ativos que suprimem a resposta imune (CCL22, CCL17, TGF- β , IL-10, IL-4) e promovem o crescimento e invasão do tumor (EGF, VEGF, MMPs).

Fonte: Figura adaptada de Mantovani et al.²³ (2002).

O fator de crescimento de transformação beta (TGF- β) é um mediador solúvel com efeitos pleiotrópicos no câncer, podendo ter influência pró- ou anti-tumoral. No TME de HNSCC, diversos tipos celulares, incluindo macrófagos, podem produzir TGF- β . As atividades pró-tumorais do TGF- β incluem a indução da transição epitélio-mesênquima (EMT, *epithelial-mesenchymal transition*), processo de ‘desdiferenciação’ das células epiteliais que é associado tanto à tumorigênese como à maior agressividade/invasividade dos tumores²⁶⁻²⁸. Além disso, TGF- β pode interferir com o ciclo celular favorecendo a proliferação; pode induzir a produção de outros mediadores angiogênicos e de MMPs; também pode apresentar um efeito supressor na resposta imune, favorecendo a evasão tumoral^{29,30}. Em modelos de câncer oral em camundongos, TGF- β promove maior agressividade (invasão de tumores), embora reduza a ocorrência de metástases^{31,32}. Expressão elevada de TGF- β é observada em lesões primárias avançadas de HNSCC, bem como em lesões metastáticas em linfonodos³³.

Os efeitos antitumorais do TGF- β incluem a supressão de lesões iniciais, devido a efeitos antiproliferativos e pró-apoptóticos³⁴. Estes efeitos pleiotrópicos do TGF- β podem estar relacionados à variações e interferências na função/atividade/expressão de seus receptores e proteínas intracelulares responsáveis pela transdução do sinal gerado pelo TGF- β extracelular até o núcleo da célula. Estas alterações e variações podem ser relacionadas à mutações/variações genéticas ou à variações epigenéticas, as quais podem afetar a expressão e atividade (funcionalidade) dos receptores e/ou proteínas sinalizadoras³⁵. Embora existam controvérsias sobre o papel de TGF- β favorecendo ou inibindo a progressão de HNSCC, existem evidências indicando que a produção de TGF- β por células tumorais está associada a pior prognóstico.

A galanina é um peptídeo de 29 resíduos de aminoácidos amplamente distribuída e com amplo espectro de efeitos biológicos³⁶. Existem três receptores distintos já identificados em ratos e humanos (GALR1, GALR2, GALR3). Em células de HNSCC, GALR2 é o receptor mais expresso e leva à maior ativação de Rap1 e das vias downstream a esta proteína, incluindo MEK/ERK, PI3kinase/Akt e β -catenin³⁷. Em modelo experimental ortotópico de HNSCC utilizando camundongos nude, o aumento da sinalização de GALR2 promove o crescimento tumoral, o que suporta um papel da expressão deste receptor como marcador prognóstico e como um mecanismo de tumorigênese^{37,38}.

As vias de sinalização associada a Rap1 estão associadas à regulação pós-transcricional (via aumento da estabilidade do mRNA) de várias citocinas e fatores de crescimento³⁹. Não se sabe se o aumento da atividade de GALR2 em células HNC aumenta a expressão de TGF- β e CSF-1, promovendo a estabilidade do mRNA. No TME, TGF- β e CSF-1 são fatores de crescimento associados a polarização no protumoral de macrófagos⁴⁰ e seu efeito pode ser regulado no nível de estabilidade do mRNA⁴¹.

Esta informação indica que a ativação mediada por GALR2 de vias de sinalização em células HNC pode afetar a expressão de vários genes de relevância para o crescimento do tumor, incluindo TGF- β e CSF-1, que estão associados à imunomodulação de macrófagos no TME.

2 PROPOSIÇÃO

A proposta considera os macrófagos como tipo celular imune de grande relevância no microambiente tumoral devido à influência decisiva na progressão do carcinoma oral de células escamosas. O objetivo central é investigar a contribuição relativa de TGF- β como componente do secretoma de células de carcinoma oral de células escamosas (OSCC) na modulação do fenótipo de macrófagos e da invasão de células tumorais influenciada por macrófagos. Também é objetivo dos estudos propostos avaliar se há uma interseção entre a expressão de GALR2 como oncogene e a produção de TGF- β pelas células tumorais.

2.1 Hipótese

A hipótese primária deste projeto é que o TGF- β secretado por células tumorais tem um papel importante na indução do fenótipo M2 em macrófagos, o que favorece a progressão do tumor / invasão das células tumorais. Como hipótese secundária, a expressão de GALR2 em células de HNSCC induzem um fenótipo pro-tumoral em macrófagos mediada pela regulação de TGF- β . A partir do desenvolvimento do projeto os resultados foram divididos em 3 objetivos/publicações.

2.2 Objetivos

O objetivo geral do projeto é verificar a influência de TGF- β como um componente do secretoma de células tumorais na biologia de macrófagos, assim como se a expressão/atividade de GALR2 por células tumorais influencia o fenótipo de macrófagos por meio da regulação de TGF- β .

Portanto, os objetivos específicos no trabalho incluem:

Artigo 1: Role of tumor cell-secreted products from OSCC cancer cell lines on macrophage biology.

O objetivo é determinar a influência do secretoma das células de linhagens celulares de OSCC com alta (H314 - câncer de células escamosas de assoalho de boca) e baixa (SCC-9 - câncer de células escamosas de língua) produção constitutiva de TGF- β no fenótipo de macrófagos, assim como na migração, quimiotaxia e invasão de células tumorais na presença de macrófagos em co-cultura.

Artigo 2: *Influence of tumor cell-derived TGF- β on macrophage phenotype and macrophage-mediated tumor cell invasion*

Neste objetivo, avaliaremos o efeito específico de TGF- β como componente do secretoma das linhagens SCC-9 e H314 (com baixo e alto nível constitutivos na produção de TGF- β , respectivamente) no fenótipo de macrófagos e na quimiotaxia e invasão (*in vitro* e *in vivo*) de células neoplásicas na presença de macrófagos. A inibição do efeito do TGF- β como componente do secretoma (CM) das linhagens tumorais foi obtida com o tratamento dos macrófagos com inibidor bioquímico da quinase associada ao receptor de TGF- β tipo I (TGF- β RIk) previamente ao estímulo com o secretoma (CM) das linhagens celulares neoplásicas.

Artigo 3: *GALR2 expression by OSCC cells influences macrophage phenotype and macrophage-mediated tumor cell invasion.*

Neste objetivo, utilizamos quatro linhagens celulares transgênicas derivadas das mesmas linhagens neoplásicas parentais utilizadas nos experimentos dos objetivos anteriores (SCC-9 e H314). Duas linhagens com alta expressão de transgênica GALR2 e duas linhagens celulares-controle (transfectadas com o mesmo vetor plasmidial de expressão - pcDNA3.1 - 'vazio'). A expressão constitutiva de TGF- β e a meia-vida do mRNA foram determinados *in vitro*, assim como o efeito do secretoma destas linhagens celulares transgênicas no fenótipo e biologia de macrófagos.

3 PUBLICAÇÕES

Os dados foram organizados em três artigos a serem submetidos, segundo os objetivos principais dos experimentos incluídos, de forma a promover a coerência e facilitar o entendimento dos resultados obtidos por meio da definição de hipótese específica em estudo para cada conjunto de experimentos.

3.1 Publicação 1*

Role of tumor cell-secreted products from OSCC cancer cell lines on macrophage biology

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Abstract

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Macrophages are the most abundant immune cell type in the tumor microenvironment (TME) of head and neck squamous cell carcinomas (HNSCC), representing up to 50% of the tumor mass. The intensity of macrophage infiltration is inversely proportional to prognosis and disease survival. In the TME, macrophages are called tumor-associated macrophages (TAM) and their phenotype is determined by molecules in the TME, including neoplastic cell secreted products. As innate immune antigen-presenting cells, macrophages have a fundamental role in maintaining homeostasis and in the response to physicochemical and microbial insults. Macrophages also have an important role in immune surveillance, detecting neoplastic cells and activating the adaptive immune response for their elimination, besides participating in the repair process after treatment. The goal of this study is to determine, in vitro, the relative contribution of HNSCC cell-secreted products in the phenotype of macrophages. We used macrophages derived from a human monocytic cell line, which were exposed to the secreted products from two HNSCC cell lines. H314 and SCC9 cells-derived products induced a characteristic ('mixed') macrophage phenotype, which included increased expression of both M1- and M2-associated markers.

Key words: Tumor-Associated Macrophages, Squamous Cell Carcinoma Of Head And Neck, Tumor Microenvironment.

Introduction

Tumor microenvironment (TME) encompasses immune cells, endothelial cells, fibroblasts, pericytes, mesenchymal stem cells and components of the extracellular matrix. The TME is currently recognized as part of the malignant tumor and may influence virtually all aspects of cancer development: oncogenesis, tumor progression, metastasis, resistance to treatment and recurrence ^{1,2}. TME has been intensely studied as a therapeutic target in cancer treatment ^{2,3}. Macrophages are the most prevalent immune cell type component of the TME in HNC ⁴ and are one of the major therapeutic targets in the TME because of their roles in tumor initiation, progression and recurrence after treatment ⁵. Macrophages in the TME are denominated tumor-associated macrophages (TAM). In HNC, an increased infiltrate of macrophages presenting an M2-like phenotype is associated with a worse prognosis (reduced survival and increased recurrence after treatment) ^{6,7}. In addition, lymph node metastasis of small and aggressive primary HNC present increased infiltration of M2 macrophages ⁸, which suggests that increased infiltration by M2-like TAMs may be an independent prognostic factor of tumor aggressiveness. This may be related with a number of macrophage-mediated mechanisms favoring tumor progression, including increased production of: (i) STAT3 activators, angiogenic and EMT-promoting mediators such as VEGF, EGF and EGF-like ligands, CCL18 and TGF- β ; (ii) extracellular matrix degrading proteases (MMP-2, MMP-9, MMP-13) that facilitate invasion and (iii) suppressive molecules that inhibit other immune cells, such as IL-10, TGF- β , CCL22, PDL1, B7/CD80 ⁹⁻¹³.

Non-activated, resting macrophages (M0 phenotype) are highly responsive to microenvironmental cues, particularly soluble mediators such as chemokines, cytokines, and growth factors. In fact, phenotypical plasticity is a major characteristic of these cells, which have been traditionally classified into two contrasting phenotypes: a pro-inflammatory, classically-activated, M1 phenotype, associated with increased expression of TNF- α , IL-1 β , IL-12 and heightened microbicidal activity; and a contrasting anti-inflammatory, alternatively activated, pro-resolution/repair phenotype, associated with increased expression of IL-10, TGF- β , VEGF and enhanced efferocytotic activity ¹⁴. In vitro, exposure of macrophages to HNC cell-secreted products or to direct contact with HNC cells has been shown to induce polarization towards an M2-like phenotype ¹⁵, albeit it is biologically plausible that this effect varies depending on the mediators expressed by different HNC cell lines. This is supported

by finding of both M1 and M2 macrophage markers in the tumor stroma of clinical HNC samples, located in the infiltrating tumor islands and also in the tumor periphery ⁸.

Recently, a 'spectrum' model for the phenotypical plasticity of macrophages has been proposed, according to which macrophages transit between the opposite extremes represented by the prototypical M1 and M2 phenotypes ^{16,17}. Phenotype of TAMs differs from the 'traditional' M2/alternative phenotype because of increased expression of pro-inflammatory TNF-alpha and other typical M1 phenotype markers ^{12,14,16,18}. M2 macrophages are associated with epithelial-mesenchymal transition (EMT, related with more invasive tumors) and pro-tumoral effects in epithelial cancers, whereas M1 macrophages are associated with anti-tumoral effects ¹⁹⁻²¹. In fact, reversing the M2-like phenotype of TAMs augmented anti-tumor response and reduced tumor progression in pre-clinical studies ²²⁻²⁴. Reversion of macrophages towards the M1 pro-inflammatory phenotype has been shown to enhance the efficacy of PD-1/PD-L1 immunotherapy ^{25,26}. Collectively, this information demonstrates the relevance of macrophages for cancer progression; however, the relative contribution of tumor cell-secreted molecules on the induction and maintenance of a pro-tumoral TAM phenotype in the TME is still unclear ²⁷. Here we report that phenotype of macrophages varies significantly after exposure to secreted products of different oral cancer cell lines. This observation further supports a personalized medicine approach in oral cancer, characterized by high heterogeneity.

Material and methods

Cell lines

The human monocytic cell line (THP-1, cat# TIB-202, ATCC) was used as macrophage precursor. These monocytic cells were maintained in RPMI1640 medium supplemented with Penicillin/Streptomycin (100 IU/mL, 100 ug/mL, respectively) and heat-inactivated fetal bovine serum (FBS) at 10% (vol:vol). THP-1 cells were differentiated into macrophages using 10 ng/mL de phorbol 12-myristate 13-acetate (PMA) for 24h. After 24h, PMA-containing medium was replaced for complete RPMI for 72h as a resting period ²⁸. The oral cancer cell lines H314 (primary anatomical site: floor of the mouth) and SCC-9 (primary anatomic site: tongue) were obtained via a material transfer agreement (MTA) and were maintained in DMEM/F12 medium supplemented with Penicillin/Streptomycin (100 IU/mL, 100 ug/mL, respectively), 400 ng/mL sodium hydrocortisone succinate and FBS at 10% (vol:vol).

Macrophage polarization

Macrophages were polarized to M1 phenotype with IFN- γ 25 ng/mL (Peprotech Inc) and LPS 100 ng/mL (Sigma-Aldrich Co.). M2 polarization was induced with IL-4 25 ng/mL (Peprotech Inc) and IL-13 25 ng/mL (Peprotech Inc) for 24 h. As control (macrophage M0) cells were maintained in blank DMEM/F12 for 24 h.

Preparation of conditioned medium (CM)

H314 and SCC-9 cell lines were seeded in 100 mm culture dishes at a density to reach 70% confluency over 24 h and then washed in blank (i.e., non-supplemented) culture medium three times over 8 h (once every 2 h). After the washes, the cells were incubated for 16-18 h in reduced volume (4 mL in a 100 mm dish) of blank DMEM/F12. Conditioned medium (CM) was collected, centrifuged (10,000 RPM, 10 min, 4°C), aliquoted and stored for up to 2 weeks in a -80°C freezer.

RT-qPCR

Total RNA was isolated using an affinity column system, according to the supplier's instructions (RNAqueous total RNA isolation kit, Ambion/Thermo Fisher Scientific). 500ng of RNA was used to synthesized cDNA using random hexamers as primers (High Capacity cDNA synthesis kit, Applied Biosystems) and used in qPCR reactions with the SYBR Green system (Power UP SYBR Green, Applied Biosystems) and species- and target-specific primers. The 20 μ L qPCR reactions were performed in a StepOne Plus (Applied Biosystems) qPCR thermocycler using pre-optimized cycling conditions and followed by a melting curve. Primers for target genes related with distinct macrophage phenotypes were designed using NIH/NCBI algorithm (Primer-Blast) and RefSeq-deposited sequences in the NCBI database (**Table 1**). Expression of a constitutive housekeeping gene (HPRT1) unaffected by the experimental conditions determined after appropriate validation²⁹ was used to normalize the results. Analysis was performed using the $\Delta(\Delta C_t)$ method.

Table 1 –RT-qPCR primers for the target genes of interest designed using the NIH/NCBI BLAST algorithm.

Gene symbol	Accession#	Sequence (5'-3')	Ta (C)	Amplicon (bp)
HPRT1	NM_000194.2	Fwd - AGGACTGAACGTCTTGCTCG Rev - GAGCACACAGAGGGCTACAA	60.04 59.68	72
CSF-1	NM_000757.5	Fwd - TTAAAAGGCTGTGCCGAGGG Rev - GAGAGGACCCAGGCAAACCTT	60.61 59.60	72
TGF- β	NM_000660.6	Fwd - TATTGAGCACCTTGGGCACTG Rev - TCTCTGGGCTTGTTTCCTCAC	60.61 59.93	127
IL-10	NM_000572.3	Fwd - GGCACCCAGTCTGAGAACAG Rev - ACTCTGCTGAAGGCATCTCG	60.32 59.82	86
TNF	NM_000594.3	Fwd - GCTGCACTTTGGAGTGATCG Rev - TCACTCGGGGTTTCGAGAAGA	59.55 60.25	108
IL-12	NM_000882	Fwd - ATGGCCCTGTGCCTTAGTAGT Rev - AGCTTTGCATTCATGGTCTTGA	62.7 60.5	77
MMP-9	NM_004994.2	Fwd - ATCCAGACTTCCTCAGGCGG Rev - AGAAGCCCCACTTCTTGTCG	59.90 59.96	92
MMP-2	NM_004530.5	Fwd - ATCCAGACTTCCTCAGGCGG Rev - ATCCAGACTTCCTCAGGCGG	61.33 58.90	71

Multiplex assays

Protein production by macrophages treated with CM from OSCC cell lines was assessed on the macrophage cell lysates by multiplex fluid-based assays. Briefly, PMA-differentiated macrophages were stimulated with CM from OSCC cell lines for 24 h, and protein transport inhibitor (Brefeldin A, 10 μ g/mL) was added in the last 4 h. Culture medium was removed, cells washed with PBS and lysed. This approach avoids cross-contamination of cytokines present in OSCC CM used to stimulate the macrophages on the cytokines produced by macrophages. Total protein as quantitated (Bradford assay, Bio-Rad Lab) and 20 μ g of total protein were used for the multiplex assay to determine production of 14 cytokines relevant for tumor growth and invasion, including IL-1, IL-6, TNF, IL-12, IL-10, MMP-2, MMP-9, Leptin receptor, CD163 (RnD Systems, BioTechne). Briefly, the samples were processed according to the instructions of the supplier of the reagents and data was acquired in a Luminex 200

system (Invitrogen, ThermoFisher Scientific) and analyzed with the Milliplex software (Millipore, Sigma-Aldrich Co).

Chemotaxis

To assess the dynamic interaction between OSCC cells and macrophages pre-treated to M0, M2 or with CM for 24 h, we used a Boyden chamber experiment. The reciprocal chemoattraction was investigated by plating either OSCC cells or macrophages in the lower compartment of transwells (12-well plates, 0.8 μ m pore polyester membranes, Corning Life Sciences Inc.) and the other cell type on the upper chamber (on top of the transwell membrane). Briefly, 1×10^5 cells (either OSCC or macrophages) were seeded in the lower compartment of the 24-well plate in a 0.5 mL volume of low FBS (1% vol:vol) medium. After 24 h, 3×10^4 (in a 0.2 mL volume) of the other cell type (either macrophages or OSCC) were seeded in FBS-free medium on the upper chamber. Negative and positive controls were prepared by adding the same number of cells in the upper chamber and only 1% (vol:vol) FBS-containing culture medium (negative control) or medium containing 10% (vol:vol) FBS (positive control) in the lower chamber. After 18h, the transwells were collected, non-migrating cells in on the upper side of the membrane were gently removed with a sterile cotton swab and the migrating cells in the lower aspect were stained with DAPI (0.5 μ g/mL) for 15 min, the membrane was mounted on glass slides with anti-fade aqueous mounting medium, visualized and stained nuclei on the 5 random 100X magnification microscopic fields/membrane were counted by a trained operator unaware of the experimental conditions on an inverted fluorescence microscope (Evos FI, AMG Micro).

Tumor cell migration– in vitro

To determinate the effect of direct cell-to-cell contact on migration of OSCC cells, we used co-cultures of OSCC cell lines with PMA-differentiated macrophages polarized to M0, M2 or MC phenotypes. Cells were plated in an approximate 3:1 proportion (2.2×10^5 OSCC cells : 8×10^4 macrophages). Controls with OSCC cells alone were prepared by plating the same total number of cells (3×10^5 cells) in each well. After 18 h, a vertical in vitro wound ('scratch') was made in the center of the wells with a sterile 200 μ L pipette tip. The wells were extensively washed with PBS to remove all non-attached cells and complete culture medium supplemented with mitomycin C (2

ug/mL) was added. Brightfield images from the same fields (determined by reference lines placed on the external surface at the bottom of the plate before starting the co-cultures) were obtained at 40X magnification on an inverted microscope (Evos FI, AMG Micro) after 4, 12 and 24h.

Tumor cell invasion – in vitro

The influence of macrophages and macrophage phenotype on neoplastic cell invasion was assessed using myogel³⁰. OSCC cells were collected and stained with green fluorescence live cell dye (5 μ M, CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes) and macrophages (M0, M2 and MC phenotypes) stained with red fluorescence live cell dye (10 μ M, CellTracker™ Red CMTPX Dye, Molecular Probes). OSCC cells and macrophages were combined at the indicated ratios (keeping the total number of cells on each well constant at 4x10⁴ cells). The cells were seeded on top of an approximately 150 μ m thick layer of myogel supplemented with collagen prepared on top of a 0.8 μ m pore polycarbonate transwell membranes. DMEM/F-12 supplemented with 1% FBS was placed in the lower compartment as chemoattractant. After 18 h, images from the center of the membranes were obtained in the green and red fluorescent channels using a confocal microscope (Carl Zeiss LSM 800). On the Z-axis, 17 images (image/slice# indicated on the images) were obtained with 8 μ m spacing for 128 μ m, beginning 32 μ m below the transwell membrane (identified at slice #5 by the presence of the transwell membrane pores on phase contrast images) and 96 μ m extending upwards.

Data Analysis

Prism 9 (GraphPad software LLC) was used for statistics. The purpose of the analyses was to compare the outcomes within each OSCC cell line according to the experimental condition of pre-stimulation of macrophages. Appropriate tests were used, according to the number of experimental conditions and nature of the outcome variable (percentage, discrete or continuous numerical). Biological reproducibility was asserted by performing three independent experiments and technical replicability was verified by analysis of each sample in duplicate. A p-value of <0.05 (95% significance level) was considered as statistically significant.

Results

Significant increases of CSF1 and TGFB1 expression by macrophages were observed after exposure to CM from both H314 and SCC-9 cells ($p < 0.05$). Treatment with CM from H314 cells, but not CM from SCC-9 cells, also increased macrophage expression of IL-10 ($p < 0.05$) and MMP-9 (non-significantly); whereas treatment of macrophages with CM from SCC-9 cells, but not CM from H314 cells, increased expression of MMP-2 ($p < 0.05$) and IL-12 (non-significantly) (**Figure 1**).

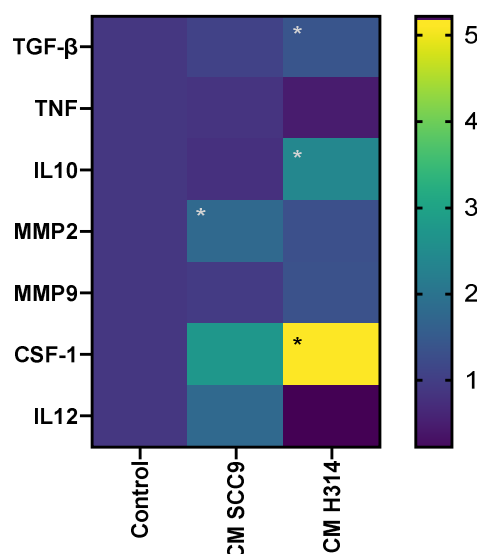


Figure 1 – Influence of secreted products from two OSCC cell lines on macrophage gene expression. PMA-differentiated macrophages were cultured in the presence CM from SCC9 and H314 cells for 24 h. RNA was extracted from cell lysates and used for RT-qPCR for the indicated target genes. Relative expression levels to the control. *indicates a statistically significant ($p < 0.05$) difference versus untreated control. Representative data from three independent experiments analyzed in duplicate.

The data indicates that CM from each OSCC cell line induces a differential response on macrophages and based on the shift in the expression of the target genes, H314 cell-secreted products skewed macrophages to an M2-like phenotype; whereas the secreted products from SCC-9 cells shifted macrophage phenotype towards the opposite (M1) side of the spectrum (**Figures 1 and 2**). Interestingly, mRNA expression / protein production was uncoupled for IL-12 but not for IL-10, two cytokines that are known to have post-transcriptional regulation. This suggests a complex regulatory mechanism, which may be related with differences in the composition and concentration of numerous other biologically-active molecules produced and secreted by the different OSCC cell lines.^{31,32}

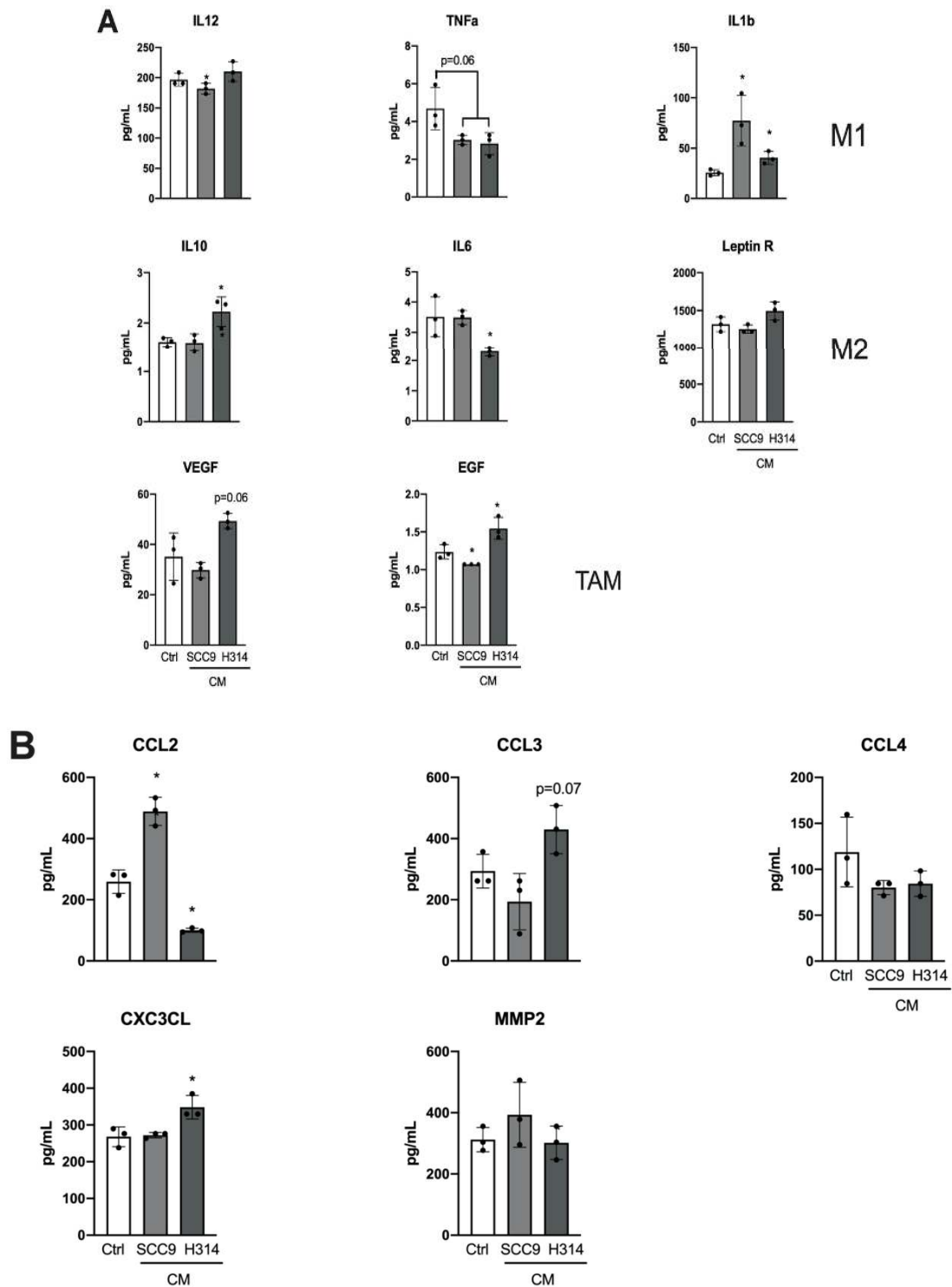
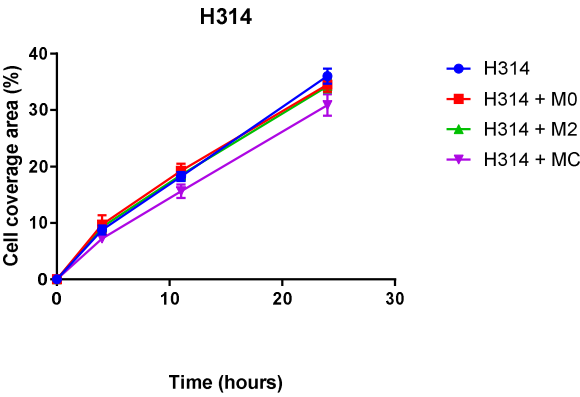
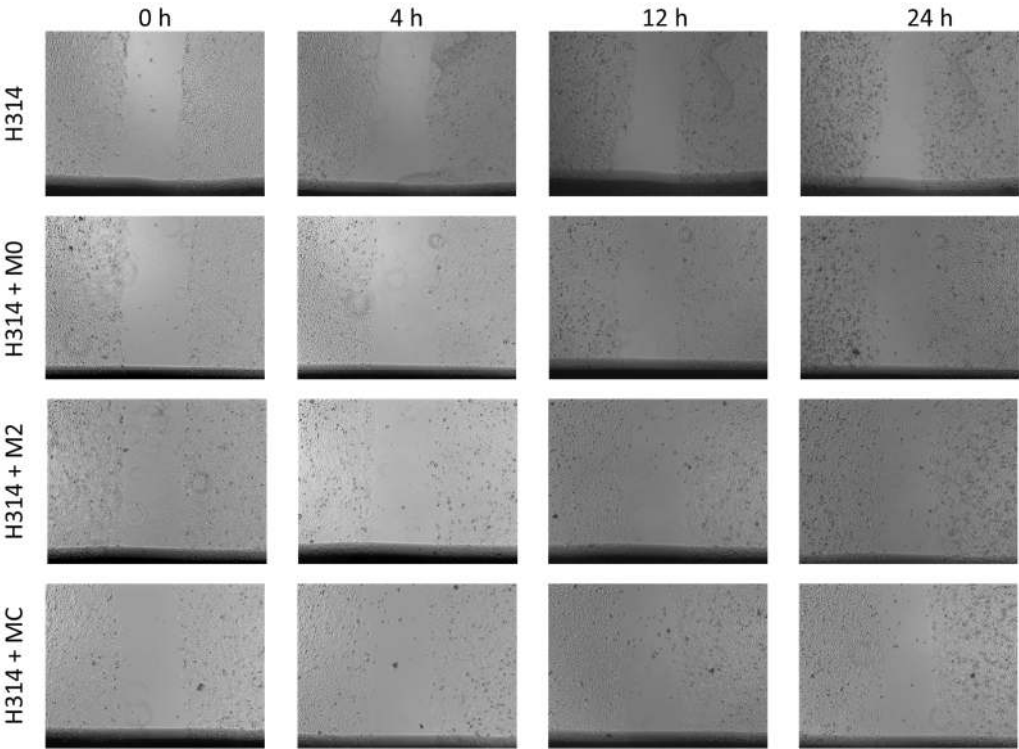


Figure 2 – Influence of secreted products from HNSCC cell lines on protein production by macrophages. (A) Cytokines and growth factors associated with different macrophage phenotypes. (B) Chemokines and matrix metalloprotease-2 that may be involved in macrophage-mediated tumor progression. Multiplex assay of proteins produced by macrophages according to the experimental condition: unstimulated (Ctrl) or stimulated with secreted products (CM, conditioned medium) from SCC9 (CM SCC9) or H314 (CM H314) cells. Representative data from three independent experiments analyzed in duplicate.

Migration of H314 and SCC-9 cells was not affected by the presence of macrophages of any phenotype (M0, M2 or MC) (**Figure 3**)



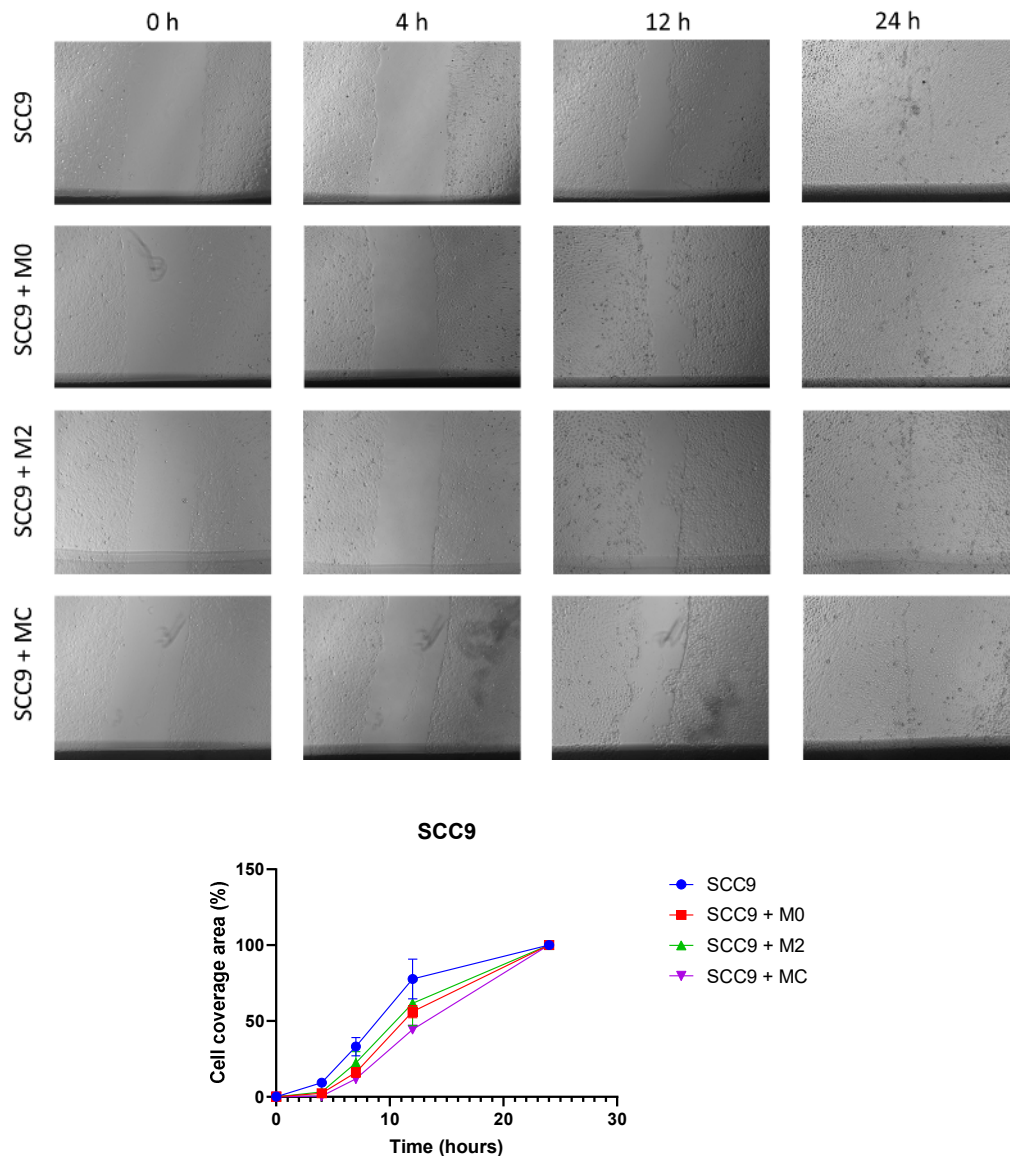


Figure 3 – Influence of macrophages of different phenotypes on migration of H314 (top) and SCC9 (bottom) cells. PMA-differentiated THP-1 macrophages of different phenotypes (M0, M2 and MC) were combined at an approximate 3:1 ratio with OSCC cell lines and plated in 24-well plates (3×10^5 total cells/well) in supplemented DMEM/F12. The clear area (exposed plastic substrate, devoid of cells) was measured in digitized images using Image J software. The extent of cell migration ('cell coverage' of the in vitro wound) is represented in the graphs relatively to the clear area of each well in the initial period (time 0 h), which was considered as 0% coverage. Data from three independent experiments performed in triplicate and including measurements of the same two microscopic fields from each wound/well in each period.

Interestingly, chemotaxis of both neoplastic cell lines was markedly enhanced by macrophage-secreted products regardless of the phenotype (M0, M2 and MC) compared to negative control (**Figure 4**). SCC9 cell-secreted products had no effect

on macrophage chemotaxis, regardless of their initial phenotype (M0, M2 or MC); however for H314 cells, there was a significant increase in chemotaxis of TAMs (**Figure 5**). These results indicate that macrophage-secreted products are chemotactic for OSCC cells, but OSCC cell lines differ in their chemotactic activity to macrophages.

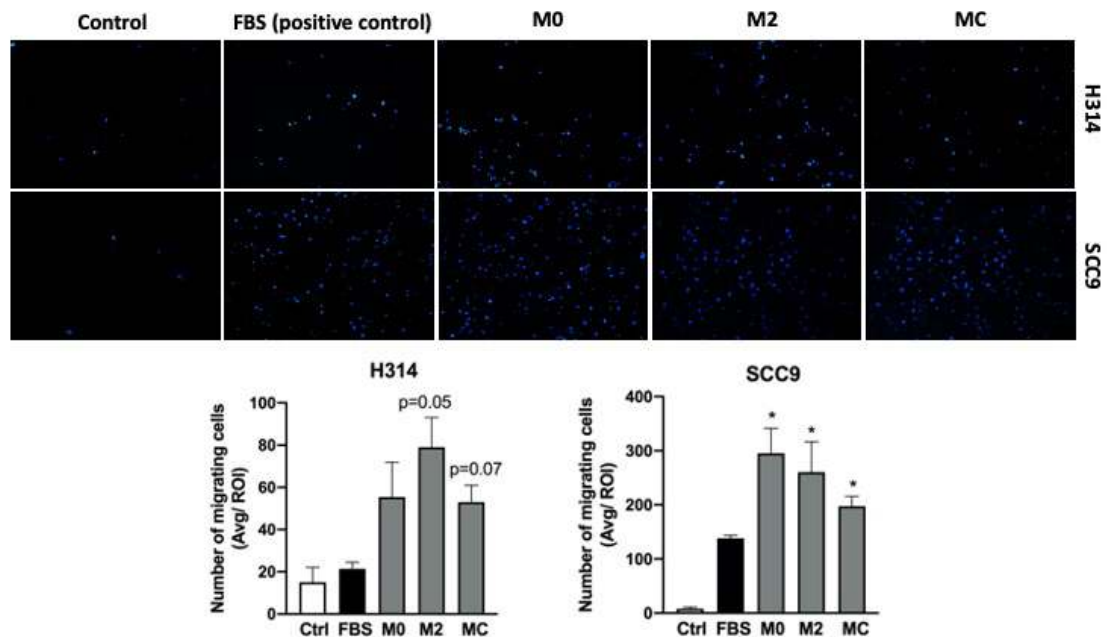


Figure 4 – Influence of macrophage-secreted products on chemotaxis of OSCC cell lines. PMA-differentiated THP-1 macrophages were plated on the lower compartment of transwells and polarized to different phenotypes (M0, M2, MC) in DMEM/F12 with low (1%, vol:vol) FBS over two days. Negative and positive controls were prepared with DMEM/F12 only (no cells) supplemented with 1% (negative control, 'Ctrl') or 10% (vol:vol) FBS ('FBS'), respectively. H314 or SCC9 cells (3×10^4 /transwell) resuspended in DMEM/F12 with low (1%, vol:vol) FBS were then seeded on the upper compartment of the transwell. After 8 h transwell membranes were removed, cells on the upper side (non-migrating) were removed with sterile cotton swabs and the membranes were stained with DAPI. Membranes were mounted onto glass slides for observation and counting of nuclei in an inverted fluorescent microscope by a trained observer unaware of the experimental conditions (representative images shown). Graphs indicate average and standard deviation of three independent experiments performed in duplicate. *indicates $p < 0.05$ and p values from analysis of variance followed by Tukey test for comparisons with the negative control (Ctrl).

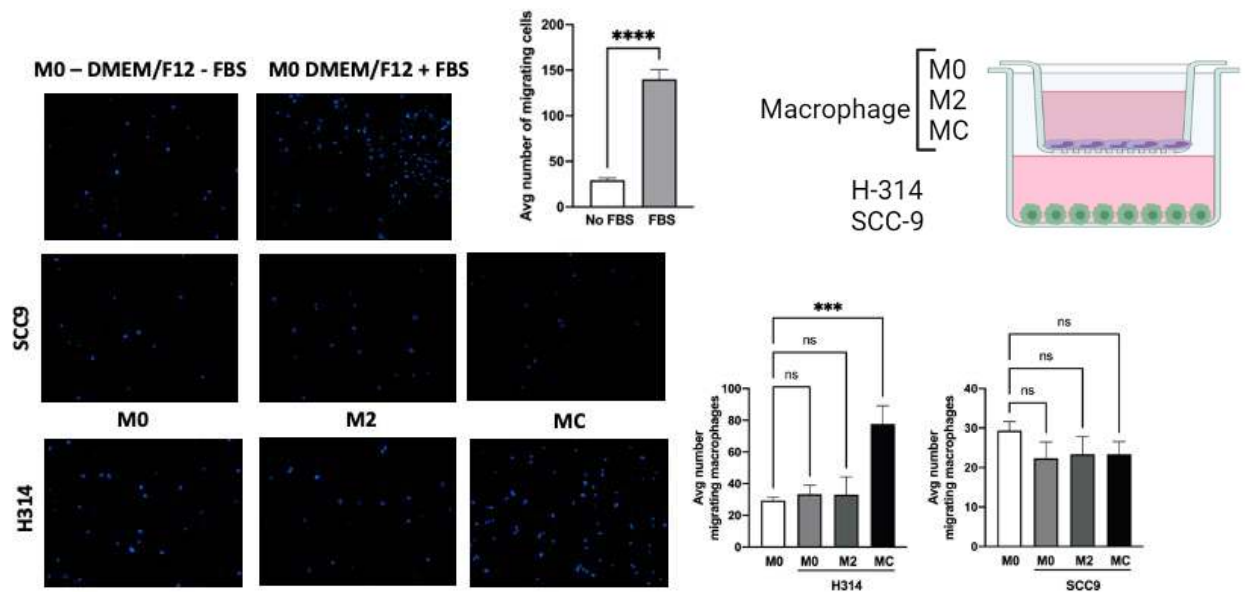


Figure 5 – Influence of tumor cell-secreted products on chemotaxis of macrophages of different phenotypes. In this experiment the chemotactic stimuli in the lower compartment were the neoplastic cell-secreted products. SCC9 or H314 cells (1×10^5 cells/well of a 24-well plate) were seeded on the lower compartment of the transwell in DMEM/F-12 supplemented with 10% FBS. After 24 h, medium was removed and cells were washed extensively with PBS. After an additional 24 h in culture in DMEM/F-12 containing 1% (vol:vol) FBS, 3×10^4 macrophages of the indicated phenotype (M0, M2 or MC) were seeded on the upper chamber. Negative and positive controls were DMEM-F-12 supplemented with 1% or 10% (vol:vol) FBS, respectively. After 18 h, cells on the upper side of the transwell were gently removed with sterile cotton swabs and the membrane stained with DAPI. The nuclei of migrating cells were counted on an inverted fluorescence microscope by a trained examiner unaware of the experimental conditions. Graphs indicate average and standard deviations of the number of macrophage nuclei counted and representative images are shown. Differences between positive and negative controls were determined by unpaired t test with Welch's correction. Differences between experimental groups and negative control were assessed by ANOVA followed by the Tukey paired (control X experimental) post-hoc test (** $p < 0.001$ vs control, 'ns' non statistically significant). Representative data from three independent experiments analyzed in duplicate.

To determine if the presence of TAMs favor tumor progression, we assessed invasion of both neoplastic cell lines in vitro in the presence and absence of macrophages treated with secreted products from the oral cancer cell lines. SCC-9 cells were markedly more invasive than H314 cells (**Figure 6**), and the presence of TAMs (macrophages pre-exposed to CM) alongside the neoplastic cells (both at 1:1 and 3:1 macrophage : neoplastic cell ratios) only enhanced invasion of H314 cells (**Figure 7A**); whereas at either ratio the presence of preconditioned macrophages slightly inhibited invasion of SCC9 cells (**Figure 7B**). This may be related with the M1

phenotype shift and greater productions of EGF induced by secreted products from H314 cells (**Figure 2A**).

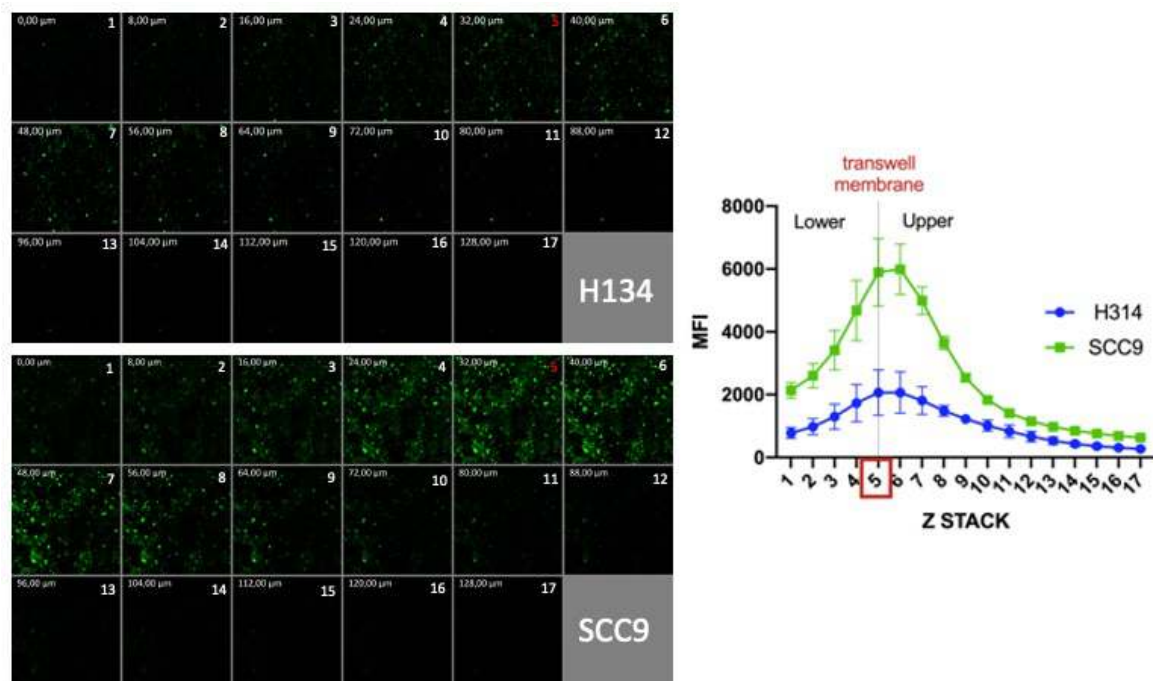


Figure 6 – In vitro invasion of H314 and SCC9 OSCC cells. Cell lines were collected, stained with green fluorescence live cell dye (5μM, CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes) and subsequently seeded on top of an approximately 150 μm thick layer of myogel supplemented with collagen placed on top of a 0.8 μm pore polycarbonate transwell (4x10⁴ cells/membrane). DMEM/F-12 supplemented with 10% FBS was placed in the lower compartment as chemoattractant. After 18 h, images from the center of the membranes were obtained in the green fluorescent channel on a confocal microscope. On the Z-axis, 17 images (image/slice# indicated on the images) were obtained with 8 μm spacing for 128 μm, beginning 32 μm below the transwell membrane (identified by the presence of the pores at the slice #5 using phase contrast). The graph indicates the median fluorescence intensity (MFI) in the green channel calculated with the confocal microscope's software at each of the 17 images in the Z-axis (image/slice# indicated on the X-axis of the graph). The MFI is directly proportional and representative of the number of cells in each slice/image. Data in graph calculated from three independent experiments analyzed in duplicate.

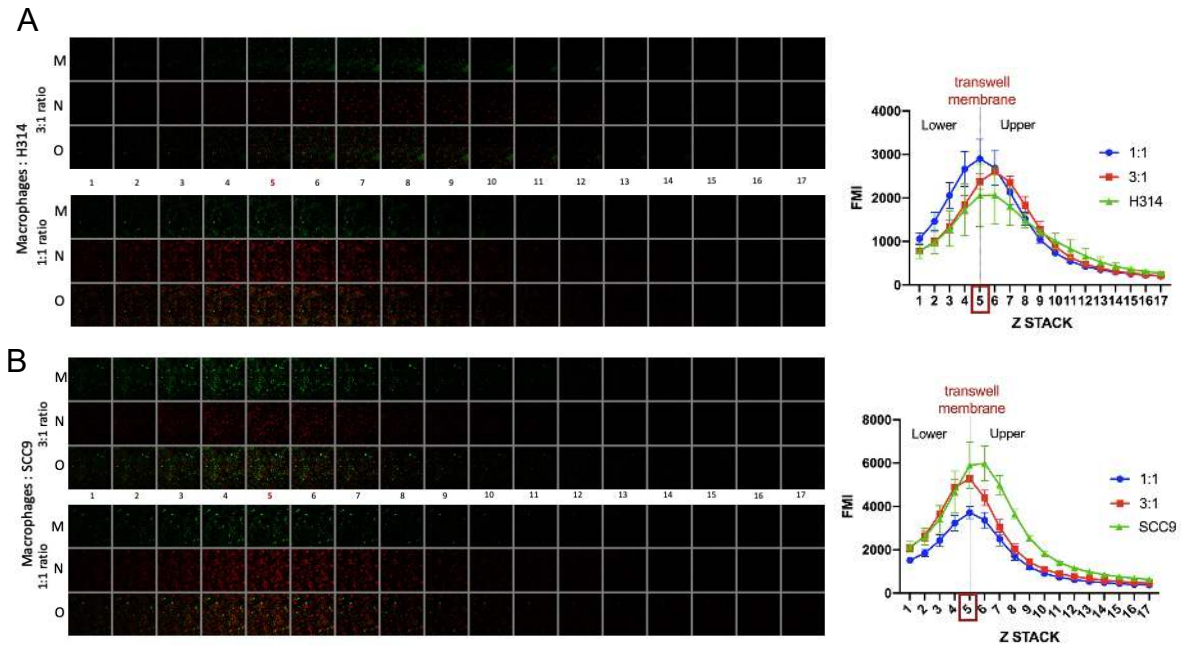


Figure 7 – Influence of macrophages on H314 and CC9 in vitro invasive capability. This experiment was performed similarly to the description on Figure 6 legend, except that macrophages cultured for 2 days in CM from H314 (**A**) or SCC-9 (**B**) cells were stained with green live cell fluorescent dye ('M', macrophages) and H314 cells stained with red live cell fluorescent cell dye ('N', neoplastic cells). Cells were mixed immediately at the indicated ratios (keep constant the total number of cells at 4×10^4 cells/membrane). Images were obtained at both green and red fluorescent channels of the confocal microscope and an overlay image of these channels ('O', overlay) is also presented. Graph indicates the median fluorescence intensity at the red fluorescent channel (neoplastic cells). Data in graph from three independent experiments analyzed in triplicate.

Discussion

Tumor cell-secreted products have a significant impact in the TME, affecting both structural constituents and cells. As the most abundant immune cell type in HNSCC stroma, macrophages have a prominent role in tumor progression, which is associated with their phenotype. In this study we investigate the influence of H314 and SCC-9 cell secreted products on phenotype of macrophages, reciprocal chemotaxis and macrophage-mediated tumor cell invasion. Our results demonstrate a different response of macrophages to H314 cell line-secreted products with a polarization to an M2-like phenotype, whereas the secretome of SCC-9 cells skewed macrophages to an M1-like phenotype. The heterogeneity of HNSCC includes anatomical tumor location, TNM stage, mutational burden, epigenetic changes and also encompasses variations in the tumor cell secretome, which subsequently influence aggressiveness of disease, response to treatment and recurrence. In response to cancer cell-secreted products, macrophages assume a mixed/unique phenotype, which may skew towards

a 'pro-inflammatory' ('M1-like') or 'pro-healing' ('M2-like'), but neither is comparable to typical M1/M2 extremes. The paradigmatic classification of macrophage phenotype into 'Classical/Inflammatory/M1' and 'Alternative/Reparative/M2'³³ generates a dichotomic pre-concept that serves little purpose in identifying the relevance of diverse dynamic phenotypical traits of macrophages for tumor progression, as many studies focus on 'conforming' the observed phenotype to either of these rather rare extremes, often considered as 'mutually exclusive'.

The realization that macrophage phenotype is both dynamic and transit in a spectrum between the M1/M2 extremes ³³, including varying levels of expression of the multiple markers and molecules associated with M1 or M2 phenotypes concomitantly ^{18,34,35}, may provide insights into novel roles for macrophages in HNSCC progression and therapeutic opportunities. Nevertheless, according to the paradigm of mutually exclusive M1/M2 and with the bulk of related literature, increased infiltration of 'M2-like' macrophages in the TME of HNSCC may favor tumor growth and invasion ^{16,23,36-39}, mostly because of wound healing-related characteristics, such as increased expression of proteases, angiogenic molecules and immunosuppressing cytokines (e.g., IL-10). However, there is evidence that macrophages cultured in tumor stroma-like conditions co-express M1 and M2 phenotypical markers ^{14,40,41}.

As a limitation of the study, it is important to consider that the experimental approach used cell lysates to assess protein expression by macrophages (and not the macrophage secreted proteins) to avoid interference with the proteins present in the CM from neoplastic cells. Nevertheless, data indicate that secreted products from two distinct HNSCC cell lines induce different shifts in macrophage phenotype. In fact, the microenvironmental cues affecting macrophages and macrophage-secreted products may have conflicting biological roles in cancer progression. For example, EGF secreted by CD206+ ('M2-like') macrophages increase proliferation and invasion of OSCC cells ³¹. There is recent evidence indicating that in the presence of conflicting external cues of phenotypical polarization, macrophages may assume a transient 'mixed' phenotype comprised of subpopulations of cells in various positions along the M1/M2 spectrum, with only a few genes being expressed in a mutually-exclusive manner ³².

Our results shows increased chemotaxis of cancer cell lines to macrophage-secreted products. This can be speculated to suggest that neoplastic cells can 'tag along' macrophages for invasion. However, we did not observe changes in the

migration of neoplastic cells in the presence of macrophages. This may be due to the nature of the substrate (cell culture-treated plastic), to the blocking of cell proliferation by mitomycin C and to the lack of a gradient in macrophage-secreted products in these bi-dimensional co-culture conditions. There is evidence that bladder cancer cells increase their proliferation and invasion when cultured with conditioned medium of macrophages, indicating a paracrine effect of macrophages. These effects were, in part, mediated by chemokines CCL5 and IL-8 ⁴². In glioblastoma, secreted products from microglial cells may promote immunevasion and enhance tumor progression ⁴³. Interestingly, macrophage chemotaxis was affected differently by the secretome of the two OSCC cell lines used: secreted products from SCC-9 cells did not enhance chemotaxis of macrophages (M0, M2 or MC); whereas the secretome of H314 cells significantly increased chemotaxis of MC macrophages (macrophages pre-conditioned with secreted products from H314 cells) (**Figure 5**). This is particularly interesting, as constitutive production of CSF-1 (the major candidate for paracrine cross-talk between neoplastic cells and macrophages) ⁴⁴ by H314 cells is lower than that of SCC-9 cells. Induction of chemokine production by macrophages may generate a positive feedback loop of cross-talk between neoplastic cells and macrophages that may promote cell recruitment and other changes in the tumor microenvironment that may enhance tumor progression and facilitate immunevasion. The in vitro invasion assay supports this notion, as invasion of H314 cells, but not of SCC-9 cells, was enhanced by the presence of macrophages. The interaction between tumor cells and macrophages is not limited to chemotaxis or invasion. For example, there was greater production of CCL2 by macrophages stimulated with secretome of SCC-9 cells (**Figure 2B**) and the axis CCR2/CCL2 has been shown to promote immunevasion via PD-1 in esophageal cancer ⁴⁵. CCL3 also has a relevant role in oral cancer ⁴⁶ and may be involved in a positive-feedback cross-talk between macrophages in neoplastic cells. CX3CL1 is a TGF- β -regulated chemokine and H314 cells have greater production of TGF- β in comparison with SCC-9 cells. Increased expression of CX3CL1 may be associated with occurrence and progression of gastric cancer ⁴⁷, and also promoted invasion of OSCC in vivo ⁴⁸.

In summary and considering the limitations of this study, the results indicate that the secretome of different OSCC cells may induce distinct phenotypes in macrophages, which may affect diverse tumor biology processes such as chemotaxis and invasion.

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3.2 Publicação 2*

Influence of tumor cell-derived TGF- β on macrophage phenotype and macrophage-mediated tumor cell invasion

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Abstract

In oral squamous cell carcinoma (OSC), macrophages are the most abundant immune cell type in the tumor microenvironment (TME). Macrophage infiltration is inversely proportional to prognosis and disease survival, particularly when these tumor-associated macrophages (TAM) assume an M2-like phenotype. This phenotype is determined by cues from the microenvironment, especially tumor cell-secreted molecules, and is associated with increased production of extracellular-matrix-degrading enzymes, angiogenic molecules and immunosuppressing cytokines. This study investigates, *in vitro* and *in vivo*, the relative contribution of OSCC cell-secreted transforming growth factor beta (TGF- β) on the phenotype of macrophages and on macrophage-facilitated tumor invasion. TCGA database shows a positive correlation between high expression of TGFB1 and macrophage infiltrate in Head and neck squamous cell carcinoma (HNSCC). THP-1 derived-macrophages were exposed to the secretome of two OSCC cell lines with and without pre-treatment with a TGF- β type I receptor kinase inhibitor (LY-364947, 10 μ M) to block the effects of TGF- β as a constituent of the OSCC secretome. RT-qPCR and ELISA determined macrophage phenotype after exposure to conditioned medium (CM) from H-314 (TGF- β^{high}) or SCC-9 (TGF- β^{low}) cell lines. The influence of TGF- β on macrophage-mediated tumor cell invasion (myogel and CAM assays) and chemotaxis (Boyden chamber) was assessed using co-cultures of macrophages and OSCC cells in which macrophages were pre-conditioned with the secretome of OSCC cells in the presence and absence of LY-364947. Blocking the effects of TGF- β skewed macrophages to the M1 end of the phenotype spectrum by reducing MMP-9 and IL-10 expression (H314 and SCC-9 secretomes) and increasing IL-12 (SCC-9 secretome). *In vitro* and *in vivo* invasion of H-314 cell line was reduced by blocking the effects of TGF- β in macrophages, whereas SCC-9 cell invasion was not affected. SCC-9/macrophage reciprocal chemotaxis were enhanced when TGF- β was blocked in macrophages, whereas only macrophage chemotaxis to H314 products was inhibited by blocking TGF- β . In summary, blocking the effects of OSCC cell-secreted TGF- β in macrophages attenuates M2-like phenotypical traits of macrophages and can impact invasion and chemotaxis of tumor cells differentially.

Key words: Tumor-Associated Macrophages, Squamous Cell Carcinoma Of Head And Neck, Tumor Microenvironment, Transforming Growth Factor Beta

INTRODUCTION

TGF- β is acknowledged as an inducer of epithelial-to-mesenchymal transition (EMT) ¹⁻³. TGF- β may also promote tumor growth and invasion by dysregulation of cyclin-dependent kinase inhibitors, increasing MMP expression and the production of angiogenic factors, and also by reducing immune surveillance due to its suppressive effects ^{4,5}. In the tumor microenvironment (TME) various cells may produce transforming growth factor beta (TGF- β), including tumor cells and macrophages. However, there is evidence for pleiotropic and contrasting effects of TGF- β in the TME. In the chemically-induced murine model of oral cancer, increased expression of TGF- β reduces metastasis but increases local invasion ^{6,7}. TGF- β may also function as a suppressor of tumor initiation in early lesions as it is associated with anti-proliferative and pro-apoptotic effects ⁸. These contrasting effects of TGF- β may be related with the contextual presence of diverse signal transduction mediators of the TGF- β signaling pathway (including receptors and intermediate signaling proteins) that are associated with genetic and epigenetic makeup of different non-neoplastic cell types in the TME, as well as mutations and epigenetic changes in neoplastic cells ⁹. On immune cells TGF- β may influence adaptive immunity facilitating tumor evasion; as well as innate immune cells, such as macrophages, changing their phenotype and enhancing tumor progression. Increased TGF- β expression is observed in more advanced primary lesions of head and neck cancer (HNC), and in lymph node metastasis ¹⁰.

TGF- β inhibits T cell proliferation, represses activation of both cytotoxic CD8+ T cells and NK cells, inhibits B cell activation and IgG secretion and promotes the differentiation of suppressive CD4+ Tregs, which contributes to inhibit anti-tumor immune response. Interestingly, in the presence of IL-6, TGF- β induces the polarization of pro-inflammatory CD4+ Th17 cells and even reverse the phenotype of Tregs to Th17 cells, which have anti-tumoral effects, indicating the complexity of the interplay among molecules in the TME ⁸.

Phenotypical polarization of tumor-associated macrophages (TAMs) is also affected by TGF- β ¹¹. In macrophages, TGF- β activates the transcription factor SNAIL via Smad2/3 and PI3K/Akt pathway. Activation of SNAIL represses the expression of pro-inflammatory TNF- α and IL-12 and increases expression of IL-10, accentuating the polarization towards the M2-like phenotype ¹². TGF- β also increases expression of VEGF by TAMs ¹³. This information supports various mechanisms by which tumor cell-secreted TGF- β can have a prominent role in tumor progression, including promotion

of EMT, increasing angiogenesis and immune suppression. Nevertheless, clinical trials of anti-TGF- β targeted therapies have been mostly disappointing ¹⁴, which emphasizes the need for further information on the biology of TGF- β in the TME. The aim of this study is to assess the influence of tumor cell-secreted TGF- β on macrophage phenotype and macrophage-mediated tumor cell chemotaxis and invasion.

METHODS

In silico analysis

The TIMER2.0 database was used to analyze overall survival and associations between TGFB1 tumor and adjacent normal tissues using TCGA database. Distributions of gene expression levels are displayed using box plots. ¹⁵. Correlation of infiltration level of macrophage in Head and neck squamous cell carcinoma (HNSCC) with high expression of TGFB1 was assessed using TIMER algorithm for estimations. The purity-adjusted Spearman's rank correlation test was used to obtain the p values and partial correlation (cor) values, and then heatmaps and corresponding scatter plots were generated ¹⁶.

Cell lines

We used a human monocytic cell line (THP-1, cat# TIB-202, ATCC) as macrophage precursor. These monocytic cell lines were maintained in RPMI1640 culture medium supplemented with Penicillin/Streptomycin (100 IU/mL, 100 ug/mL, respectively) and heat-inactivated fetal bovine serum (FBS) at 10% (vol:vol). Macrophage differentiation was performed by treatment with 10ng/mL of phorbol 12-myristate 13-acetate (PMA) in RPMI culture medium for 24 h. After 24 h, culture medium was replaced by supplemented RPMI without PMA for 72 h and the cells recovered from PMA stimulation, generating 'M0' macrophages ¹⁷. Macrophage differentiation was verified by morphological and functional changes (increased cell size, attachment to the culture substrate and increased expression of CD11b – data not shown). Oral squamous cell carcinoma cell lines H314 (floor of the mouth) and SCC-9 (tongue) were maintained in DMEM/F12 culture medium supplemented with Penicillin/Streptomycin (100 IU/mL, 100 ug/mL, respectively), 400 ng/mL sodium hydrocortisone succinate and heat-inactivated FBS at 10% (vol:vol). Cancer cell lines were obtained via a material transfer agreement (MTA) established with directly with a researcher (SCC-9) or commercially (H314, ECACC).

Preparation of OSCC secretome / conditioned medium (CM)

2.5x10⁶ OSCC cells were plated in complete culture medium in 100 mm cell culture dishes. After 24 h cells were extensively washed in blank (i.e., non-supplemented) culture medium three times over 6 h (once every 2 h). After the washes, the cells were incubated for 16-18 h in small volume (4 mL in a 100 mm culture dish) of blank DMEM/F12 culture medium. At the end of this period the culture medium was collected as conditioned medium (CM), centrifuged (10,000 RPM, 10 min, 4°C), aliquoted and stored for up to 2 weeks in a -80°C freezer.

Macrophage polarization

Macrophages were polarized to M2 phenotype with IL-4 (25 ng/mL, Peprotech Inc) and IL-13 (25 ng/mL, Peprotech Inc) in blank DMEM/F-12 culture medium for 24 h. As controls, culture medium of M0 macrophages were changed to blank DMEM/F12 for 24 h.

Inhibition of TGF- β

Macrophages were pre-treated for 4 h with TGF- β type I receptor kinase inhibitor (LY-364947, 10 μ M, Sigma-Aldrich Co) or with the same volume of DMSO vehicle as control, before changing the culture medium to CM. Macrophages were treated with CM from either OSCC cell line for 24 h for the assessment of the polarization outcomes (gene expression and phenotypical markers).

Western blot

Total protein extracts were prepared by lysing cells in detergent-containing buffer (M-Per, Pierce/ThermoFisher Scientific) supplemented with protease and phosphatase inhibitors (Pierce Protease and Phosphatase Inhibitor Mini Tablets/ThermoFisher Scientific) and subsequently quantitated by the Bradford method. Equal concentrations (20 – 40 μ g) of total protein were loaded onto 4-12% gradient polyacrylamide gels and electrophoresed in denaturing conditions (samples heated to 95°C in the presence of 0.1 M DTT and run in SDS-containing buffers and gel). After electro-transfer to 0.2 μ m nitrocellulose membranes, the proteins were blocked with 5% BSA in tris-buffered saline containing 1% Tween-20 (TBS-T) and incubated overnight with primary antibodies to detect phosphorylated or total forms of

the proteins of interest Smad2/3 and p38 MAPK. Detection of target proteins were based on chemiluminescence reaction between HRP conjugated to species-specific secondary antibodies and a high sensitivity substrate system (SuperSignal West Pico, Pierce/ThermoFisher Scientific) detected on a digital documentation system (Chemi-Doc XR, Bio-Rad Laboratories). The relative activation status was determined by densitometric analysis with normalization of phosphorylated forms to the total forms of each signaling intermediate.

RT-qPCR

Total RNA was isolated using an affinity column system, according to the supplier's instructions (RNAqueous total RNA isolation kit, Ambion/Thermo Fisher Scientific). cDNA was synthesized using random hexamers as primers (High Capacity cDNA synthesis kit, Applied Biosystems) and used in qPCR reactions with the SYBR Green system (Power UP SYBR Green, Applied Biosystems) and species- and target-specific primers. The 20 μ L qPCR reactions were performed in a StepOne Plus (Applied Biosystems) qPCR thermocycler using pre-optimized cycling conditions and followed by a melting curve. Primers designed using NIH/NCBI algorithm (Primer-Blast) and RefSeq-deposited sequences in the NCBI database (**Table 1**) for the assessment of macrophage phenotype. Expression of a constitutive housekeeping gene (HPRT1) that was unaffected in the experimental conditions (determined after appropriate validation ¹⁸) was selected to normalize the results. Analysis was performed using the $\Delta(\Delta C_t)$ method.

Table 1 – RT-qPCR primers for human target genes designed using the NIH/NCBI BLAST algorithm.

Gene symbol	Accession#	Sequence (5'-3')	Ta (C)	Amplicon (bp)
HPRT1	NM_000194.2	Fwd - AGGACTGAACGTCTTGCTCG Rev - GAGCACACAGAGGGCTACAA	60.04 59.68	72
CSF-1	NM_000757.5	Fwd - TTAAAAGGCTGTGCCGAGGG Rev - GAGAGGACCCAGGCAAACCTT	60.61 59.60	72
TGF- β	NM_000660.6	Fwd - TATTGAGCACCTTGGGCACTG Rev - TCTCTGGGCTTGTTTCCTCAC	60.61 59.93	127
IL-10	NM_000572.3	Fwd - GGCACCCAGTCTGAGAACAG Rev - ACTCTGCTGAAGGCATCTCG	60.32 59.82	86
IL-12	NM_000882	Fwd - ATGGCCCTGTGCCTTAGTAGT Rev - AGCTTTGCATTCATGGTCTTGA	62.7 60.5	77
TNF	NM_000594.3	Fwd - GCTGCACTTTGGAGTGATCG Rev - TCACTCGGGGTTTCGAGAAGA	59.55 60.25	108
MMP-9	NM_004994.2	Fwd - ATCCAGACTTCCTCAGGCGG Rev - AGAAGCCCCACTTCTTGTCTG	59.90 59.96	92
MMP-2	NM_004530.5	Fwd - ATCCAGACTTCCTCAGGCGG Rev - ATCCAGACTTCCTCAGGCGG	61.33 58.90	71

ELISA

Macrophages were stimulated with CM from OSCC cell lines for 24 h, in the presence and absence of TGF- β type I receptor kinase inhibitor. Supernatants from macrophages were collected after 24 h for ELISA. To avoid bias from neoplastic cell-derived cytokines in the CM, an aliquot from same CM used to stimulate macrophages was also submitted to ELISA in parallel with macrophage supernatants. Concentration of target cytokines in the CM was subtracted from the concentration determined in samples of macrophage supernatants. ELISAs for TGF- β (RnD Systems, BioTechne), VEGF (RnD Systems, BioTechne), IL-10 (Peprotech Inc) and IL-6 (Peprotech Inc) were performed according to the instructions provided by the suppliers, data obtained on a multiwell plate reader (Spectramax M, Molecular Devices) and analyzed with the equipment's software (SoftMax Pro, Molecular Devices).

Chemotaxis – in vitro

The influence of tumor cell-secreted TGF- β on the dynamic interaction between OSCC cells and macrophages was assessed in a Boyden chamber experiment. The reciprocal chemoattraction was investigated by plating either OSCC cells or macrophages in the lower compartment of the transwells (12-well plates, 0.8 μ m pore polyester membranes, Corning Life Sciences Inc.) and the other cell type on the upper chamber (on top of the transwell membrane). Briefly, 1×10^5 cells (either macrophages or OSCC cells) were seeded in the lower compartment of the 24-well plate in a 0.5 mL volume of low FBS (1% vol:vol) medium. After 24 h, 3×10^4 (in a 0.2 mL volume) of the other cell type were seeded in FBS-free medium on the upper chamber. Negative and positive controls were prepared by adding the same number of cells in the upper chamber and only 1% (vol:vol) FBS-containing culture medium (negative control) or medium containing 10% (vol:vol) FBS (positive control) in the lower chamber. After 18h, the transwells were collected, non-migrating cells in on the upper side of the membrane were gently removed with a sterile cotton swab and the migrating cells in the lower aspect were stained with DAPI (0.5 μ g/mL) for 15 min, the membrane was mounted on glass slides with anti-fade aqueous mounting medium, visualized and stained nuclei on the 5 random 100X magnification microscopic fields/membrane were counted by a trained operator unaware of the experimental conditions on an inverted fluorescence microscope (Evos FI, AMG Micro).

Tumor cell invasion – in vitro

Neoplastic cell invasion in the presence and absence of macrophages was assessed in a Boyden chamber using collagen-supplemented myogel as extracellular matrix¹⁹. Neoplastic cells were previously stained with green fluorescence live cell dye (5 μ M, CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes). Macrophages were pre-treated for 4 h with 10 μ M of LY-364947 or same volume of DMSO (vehicle) as control before the 24 h stimulation with: 1. CM from OSCC cells, 2. M2-polarizing conditions, or 3. low FBS (1%, vol:vol) containing DMEM/F12 as an 'M0' / control condition. After 24 h, macrophages were collected by gentle enzymatic dissociation and stained with red fluorescence live cell dye (10 μ M, CellTracker™ Red CMTPX Dye, Molecular Probes). Macrophages and OSCC cells (matching the cell line generating CM used in the 24 h stimulation) were mixed in a 3:1 (neoplastic cells : macrophage) ratio, keeping the total number of cells constant at 4×10^4 cells/well. The

macrophage : OSCC cell mixture was seeded on top of an approximately 150 μm thick layer of myogel prepared on top of a 0.8 μm pore polycarbonate transwells. DMEM/F-12 supplemented with 1% FBS was placed in the lower compartment as chemoattractant. After 18 h, images were obtained at the center of the transwell membranes on green and red fluorescent channels in a confocal microscope (Carl Zeiss LSM 800). On the Z-axis, 17 images (image/slice# indicated on the images) were obtained with 8 μm spacing for 128 μm , starting 32 μm below the transwell membrane (identified on phase contrast at slice #5 by the presence of the polycarbonate membrane pores).

Tumor cell invasion – in vivo

The basic protocol for this assay is described by Liu et al., 2013²⁰. Commercially-acquired fertilized chicken eggs were incubated for 8 days before their use in the experiment. Briefly, the chorioallantoic membrane were exposed with the creation of a window on the eggshell that enables seeding the cells on top of the untouched chorioallantoic membrane epithelium. Macrophages were collected after treatment for 24 h with CM (in the presence and absence of TGF- β type I receptor kinase inhibitor), then mixed with the neoplastic cell lines (matching the cell line generating the CM used in the 24 h treatment) at the ratio of 3 neoplastic cells: 1 macrophage (total 5×10^5 cells/CAM). Cells were resuspended in a total final volume of 5 μL of HBSS for seeding onto the CAM. The egg shell openings were protected with a flexible wound dressing that allows for air exchange and maintained in an appropriate incubator at 37 C for 3 days for the assessment of tumor growth/invasion. After 3 days, CAM samples (upper membrane) were collected and rinsed in PBS, then fixed in 4% paraformaldehyde for 4 h at 4°C, transferred to PBS and kept at 4°C for the subsequent analyses. Upper CAMs were trimmed to include only the areas with seeded cells (neoplastic cell lines / macrophages), embedded in paraffin and semi-serial 5 μm thick sections were obtained for histological analyses. Histological sections were stained with hematoxylin and eosin for descriptive analysis and used in immunohistochemistry.

Immunohistochemistry

Briefly, after deparaffinization in two successive incubations in xylene (8 min/incubation), sections were gradually rehydrated in decreasing concentrations of ethanol and submitted to a final rehydration step in milliQ water. Activity of endogenous

peroxidase was quenched with 1% peroxide in methanol for 30 min. Sections were heated to 92-95 °C for 15 min in citrate buffer (pH 6.0) for antigen retrieval, blocked with 1% BSA in PBS (or with 10% non-immune serum from the animal species in which secondary antibodies were raised) for 1 h at room temperature, washed and incubated with the optimized dilutions of primary antibodies (**Table 2**) overnight (16 hours) at 4°C. After removal of primary antibody solution and washing, sections were incubated with HRP streptavidin (20 min at room temperature), washed again and then incubated with DAB/chromogen substrate (60 seconds). Sections were counter-stained in Mayer's hematoxylin for 30 seconds, rinsed in running water and mounted with coverslips. Visualization and digital images of the slides were obtained using a conventional microscope (Leica Microsystem GmbH, Wetzlar, Germany) coupled to a digital camera (DP71, Olympus, Tokyo, Japan) and analyzed by counting the number of positively-stained cells in 10 high-power (200X magnification) microscopic fields by a trained examiner blind to the experimental conditions.

Table 2 – Antibodies and outcomes assessed by immunohistochemistry.

Target protein	Supplier	Function	Dilution (in PBS 1X)
CD163	Novus biological, NB110-40686	M2 marker	1:100
CD68	Novus biological, NBP2-32831	M1 marker	0,5 ug/ml
Cytokeratin	Novus biological, NBP2-44951	Epithelial cells	1 ug/ml

Data Analysis

Prism 9.0 (GraphPad Software LLC) was used for statistics. The goal was to compare the outcomes of interest according to the experimental conditions, considering data distribution and type of variable (discrete, continuous, proportion). Parametric and non-parametric tests (ANOVA, Kruskal-Wallis followed by pairwise tests) were used and the significance level was set to 95% ($p < 0.05$).

RESULTS

According to the TCGA database, TGFB1 is overexpressed in tumors compared to normal tissues in HNSCC (Figure 1A). We next analyzed overall survival in HNSCC patients with high or low expression of TGFB1 (Figure 1B). Low expression of TGFB1 increased overall survival compared to patients with high expression of TGFB1. Finally, a positive correlation between high expression of TGFB1 and macrophage infiltrate in the tumor was observed (Figure 1C).

Using in vitro experiments, we initially checked constitutive expression (mRNA) and production (protein) of TGF- β by a panel of five OSCC cell lines (**Supplementary data 1**) and then the lowest effective concentration of TGF- β kinase I inhibitor, LY-364947 (**Supplementary data 2**). Based on the data for protein production, we chose to use H314 (floor of mouth, TGF- β^{high}) and SCC-9 (tongue, TGF- β^{low}) OSCC cell lines for subsequent experiments.

Efficacy of the TGF- β type I receptor kinase inhibitor in macrophages was verified by western blot and RT-qPCR (**Supplementary data 2**), and in all subsequent experiments pre-treatment of macrophages was carried out for 4 h with 10 μM of LY-364947, which was the lowest concentration that effectively reduced activation of downstream targets of TGF β IR kinase in macrophages in the experimental conditions used.

Next, we evaluated the effect of TGF- β type I receptor kinase (TGF- β RIk) signaling on the response of macrophages to CM from neoplastic cell lines. Inhibition of TGF- β RIk reduced expression of MMP-9 and IL-10 mRNA by macrophages stimulated with the CM from both H314 and SCC-9 neoplastic cell lines, indicating that tumor cell-secreted TGF- β (at both 'high' and 'low' levels) is important for the expression of these M2-associated genes by macrophages, and suggesting that inhibiting tumor cell derived TGF- β may skew macrophages towards the M1 end of the phenotype spectrum.

Interestingly, there was no influence of tumor cell-secreted TGF- β on the expression of TGFB1 mRNA by macrophages stimulated with CM from H314 cells, suggesting the absence of a positive feedback loop (**Figure 2A**). Assessment of protein production by macrophages stimulated with CM from H314 and SCC-9 cells indicated that tumor cell-secreted TGF- β is important for production of IL-10 by macrophages (**Figure 2B**). On the other hand, inhibition of TGF- β RIk increased

production of VEGF by macrophages stimulated with CM from H314 cells, indicating that H314 cell-secreted TGF- β reduces VEGF production by macrophages.

TGF- β RI inhibition in macrophages increased reciprocal chemotaxis between SCC-9 / macrophages (**Figure 3A and B**), suggesting that SCC-9 tumor cell secreted TGF- β reduced production of chemotactic molecules by macrophages and also that tumor cell-secreted TGF- β reduces macrophage chemotaxis to tumor cell-secreted products. In contrast, blocking the influence of TGF- β secreted by H314 cells did not affect the chemotaxis of these cells towards macrophages (**Figure 3A**). Furthermore, inhibiting the effects of H314-secreted TGF- β significantly decreased macrophage chemotaxis to H314 secretome (**Figure 3B**). These results indicate that inhibiting the influence of tumor cell-secreted TGF- β on macrophages can either increase or decrease reciprocal chemotactic activity, likely depending on the influence of other constituents of the tumor cell secretome.

In spite of the lack of influence on chemotaxis of H314 cells, blocking TGF- β in macrophages tended to reduce macrophage-mediated in vitro invasion of H314 cells (**Figure 4A and B**). In contrast, whereas blocking the influence of SCC-9 cell-secreted TGF- β increased chemotaxis of SCC-9 cells, it did not affect macrophage-mediated invasion of SCC-9 cells (**Figure 4C and D**). This suggests that high constitutive expression of TGF- β by cancer cells favors macrophage-mediated invasion, and we speculate that differential mechanisms are involved in TGF- β modulation of macrophage-mediated chemotactic activity and invasion.

In vivo (CAM model), blocking the influence of tumor cell-secreted TGF- β on macrophages by pre-treating with TGF- β RI inhibitor previously to seeding of the mixture tumor cells : macrophages (3:1 ratio) onto the CAM reduced tumor cell invasion in vivo (assessed histologically by the depth reached by tumor cells from the surface of the CAM epithelium) more prominently ($p=0.06$ vs vehicle) for H314 (TGF- β^{high}) cells, but not for SCC-9 (TGF- β^{low}) cells (**Figure 5A and B**). These results from in vivo invasion model support the data obtained in vitro, and further indicate that in tumor cells with high constitutive production of TGF- β , blocking the influence of TGF- β in macrophages reduces macrophage-mediated tumor cell invasion.

Inhibition of TGF- β RI markedly decreased CD68 staining in SCC-9-derived tumors (but not in H314-derived tumors); however there were no noticeable differences in CD163 or Cytokeratin staining in either in SCC-9- or H314-derived tumors (**Figures 5C-F**).

DISCUSSION

TGF- β has crucial roles in oncogenesis and tumor progression, particularly by promoting angiogenesis and EMT ²¹. In this study we investigated the influence of OSCC cell-secreted TGF- β on macrophages. The experimental approach was based on the blocking of the biological effects of TGF- β , as a constituent of tumor cell secretome, on macrophages by inhibiting TGF- β type I receptor kinase (TGF- β RIk). We used two OSCC cell lines with contrasting levels of constitutive production of TGF- β : H314 (high expression of TGF- β , TGF- β^{high}) and SCC-9 (low expression of TGF- β , TGF- β^{low}). This approach allowed for the use of the exact same, undisturbed, tumor cell secretome aliquot when stimulating macrophages. In co-culture experiments, macrophages were pre-treated with TGF- β RIk to block the biological effects of TGF- β secreted by OSCC cells.

Blocking the effects of tumor cell-secreted TGF- β in macrophages attenuated traits of the anti-inflammatory (M2-like) phenotype observed in response to exposure to the secretome of neoplastic cells. This indicates the relevance of TGF- β as a tumor cell-derived immunosuppressive mediator and is in agreement with the role of TGF- β in CD8+ T cells ²². Interestingly different mechanisms were involved in this attenuation of suppressive phenotype by blocking the influence of tumor cell-derived TGF- β in macrophages: in the presence of TGF- β^{low} SCC-9 secretome, blocking the influence of TGF- β skewed macrophages towards a M1-like phenotype, with increased expression of IL-12 and decreased expression of TGFB1. On the other hand, in the presence of TGF- β^{high} H314 secretome, blocking the influence of TGF- β in macrophages reduced expression of IL-10 and MMP-9. Besides the possibility of a partial efficacy of TGF- β RIk inhibitor in the presence of a greater concentration of TGF- β in the secretome of H314 cells, it is also possible that even partially blocking the effects of TGF- β allowed for a greater influence of other biologically active molecules in the secretome from H314 cells.

Gene expression data is supported by protein production, as inhibiting the influence of tumor cell-secreted TGF- β from both OSCC cell lines reduced IL-10 production by macrophages. On the other hand, production of VEGF induced by tumor cell secretome in macrophages was increased with blocking the effects of TGF- β in the secretome of H314 TGF- β^{high} , which may be related with the enhancement of the influence of other molecule constituents produced by H314 cells. A study using bone marrow primary macrophages treated with CM from tongue squamous cell carcinoma

for 24 h shows an increase of VEGF. Subsequently, they demonstrate that neutralizing TGF- β 1 led to a decreased VEGF expression. Finally, they demonstrate that TGF- β increased VEGF secretion via TGF β RII/Smad3 signaling ¹³. This discrepancy with our results may be associated with the use of murine or human macrophages, different OSCC cell lines used and to the distinct approaches in inhibiting the influence of tumor cell-secreted TGF- β , namely neutralizing antibodies or biochemical inhibitor of TGF β IRk. Furthermore, in this study we assessed protein production by macrophages using cell lysates, not the secreted products to avoid introduction of bias by tumor cell-secreted proteins in the culture supernatants.

Co-culture of murine macrophages with a colon cancer cell line increased migration of cancer cells compared to macrophage alone. In the same study, was confirmed that cell secreted products from macrophages and cancer cell lines promoted a reciprocal chemotaxis in vitro ²³, which we also investigated. This reciprocal macrophage/tumor cell chemotaxis may be relevant both for tumor growth (and shaping the tumor microenvironment) and progression (in macrophage-mediated tumor cell invasion). Blocking the influence of TGF- β reduced chemotaxis of macrophages to secreted products from TGF- β^{high} H-314 but not of H314 cells to macrophage-secreted products. In contrast inhibiting the effects of TGF- β in macrophages increased reciprocal chemotaxis between TGF- β_{low} SCC9 and macrophages. Migrating macrophages can enhance the speed with which of tumor cells travel in the stroma, whereas sessile/non-migrating perivascular macrophages may facilitate intravasation, dissemination and extravasation of tumor cells in the metastatic site. This was demonstrated in a mouse model of breast cancer, in which macrophages were recruited to the TME via CCR2 and then transitioned to the perivascular region in the TME by CXCR4 up-regulated by tumor cell-produced TGF- β responding to CXCL12 produced by fibroblasts ²⁴.

It is tempting to speculate that TGF- β has an important immunosuppressive effect in tumors with high or low expression of TGF- β , and that inhibiting its influence in macrophages may attenuate the pro-tumoral M2-like phenotype. The experimental approach of inhibiting the biological effect of tumor cell-secreted TGF- β on macrophages using a TGF- β IRk inhibitor did not interfere with other biologically-active molecules secreted by tumor cells, as the goal was to assess the relative importance of TGF- β as a component of the tumor cell secretome on the phenotype of macrophages and on macrophage-mediated tumor cell invasion. This approach is

limited as multiple other biologically-active molecules may have either synergistic or antagonist effects on these outcomes; however it allows insight into the potential of targeting TGF- β as a single tumor cell-derived target. Even though using rhTGF- β as a single stimulus on macrophages would allow for the investigation of the biological mechanisms involved in the modulation of macrophage phenotype by this growth factor, it would not account for the complexity of neoplastic cell secretome in which even minute concentrations of TGF- β can still have a biological impact on immune cells²². By attenuating the M2-like phenotype, targeting the effect of tumor cell-secreted TGF- β may further enhance the rescue of anti-tumor immunity in other therapeutic approaches, as reported for CD8⁺ T cells. Subsequent studies may assess the synergistic effect of blocking TGF- β in the tumor microenvironment in association with other therapeutic approaches, particularly with immune checkpoint inhibitors.

The secretome in oral cancer ultimately determines the characteristics of the tumor microenvironment and phenotype of immune infiltrating cells, including macrophages. The results indicate that TGF- β as a component of OSCC secretome, at both low and high concentrations, has a prominent role in inducing an M2-like, pro-tumoral phenotype in macrophages. Blocking the influence of tumor cell-secreted TGF- β in macrophages reduced macrophage-mediated invasion of TGF- β^{high} H314 cells, but not of TGF- β^{low} SCC-9 cells, indicating that the relevance of TGF- β as a component of tumor cell secretome can vary in diverse tumor microenvironments. This highlights the relevance of a personalized approach in choosing novel treatment strategies, as opposed to the ‘tumor size determines all’ approach with cytotoxic-based chemo/radiotherapies.

We speculate that the limited success of TGF- β kinase inhibitors may relate with the heterogeneity in the composition of the tumor cell secretome and the lack of specificity in targeting a given cell type in vivo. This may result in serious adverse effects, as TGF- β has an important role in homeostasis.

In summary, our data indicates that inhibition of TGF- β RI kinase in macrophages only reduces macrophage-enhanced invasion TGF- β^{high} OSCC cells, but attenuated the pro-tumoral, M2-like phenotype induced in macrophages by tumor cell-secreted products.

FIGURES

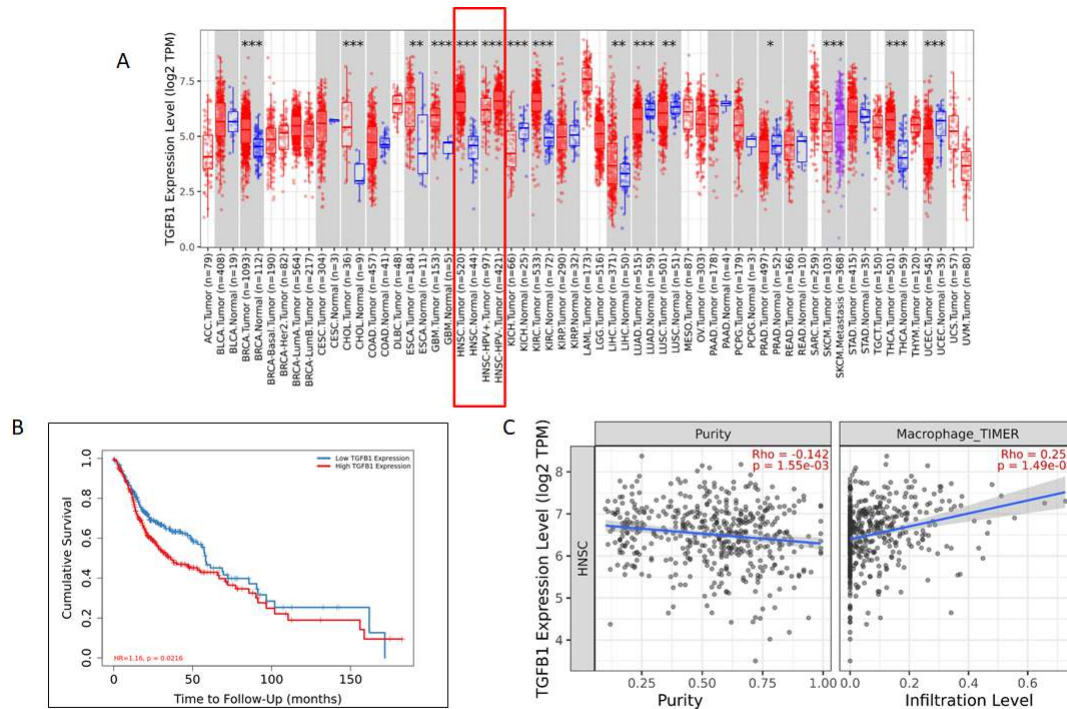


Figure 1 - TGFB1 is associated with a significant reduction in overall survival (A) Box plots of differential expression of TGFB1 between HNSCC tumor and adjacent normal tissues in the TCGA database. The statistical significance computed by the Wilcoxon test is annotated by the number of stars (*: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001). (B) Kaplan-Meier plot shows the difference of overall survival among HNSCC patients stratified with high or low expression of TGFB1. (C) Scatter plots of depicting positive correlation between TGFB1 expression with tumor purity (left) and the infiltration of macrophages in HNSCC estimated by TIMER (right).

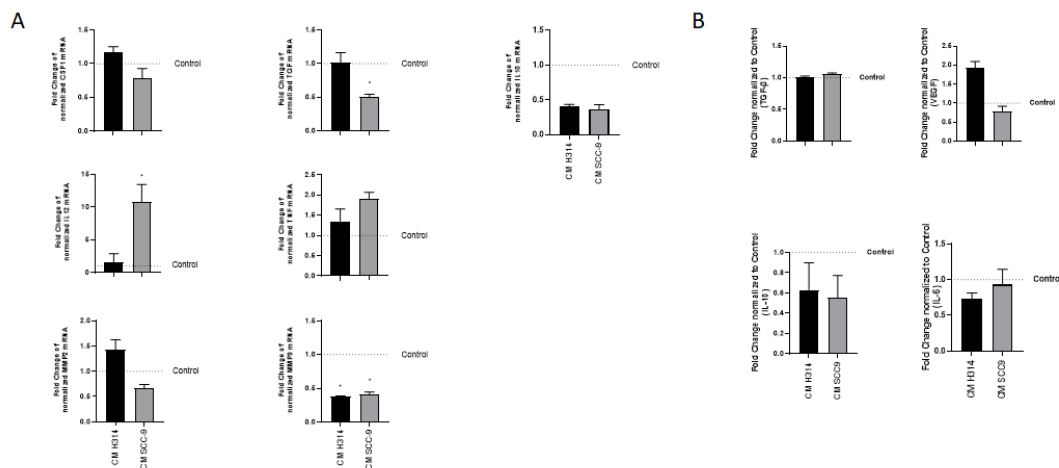


Figure 2 – Influence of TGF- β RIK activity on regulation of selected candidate gene expression and protein production by macrophages treated with CM from OSCC cells with different constitutive production of TGF- β . Macrophages were pre-treated for 4 h with TGF- β RIK inhibitor (LY-364947, 10 μ M) or with the same volume of DMSO vehicle ('control') and subsequently stimulated with CM from H314 and SCC-9 OSCC cells for 24 h. **(A)** mRNA extracted from macrophages was used to synthesize cDNA and relative expression of candidate genes of interest was determined by qPCR. **(B)** Supernatants from macrophages stimulated for 24 h with CM from OSCC cells were used to assess TGF- β , VEGF, IL-10 and IL-6. As 'background' control of neoplastic cell-derived cytokines in the CM, an aliquot from the same neoplastic cell CM used to stimulate the macrophages was assessed in parallel by ELISA along with macrophage supernatant samples. The concentration of target cytokines in the CM aliquot was subtracted from the results of macrophage supernatants. ELISAs were performed according to the supplier's instructions. Data show relative change (fold change) indicative of regulation of gene expression **(A)** or protein production **(B)** in the presence of TGF- β RIK inhibitor. In both **(A)** and **(B)**, graphs show data from three independent experiments analyzed in duplicate.

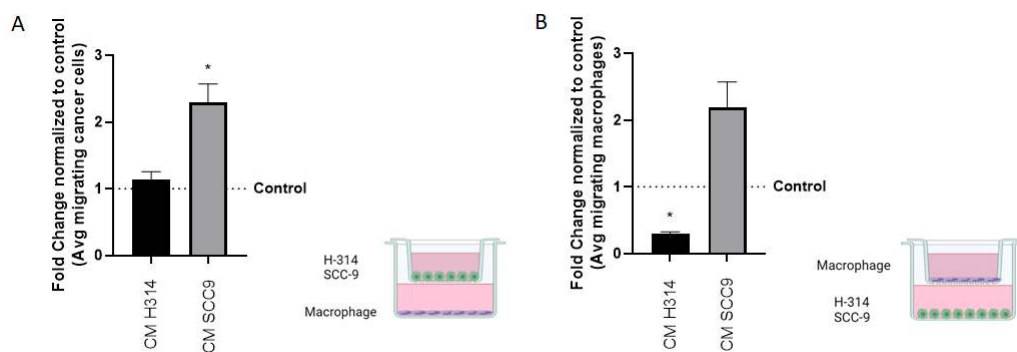


Figure 3 – Influence of tumor cell-secreted TGF- β on chemotaxis of OSCC cells to macrophages and of macrophages to OSCC cells. **(A)** Macrophages were plated on the lower compartment of transwells and treated for 4 h with TGF- β RIK inhibitor (LY-364947, 10 μ M) or with the same volume of DMSO vehicle and subsequently stimulated with CM from H314 and SCC-9 cell lines for 24 h. H314 or SCC-9 cells (3×10^4 /transwell) resuspended in DMEM/F12 with low (1%, vol:vol) FBS were then seeded on the upper compartment of the transwell. **(B)** SCC-9 or H314 cells (1×10^5 cells/well of a 24-well plate) were seeded on the lower compartment of the transwell in DMEM/F-12 supplemented with 10% FBS. After 24 h, medium was removed, cells were washed extensively with PBS and cultured for an additional 24 h in DMEM/F-12 containing 1% (vol:vol) FBS. 3×10^4 macrophages pre-treated for 4 h with TGF- β RIK inhibitor (LY-364947, 10 μ M) or with the same volume of DMSO vehicle and

subsequently stimulated with CM from H314 and SCC-9 cell lines for 24 h were seeded on the upper chamber. In both **(A)** and **(B)**, after 18 h, cells on the upper side of the transwell were gently removed with sterile cotton swabs and the membrane stained with DAPI. The nuclei of migrating cells were counted on an inverted fluorescence microscope by a trained examiner unaware of the experimental conditions. Data is presented as regulation (fold change) of OSCC **(A)** or macrophage **(B)** chemotaxis activity by blocking of the influence of tumor cell-secreted TGF- β in macrophages with LY-364947 pre-treatment. In both **(A)** and **(B)**, graphs indicate average and standard deviation of three independent experiments performed in duplicate. * indicates $p < 0.05$ and p values from analysis of variance followed by Tukey test for comparisons with the control (Vehicle + CM).

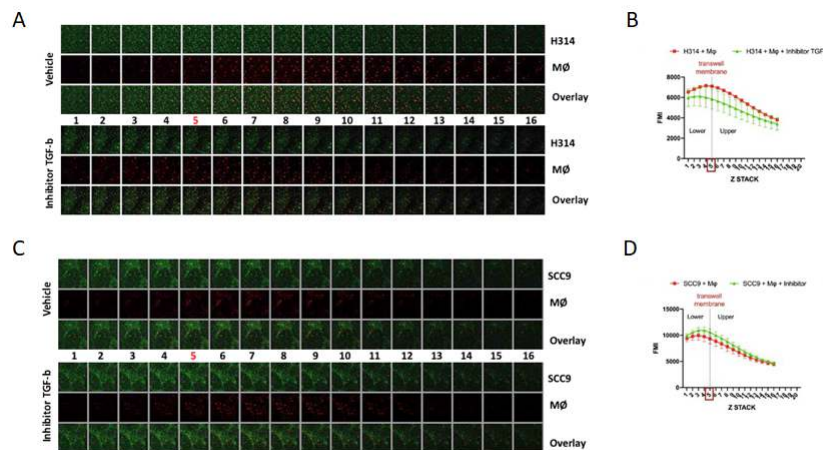


Figure 4 – Influence of tumor cell-secreted TGF- β on macrophage-mediated invasion of H314 and SCC-9 cells in vitro. **(A)** and **(C)** Representative images obtained with the confocal microscope at each slice in the Z-axis (spanning 128 μ m of the thickness of myogel). On the top images, macrophages were pre-treated with DMSO vehicle and on the bottom with 10 μ M of LY-364947 for 4 h before stimulation with CM from H314 or SCC-9 cells for 24 h. Macrophages and cancer cells were harvested and separately stained with red and green fluorescent live cell dyes (CellTracker™ Red CMTPX Dye and CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes), respectively. OSCC cell lines and macrophages were combined at a 3:1 ratio and then seeded on top of myogel. After 18 h, tumor cells and macrophages were visualized on a confocal microscope in the green and red channels, respectively. **(B)** and **(D)** Quantitation of median fluorescence intensity (MFI) in the green channel (tumor cells) at each of the 16 Z-axis images (image/slice #5, highlighted in red and with the vertical dotted line, corresponds to the level of the transwell membrane: the left of the dotted line corresponds to the lower portion of the membrane the right, the upper portion / myogel layer). Fluorescence intensity is directly proportional to the number of invading H314 or SCC-9 cells. Data on graph represents average and standard deviation of MFI in three independent experiments.

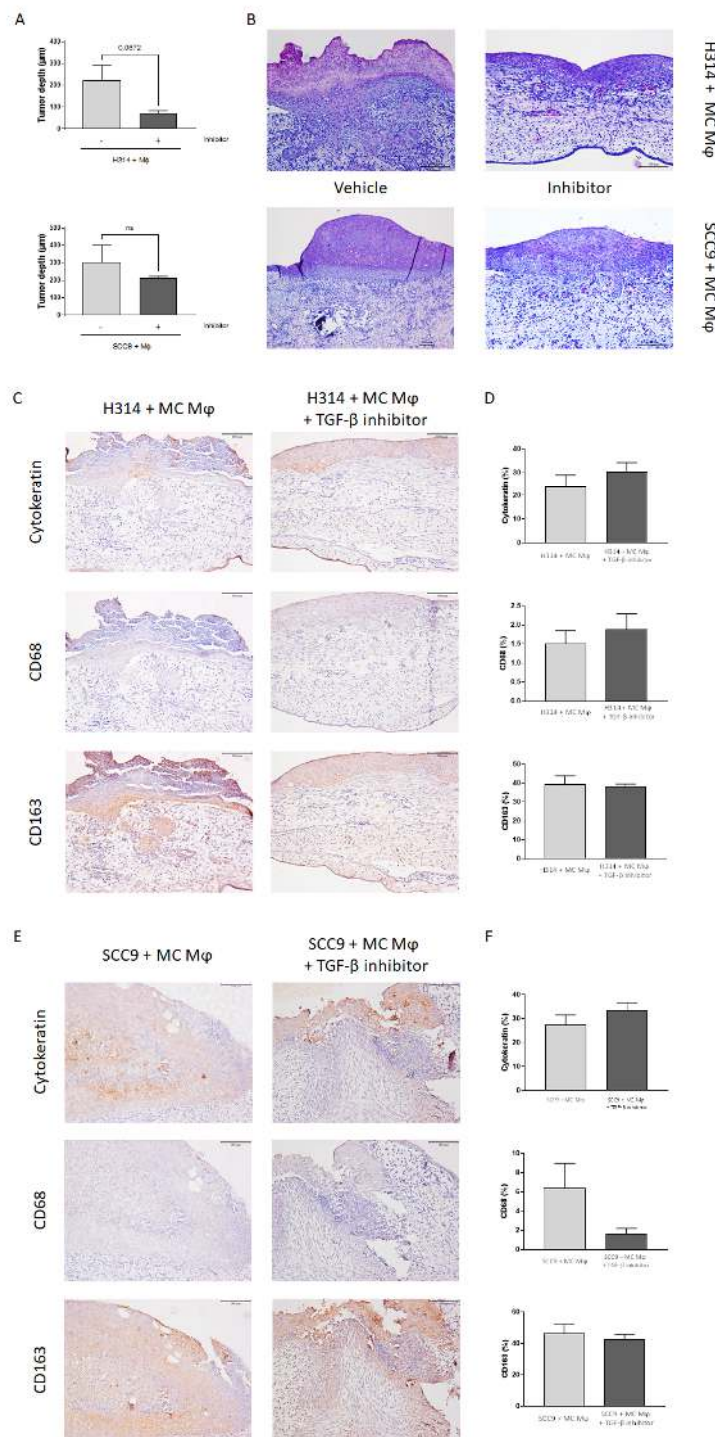
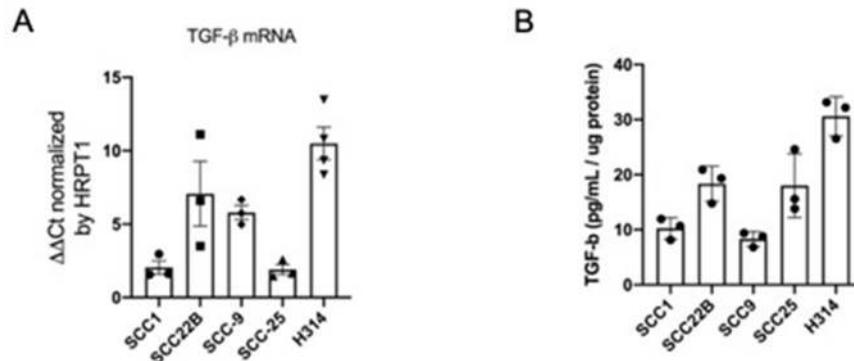


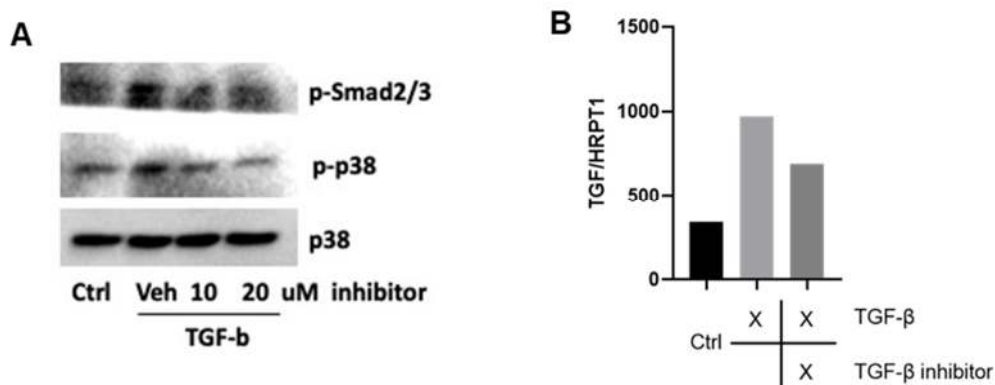
Figure 5 – Influence of blocking the influence of tumor cell-secreted TGF- β on macrophages on macrophage-mediated tumor cell invasion in vivo (CAM model). Macrophages were treated with DMSO vehicle of LY-364947 (10 μ M) for 4 h before a 24 stimulation with CM from the OSCC cells. Macrophages and neoplastic cells were then combined at a 1:1 ratio and a total of 5×10^5 cells were seeded onto the CAMs, which were harvested after 3 days. **(A)** Averages and standard deviations of tumor cell depth measured in H/E-stained sections of tumors derived from H314 and SCC-9 cells. Data are expressed as the mean $\pm$ standard error of the mean. Unpaired Student t test of with Welch's correction for unequal variances. data from 4 different CAMs for each experimental condition. **(B)** Representative H/E-stained sections of CAM with H314/macrophage and SCC-9/macrophage-

derived tumors. **(C)** and **(E)** Immunohistochemical staining of CD68, CD163 and Cytokeratin in H314- and SCC-9-derived tumors in vivo (CAM model) in the presence of macrophages with and without inhibition of TGF- β RIk **(D)** and **(F)** Semi-quantitative analysis of expression of each candidate protein of interest in sections of H314- and SCC-9-derived tumors in vivo. Graphs indicate average and standard deviations of 3 sections from each of 4-5 different CAMs in each experimental condition.

Supplementary data



Supplementary data 1 – Assessment of constitutive expression of TGF-β by a panel of five OSCC cell lines. (A) RT-qPCR assessment of the expression of TGFB1 by unstimulated neoplastic cell lines. (B) Normalized production of TGF-β by unstimulated neoplastic cell lines assessed by ELISA. Data presented from three independent samples (RNA, conditioned medium) collected from 3 non-consecutive passages of the indicated cell lines, assessed in duplicate. Symbols indicate individual experiment data points on the bars of the graphs.



Supplementary data 2 – Efficacy of TGF-β type I receptor kinase inhibitor in macrophages. (A) Effectiveness of LY-364947 to inhibit TGF-β type I receptor signaling in PMA-differentiated THP-1-derived macrophages. Macrophages were treated with LY-364947 (10 and 20 μM) or the equivalent volume of DMSO vehicle ('Veh') for 4 h, washed with PBS and stimulated with 2 μM of rhTGF-β for 24 h. 20 μg of total protein from whole cell lysates were used in SDS-PAGE for detection of activated (phosphorylated) forms of Smad2/3 and p38 MAPK (downstream targets of TGFb type I receptor kinase). (B) Expression of TGFB1 mRNA by PMA-differentiated THP-1-derived macrophages. Cells were treated for 4 h with TGF-β type I receptor kinase inhibitor (LY-364947, 10 μM) or with the same volume of DMSO vehicle and subsequently stimulated with 2 μM of rhTGF-β for 24 h. mRNA extracted from cell lysates was used to synthesize cDNA and relative expression of TGFB1 and HPRT1 was determined by qPCR. Data from a single experiment assessed in duplicate.

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3.3 Publicação 3*

GALR2 expression by OSCC cells influences macrophage phenotype and macrophage-mediated tumor cell invasion

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Abstract

Increased signaling via GALR2 has been shown to promote tumor growth, which supports expression of this receptor as a marker of tumor aggressiveness and as a possible therapeutic target to inhibit progression of Head and neck squamous cell carcinoma (HNSCC). Recently there has been great interest in studying the interaction of tumors with the immune system, to recover or enhance anti-tumor immune activity. The relevance of macrophages in the tumor microenvironment (TME) of HNSCC derive from its relative abundance, representing up to 50% of the tumor mass, and also from the inverse correlation between macrophage infiltrate and prognosis and disease survival. In the TME, macrophages are known as tumor-associated macrophages (TAM) and their phenotype is determined by molecules in the TME, especially neoplastic cell-secreted products. In this project, we used four transgenic cell lines from SCC-9 and H-314 cell lines (SCC-9 pcDNA/GALR2 and H314 pcDNA/GALR2). Differences in tumor cell constitutive expression of TGF- β and CSF-1 and mRNA half-life were determined in vitro. To determine the influence of increased expression of GALR2 in tumor cells on macrophage phenotype and biology, THP-1-differentiated macrophages were exposed to the secretome of GALR2-overexpressing and control neoplastic cell lines. Our results demonstrate that secreted products from GALR2-overexpressing neoplastic cells had discrete effects on macrophage gene expression, and modulated production of proteins relevant for tumor progression, such as IL-10, EGF and VEGF.

Keywords: Receptor, Galanin, Type 2, Squamous Cell Carcinoma of Head and Neck, Tumor-Associated Macrophages

Introduction

Galanin, originally identified as a neuropeptide, has been implicated in multiple physiological and pathological processes ¹. These include biological effects in the nervous system (neural stem cells and non-neuronal cells in the brain) and extend to endocrine systems, metabolism, energy homeostasis, and paracrine effects in bone tissue ². Galanin regulates cell activity by binding to three G-protein coupled receptors: galanin receptors 1, 2 and 3 (GALR1, GALR2 and GALR3) ^{1,3}. Galanin production is increased in cells overexpressing GALR2 in an auto-regulatory loop.

Increased expression of GALR2 in head and neck cancer (HNC) cells correlates with increased activation of the small GTPase Rap1 ⁴, which triggers various downstream signaling pathways, including p38 and ERK MAPK and PI3K/Akt. These Rap1-influenced signaling pathways, particularly MAPKinases (most notably p38 and ERK), are associated with post-transcriptional regulation (via increased mRNA stability) of various cytokines and growth factors ⁵, including IL-6, TNF, VEGF and COX-2 of relevance in HNC progression ^{6,7}.

In the tumor microenvironment (TME), TGF- β and CSF-1 are important mediators of the M2-like phenotype of tumor-associated macrophages (TAMs) ⁸; and both growth factors are partly post-transcriptionally regulated by mRNA stability ⁹. It is not known if increased GALR2 activity in HNC cells enhances expression of TGF- β and CSF-1 by increasing mRNA stability. Regulation of CSF-1 expression is complex and involves different posttranscriptional mechanisms, including regulation of mRNA stability by interaction of RNA-binding proteins and miRNAs with its 3'UTR region ⁹⁻¹¹. TGF- β also has complex regulation and involves both transcriptional and posttranscriptional regulation, which may include repression of translation and mRNA stability and degradation mediated by cis-regulatory sequences in both the 5' and 3'UTRs ¹²⁻¹⁴. Data from various cell types indicate that TGF- β is auto-regulated by post-transcriptional mechanisms (positive feedback loop) and also participates in post-transcriptional regulation of various other genes of relevance for tumor progression, including collagen (increase), MMPs 2 and 9 (increase), IL-6 (increase), IL-12 (decrease), IFN-gamma (decrease), nerve growth factor (increase), iNOS (decrease), COX-2 (increase) ¹⁵.

Importantly, targeting common gene regulation mechanisms (such as mRNA stability or specific signaling pathways) regulated by GALR2 may affect the expression of various other biological mediators involved in diverse aspects of tumor growth and

progression, including angiogenesis, extracellular matrix degradation ¹⁶ and suppression and subversion of the immune response.

In summary, this information indicates that GALR2-mediated activation of signaling pathways in HNC cells may affect the expression of multiple genes of relevance for tumor growth, including TGF- β and CSF-1, which are associated with immunomodulation of macrophages in the TME. The central hypothesis of this study is that GALR2 expression/activity in HNC cells increases TGF- β and CSF-1 production by tumor cells. Thus, increased activity of GALR2 can function as a tumor promotor regulating expression of mediators that enhance the pro-tumoral phenotype of macrophages and favor tumor progression.

Material and Methods

Cell lines

We used a human monocytic cell line (THP-1, cat# TIB-202, ATCC) as a source of macrophages. These monocytic cells were maintained in RPMI1640 culture medium supplemented with Penicillin/Streptomycin (100 IU/mL, 100 ug/mL, respectively) and heat-inactivated fetal bovine serum (FBS) at 10% (vol:vol). Macrophages were obtained by stimulation with phorbol 12-myristate 13-acetate (PMA, 10 ng/mL) for 24h. After 24h, culture medium was replaced for complete RPMI without PMA and supplemented with 10% (vol:vol) of heat-inactivated FBS for 72h ¹⁷.

Oral cancer cell lines H314 and SCC-9 (obtained commercially from ECACC and via a material transfer agreement, respectively) were maintained in DMEM/F12 culture medium supplemented with Penicillin/Streptomycin (100 IU/mL, 100 ug/mL, respectively), 400 ng/mL sodium hydrocortisone succinate and 10% (vol:vol) heat-inactivated FBS.

Transgenic cell lines overexpressing GALR2 (denominated 'GALR2 cells') were prepared by stable transfection with a plasmid construct containing a CMV-driven human GALR2 ORF. Controls (denominated 'pcDNA cells') were transfected with the same 'empty' (no sequence cloned in the ORF) plasmid vector (pcDNA3.1, Invitrogen Corp). The stable cell lines were selected over 10 passages in geneticin (resistance gene included in the plasmid construct), with overexpression verified by RT-qPCR and western blot. These cell lines were maintained under selective pressure in 150 ug/mL geneticin (G418, Invitrogen Corp). All cell lines were checked for overexpression of GALR2 by western blot every 6 months.

Preparation of conditioned medium (CM)

GALR2 and pcDNA cells were seeded in 100 mm culture dishes at empirically-determined densities to reach 70% confluency in 24 h and then washed in blank (i.e., non-supplemented) culture medium three times over 6 h (once every 2 h). After washing, cells were incubated for 16-18 h in reduced volume of culture medium (4 mL in 100 mm dish) of blank DMEM/F12. Conditioned medium (CM) was collected, centrifuged (10,000 RPM, 10 min, 4°C), aliquoted and stored for up to 2 weeks in a -80°C freezer.

RNA stability

Stability of mRNA was assessed by reverse pulse-chase experiments¹⁸ using 5 µg/mL of actinomycin-D to inhibit RNA polymerase, preventing transcription. Briefly, 5x10⁵ GALR2-overexpressing or pcDNA cells were plated in 35 mm cell culture dishes and after 16-18 h, treated with actinomycin-D at 0, 1, 2, 4, 6, 8 and 12 h. At the end of the 12 h period, RNA was isolated from cell lysates (as described in RT-qPCR section below) and expression of TGFB1 and CSF-1 mRNA at each experimental period were detected by RT-qPCR (see below), normalized to the expression of HPRT1 RNA (long-lived mRNA, > 8 h half-life). Results of normalized TGFB1 and CSF-1 mRNA are plotted as a percentage of the normalized expression level in the samples treated with actinomycin-D at 12 h.

RT-qPCR

Total RNA was isolated using an affinity column system, according to the supplier's instructions (RNAqueous total RNA isolation kit, Ambion/Thermo Fisher Scientific). cDNA was synthesized using random hexamers as primers (High Capacity cDNA synthesis kit, Applied Biosystems) and used in qPCR reactions with the SYBR Green system (Power UP SYBR Green, Applied Biosystems) and species- and target-specific primers. The 20 µL qPCR reactions were performed in a StepOne Plus qPCR thermocycler (Applied Biosystems) using pre-optimized cycling conditions and followed by a melting curve. Primers designed using NIH/NCBI algorithm (Primer-Blast) and RefSeq-deposited sequences in the NCBI database (**Table 1**) for target genes characteristic of distinct macrophage phenotypes. Expression of a constitutive housekeeping gene (HPRT1) unaffected in the experimental conditions (determined after appropriate validation¹⁹) was selected to normalize the results. Analysis were performed using the $\Delta(\Delta C_t)$ method.

Table 1 – Human target-specific RT-qPCR primers designed using the NIH/NCBI BLAST algorithm.

Gene symbol	Accession#	Sequence (5'-3')	Ta (C)	Amplicon (bp)
HPRT1	NM_000194.2	Fwd - AGGACTGAACGTCTTGCTCG Rev - GAGCACACAGAGGGCTACAA	60.04 59.68	72
CSF-1	NM_000757.5	Fwd - TTAAAAGGCTGTGCCGAGGG Rev - GAGAGGACCCAGGCCAACTT	60.61 59.60	72
TGF- β	NM_000660.6	Fwd - TATTGAGCACCTTGGGCACTG Rev - TCTCTGGGCTTGTTTCCTCAC	60.61 59.93	127
IL-10	NM_000572.3	Fwd - GGCACCCAGTCTGAGAACAG Rev - ACTCTGCTGAAGGCATCTCG	60.32 59.82	86
TNF	NM_000594.3	Fwd - GCTGCACTTTGGAGTGATCG Rev - TCACTCGGGGTTCGAGAAGA	59.55 60.25	108

Multiplex assays

To assess protein production by macrophages after treatment for 24 h with CM, macrophages were stimulated with CM from OSCC cell lines for 24 h, and protein transport inhibitor (Brefeldin A, 10 μ g/mL) was added in the last 4 h. Cell lysates were harvested, quantitated and 20 μ g were used for the multiplex assay to determine production of 14 cytokines relevant for tumor growth and invasion, including IL-1, IL-6, TNF, IL-12, IL-10, MMP-2, MMP-9, Leptin receptor, CD163 (RnD Systems, BioTechne). Briefly, the samples were processed according to the instructions of the supplier of the reagents and data was acquired in a Luminex 200 system (Invitrogen, ThermoFisher Scientific) and analyzed using Milliplex software (Millipore, Sigma-Aldrich Co).

Tumor cell invasion – in vitro

The influence of macrophages on neoplastic cell invasion was investigated in vitro using direct co-cultures of tumor cells and macrophages. Human leiomyoma extracellular matrix (myogel)²⁰ supplemented with collagen was used as extracellular matrix in these experiments. OSCC cells were collected and stained with green fluorescent live cell dye (5 μ M, CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes). Macrophages were pre-treated for 24 h with CM from the same OSCC cell

line used in the co-culture, M2 or M0 conditions, collected by gentle enzymatic dissociation and stained with red fluorescence live cell dye (CellTracker™ Red CMTPX Dye, Molecular Probes). After staining and washing, macrophages and OSCC cells were mixed in ratio 3:1 (neoplastic cells : macrophage) or transgenic cells alone, maintaining the total number of cells constant at 4×10^4 cells seeded on top of an approximately 150 μm thick layer of myogel prepared on top of a polycarbonate membrane with 0.8 μm pores in a 24-well transwell. DMEM/F-12 supplemented with 1% FBS was placed in the lower compartment as chemoattractant. After 18 h, images from the center of the membranes were obtained in the green and red fluorescent channels using a confocal microscope (Carl Zeiss LSM 800). On the Z-axis, 17 images (image/slice# indicated on the images) were obtained with 8 μm spacing for 128 μm , beginning 32 μm below the transwell membrane (identified on phase contrast images by the presence of the 0.8 μm pores at the slice #5).

Results

Initially, we prepared six transgenic cell lines H314, SCC-25 and SCC-9 (GALR2-overexpressing and respective empty-vector controls). We chose to use four transgenic cells (SCC-9 pcDNA and SCC-9 GALR2; and H314 pcDNA and H314 GALR2) based on the presence of a positive feedback loop between GALR2 overexpression and expression of Galanin mRNA, which indicates increased activity of GALR2 in these cells (**Figure 1**).

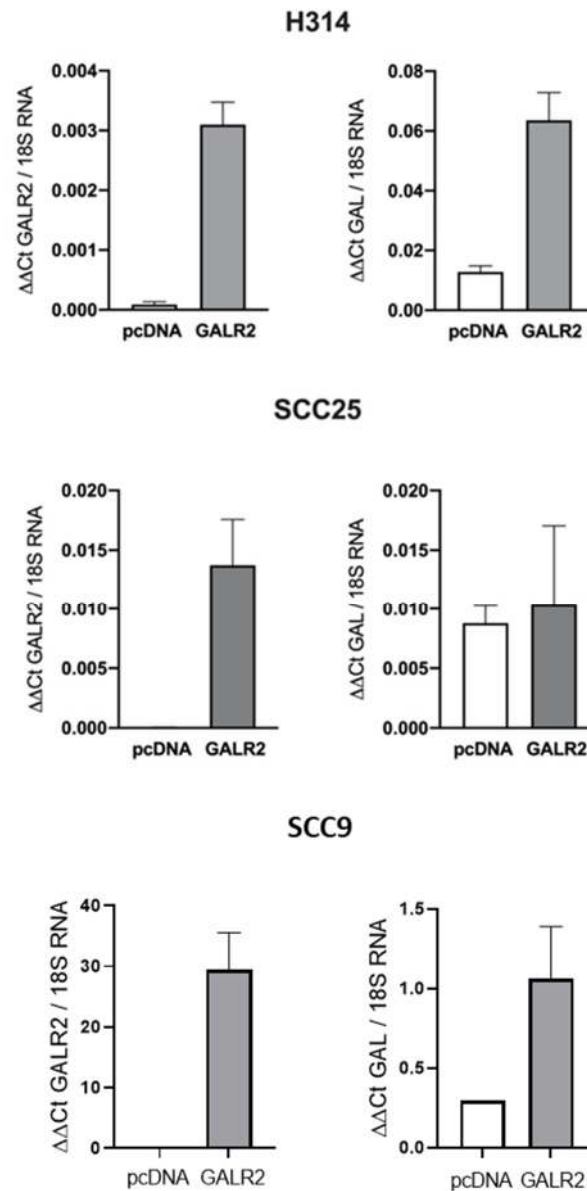


Figure 1 – Transgenic cell lines with overexpression of GALR2 and their respective empty-vector controls established from H314, SCC9 and SCC25 OSCC cell lines. RT-qPCR for GALR2 and Galanin expression. Representative data from three independent experiments analyzed in duplicate.

In both H314 and SCC-9 cells, overexpression of GALR2 did not increase the production of either TGF- β or CSF-1 (**Figure 2A**). In fact, in H314-GALR2, there was a decrease in the half-life of TGF β 1 and CSF-1 mRNAs (**Figure 2B**).

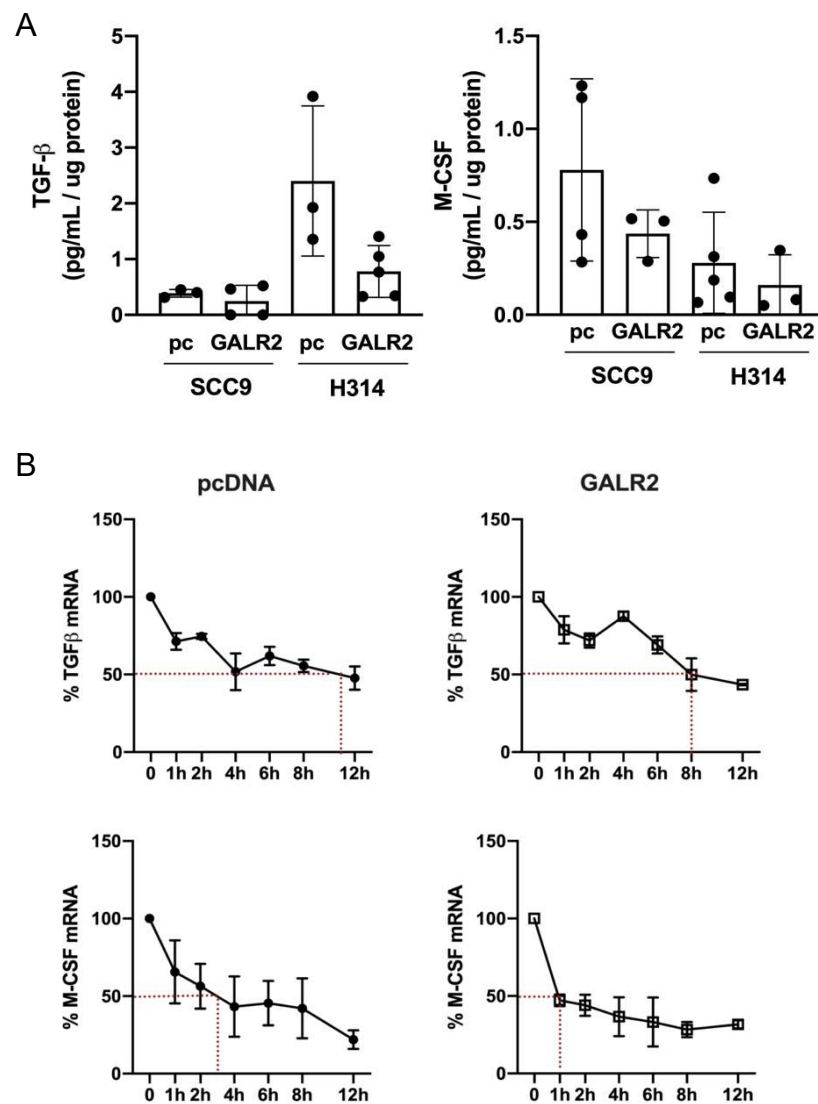


Figure 2 – Effect of GALR2 overexpression on protein production and mRNA stability of CSF1 and TGF β 1 in OSCC cells. (A) Normalized concentrations of CSF1 and TGF β in cell culture supernatants from transgenic SCC9 and H314 cells. In H314 cells, overexpression of GALR2 unexpectedly reduced both TGF β 1 and CSF1. (B) Actinomycin-D reverse pulse-chase experiments showing that overexpression of GALR2 reduced the half-life of TGF β 1 (from 11 to 8h) and CSF1 (from 3 to 1h) mRNAs in H314 cell line. Data from a minimum of three independent experiments for each condition/timepoint. For mRNA stability experiments cells were treated with 2.5 μ g/mL of actinomycin-D.

Next, we assessed how overexpression of GALR2 in OSCC cells affected macrophages. Stimulation of macrophages with secreted products ('CM') from these transgenic cell lines influenced the phenotype of macrophages, and the most noticeable changes were increased mRNA of TGF β 1, IL10 and TNF by macrophages stimulated with CM from H314-GALR2 (but not H314-pcDNA) cells. Only CSF1 mRNA was increased by CM from SCC-9-pcDNA cells (but not CM from SCC-9-GALR2 cells) (**Figure 3**). However, CM from SCC-9 cells overexpressing GALR2 had prominent (i.e.,

relative change of 30% or more in comparison to controls) effects on protein production by macrophages, as IL-10, VEGF and EGF were increased, whilst IL-6 and Leptin R were reduced. Macrophages exposed to CM from H314-GALR2 cells reduced production of TNF, IL-1 β and of the chemokines CCL2, 3, 4 and CX3CL1 (**Figure 4**).

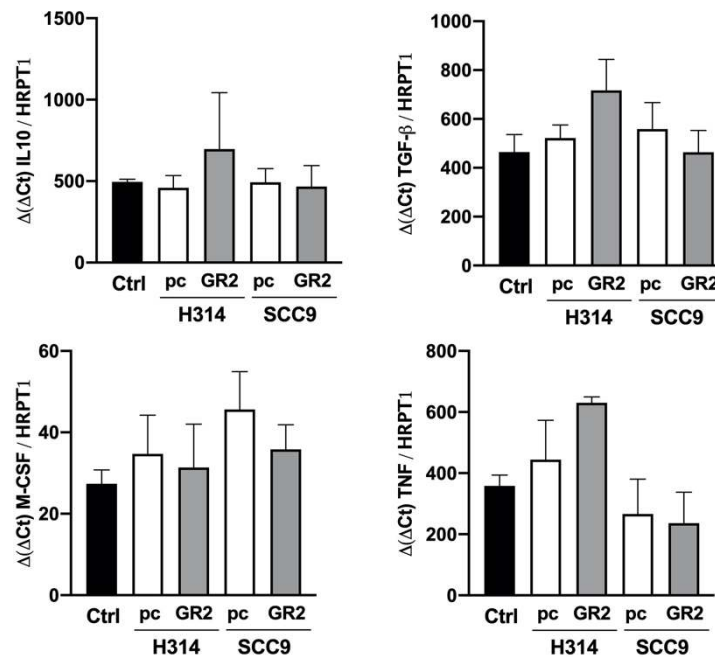


Figure 3 – Effects of secreted products from neoplastic cells overexpressing GALR2 on candidate gene expression by macrophages. PMA-differentiated macrophages were cultured in the presence of secreted products (CM) from SCC-9 pcDNA/GALR2 and H314 pcDNA/GALR2 cells for 24 h. RNA was extracted from cell lysates and used for RT-qPCR for the indicated target genes. Bars and vertical lines are averages and standard deviations of three independent experiments assessed in duplicate.

This data indicates that secreted products from GALR2-overexpressing OSCC cells influence macrophage phenotype. To assess if GALR2 affected tumor cell biology, initially we compared in vitro invasion of the OSCC cells overexpressing GALR2. Invasion of H314 cells was not changed with overexpression of GALR2, but in SCC-9 cells overexpression of GALR2 markedly reduced in vitro invasion. In the presence of macrophages pre-conditioned with the CM from the OSCC cells, invasion of H314-GALR2 cells was discretely enhanced, whilst there was no influence on invasion of SCC-9-GALR2 cells (**Figures 4 and 5**).

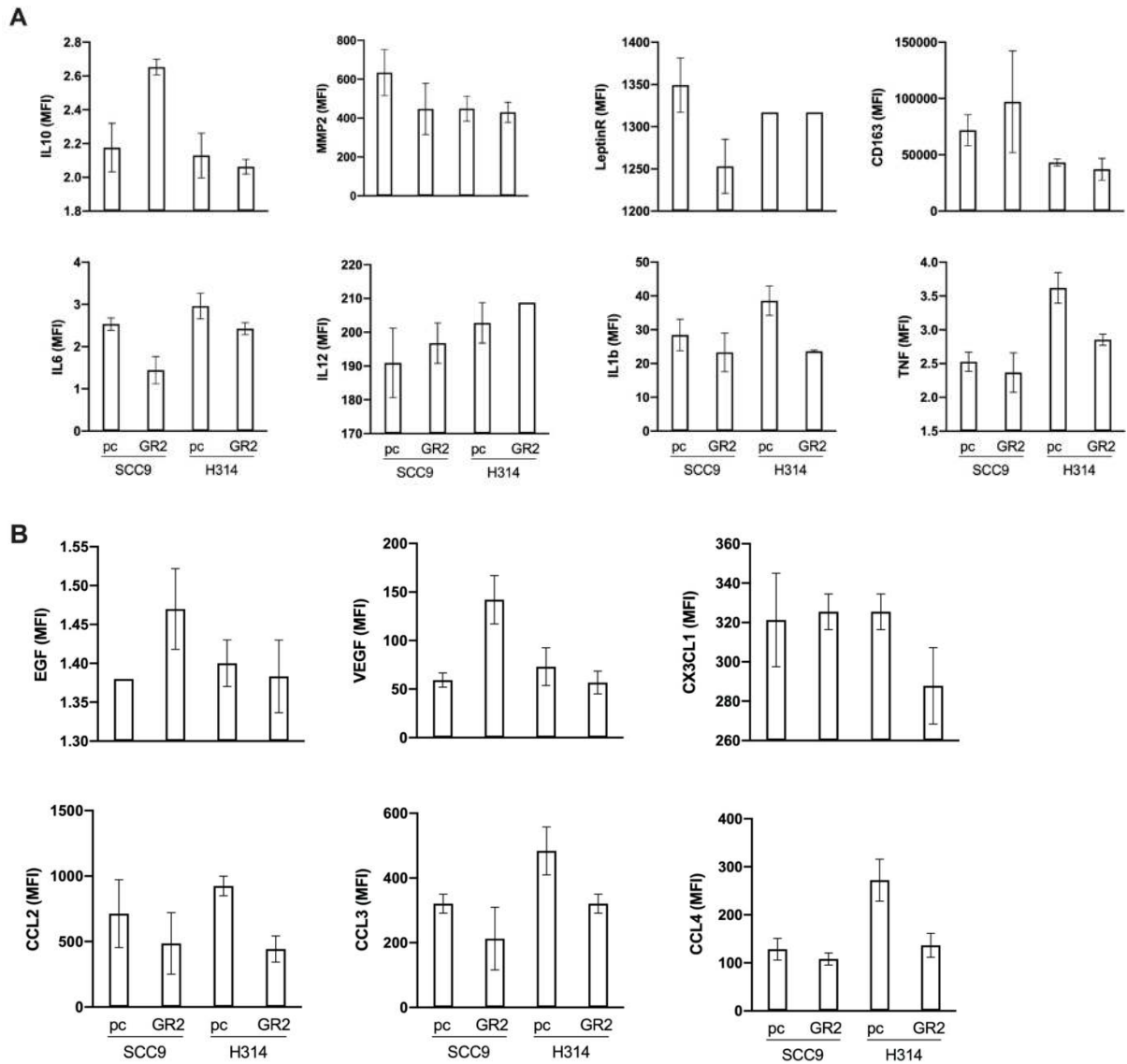


Figure 4 – Effect of overexpression of GALR2 by neoplastic cells on the production of selected candidate molecules by macrophages stimulated with tumor cell-secreted products (CM). PMA-differentiated THP-1-derived macrophages were stimulated with CM from H314-pcDNA ('pc') H314-GALR2 ('GR2') or SCC-9-pcDNA, SCC-9-GALR2 transgenic cell lines for 24 h. In the last 4 h of the 24 h stimulation, macrophages were treated with a protein transport inhibitor (Brefeldin A, 10 µg/mL). Cell lysates were prepared and 40 µg of total protein (for normalization across different experimental conditions) were used in the multiplex assay according to the supplier's instructions. Graphs present average and standard deviations of median fluorescence intensity (MFI), which is proportional to the concentration of the indicated target molecule. Data from three independent experiments for each experimental condition/treatment.

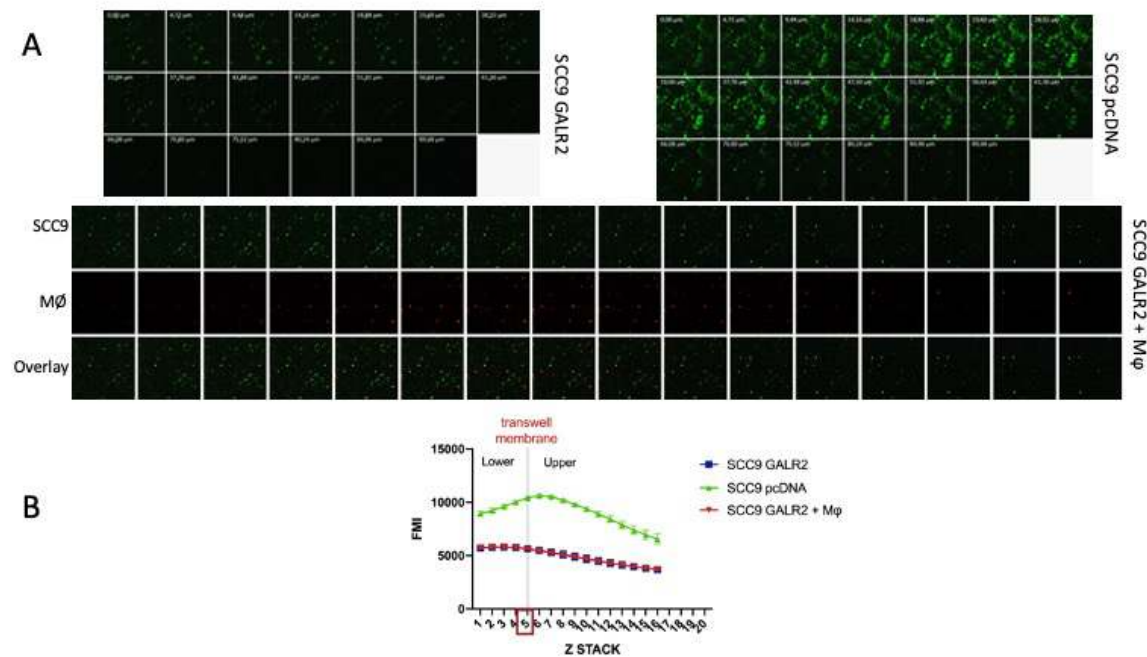


Figure 5 – In vitro invasion of SCC-9 cells overexpressing GALR2 in the presence and absence of macrophages. SCC cells were collected, stained with green fluorescent live cell dye (CellTrace™ CFSE Cell Proliferation Kit , Molecular Probes). On the same day, macrophages pre-conditioned for 2 days in CM from SCC-9-GALR2 cells were stained with red fluorescent live cell dye (CellTracker™ Red CMTPX Dye , Molecular Probes). Macrophages and SCC-9 cells were combined at a 3:1 ratio and subsequently seeded on top of a 150 μm thick layer of myogel prepared on top of polycarbonate membrane with 0.8 μm pores in 24-well transwells (1×10^4 cells/membrane). DMEM/F-12 supplemented with 1% FBS was placed in the lower compartment as chemoattractant. After 18 h, images from the center of the membranes were obtained in the green fluorescent (or in the green and red fluorescent channels in the presence of macrophages) channel using a confocal microscope. On the Z-axis, 17 images (from left to right in the images) were obtained with 8 μm spacing for 128 μm , beginning 32 μm below the transwell membrane (identified on phase contrast images by the presence of the membrane pores at the image/slice #5). The graph indicates the median fluorescence intensity (MFI) in the green channel calculated with the confocal microscope's software at each of the 17 images in the Z-axis (image/slice3 indicated on the X-axis of the graph). The MFI is directly proportional and representative of the number of cells in each slice/image. Data in graph calculated from three independent experiments.

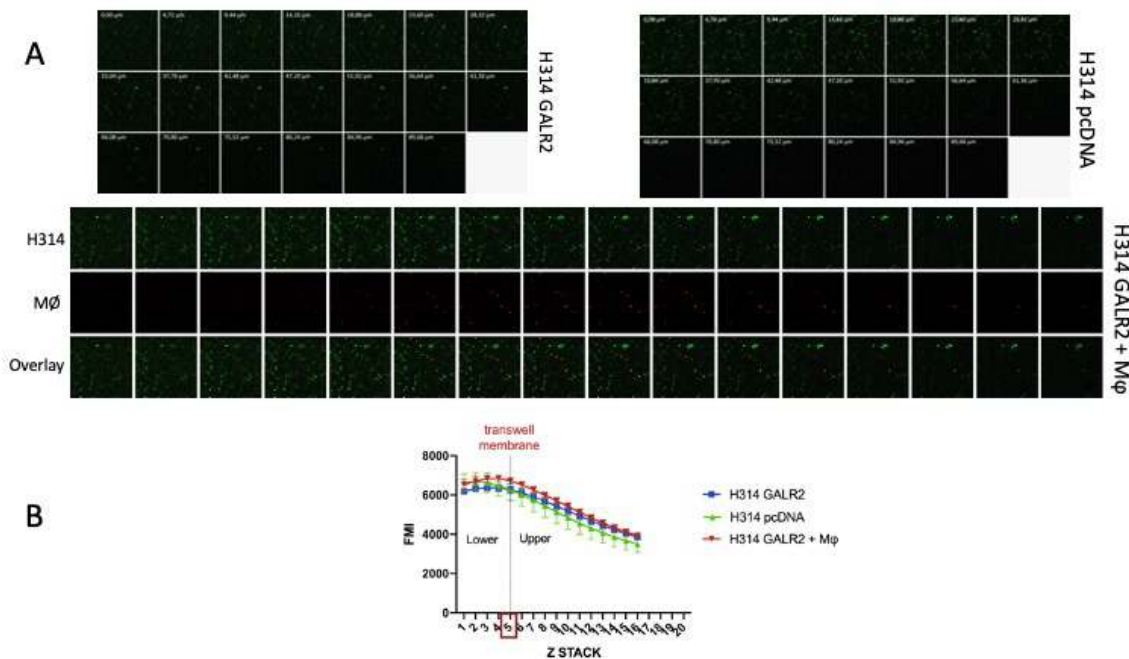


Figure 6 – In vitro invasion capability of H314 cells overexpressing GALR2 in the presence and absence of macrophages. Experiment was performed as described in the legend of Figure 5.

Discussion

In this study we demonstrate the effect of GALR2 expressed by OSCC cell lines on the phenotype of macrophages. Initially, we overexpressed GALR2 in SCC-9 and H314 cell lines and evaluated the effect in TGF- β and CSF-1 expression and protein production by neoplastic cells. Our initial hypothesis was of a direct relationship between high expression of GALR2 in tumor cell lines and production of pro-tumoral TGF- β and CSF-1 by these cells.

In HNSCC, increased TGF- β is associated to worse prognosis due to greater disease aggressiveness^{21,22}. CSF-1 is also associated with pro-tumoral properties, such as increased proliferation and invasion of tumor cells²³⁻²⁵. These features of tumor cell biology can be mediated by ERK MAPK activation downstream of CSF1R and GALR2. We rationalized that pro-tumoral properties of high GALR2-expressing neoplastic cells were mediated by increased activation of downstream p38 and ERK MAPK and enhanced expression/production of TGF- β and CSF-1. Our results demonstrate that overexpression of GALR2 reduced expression and production (protein secretion) of TGF- β and CSF-1 by SCC-9 and H-314 cell lines, which indicate no association between increased expression/activity of GALR2 and expression/production of TGF- β and/or CSF-1 by these neoplastic cell lines. In small cell lung cancer cells, GALR2 simultaneously activates multiple classes of G proteins

and downstream signaling pathways ²⁶. The experimental CMV-driven overexpression may not be representative of increased expression driven by gene copy number or epigenetic mechanisms. Also, this result may be peculiar to the cell lines used in this study and may vary according to the genetic/epigenetic landscape and composition of the tumor cell secretome with its auto-and paracrine effects.

In sharp contrast to our expectations, the reduction in expression observed in H314-GALR2 cells is associated to increased degradation of mRNA (shorter mRNA half-life) of TGFB1 and CSF1. These results may be interpreted as indicative that high expression of GALR2 may attenuate tumor aggressiveness. Results from Ichimura et al demonstrated that GALR2 attenuate cancer aggressiveness by reducing cell proliferation ²⁷.

In spite of these unexpected results, our main goal can be defined as determining the influence of increased expression/activity of GALR2 on cancer cell secretome and the subsequent effect on macrophage phenotype. We used two OSCC cell lines (H314 and SCC-9) and macrophages were stimulated with the secretome (CM) of GALR2-overexpressing and control cells. TGF- β , IL-10 and TNF were increased in macrophages treated with the secretome of H314-GALR2 cells, suggesting that overexpression of GALR2 in this cell line enhances the pro-tumoral phenotype of macrophages. HNSCC is highly heterogeneous cancer in terms of progression and prognosis, which are known to be influenced by stage, anatomical location, patient-associated characteristics, genetic and epigenetic changes. We used only two OSCC cell lines (H314 - floor of the mouth; and SCC-9 – tongue) and the results already indicate the heterogeneity of the secretome, biological characteristics and influence on macrophages. Of note, we did not observe an influence of GALR2 on in vitro invasion, but there was marked differences in invasiveness between the SCC-9 and H314 cell lines. This highlights the importance of a personalized approach in the implementation of targeted therapeutic approaches, including immunomodulating strategies.

In conclusion, our results indicate that overexpression of GALR2 in OSCC cells influences gene expression and protein production by macrophages in a cell line-specific manner. Also, increased GALR2 may either increase or decrease tumor cell invasion.

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4 CONCLUSÃO

O secretoma de diferentes células de carcinoma oral de células escamosas (OSCC); a influência específica do TGF- β como um componente do secretoma de diferentes células de OSCC e a influência da expressão e atividade de GALR2 em diferentes células de OSCC exercem efeitos distintos sobre macrófagos.

Coletivamente, os diferentes estudos conduzidos indicam que:

O secretoma de células de carcinoma oral de células escamosas influencia o fenótipo de macrófagos;

A modulação do fenótipo de macrófagos pode alterar a capacidade de invasão das células tumorais;

TGF- β , como um componente do secretoma das linhagens H314 e SCC-9 tem efeito supressor de fenótipo pró-inflamatório de macrófagos;

A inibição da influência de TGF- β em macrófagos pode favorecer ou inibir a quimiotaxia e invasão de diferentes linhagens celulares tumorais;

A expressão/atividade aumentada de GALR2 reduz a expressão de CSF-1 e TGF- β pela linhagem H314;

Isoladamente, a expressão aumentada de GALR2 não promove a invasividade das linhagens H314 e SCC-9.

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APÊNDICE A - MATERIAL E METODO

Cultura celular

Foi utilizado a linhagem celular monocítica humana THP-1 para diferenciar em macrófagos, as células foram mantidas em meio RPMI1640 (cat# 11875119, Gibco, Thermo Fisher Scientific, San Jose, CA, USA) suplementado com penicilina (100 UI/mL) e estreptomicina (100 µg/mL) (cat# 15140122, Gibco, ThermoFisher Scientific) na presença de 10% (vol:vol) de soro fetal bovino (FBS) inativado por calor (cat# 16000044, Gibco, ThermoFisher Scientific). As linhagens celulares neoplásicas H-314 e SCC-9 foram mantidas em meio de cultura DMEM/F12 (cat# 12100046, Gibco, ThermoFisher Scientific) suplementado com penicilina e estreptomicina (100 U/mL e 100 µg/mL, respectivamente) na presença de 10% (vol:vol) de FBS inativado por calor e hidrocortisona 400ng/mL. O meio de cultura destas linhagens celulares foi renovado a cada 3 dias e era realizada passagem quando a densidade celular atinge 70-80% da superfície de área de cultura.

Diferenciação de macrófagos

O meio de cultura das células THP-1 foi renovado a cada três dias até que a densidade de células atingiu 1×10^6 céls/mL, e então foram realizadas as subculturas. Para a diferenciação foram plaqueadas 3×10^6 células em placas de 100mm de diâmetro e diferenciadas em macrófagos por tratamento com 10 ng/mL de forbol-miristil-acetato (PMA, cat# P1585, Sigma-Aldrich Co., Saint Louis, MO, USA) por 24 horas. Após 24 horas de tratamento com PMA, o meio de cultura foi removido, as células aderidas ao substrato plástico de cultura lavadas gentilmente com tampão fosfato (PBS, pH 7.2, cat# 20012-027, Gibco, ThermoFisher Scientific), e o meio RPMI1640 suplementado com antibióticos e FBS (sem PMA) foi acrescentado às placas de cultura por 72 h. Para confirmar o protocolo de diferenciação para os experimentos propostos no projeto, a diferenciação foi verificada pelas alterações morfológicas (tamanho e adesão ao substrato de cultura).

Meio condicionado (MC)

As linhagens de HNSCC H-314 e SCC-9 foram plaqueadas em concentração de 2.5×10^6 células/placa de 100 mm de diâmetro durante 24h, e então submetidas à três lavagens sucessivas (2 h / lavagem, utilizando 8 mL de meio/placa de 100 mm)

em meio de cultura DMEM/F12 'branco' (não suplementado com antibióticos ou FBS) para desindução das células. Após a última lavagem, foi acrescentado volume reduzido (4 mL/placa de 100 mm) e as células cultivadas por 16-18 h (para secreção dos produtos expressos constitutivamente pelas linhagens celulares tumorais). O meio condicionado contendo os produtos secretados pelas linhagens tumorais foi coletado em tubo de 15 mL, centrifugado (10.000 RPM, 4°C, 10min) para remoção de células e debris celulares em suspensão, aliquotado e armazenado em freezer -80C (armazenamento por até 2 semanas).

Estimulação dos monócitos e macrófagos com CM das linhagens celulares tumorais

Macrófagos e monócitos foram estimulados por 24h com MC contendo produtos secretados pelas células tumorais SCC-9 e H-314. Após 24 horas de cultura com o meio condicionado, foi realizada a extração de RNA ou extração de proteínas das células para posterior análise.

Polarização de macrófagos

A polarização fenotípica para o perfil M1 foi induzida por tratamento dos macrófagos com interferon-gama (25 ng/mL, cat# 300-02, Peprotech Inc), LPS (100 ng/mL, cat# L6529, Sigma-Aldrich Co.). A polarização para o perfil M2 foi realizada pelo tratamento dos macrófagos com IL-4 (20 ng/mL, cat# 200-04, Peprotech Inc) e IL-13 (20 ng/mL, cat# 200-13, Peprotech, Inc). Estes tratamentos foram mantidos por 24 h. Macrófagos não polarizados (perfil fenotípico "M0") foram preparados em paralelo, mantendo as células em meio de cultura DMEM/F12 puro.

RT-qPCR

O RNA total foi purificado de lisados celulares preparados ao final do período de cada experimento utilizando sistema de colunas de afinidade incluindo tratamento com DNase (RNAqueous-4-PCR, Ambion, ThermoFisher Scientific), de acordo com o protocolo do fabricante. A quantificação do RNA total isolado de cada amostra foi avaliada em espectrofotômetro de microvolumes (NanoVue Plus, GE Healthcare), pela absorbância em 260 nm. A síntese do DNA complementar (cDNA) foi realizada utilizando 500 ng de RNA total, segundo as instruções do fornecedor dos reagentes (High Capacity cDNA reverse transcription kit, Applied Biosystems, ThermoFisher

Scientific) e utilizando hexâmeros aleatórios como primers. A detecção dos genes-alvo de interesse foi realizada utilizando as sequências de oligonucleotídeos para os genes-alvo, obtidas do banco de primers online da Universidade de Harvard.

Para a reação de amplificação foi utilizado o master mix do sistema SYBR Green (PowerUp SYBR Green, Applied Biosystems, ThermoFisher Scientific), segundo as orientações do fornecedor do reagente. A expressão dos genes-alvo (Quadro 1) de interesse foi normalizada usando o gene constitutivo HPRT1, e os dados analisados pelo método $\Delta(\Delta CT)$.

Quadro 1 - Sequência ('Fwd'-forward; 'Rev'-reverse) dos primers utilizados para avaliação da expressão gênica por RT-qPCR com o sistema SYBR Green

Gene symbol	Accession#	Sequence (5'-3')	Ta (C)	Amplicon (bp)
HPRT1	NM_000194.2	Fwd - AGGACTGAACGTCTTGCTCG	60.04	72
		Rev - GAGCACACAGAGGGCTACAA	59.68	
CSF-1	NM_000757.5	Fwd - TTAAAAGGCTGTGCCGAGGG	60.61	72
		Rev - GAGAGGACCCAGGCAAACCTT	59.60	
TGF- β	NM_000660.6	Fwd - TATTGAGCACCTTGGGCACTG	60.61	127
		Rev - TCTCTGGGCTTGTTTCCTCAC	59.93	
IL-10	NM_000572.3	Fwd - GGCACCCAGTCTGAGAACAG	60.32	86
		Rev - ACTCTGCTGAAGGCATCTCG	59.82	
TNF	NM_000594.3	Fwd - GCTGCACTTTGGAGTGATCG	59.55	108
		Rev - TCACTCGGGGTTCGAGAAGA	60.25	
IL-12	NM_000882	Fwd - ATGGCCCTGTGCCTTAGTAGT	62.7	77
		Rev - AGCTTTGCATTCATGGTCTTGA	60.5	
MMP-9	NM_004994.2	Fwd - ATCCAGACTTCCTCAGGCGG	59.90	92
		Rev - AGAAGCCCCACTTCTTGTCG	59.96	
MMP-2	NM_004530.5	Fwd - ATCCAGACTTCCTCAGGCGG	61.33	71
		Rev - ATCCAGACTTCCTCAGGCGG	58.90	

Fonte: Elaboração própria.

Extração de proteínas

Neste experimento, para evitar que as proteínas secretadas pelas linhagens tumorais presentes no MC fossem detectadas juntamente com as proteínas produzidas pelos macrófagos (já que tanto as linhagens neoplásicas quanto os macrófagos são de origem humana), foram detectadas proteínas produzidas especificamente pelos macrófagos evitando a sua secreção no meio de cultura e

coletando os lisados celulares (e não o meio de cultura com proteínas secretadas). Para isso, os macrófagos foram tratados com inibidor de transporte de proteína (Brefeldin A, 10 µg/mL) nas últimas 4 h antes de completadas as 24 h de estímulo com MC das linhagens neoplásicas. O lisado celular dos macrófagos foi coletado e a extração de proteínas realizado com tampão de lise (Cell lysis Buffer, cat. 9803, Cell Signaling Tech., Inc.) suplementado com inibidores de protease e fosfatase (cat. A32961, Pierce Protease and Phosphatase Inhibitor Mini Tablets, EDTA-free, ThermoFisher Scientific). A quantificação das proteínas foi realizado usando o método de Bradford (Bradford assay, Bio-Rad Lab),

Análise multi-ligante (multiplex)

Depois de quantificar as proteínas, 20 µg de proteína total foram usados para a análise de multiplex. A concentração de 14 diferentes marcadores fenotípicos de macrófagos foi determinada por ensaio multiplex (R&D Biosystems, Biotechne) realizado segundo as instruções do fornecedor, com obtenção dos dados em equipamento Luminex xMAP 200 (Invitrogen, ThermoFisher Scientific) e software de análise Milliplex multianalyst (Millipore, Merck). Nesta análise, foram avaliados os seguintes alvos: IL-1, IL-6, TNF, IL-12, IL-10, MMP-2, MMP-9, Leptin receptor, CD163

Quimiotaxia

Neste experimento, as linhagens tumorais foram plaqueadas em placas de 24 poços e após 24 h (para permitir a aderência celular ao substrato plástico de cultura) foram lavadas com PBS 1X e mantidas com meio de cultura DMEM/F-12 suplementado com 1% FBS. Após 24 h, foram plaqueados os macrófagos diferenciados a partir de células THP-1 sobre a membrana (poros de 0.8 µm) do transwell, de forma que não havia contato físico direto entre as células tumorais e macrófagos.

Macrófagos foram diferenciados em placas de 6-well, iniciando com a diferenciação de células monocíticas THP-1 com 10 ng/mL de PMA. Após o período de descanso sem PMA, os macrófagos foram estimulados com MC das linhagens tumorais por 24 h. Após 24 h, estes macrófagos foram coletados por dissociação enzimática suave (para evitar a remoção de proteínas/receptores presentes na membrana celular) e colocados sobre transwells de membrana de policarbonato com poros de 0.8 µm. Sob os transwells estavam em cultura há 36 h as linhagens celulares

tumorais (as últimas 24 h de cultura em meio DMEM/F12 não suplementado, contendo os produtos secretados pelas células). A quimiotaxia dos macrófagos é avaliada pelo número de células que passam pelos poros da membrana do transwell, chegando à porção inferior da membrana. Ao final do experimento, as células que não passaram pela membrana (presentes em sua porção superior, onde foram inicialmente semeadas) são removidas mecanicamente (com um cotonete esterilizado) e as membranas são expostas a uma substância química (DAPI, 2 µg/mL) por 10 min para marcação do núcleo celular. Esta substância (DAPI) tem as propriedades de se ligar (intercalar) em DNA dupla-fita (DNA genômico, presente no núcleo celular) e emitir fluorescência quando excitada por luz de um certo comprimento de onda, possibilitando a visualização dos núcleos em microscópio de fluorescência. Quanto mais células passarem pela membrana e forem detectadas na porção inferior desta, maior o estímulo quimiotático. Como controles negativo e positivo foi utilizado apenas o meio de cultura (DMEM/F-12) não suplementado (controle negativo) ou suplementado com soro fetal bovino (FBS) na proporção de 10% (vol:vol) (controle positivo).

Migração– in vitro

Para determinar o efeito do contato direto célula a célula na migração de células OSCC, realizamos co-cultura de células H-314 o SCC-9 com macrófagos previamente polarizados para o fenótipos M0, M2 ou tratados com MC por 24 h . As células foram plaqueadas em uma proporção aproximada de 3:1 ($2,2 \times 10^5$ células OSCC: 8×10^4 macrófagos). Como controle, o total de células tumorais (3×10^5 células) foram plaqueadas em cada poço. Após 18 h, uma ferida vertical in vitro (“risco”) foi feita no centro dos poços com ponta de pipeta estéril de 200 µL. Os poços foram lavados com PBS para remover todas as células não aderidas e meio de cultura completo suplementado com mitomicina C (2 µg/mL) foi adicionado. Imagens de campo claro dos mesmos campos (determinadas por linhas de referência marcadas na superfície externa na parte inferior da placa antes de iniciar as co-culturas) foram obtidas com ampliação de 40X em um microscópio invertido (Evos FI, AMG Micro) após 4, 11 e 24h.

Invasão – in vitro

A influência de macrófagos e fenótipo de macrófagos na invasão de células neoplásicas foi avaliada usando myogel. As células tumorais foram coletadas e coradas com corante com um marcador de viabilidade de fluorescência verde (5µM, CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes) e os macrófagos (M0, M2 e MC) corados com marcador de viabilidade de fluorescência vermelha (10 µM, CellTracker™ Red CMTPX Corante, Sondas Moleculares). Células tumorais e macrófagos foram combinados nas proporções indicadas (mantendo o número total de células em cada poço constante em 4×10^4 células). As células foram plaqueadas no topo do miogel suplementado com colágeno e mantido sobre as membranas do transwell (poros de 0,8 µm). DMEM/F-12 suplementado com 1% de FBS foi acrescentado na parte inferior como quimioatraente. Após 18 h, imagens do centro das membranas foram obtidas nos canais fluorescentes verde e vermelho usando um microscópio confocal (Carl Zeiss LSM 800). No eixo Z, 17 imagens foram obtidas com espaçamento de 8 µm para 128 µm, começando 32 µm abaixo da membrana transwell (identificada na imagem/fatia # 5 pela presença dos poros da membrana transwell nas imagens de contraste de fase).

ELISA

Como metodologia alternativa à análise multi-ligante (multiplex) foi realizado o ELISA ('single-plex', para detecção de uma única proteína-alvo) do lisado dos macrófagos e analisada a expressão de TGF-β (RnD Systems, BioTechne) e CSF-1 (RnD Systems, BioTechne). O ELISA foi realizado de acordo com as instruções fornecidas pelo fornecedor dos reagentes, os dados obtidos em um leitor de placa multipos (Spectramax M, Molecular Devices) e analisados com o software do equipamento (SoftMax Pro, Molecular Devices).

Inibição de TGF-β

Macrófagos derivados de THP-1 diferenciados por PMA foram pré-tratados por 4 h com inibidor da quinase associada ao receptor tipo I de TGF-β (LY-364947, 10uM, Sigma-Aldrich Co) ou com o mesmo volume de veículo DMSO como controle, antes de tratar as células com CM. Os macrófagos foram tratados com CM por 24 h para avaliação dos desfechos (expressão gênica e marcadores fenotípicos).

Western blot

Após a quantificação das proteínas com o método de Bradford, 20 µg de proteína total foram diluídos em SDS Sample Buffer 4X (NuPAGE, cat NP0007, Invitrogen) contendo DTT e as proteínas foram desnaturadas por aquecimento a 95°C durante 10 min. A separação eletroforetica foi realizada em gel de poliacrilamida a 12%, seguido de transferência para uma membrana de nitrocelulose de 0.2 µm. Após a eletrotransferência, as membranas foram bloqueadas com 5% de BSA em TBS-T durante 2 horas para evitar ligações não específicas. Posteriormente, a membrana foi incubada durante 18 h com anticorpos primários para detectar formas fosforiladas ou totais das proteínas de interesse Smad2 /3 e p38 MAPK. A detecção de proteínas-alvo foi baseada na reação de quimioluminescência (SuperSignal West Pico, Pierce/ThermoFisher Scientific) detectado em um sistema de documentação digital (Chemi-Doc XR, Bio-Rad Laboratories).

CAM

Ovos de galinha fertilizados adquiridos comercialmente foram incubados por 8 dias antes do uso no experimento. Resumidamente, a membrana corioalantóica foi exposta com a abertura de uma janela na casca do ovo que permite semear as células no epitélio da membrana corioalantóica. Os macrófagos foram coletados após tratamento por 24 h com CM (na presença e ausência de inibidores de TGF-β), depois misturados com as linhagens de H-314 ou SCC-9 (proporção de células células neoplásicas 3 : 1 macrófago) no total 5x10⁵ células/CAM. As células foram re-suspendidas em volume final de 5 µL de HBSS e depois deixadas delicadamente sobre a membrana no CAM. Os ovos foram protegidos com um curativo flexível que permite a troca de ar e mantidos na incubadora de ovos por 3 dias para avaliação do crescimento/invasão tumoral. Após 3 dias, as membranas foram coletadas e lavadas em PBS, depois fixadas em paraformaldeído a 4% por 4 h a 4°C, depois foram mantidas em PBS a 4°C para as análises subsequentes. Posteriormente, as membranas foram incluídas em parafina e foram realizados cortes semi-seriados de 5 µm de espessura para análises histológicas. Os cortes histológicos foram corados com hematoxilina e eosina para análise descritiva e utilizados para imuno-histoquímica.

Imuno-histoquímica

Para análise de imuno-histoquímica, foi realizada a desparafinação em duas incubações sucessivas em xilol (8 min/incubação), seguida de reidratação gradual em concentrações decrescentes de etanol e reidratação final em água milliQ. A recuperação antigênica foi realizada aquecendo as amostras de tecido a 92-95°C em tampão citrato (pH 6,0) por 15 min. Os cortes foram bloqueados com 1% de BSA em PBS por 1 h à temperatura ambiente, lavados e incubados com as diluições otimizadas de anticorpos primários (Quadro 2) overnight (16 horas) a 4°C. Após a lavagem, os cortes foram incubados com solução de estreptavidina HRP (20 min à temperatura ambiente), lavados novamente e depois incubados com o substrato cromógeno (60 segundos). Os cortes foram finalmente, corados com hematoxilina de Mayer por 30 segundos, lavados em água corrente e montados com lâminulas. A visualização e as imagens digitais das lâminas foram obtidas em microscópio convencional (Leica Microsystem GmbH, Wetzlar, Alemanha) acoplado a uma câmera digital (DP71, Olympus, Tóquio, Japão).

Quadro 2 - Anticorpos e objetivos avaliados por imuno-histoquímica

Proteína	Marca comercial	Função
CD163	Novus biological, NB110-40686	M2 marker
CD68	Novus biological, NBP2-32831	M1 marker
Cytokeratin	Novus biological, NBP2-44951	Epithelial cells

Fonte: Elaboração própria.

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