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BIANCA DE BEM PRUNES

ACIDEZ TUMORAL AUMENTA AGRESSIVIDADE, MIGRAÇÃO CELULAR, RESISTÊNCIA AO TRATAMENTO NO CARCINOMA ESPINOCELULAR ORAL E ALTERA A RESPOSTA IMUNOLÓGICA ANTITUMORAL

Tese de Doutorado

PORTO ALEGRE
2022

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Com muito orgulho, nos entrego ao mundo.

Coisas que a gente se esquece de dizer
Frases que o vento vem as vezes me lembrar
Coisas que ficaram muito tempo por dizer
Na canção do vento não se cansam de voar

Você pega o trem azul, o Sol na cabeça
O Sol pega o trem azul, você na cabeça
O Sol na cabeça
Milton Nascimento, O Trem Azul, 1978

Resumo

A presença de lactato e acidificação extracelular são características marcantes do microambiente de tumores sólidos, que decorrem de alterações no metabolismo celular. O pH de tumores de cabeça e pescoço é ácido, variando entre 6.2 e 6.9. Tais condições observadas no microambiente tumoral são correlacionadas na literatura à maior agressividade, disseminação e resistência ao tratamento, bem como ao comprometimento de diversas funções efetoras do sistema imune. Há indicativos de que a capacidade adquirida pelas células tumorais em alterar seu metabolismo energético regulando a secreção de ácido lático e a resultante acidificação do meio extracelular, compõem o grupo de estratégias que culminam no mecanismo de evasão das células tumorais à ação antitumoral do sistema imune. Considerando que o papel do lactato/acidez tumoral no carcinoma espinocelular oral segue pouco explorado, com o primeiro artigo da presente tese, avaliamos *in vitro* o comportamento de células de carcinoma espinocelular oral (SCC4 e SCC25) expostas à acidez extracelular. Foram analisadas migração, indiferenciação e resistência ao tratamento quimio e radioterápico das linhagens supracitadas cultivadas em diferentes esquemas experimentais, os quais consistiram em três grupos principais: Exposição contínua ao meio de cultura neutro (pH 7.4), considerado o controle e denominado pH 7.4; Exposição contínua ao meio de cultura celular acidificado (pH 6.8) por um período de 7 dias, foi denominado pH 6.8; Exposição intermitente, inicialmente cultivadas em meio de cultura acidificado (pH 6.8) durante 7 dias, seguidos de mais 7 dias de cultivo em meio neutro (pH 7.4), denominado pH 6.8 $\rightarrow$ pH 7.4, na tentativa de mimetizar as flutuações de pH observadas no microambiente tumoral. Além desses, um grupo mimetizando a acidez de forma crônica, induzida pela exposição das células ao meio de cultura acidificado (pH 6.8) por um período de 21 dias, denominado pH 6.8, foi estabelecido para confirmação de alguns achados. Após exposição à acidez, as células adquiriram um fenótipo fusiforme, achado confirmado por meio da análise da razão de polaridade. A análise de viabilidade celular indicou, para ambas as linhagens celulares, uma diminuição estatisticamente significativa ($p<0,0001$) do potencial de sobrevivência após exposição ao pH 6.8, o qual foi em parte recuperado após recondicionamento das células em pH 7.4. Observou-se uma redução significativa da expressão de E-Caderina acompanhada de um aumento da expressão dos marcadores de EMT N-Caderina e Vimentina nos grupos teste ($p<0,05$). O aumento da capacidade migratória do grupo pH 6.8 $\rightarrow$ pH 7.4 (SCC4) foi observado por meio dos ensaios de cicatrização de ferida ($p<0,0001$), capacidade invasiva em transwell ($p=0,022$) e potencial de motilidade ($p<0,05$) em ensaio de time-lapse. Observou-se um aumento da expressão dos marcadores de pluripotência CD44 e NANOG ($p<0,05$) no grupo de pH 6.8. Com relação aos tratamentos quimio e radioterápicos, as células tumorais demonstraram maior resistência à Cisplatina ($p<0,05$) e à radiação ($p<0,0001$) durante a exposição à acidez extracelular. Para melhor compreensão dos efeitos do lactato e da acidez presentes no microambiente tumoral sobre

a resposta imunológica, desenvolvemos uma revisão de escopo sistemática da literatura. Foram incluídos 96 estudos os quais apontaram uma correlação entre acidez extracelular e presença de lactato no microambiente tumoral e comprometimento de diversas funções celulares de imunócitos.

Palavras-chave: Câncer bucal. Acidez tumoral. Migração celular. Pluripotência. Sistema Imune. Evasão Tumoral. Radioterapia. Quimioterapia.

Abstract

The presence of lactate and extracellular acidification are solid tumors' microenvironment hallmarks happening as a consequence of alterations in cellular metabolism. The pH of head and neck tumors is makedly low varying between 6.2 and 6.9. These conditions observed in the tumor microenvironment (TME) have been correlated in the literature with increased aggressiveness, dissemination and resistance to treatment, and impaired immune functions. There are evidence indiating that the capacity acquired by tumor cells to alter their energy metabolism by regulating the secretion of lactic acid and the resulting acidification of the extracellular milieu build the group of strategies culminating in neoplastic cells' evasion from antitumor actions of the immune system. Considering that the role of lactate/tumor acidity in oral squamous cell carcinoma remains little explored, in the first article of this thesis, we evaluated the behavior of human oral squamous cell carcinoma cells (SCC4 and SCC25) exposed to extracellular acidity in vitro. Migration, undifferentiation, and resistance to chemo- and radiotherapy treatment of the strains mentioned above cultured in different experimental schemes were analyzed. Attempting to mimic the pH fluctuations observed in the TME, three main groups were established: Continuous exposure to neutral culture medium (pH 7.4), considered the control and named pH 7.4; Continuous exposure to acidified cell culture medium (pH 6.8) for a period of 7 days, was named pH 6.8; Intermittent exposure, initially cultured in acidified culture medium (pH 6.8) for 7 days, followed by another 7 days of culture in neutral medium (pH 7.4), named pH 6.8 $\rightarrow$ pH 7.4. Furthermore, acidity in a chronic manner, induced by exposing a group of cells to acidified cell culture medium (pH 6.8) for a period of 21 days, termed pH 6.8, was established in order to confirm some findings. After exposure to acidity, the cells acquired a fusiform phenotype, a finding that was confirmed by polarity ratio analysis. Cell viability analysis indicated, for both cell lines, a statistically significant ($p<0.0001$) decrease in survival potential after exposure to pH 6.8, which was partially recovered after reconditioning cells at pH 7.4. We observed a significant reduction of E-Cadherin expression accompanied by an increased expression of the EMT markers N-Cadherin and Vimentin in the test groups ($p<0.05$). The increased migratory capacity of the pH 6.8 $\rightarrow$ pH 7.4 (SCC4) group was observed via wound healing assays ($p<0.0001$), transwell invasive capacity ($p=0.022$), and motility potential ($p<0.05$) in time-lapse assays. Increased expression of pluripotency markers CD44 and NANOG ($p<0.05$) was observed for the pH 6.8 group. Regarding chemo- and radiotherapy treatments, tumor cells showed increased resistance to Cisplatin ($p<0.05$) and radiation ($p<0.0001$) during exposure to extracellular acidity. To better understand the effects of lactate and acidity present in the tumor microenvironment on the immune response, we developed a systematic scoping review of the literature. Ninety-six studies were included and a correlation between extracellular acidity/lactate abundance at the tumor microenvironment and impairment of various immunocyte cellular functions were observed.

Keywords: Oral cancer. Tumor acidity. Cell migration. Pluripotency. Immune System. Tumor Evasion. Radiotherapy. Chemotherapy.

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1 INTRODUÇÃO

1.1 Câncer

Considerado um desafio para as políticas públicas em saúde, o câncer está dentre as principais causas de óbito da população mundial e estima-se que suas taxas de incidência cresceram em 20% na última década, totalizando mais de 27 milhões de novos casos mundialmente até o ano de 2030 (WARNAKULASURIYA, 2010; INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2020). De acordo com os últimos dados levantados pelo Instituto Nacional do Câncer, 626 mil novos casos de câncer ao ano, para o triênio 2020-2022, foram estimados (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2020).

O termo ‘câncer de boca’ remete às neoplasias malignas que acometem a cavidade bucal. Nesse contexto, o carcinoma espinocelular oral é descrito como a neoplasia maligna de maior prevalência, representando cerca de 95% do total das malignidades que atingem este sítio (NEVILLE et al., 2009; INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2020). O carcinoma espinocelular oral está entre os tipos mais comuns de neoplasias malignas que atingem a população brasileira ocupando o quinto lugar em prevalência em homens. Estimou-se 11.200 novos casos da doença em indivíduos do sexo masculino e 4.010 na população feminina brasileira para o ano de 2020 (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2020). Essa transformação maligna possui como fatores de risco o hábito de fumar, o consumo excessivo de álcool e também a infecção por subtipos de papilomavírus humano de alto risco (LEEMANS; SNIJDERS; BRAKENHOFF, 2018).

O tratamento quimioterápico possui papel coadjuvante, baseando-se majoritariamente na atividade proliferativa das células tumorais e falhando em aumentar as taxas de sobrevida dos pacientes (FURNESS et al., 2011). A remoção cirúrgica agressiva, por vezes mutiladora, permanece como abordagem predominante no tratamento de indivíduos portadores dessa neoplasia, mesmo com a doença sendo detectada em estágios iniciais (SCIUBBA, 2001). Levando em consideração as características da doença e buscando modificar indicadores epidemiológicos que seguem inalterados ao longo de décadas, um grande número de pesquisas científicas são desenvolvidas em busca de soluções mais eficazes em termos de tratamento (HANAHAN; WEINBERG, 2011; SMALLBONE et al., 2005).

Figura 1 – Aspecto clínico de lesão de carcinoma espinocelular bucal em bordo lateral/base de língua. Úlcera de bordos elevados e endurecidos.

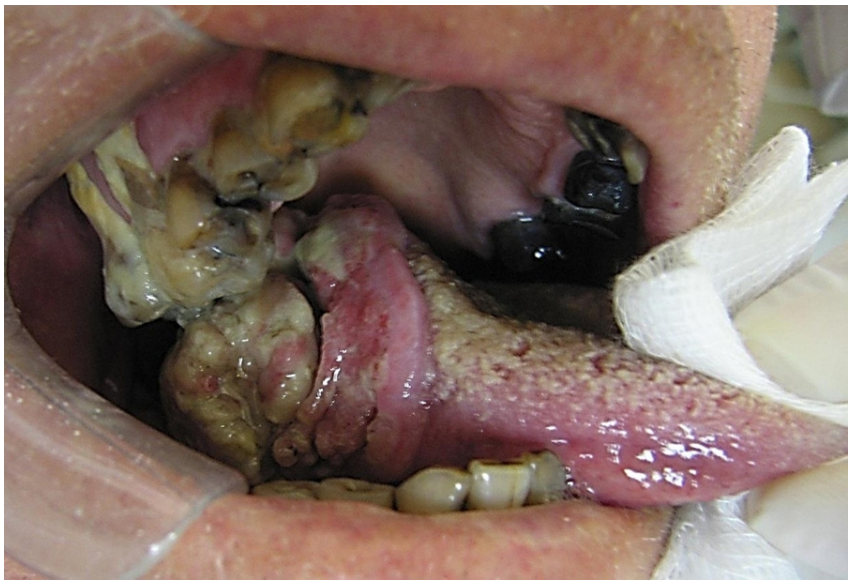

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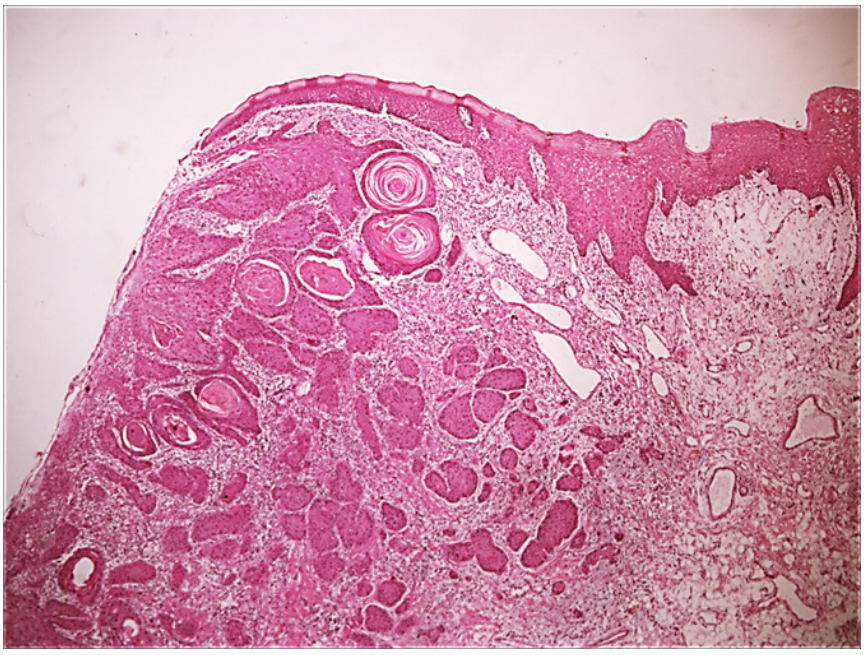
Figura 2 – Corte histológico de lesão de carcinoma espinocelular bucal apresentando ilhas de epitélio escamoso invadindo o tecido conjuntivo. No interior das ilhas observa-se a formação de pérolas de ceratina.



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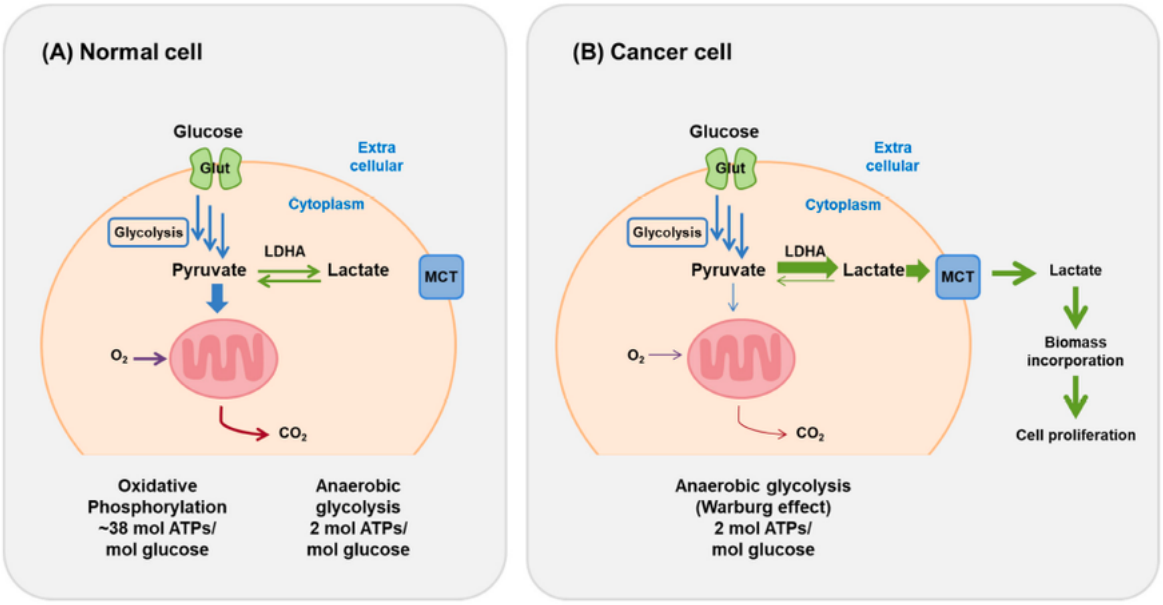
1.2 Microambiente Tumoral

Dentre as razões pelas quais frequentemente observa-se falha no tratamento quimio e radioterápico, destaca-se a alta complexidade da doença, que é determinada pela

existência de um MT no qual ocorrem recíprocas interações entre células cancerosas e seu estroma. Nesse contexto, são considerados fibroblastos, células endoteliais e células do sistema imunológico as quais, ao interagirem com o componente tumoral, contribuem para a progressão do tumor e modulação da resposta ao tratamento (GILLIES et al., 2002; HANAHAN, 2022; HANAHAN; WEINBERG, 2011). Nesse cenário, diversas capacidades adquiridas pelas células neoplásicas, tais como importantes alterações metabólicas, atuam favorecendo o recrutamento de uma comunidade de células não tumorais que atuam em prol do avanço da doença (HANAHAN, 2022; HANAHAN; WEINBERG, 2011).

Frequentemente as células tumorais modificam seu fluxo de produção energética, optando pela via glicolítica em detrimento da fosforilação oxidativa. Inicialmente, essa inversão ocorre em resposta à privação transitória de oxigênio e nutrientes naquelas regiões da massa tumoral que ainda encontram-se pobremente vascularizadas. Entretanto, mesmo quando há suporte vascular satisfatório, as células mutadas optam pela via da glicólise, configurando o fenômeno chamado “glicólise aeróbica” primeiramente descrito pelo pesquisador Otto Warburg. A secreção de íons hidrogênio e lactato gerada por essa inversão metabólica tem como principal consequência o estabelecimento de um meio extracelular marcadamente ácido (DA SILVA et al., 2018; YANG; HU; MO, 2019).

Figura 3 – Diferenças nas vias de glicólise comparando células normais e células tumorais. (A) Na presença de oxigênio, as células normais produzem até 38 ATPs por molécula de glicose através da fosforilação oxidativa. Em um ambiente hipóxico, o piruvato acumula-se e é então convertido em ácido láctico, produzindo apenas 2 ATPs. (B) As células neoplásicas a via da glicólise, independentemente da presença ou ausência de oxigênio; apenas 2 ATPs são produzidos por molécula de glicose.

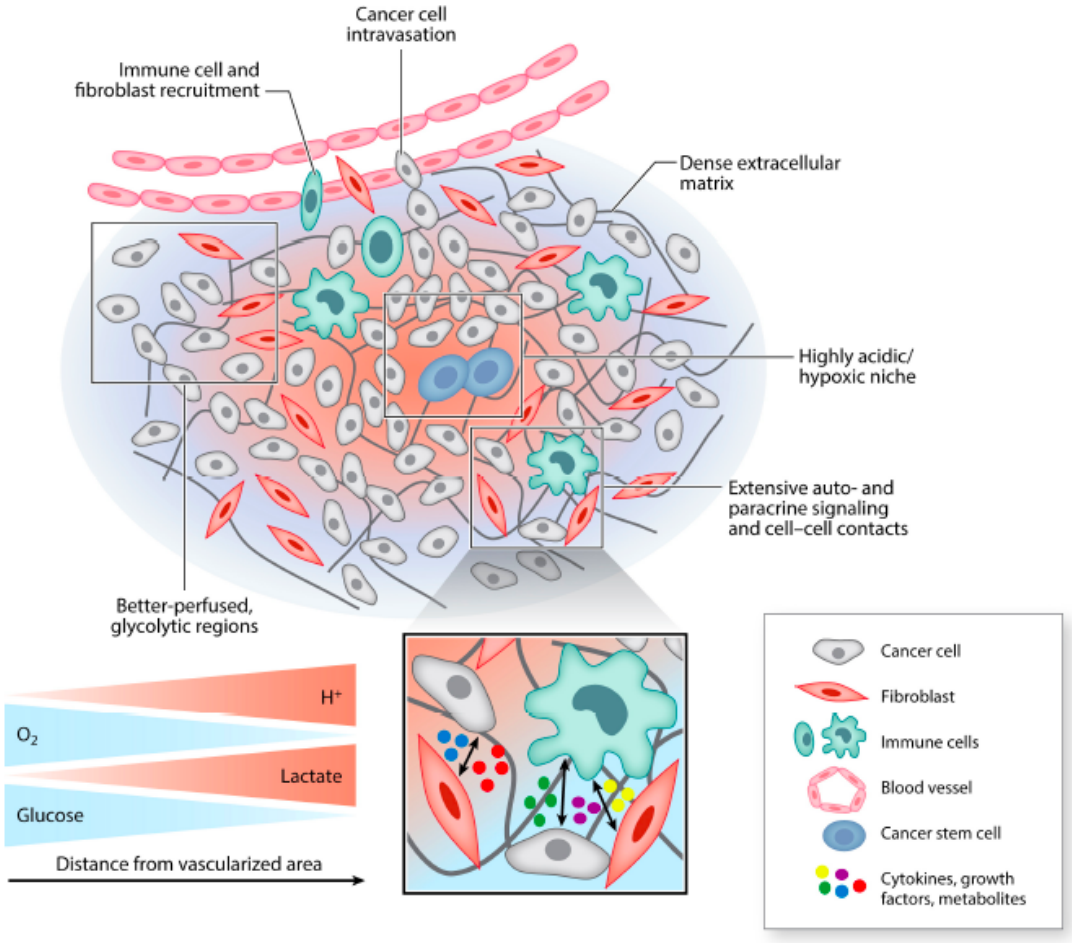


Kim; Baek, 2021

O pH extracelular de tumores sólidos chega a atingir valores entre 6,2 e 6,9, no-

tavelmente mais baixos quando em comparação ao pH fisiológico dos tecidos (IBRAHIM HASHIM et al., 2011; YANG; HU; MO, 2019). Por conseguinte, instituí-se um microambiente hostil que resulta, em última instância, na morte ou comprometimento funcional irreversível de células do sistema imune e, até mesmo, de células cancerosas não-adaptadas. Por outro lado, as células tumorais capazes de resistir a essa alta pressão seletiva gerada pelo estresse ácido demonstram-se mais agressivas (ALLISON; COOMBER; BRIDLE, 2017).

Figura 4 – Desenho esquemático de um tumor sólido demonstrando os componentes microambiente tumoral. Baixo aporte de oxigênio e glicose seguidos de elevadas concentrações de íons H+ e lactato.



Boedtkjer; Pedersen, 2020

1.3 Efeitos do Microambiente Tumoral sobre fenótipos adaptativos agressivos

Os eventos relacionados à adaptação ao MT biologicamente hostil não ocorrem de forma passiva e impactam permanentemente o fenótipo das populações celulares sobreviventes. Devido a alta complexidade dos processos relacionados ao desafio adaptativo gerado pela acidez e presença de lactato no microambiente, maiores detalhes a respeito das mudanças fenotípicas observadas seguem imprecisos ou ainda pouco explorados. Relatos

prévios da literatura abordam uma série de alterações fenotípicas observadas durante a adaptação das células neoplásicas malignas ao microambiente ácido, dentre elas destacam-se o aumento do acúmulo de proteínas lisossômicas na membrana plasmática, presença de atividade autofágica crônica, crescimento da subpopulação de células pobremente diferenciadas e pluripotentes com características que se assemelham a células tronco e aumentada agressividade e invasividade celular (ALLISON; COOMBER; BRIDLE, 2017; AVNET et al., 2017; DAMAGHI et al., 2015; DAMAGHI; GILLIES, 2016; SADEGHI et al., 2020; WOJTKOWIAK et al., 2012). Além disso, a acidose tumoral comprovadamente promove a angiogênese ao estimular a liberação VEGF, contribui com a remodelação da matriz extracelular facilitando a invasão do estroma por parte das células tumorais e compromete profundamente a vigilância imunológica (DAMAGHI et al., 2015; DAMAGHI; WOJTKOWIAK; GILLIES, 2013; ESTRELLA et al., 2013; GATENBY; GILLIES, 2008; LARDNER, 2001; PILON-THOMAS et al., 2016; XU; FUKUMURA; JAIN, 2002).

Nesse sentido, ao longo do processo de adaptação e com o objetivo de sobreviver às pressões impostas pelo microambiente, as células tumorais frequentemente utilizam-se de mecanismos que são muito importantes em processos não patológicos (SUIJKERBUIJK; VAN RHEENEN, 2017). Um exemplo significativo dessa afirmativa é o processo por meio do qual as células tumorais sofrem múltiplas alterações biológicas, adquirindo características fenotípicas próprias de células mesenquimais denominado Transição Epitélio Mesênquima (TEM), um mecanismo fisiologicamente crítico durante a embriogênese e nos eventos relacionados à cicatrização de feridas (KALLURI; WEINBERG, 2009; KARACOSTA et al., 2019; NIETO, 2013). Durante essa transição, com a perda total ou parcial de suas características epiteliais e ganho de características mesenquimais, as células neoplásicas desenvolvem características tais como aumento da motilidade e maior expressão de metaloproteinases responsáveis pela degradação da matriz extracelular, o que favorece os eventos migratórios, a invasão aos tecidos e o estabelecimento de metástases regionais ou à distância (MANI et al., 2008; SUZUKI et al., 2014). Apesar de denominada transição, mais recentemente emergiu o conceito de que a TEM deve ser analisada como um espectro de estados de transição e não mais como um estado dicotômico, podendo então compreender tanto fenótipos epiteliais quanto mesenquimais (KARACOSTA et al., 2019; LOVISA; ZEISBERG; KALLURI, 2016).

Já foi demonstrado na literatura que tumores malignos de mama, próstata, pulmão e melanoma, quando expostos a um microambiente ácido, desenvolvem maior capacidade de invasão e migração, acompanhados de um aumento na expressão de marcadores mesenquimais (PEPPICELLI et al., 2015; ROFSTAD et al., 2006; SUTOO et al., 2020). Além disso, uma assinatura genética mesenquimal vem sendo associada na literatura a um pior prognóstico para os pacientes com carcinomas (SCHLIEKELMAN et al., 2015; SOWA et al., 2015). Estudos in vivo demonstram que terapias capazes de reverter a acidose tumoral proporcionam a redução no crescimento do tumor associada a um menor risco

de estabelecimento de metástases e, consequentemente, ao aumento da sobrevida (DA SILVA et al., 2018; ROBEY et al., 2009). Aprofundar esse conhecimento poderá nos levar um passo adiante nas estratégias terapêuticas individualizadas destinadas justamente a essas subpopulações mais agressivas e resistentes (SADEGHI et al., 2020).

Outro importante mecanismo biológico do qual as células neoplásicas apropriam-se é a pluripotência, característica própria de células tronco, as quais são requeridas em diversas condições fisiológicas. Definem-se como tronco as células com capacidade de perpetuar-se por meio de processos de autorrenovação e que dão origem, através da maturação, a células diferenciadas que irão compor os diversos tecidos do organismo (REYA et al., 2001; ZHOU et al., 2021). Em condições de normalidade, as células tronco estão envolvidas em diversos processos fisiológicos: durante o desenvolvimento embrionário células-tronco pluripotentes originam células precursoras teciduais; a geração e regeneração de células sanguíneas e do sistema imunológico; manutenção da homeostase e regeneração tecidual (BLANPAIN; FUCHS, 2014; BLANPAIN; SIMONS, 2013; REYA et al., 2001; ZHOU et al., 2021). Justamente por meio do descrito potencial de autorrenovação - característica de alto valor às células tronco - podemos traçar paralelos biológicos entre essas e algumas populações de células tumorais as quais compartilham dessa habilidade adquirida e, consequentemente, possuem similares vias de sinalização. Essa subpopulação de células vem recebendo diversas denominações na literatura ao longo dos anos, tais como células tronco tumorais (CTT), células propagadoras, progenitoras ou, até mesmo, células iniciadoras tumorais. Em suma, a definição mais aceita para as CTTs é: rara população de células neoplásicas malignas munidas da capacidade de auto renovar-se e gerar tumores (REYA et al., 2001; ZHOU et al., 2021). Existem técnicas capazes de detectar, quantificar e até mesmo isolar essas células utilizando-se biomarcadores específicos presentes tanto na superfície quanto a nível intracelular (ZHOU et al., 2021). Um importante marcador de superfície é o CD44, uma glicoproteína transmembrana, utilizado ao longo dos anos como marcador de CTT em diversos tipos de tumores (ALVERO et al., 2009; DALERBA et al., 2007; DU et al., 2008; LEE; DOSCH; SIMEONE, 2008; ZHOU et al., 2021). Quando presente sozinha, ou juntamente com outros marcadores de CTT, o CD44 já foi associado na literatura ao aumento do potencial tumorigênico à capacidade de auto-renovação celular e de originar células progenitoras diferenciadas CD44 negativas, sendo elencado dentre os marcadores de maior especificidade para detecção e isolamento de subpopulações de CTTs de câncer de cabeça e pescoço resistentes ao tratamento quimioterápico (ABBASIAN et al., 2019; LI et al., 2011; NGUYEN et al., 2017; TAKAISHI et al., 2009).

Também são descritos como biomarcadores os fatores de transcrição Oct4 e Nanog os quais possuem imprescindível papel fisiológico nos estágios iniciais do desenvolvimento embrionário (ZHANG et al., 2008). O Oct4, o qual funciona como fator de transcrição que regula a expressão de diversos genes, em conjunto com outros fatores, já foi associado à conversão de células diferenciadas em células pluripotentes (PAPP; PLATH, 2011; SCHÖ-

LER; CIESIOLKA; GRUSS, 1991). Foi inicialmente associado ao câncer por um grupo de pesquisadores no ano de 2001 (55) e, mais adiante, seu potencial de dar origem a novos tumores foi descrito para o câncer de mama (MONK; HOLDING, 2001; PONTI et al., 2005). Até o presente momento, esse fator de transcrição vem sendo utilizado para isolamento de CTTs em carcinomas de mama, pulmão e fígado, dentre outros (GHANEI et al., 2020; LIU et al., 2020; PÁDUA et al., 2020; WANG et al., 2018). Em suma, células tumorais com alta expressão desse biomarcador, possuem características que se assemelham às das células tronco, tais como potencial auto-renovativo, capacidade de formação de novos focos tumorais e quimiorresistência (KOO et al., 2015).

O Nanog, que também possui papel decisivo durante o desenvolvimento embrionário, é uma molécula ausente em células maduras. Necessário para a manutenção de um fenótipo pluripotente, a expressão desse fator de transcrição é diretamente correlacionada na literatura com a presença de CTTs (BOTCHKINA et al., 2010; CAVALERI; SCHÖLER, 2003; CHAMBERS et al., 2003; GHANEI et al., 2020; MITSUI et al., 2003). Como importantes consequências observadas clinicamente, descreve-se a associação entre Nanog em amostras teciduais tumorais com resistência ao tratamento radio e quimioterápico (DEHGHAN HARATI; RODEMANN; TOULANY, 2019; LAI et al., 2019). Outro marcador altamente expresso em neoplasias de cabeça e pescoço é o Bmi-1, uma proteína do grupo polycomb, a qual compõe a maquinaria de controle transcricional, operante em modificações relacionadas à organização da cromatina (ALKEMA et al., 1993; SIDDIQUE; SALEEM, 2012). Biologicamente relacionado à capacidade de auto-renovação das CTTs, sua superexpressão em neoplasias é correlacionada com a TEM e elevada marcação por Ki-67 (CUI et al., 2007; HUBER et al., 2011; MIHIC-PROBST et al., 2007; SALOMONE, 2020). No espectro clínico, sua presença em amostras teciduais é associada ao estabelecimento de metástases regionais em linfonodos da cadeia cervical nos tumores malignos de cabeça e pescoço, reduzidas taxas de sobrevida, bem como foi descrito como indicador de prognóstico independente no carcinoma espinocelular oral. Alguns estudos experimentais demonstram que o Bmi-1 está relacionado com maior resistência à radioterapia e aumentada probabilidade de metástases à distância (CURTARELLI et al., 2018; JAKOB et al., 2021; KURIHARA et al., 2016; LI et al., 2014; YU et al., 2011). Em suma, um crescente número de evidências científicas ilustram que a expressão de marcadores compatíveis com o fenótipo de CTT está correlacionada à aumentada resistência à terapia anti-tumoral e, consequentemente, demonstram-se determinantes no curso da doença e em seus diversos desfechos clínicos. Diferentes mecanismos intrínsecos (fatores específicos da neoplasia que estão presentes antes de qualquer tratamento) ou adquiridos (desenvolvidos durante o tratamento) determinam a resposta das CTTs à terapia. Durante ou posteriormente à abordagem terapêutica, as CTTs apresentam múltiplas propriedades envolvidas com quimio e radioresistência, tais como: hiperativação da capacidade de reparo do DNA; deter eventos apoptóticos; elevados níveis de espécies reativas de oxigênio; altas taxas de efluxo de

drogas; formação de canais que mimetizam vasos sanguíneos sem o envolvimento de células endoteliais (mimetização vasculogênica) (BAUMANN; KRAUSE; HILL, 2008; CHEN et al., 2011; DEAN; FOJO; BATES, 2005; EPPERT et al., 2011; RICCI-VITIANI et al., 2006; ZHOU et al., 2021).

Todas essas alterações celulares e moleculares ocasionadas pela acidez do MT culminam, em última instância, com o desenvolvimento de resistência frente a múltiplas abordagens terapêuticas, tendo esta correlação sido previamente estabelecida para neoplasias malignas em pele, mama, próstata, cólon bem como para diferentes linhagens celulares de carcinoma espinocelular oral (AVNET et al., 2016; BÖHME; BOSSERHOFF, 2020; DA SILVA et al., 2018; FEDERICI et al., 2014; PELLEGRINI et al., 2014; PEPPICELLI; BIANCHINI; CALORINI, 2014; SAUVANT et al., 2008; VISIOLI et al., 2014). Dentre os mais relevantes mecanismos adquiridos por fenótipos celulares resistentes a agentes quimioterápicos, são considerados a presença e ativação de estruturas celulares capazes de promover o efluxo das drogas administradas; o aumento da expressão de bombas de prótons responsáveis por trocas iônicas e a ativação da via de resposta de proteínas mal dobradas, originalmente denominada Unfolded Protein Response (UPR). Considerando-se que a pressão seletiva imposta pela acidez extracelular elege uma subpopulação de células com alta expressão de transportadores de efluxo de drogas, o influxo e a ação desses agentes é massivamente comprometido, prejudicando assim sua capacidade de combater as células tumorais (DA SILVA et al., 2018; MAHONEY et al., 2003; SIMON; SCHINDLER, 1994).

Alterações de pH observadas no MT estão ligadas a outro fenômeno relevante envolvido com a resistência a drogas quimioterápicas, o aprisionamento dos agentes químicos por íons, denominado íon trapping. Como consequência, a modificação na distribuição das drogas alcalinas, as quais ficam aprisionadas em regiões mais ácidas do tumor, prejudica o efeito terapêutico esperado. A presença de maiores quantidades de bombas de prótons, como as V-ATPases, afetam de forma direta a manutenção de um meio extracelular ácido e, por tanto, corroboram para a manutenção do efeito de ion trapping (LU et al., 2013; MAHONEY et al., 2003; RAGHUNAND; GILLIES, 2000; RAGHUNAND; MAHONEY; GILLIES, 2003).

No que diz respeito à radioresistência, supõe-se que a presença de acidez e de lactato no MT exerce um efeito protetor às células tumorais, baseado na alteração de disponibilidade de espécies reativas de oxigênio (ERO). Já que as EROs são necessárias para a radioindução de danos ao DNA, acredita-se que a atividade antioxidante do lactato, por exemplo, exerça um efeito protetor sobre as células cancerígenas. A aprofundada elucidação desses mecanismos e de sua correlação com características do MT e dos motivos pelos quais, em muitos casos, observa-se a falha dos tratamentos aplicados nos pacientes, contribui para a evolução dos protocolos terapêuticos (BROWN; WILSON, 2004; GROUSSARD et al., 2000; SATTLER et al., 2010; SERTORIO et al., 2018).

1.4 Sistema imunológico e câncer

Durante o crescimento tumoral diversos mecanismos de defesa do hospedeiro podem ser ativados na tentativa de barrar o estabelecimento e desenvolvimento de células que apresentam danos críticos ao seu material genético. Dentre as principais estratégias estão os mecanismos de defesa inerentes às células, responsáveis, por exemplo, pela morte celular programada de células tumorais incipientes. Outra importante linha de defesa dos organismos contra o câncer reside no sistema imune (WEINBERG, 2013). A partir do desenvolvimento de técnicas e imunomarcadores capazes de distinguir os diversos tipos de células de defesa, tornou-se evidente a presença de diferentes densidades de infiltrado inflamatório de caráter heterogêneo no microambiente dos tumores sólidos, o que indicaria uma possível resposta antitumoral do sistema imune na tentativa de combater as células neoplásicas malignas (HANAHAN; WEINBERG, 2011; PAGÈS et al., 2010). Ainda na primeira década do século XIX, o pesquisador Paul Ehrlich levantou a hipótese de que, dentre as funções do sistema imune em proteger o hospedeiro, estaria a resistência ou erradicação do estabelecimento e progressão de tumores desde seus estágios iniciais, avançados e micrometástases. Tal hipótese não foi testada a nível experimental devido, principalmente, à falta de conhecimentos e técnicas acerca do sistema imunológico (EHRLICH, 1908). Alguns anos depois, com o desenvolvimento dos conhecimentos relacionados à imunologia, Burnet e Thomas propuseram a teoria de que populações de células tumorais eram constantemente eliminadas pelos linfócitos barrando o surgimento dos tumores, determinando o conceito de imunovigilância no câncer (BURNET, 1971, 1957, 1964).

Um crescente número de estudos recentes envolvendo o uso de animais geneticamente modificados, bem como estudos epidemiológicos nos levam a crer que, de fato, o sistema imune atua impedindo o estabelecimento e progressão dos tumores. Quando comparados tumores induzidos em animais imunodeficientes com animais controle imunocompetentes, observou-se uma maior frequência e velocidade de crescimento tumoral nos animais com resposta imune deficiente. Já em pacientes portadores de tumores de cólon, ovário e melanoma observou-se um melhor prognóstico naquelas amostras com alta densidade de linfócitos T citotóxicos e Natural Killer (NELSON, 2008; OSTRAND-ROSENBERG, 2008; PAGÈS et al., 2010).

Sob essa ótica, quando os tumores se estabelecem, as células neoplásicas, ou parte delas, adquiriram a capacidade de evadir a constante “vigilância” dos diversos braços do sistema imune. As dificuldades enfrentadas pelo sistema imune no reconhecimento das células tumorais residem no fato de que este atua na eliminação de agentes estranhos ao organismo, logo, o reconhecimento de células nativas do organismo, ainda que alteradas, representa um desafio ao hospedeiro. A detecção e eliminação de clones celulares neoplásicos altamente imunogênicos ocorre constantemente, no entanto, ao que tudo indica, populações clonais pobremente imunogênicas não são detectadas e subsequentemente

expandem-se, estabelecendo as massas tumorais sólidas (HANAHAN, 2022; HANAHAN; WEINBERG, 2011).

Esse processo, no câncer, é denominado imunoedição e é composto por três fases: eliminação, equilíbrio e evasão. A fase de eliminação consiste na imunovigilância do câncer; o equilíbrio é um período de latência que ocorre após a destruição incompleta das células tumorais, apenas de sua subpopulação fortemente imunogênica; e a evasão caracteriza-se pela expansão dos clones menos imunogênicos os quais driblaram o sistema imune nas fases anteriores (DUNN; OLD; SCHREIBER, 2004). Além disso, é muito provável que, mesmo células neoplásicas altamente imunogênicas sejam capazes de driblar a vigilância do sistema imune através da secreção de citocinas imunossupressivas (HANAHAN; WEINBERG, 2011).

Figura 5 – QUADRO 1. Mecanismos imunoevasivos das células tumorais

Estratégia	Mecanismo	Células do sistema imune
Impedir a detecção	Repressão de antígenos tumorais e proteínas MHC I	Linfócitos T citotóxicos
Supressão do estresse	Repressão de proteínas de superfície relacionadas ao estresse celular	Células Natural Killer
Inativação de imunócitos	Destruição e saturação de receptores de imunócitos	Células Natural Killer Linfócitos T citotóxicos
Inibição da apoptose	Inibição da cascata de caspases Resistência a apoptose mediada por FasL	-
Indução da apoptose de imunócitos	Liberação de FasL solúvel e citocinas IL-10 e TGF-ß	Linfócitos T citotóxicos Células dendríticas Macrófagos
Neutralização do sistema complemento	Superexpressão de proteínas regulatórias do complemento ligadas à membrana (mCRPs).	Sistema complemento

Adaptado de Weinberg, 2013.

O estabelecimento de células malignas têm o potencial de ativar a resposta imune do hospedeiro, o que resulta na destruição das células mutadas. A presença de um infiltrado inflamatório rico em linfócitos T vem sendo correlacionada na literatura com melhores prognósticos em diversos tipos de tumores, tais como câncer de mama, ovário e melanoma,

bem como em tumores malignos de cabeça e pescoço (PAGÈS et al., 2010). As células T helper CD4+, T citotóxicas CD8+ e natural killer (NK) são descritas como as principais envolvidas na destruição das células tumorais e estão relacionadas com a memória do sistema imune, evitando tanto a recorrência do tumor, quanto o estabelecimento de metástases (OSTRAND-ROSENBERG, 2008).

Os macrófagos podem exercer diferentes papéis durante a progressão tumoral a depender do seu fenótipo. Os macrófagos do tipo 1 (M1) são ativados por interferon γ (IFNγ), lipopolissacarídeos bacterianos (LPS), fator de necrose tumoral α (TNF-α) e fator estimulador de colônias de monócitos granulócitos (GM-CSF). Essas células atuam eliminando células tumorais por meio da secreção de óxido nítrico e outros mediadores químicos, bem como ativam a resposta citotóxica de linfócitos T CD8+ (DENARDO; ANDREU; COUSSENS, 2010; OSTRAND-ROSENBERG, 2008).

Linfócitos T CD8+ apresentam um papel bem estabelecido dificultando o desenvolvimento do câncer. Atuam diretamente sobre as células tumorais detectando-as a partir do MHC de classe I e induzindo sua apoptose via Fas e FasL, ou através da liberação de proteínas citotóxicas, como as perforinas e granzimas (COHEN; BLASBERG, 2017; WEINBERG, 2013). Tumores que apresentam um denso infiltrado desse subtipo linfocitário possuem desfechos clínicos favoráveis (NELSON, 2008).

Células T CD4+ do tipo 1 (Th1), ativadas por interleucina (IL) 12, apresentam papel antitumoral, responsáveis pela estagnação ou regressão do tumor em formação. Além disso, estas células influenciam a ativação das células T citotóxicas CD8+, facilitando assim a destruição de células tumorais (DENARDO; ANDREU; COUSSENS, 2010; OSTRAND-ROSENBERG, 2008). A regulação da vigilância imune se dá através da secreção de citocinas pelas células Th1. Quando ativadas, essas células secretam mediadores químicos como IFNγ, fator de necrose tumoral α (TNFα), IL-2 e 12. Essas citocinas reguladoras estão relacionados ao processamento e apresentação de antígenos nas moléculas do complexo de histocompatibilidade (MHC) de classe I e II por células apresentadoras de antígenos (APCs) as quais, por sua vez, influenciam na duração e magnitude da resposta citotóxica dos linfócitos T CD8+ no ataque às células neoplásicas. A secreção de IFNγ pelas células Th1 promove a indução de um fenótipo M1 em macrófagos. Além disso, as células Th1 podem matar diretamente células tumorais por meio da liberação de altas concentrações de IFNγ, TNFα e grânulos citolíticos. Ou seja, as respostas de Th1 podem direta e/ou indiretamente impedir o desenvolvimento do câncer (DENARDO; ANDREU; COUSSENS, 2010).

As células NK são capazes de reconhecer proteínas de superfície relacionadas ao estresse celular, bem como reconhecer células tumorais as quais tenham suprimido a expressão de MHC I, desencadeando uma resposta citotóxica (WEINBERG, 2014). Um aumento da resposta antitumoral desempenhada por essas células se dá através da superexpressão de receptores NK e seus ligantes nas células alvo, isso ocorre frente a

expressão de IL12 e IFNγ pelas células Th1. Sendo assim, a resposta antitumoral que é desempenhada tanto por macrófagos, quanto por células NK, parece ser favorecida por Th1 (DENARDO et al., 2010).

Os linfócitos T apresentam uma outra subclasse a qual tem revelado um importante papel durante o processo de carcinogênese, os linfócitos T reguladores (Tregs). Tal subpopulação linfocitária CD4+, pode ser mais precisamente identificada pela presença de grandes concentrações do receptor de superfície CD25 o qual é destinado à IL-2. Além disso, esses linfócitos também apresentam o fator de transcrição Foxp3 como marcador de linhagem. Apesar de serem muito associados a eventos que favorecem a progressão tumoral, os linfócitos Tregs foram correlacionados a bons prognósticos e a um melhor controle locorregional em carcinomas de cabeça e pescoço e outros tumores (PAGÈS et al., 2010). Há, ainda, relatos na literatura de que esses linfócitos possuem uma atividade regulatória supressora de macrófagos (M2) e neutrófilos do tipo 2, conhecidos por sua ação pró-tumoral que se estabelece através da liberação de IL6, IL23, IL1 e TNFα. Assim macrófagos e neutrófilos do tipo 2 induziriam a produção, por outras células do sistema imune, de interleucinas (IL17, IL21 e IL22) envolvidas com proliferação e sobrevivência de células tumorais (WANG; VELLA, 2016).

Um crescente número de evidências nos leva a compreender que as células do sistema imune parecem desempenhar papéis conflitantes durante o estabelecimento e desenvolvimento do câncer (DENARDO; ANDREU; COUSSENS, 2010). Por outro lado, a presença de um infiltrado inflamatório no sítio tumoral pode contribuir, por meio da liberação de citocinas, para a manutenção de sinais proliferativos, sobrevivência celular, angiogênese, invasão e metástase (COLOTTA et al., 2009; DENARDO; ANDREU; COUSSENS, 2010; GRIVENNIKOV; GRETEN; KARIN, 2010; QIAN; POLLARD, 2010). Além disso, o recrutamento de algumas subclasses de células mielóides que, ao serem expostas a determinados estímulos do microambiente, tornam-se capazes de promover a carcinogênese, tais como mastócitos, monócitos, granulócitos, macrófagos e linfócitos parece ser mais uma estratégia das células tumorais (DENARDO; ANDREU; COUSSENS, 2010).

Como visto anteriormente, os macrófagos exercem papéis ambíguos durante a progressão tumoral a depender do seu fenótipo. Um fenótipo do tipo 2 (M2) é adquirido após a exposição de monócitos à IL4, IL13, IL10 e fator de crescimento tumoral (TGFß) (DENARDO; ANDREU; COUSSENS, 2010; OSTRAND-ROSENBERG, 2008; WEINBERG, 2013). Uma vez tendo adquirido tais características, os M2 liberam moléculas envolvidas com o crescimento, sobrevivência, ativação da angiogênese, remodelação da matriz extracelular, invasão, bloqueio da ativação de linfócitos T citotóxicos, bem como induzem a produção de fatores de crescimento e alteram a resposta inflamatória por meio da diminuição de citocinas pró-inflamatórias como IL6 e TNFα (DENARDO; ANDREU; COUSSENS, 2010; OSTRAND-ROSENBERG, 2008; PAGÈS et al., 2010).

Células T CD4+ do tipo 2 (Th2), ativadas por IL14, expressam altos níveis de IL4, IL5,

IL6, IL10, IL13 e são correlacionadas na literatura com a inibição da citotoxicidade de células T e promoção da ativação de células B. Além disso, a inibição de eventos apoptóticos e indução de atividade proliferativa por meio da liberação de IL4 também é atribuída aos M2. Clinicamente, tais células foram correlacionadas com maior tamanho, estadiamento e número de metástases em linfonodos em câncer de mama (DENARDO; ANDREU; COUSSENS, 2010; OSTRAND-ROSENBERG, 2008). Outra subclasse de linfócitos com papel pró-tumoral são os linfócitos T helper CD4+ produtoras de IL17 (Th17), os quais são ativados pela combinação de IL6 e TGFß e tem seus efeitos mediados pela secreção de IL17, IL21 e IL22. Tais células foram descritas como presentes em pacientes portadores de câncer em ovários, cólon, próstata e carcinoma hepatocelular para os quais altas taxas de IL17 é correlacionada com piores prognósticos (DENARDO; ANDREU; COUSSENS, 2010; WANG; VELLA, 2016).

Há indícios de que as células tumorais recrutam para o sítio tumoral linfócitos Tregs onde estes, por sua vez, inibirão a atividade antitumoral de linfócitos T infiltrantes da massa tumoral, bloquearão a morte celular mediada por células NK e suprimirão a atividade de células dendríticas e células apresentadoras de antígenos (através da indução da ligação CTLA-4 e CD80/86). Além disso, esses linfócitos promovem o aumento das citocinas imunossupressivas IL10, IL35 e TGFß bloqueando a ativação de M1 e promovendo fenótipos mielóides imunossupressivos. Clinicamente, a presença dessas células é correlacionada a estágios mais avançados de doença e piores sobrevidas dos pacientes (OSTRAND-ROSENBERG, 2008; PAGÈS et al., 2010; WANG; VELLA, 2016). Por último devemos considerar as células B, responsáveis pela regulação de diversas vias que envolvem a secreção de IL10 e TGFß transformando o microambiente tumoral e o estroma celular em um meio que favorece a carcinogênese (DENARDO; ANDREU; COUSSENS, 2010; OSTRAND-ROSENBERG, 2008).

As células tumorais, por sua vez, expressam moléculas de reconhecimento em sua superfície, as quais desencadeiam diferentes desfechos que promovem a eliminação ou manutenção celular. Dentre essas moléculas de superfície destacam-se a calreticulina (CRT) e o cluster de diferenciação 47 (CD47). Um complexo protéico formado entre a CRT e ERp57 é translocado à membrana plasmática e promove um sinal de morte facilitando a ação fagocítica de macrófagos e células dendríticas. Em oposição a esse sinal de morte celular, está a expressão, na membrana plasmática, do CD47 o qual emite uma informação de proteção das células tumorais evitando a ação fagocítica de macrófagos (HERRERA; BOURHIS; COUKOS, 2017).

1.5 Efeitos do lactato/acidez do microambiente tumoral sobre a resposta imunológica

Dentre as vantagens adaptativas decorrentes das inóspitas condições do microambiente tumoral, a presença de lactato e acidez do compartimento extracelular estão correlacionadas como comprometimento funcional de células do sistema imune, enfraquecendo a imunovigilância contribuindo, em última instância, para a consolidação de mecanismos de escape imunológico (ESTRELLA et al., 2013; GATENBY; GILLIES, 2004; HJELMELAND et al., 2011; MOELLERING et al., 2008; SMALLBONE et al., 2005; ZHANG; LI, 2020). Não somente a atividade citotóxica de diversos imunócitos é prejudicada, como também há um favorecimento de células do sistema imune com potencial pró-tumoral, beneficiando células neoplásicas malignas no processo de progressão do tumor (BOHN et al., 2018; ZHANG; LI, 2020).

A acidez do microambiente tumoral é capaz de suprimir a atividade antitumoral de células T citotóxicas provocando a redução de citocinas inflamatórias e diminuindo a capacidade citolítica desse subtipo celular (BALGI et al., 2011). Em macrófagos, o pH ácido gerado pelas células tumorais aumenta a expressão de marcadores característicos de do fenótipo M2, descrito como pró-tumoral e diminui a expressão de marcadores relacionados ao fenótipo M1, de atividade descrita como antitumoral, tais como IL-6, iNOS e CCL2 (XIE; ZHU; BAI, 2016). Em consonância com esses achados, foi descrito na literatura um efeito prejudicial da acidez extracelular sobre a atividade antitumoral de células NK (LANGIN, 2010).

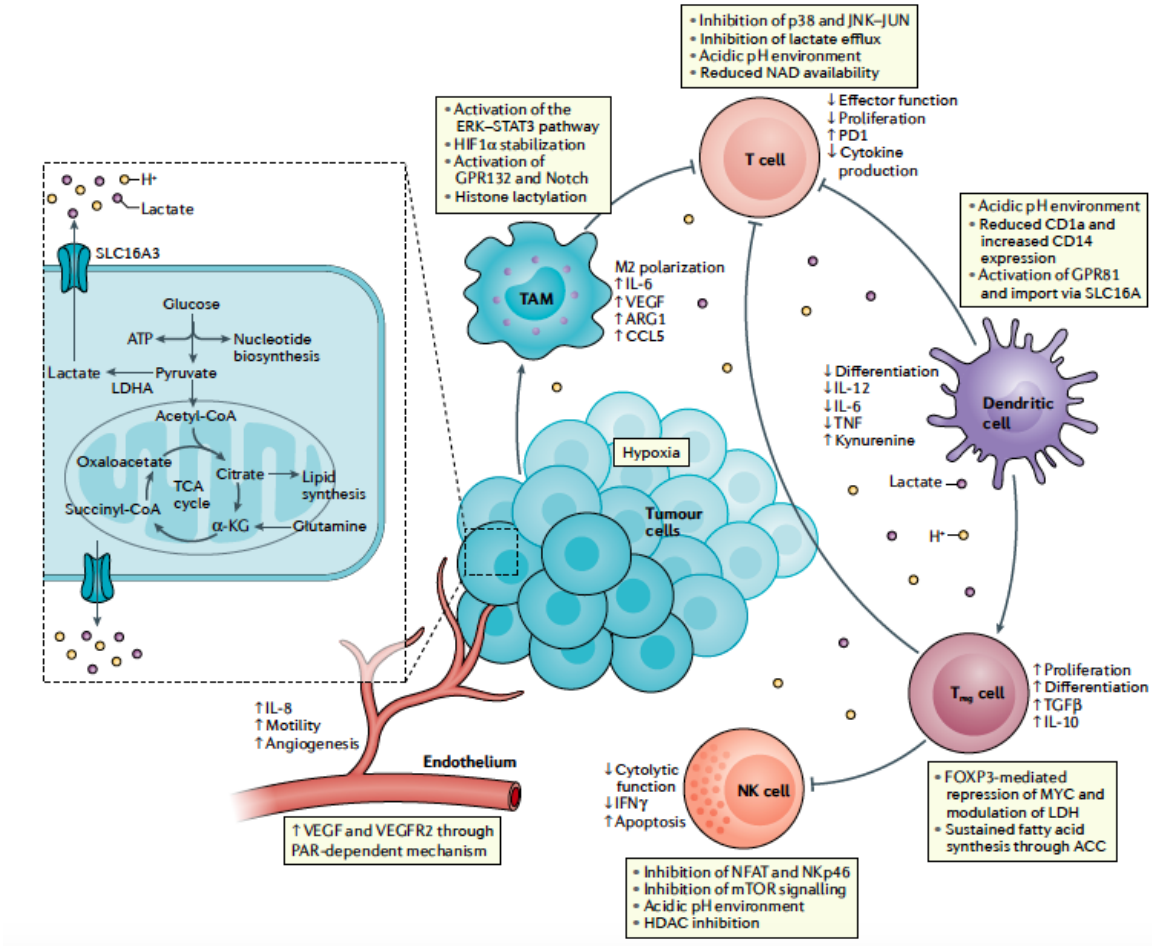
Nesse contexto, foi demonstrado que o lactato, como subproduto do metabolismo celular tumoral, é capaz de induzir a polarização de monócitos humanos em macrófagos M2. Nesse processo, explora-se na literatura o envolvimento da via de sinalização ERK-STAT3, bem como a ativação de receptores acoplados à proteína G, como GPR132, presentes na membrana celular de macrófagos, comprovadamente correlacionados com a ativação de um fenótipo M2 (CHEN et al., 2017; IPPOLITO et al., 2019). Recentemente vem sendo discutida a participação de grupos lactil, derivados do lactato, em alterações nas histonas a nível pós translacional, a partir das quais observa-se aumento na expressão de genes característicos do fenótipo M2, como IL6 e ARG1. Além disso, foi observado que esses metabólitos levam à inibição da diferenciação de monócitos em células dendríticas, apontando para o lactato como fator dificultador da diferenciação e acúmulo desse tipo celular no microambiente de tumores (GOTTFRIED et al., 2006; PUIG-KRÖGER et al., 2003; ZHANG et al., 2019).

Em células T, os altos níveis de lactato no compartimento extracelular geram um desequilíbrio osmótico, impedindo o efluxo do metabólito o qual acumula-se a nível intracelular. Como consequência desse processo, observam-se a diminuição da produção de citocinas e o profundo comprometimento das funções citotóxicas desses linfócitos (FISCHER et al., 2007; XIA et al., 2017). O lactato presente em abundância no microambiente tumoral possui

um efeito inibitório das funções citolíticas clássicas de células NK, aumentando a concentração de mielócitos imunossupressores, os quais são capazes de prejudicar o potencial citotóxico das células natural killer (HUSAIN; SETH; SUKHATME, 2013). Adicionalmente aos efeitos relacionados à diminuição da citotoxicidade nos dois imunócitos, o lactato leva à redução na produção de IFNγ, por meio da inativação de NFAT, em ambas os tipos celulares (PENG et al., 2016). Por outro lado, observou-se em estudo recente, que a expressão de FOXP3 em células T reguladoras - as quais possuem atividade regulatória sobre outras células do sistema imune - é parte de adaptações metabólicas vitais para sobrevivência e manutenção das funções imunossupressoras exercidas por esse imunócito (ANGELIN et al., 2017).

Apesar de bem estabelecido na literatura, o efeito imunossupressor do microambiente tumoral compreende uma ampla gama de mecanismos que seguem sendo explorados e descritos ao longo das últimas décadas (DAMAGHI; WOJTKOWIAK; GILLIES, 2013; GIATROMANOLAKI et al., 2017, 2019; HUBER et al., 2017; PILON-THOMAS et al., 2016). O avanço dos conhecimentos produzidos nessa área converte-se, na esfera clínica, em uma melhor compreensão acerca dos processos e fatores envolvidos na malignidade tumoral e, em última instância, na previsão de melhores ou piores prognósticos clínicos. Além disso, no que diz respeito às opções terapêuticas disponíveis na atualidade, sabe-se que, em muitos casos, a resposta aos agentes imunoterápicos não é satisfatória, principalmente no que concerne à eficácia e manutenção dos efeitos antineoplásicos obtidos. Sendo assim, o esclarecimento dos efeitos do microambiente tumoral sobre a resposta aos agentes imunoterápicos é de grande importância na busca por melhores índices de sucesso terapêutico.

Figura 6 – Lactato no microambiente tumoral. O microambiente tumoral é composto por diversos componentes, incluindo diferentes tipos de células, bem como componentes do estroma. Neste microambiente, como consequência do efeito Warburg, as células tumorais secretam grandes quantidades de lactato no compartimento extracelular, levando à acidificação do meio e favorecendo angiogênese e imunossupressão. O lactato, um dos metabólitos mais proeminentes do microambiente tumoral, atua modulando o metabolismo das células do sistema imune, inibindo a ativação e proliferação de células CD8+ T, células natural killer e células dendríticas. Além disso, o lactato afeta positivamente o perfil metabólico das células T reguladores CD4+CD25+ (Treg), potenciando as suas funções imunossupressoras e permitindo a sua sobrevivência mesmo em condições hostis. A polarização dos macrófagos de fenótipo protumoral do tipo M2 é favorecido o que colabora com angiogênese, remodelação de tecidos, promovção do crescimento e invasão tumoral.



Certo et al., 2021

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar a influência da acidez e da presença do lactato no microambiente tumoral do carcinoma espinocelular bucal sobre os desfechos de proliferação, migração celular, resposta a tratamento quimioterápico e radioterápico, assim como sobre a resposta imune antitumoral.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar diferentes condições experimentais relacionadas à acidez, comparando a exposição celular ao pH ácido de caráter contínuo e intermitente.
- Avaliar a adaptação de células tumorais após a exposição ao microambiente ácido.
- Avaliar a influência das condições da acidez do microambiente tumoral sobre a capacidade migratória de células tumorais, assim como vias de sinalização relacionadas a esse fenômeno.
- Analisar alterações na resposta ao tratamento quimio e radioterápico nas diferentes condições experimentais que mimetizam o microambiente ácido.
- Realizar uma revisão sistemática da literatura sobre o impacto da acidez tumoral na resposta imunológica antitumoral.

3 ARTIGOS CIENTÍFICOS

3.1 ARTIGO CIENTÍFICO I

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The role of tumor acidification in aggressiveness, cell dissemination and treatment resistance of oral squamous cell carcinoma

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ABSTRACT

Aims: To investigate the role of tumor acidification in cell behavior, migration, and treatment resistance of oral squamous cell carcinoma (OSCC).
Main methods: The SCC4 and SCC25 cell lines were exposed to acidified (pH 6.8) cell culture medium for 7 days. Alternatively, a long-term acidosis was induced for 21 days. In addition, to mimic dynamic pH fluctuation of the tumor microenvironment, cells were reconditioned to neutral pH after experimental acidosis. This study assessed cell proliferation and viability by sulforhodamine B and flow cytometry. Individual and collective cell migration was analyzed by wound healing, time lapse, and transwell assays. Modifications of cell phenotype, EMT induction and stemness potential were investigated by qRT-PCR, western blot, and immunofluorescence. Finally, resistance to chemo- and radiotherapy of OSCC when exposed to acidified environmental conditions (pH 6.8) was determined.
Key findings: The exposure to an acidic microenvironment caused an initial reduction of OSCC cells viability, followed by an adaptation process. Acidic adapted cells acquired a mesenchymal-like phenotype along with increased migration and motility indexes. Moreover, tumoral extracellular acidity was capable to induce cellular stemness and to increase chemo- and radioresistance of oral cancer cells.
Significance: In summary, the results showed that the acidic microenvironment leads to a more aggressive and treatment resistant OSCC cell population.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent malignancy in the head and neck region and accounts for 90% of malignant neoplasms affecting the oral cavity [1–3]. Its initiation and progression are due to genetic alterations usually caused by environmental factors like tobacco and alcohol consumption [4,5]. OSCC survival rate is around 50% in 5 years and has poorly improved in recent decades [6–10]. During initial diagnosis most of the OSCC patients present a locally advanced disease and are treated with an interdisciplinary therapy consisting of surgery, radio- and chemotherapy [11,12]. This highly aggressive approach often decreases patient's quality of life, due to the high morbidity. To achieve more effective therapies, it is necessary to better understand its biological behavior [13–15].

One of the main reasons for OSCC treatment failure is the high complexity of this disease, which is not only defined by a set of genetically modified cancer cells but also by the existence of a tumor microenvironment [16]. Solid cancers are affected by a set of metabolic

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alterations, which contributes to promote tumor growth and progression as well as modulating treatment response [16,17]. An important hallmark of solid tumors is extracellular acidity. Previous evidence suggests that tumor acidosis is caused by low blood perfusion, hypoxia, and reprogramming of cellular energy metabolism [18]. Tumor cells often reverse their energy production increasing glycolysis rates rather than oxidative phosphorylation. At first, this inversion occurs in response to hypoxia. However, in well-established tumors with vascular support, the phenomenon called 'aerobic glycolysis' has been described. The secretion of hydrogen ions and lactate generated by increased glycolysis and lactic fermentation rates results in acidification of the extracellular milieu [18].

The extracellular pH of solid tumors is known to be remarkably low, reaching values between 6.2 and 6.8 [18,19]. As a result of this acidic stress, host stromal and immune cells and even non-adapted cancer cells die. On the other hand, the acidic pH adapted tumor cells gain invasiveness and metastatic capacity [20]. In addition, the presence of lactic acid and low pH levels in the tumor microenvironment is correlated with the decrease of several immune effector cell functions contributing to immune escape and cancer progression [20–22]. In this context, chronic exposure of tumor cells to acidic pH may result in the selection of more aggressive phenotypes [23–26]. *In vivo* studies demonstrate that when treating animals with pH buffering experimental therapies, such as lactate dehydrogenase A (LDHA) inhibition [27], inhibition of the lactate monocarboxylate transporter 1 (MCT1) [28], and oral administration of sodium bicarbonate [26], among others, can lead to a decreased tumor growth and an increased survival rate [29–32]. Therefore, therapies aiming to reverse tumoral acidification mechanisms and targeting molecules of the glycolytic metabolism have been developed over the past years [33].

Although the literature has already demonstrated that the extracellular pH of OSCC is also acidic, the profile of its behavior under an acidic microenvironment has not yet been elucidated [34]. Hence, the aim of the present study was to demonstrate the influence of an acidic microenvironment over OSCC cells behavior in regard to its proliferation and migratory capacities, stemness potential, and therapy resistance.

2. Materials and methods

2.1. Cell culture

The well-established human cell lines SCC4 and SCC25 derived from tongue OSCC (ATCC, https://www.lgcstandards-atcc.org/), human keratinocyte HaCat (human aneuploid immortal keratinocyte - BCRJ) and primary oral fibroblasts were used. Primary oral fibroblasts were obtained through the explant cell culture method and characterized by its typical spindle morphology and e-cadherin lack of expression [35]. For SCCs and HaCat cell lines, three main groups were established: Group 1 was continuously exposed to neutral (pH 7.4) cell culture medium being considered the control and was called pH 7.4; Group 2 was continuously exposed during 7 days to acidified (pH 6.8) cell culture medium and was named pH 6.8; and Group 3 was exposed to acidified (pH 6.8) cell culture medium for 7 days being later reconditioned to neutral (pH 7.4) medium for additional 7 days and was named pH 6.8 → pH 7.4 (Fig. 1A). Alternatively, a long-term acidosis was induced by exposing cells to acidified (pH 6.8) cell culture medium for 21 days (Fig. 1B).

The neutral culture medium was based on Dulbecco modified Eagle medium (DMEM, Thermo Scientific, Waltham, MA, EUA), supplemented with 10% fetal calf serum (Thermo Scientific), 5 mM Hepes (Thermo Scientific), 3.7 g sodium bicarbonate (Sigma-Aldrich, St. Louis, Missouri, United States), 100 U/mL penicillin, and 100 mg/mL of streptomycin (Thermo Scientific).

The acidified cell culture medium was prepared by adding 25 mM Pipes Buffer (Sigma-Aldrich) instead of sodium bicarbonate in DMEM medium (Thermo Scientific). The solution was then acidified with 5 M hydrochloric acid (Sigma-Aldrich) and supplemented with 10% fetal calf serum (Thermo Scientific), 5 mM Hepes (Thermo Scientific), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Thermo Scientific) [36,37]. pH stability was measured with a pH meter every two days. All

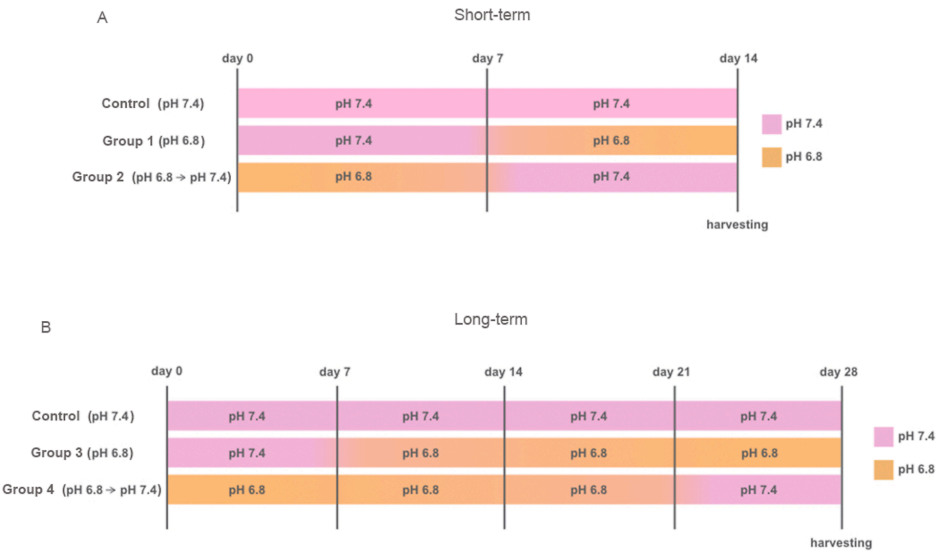


Fig. 1. Methodological definition of experimental groups. Different cell exposure patterns to the acidified culture medium: A – Cell exposure to acidified culture medium with (Group 2) and without (Group 1) reconditioning to neutral culture medium; B – Long-term cell exposure (21 days) to acidified culture medium with (Group 5) and without (Group 4) reconditioning to neutral culture medium. The Controls were kept in pH 7.4 during the indicated time points.

groups were cultivated at 37 °C and 5% CO2. Cells were monitored daily, by an experienced researcher, under an inverted phase microscope. Conditions such as cell confluence, health, and absence of contamination signs were constantly checked. All tests were performed in triplicate and analyzed by a single blind evaluator.

Cell growth and multiplication was analyzed through cumulative population doubling (CPD) method according to the protocol proposed by Silva et al. [38] for the analysis of long-term population growth in cancer. Cells were harvested when confluence was between 20 and 80%. A defined number of cells was seeded and maintained under specific cell culture medium pH. Cells were counted using trypan blue dye to exclude dead cells at established time points or whenever a maximal 80% confluence was reached. Next, a given number of cells were reseeded, and previous steps were repeated during the whole experiment. Each interval gave rise to an initial number (IN) and a final number (FN) of cells that were applied in the population doubling (PD) formula to quantify how many times the population doubles in a given interval. The sum of consecutive population doublings (PD) produces the CPD.

2.2. Flow cytometry

Cell cycle analysis and apoptosis/necrosis were performed with the SCC4 cell line. For the cell cycle analysis, 2×10^5 cells had their DNA stained with propidium iodide (PI). To this end, 50 μg/mL of propidium iodide (Sigma Aldrich), 0.1% TritonX (Sigma Aldrich) and 0.1% sodium citrate (Sigma Aldrich) were added to the cells and the solution was read in the flow cytometer (Attune Nxt, ThermoScientific). For the apoptosis/necrosis assay 1×10^5 cells had apoptosis induction with 2 mM hydrogen peroxide and necrosis induction with 4 mM hydrogen peroxide 4 h before the experiment. All the content from the culture medium, the washing PBS and trypsinization solution were collected and centrifuged. The supernatant was resuspended with Binding Buffer (BD) and 100 μL of the solution was transferred to a microtube, where 5 μL of PI (50 μg/mL) and 5 μL of BD were added. After incubation, 400 μL of Binding Buffer was added and a flow cytometer reading was performed.

2.3. Polarity ratio assay

At the end of the experimental timepoints, approximately 100 cells in culture plates were photographed (Zeiss Axioscan Observer z.1). The polarity index was calculated as the length of the major axis divided by the length of the perpendicular axis that intersects the center of the cell nucleus using ImageJ Software [39]. An increased polarity ratio is related to a spindle cell morphology.

2.4. Real time PCR

The gene expression of stemness Bmi-1, CD44 and Nanog and EMT markers E-cadherin and N-cadherin of the SCC4 cell line groups were analyzed and compared. The mRNA was extracted with Trizol (Invitrogen, Carlsbad, OH, USA), followed by phenol and chloroform. The cDNA was prepared with the superscript III reverse transcriptase kit (Invitrogen). The Taqman (Thermo Scientific) primers were used to perform the real time PCR reaction: Bmi-1 (Hs00409825_g1); CD44 (Hs01073861_m1), Nanog (Hs04260366_g1), E-cadherin (Hs01023894_m1); N-cadherin (Hs00983056_m1). All reactions were performed in triplicate and standardized for GAPDH (Hs02758991_g1).

2.5. Wound healing assay

To assess the migration capacity, 7×10^5 cells on six-well plates were seeded and the scratch wound healing assays were performed. After reaching 100% confluence, two scratches forming a cross in the center of the plate were produced with the aid of a white tip. The cells were washed three times with PBS and neutral culture medium (pH 7.4) was added. Photographs of the four points adjacent to the central cross (meeting point between the two traces) were taken at different experimental time points (0 h, 10 h and 24 h). Each picture was analyzed, and the wound closure was measured using ImageJ software.

2.6. Time lapse assay

Cell's individual migration pattern was analyzed by a time lapse test. The cells were seeded at 1×10^5 concentration. Twelve hours later the plate was filled with neutral culture medium (pH 7.4) and had its lid sealed with solid vaseline. In an inverted microscope (Zeiss Axioscan Observer z.1), an area containing between ten and twenty cells was selected and a time lapse video was performed by taking one picture every ten minutes over a twenty-hour interval. The material obtained was analyzed and the movement of each cell on the plate was mapped using ImageJ software.

2.7. Transwell assay

Cell invasion was examined using transwell chambers (Corning, USA) with 8 μm-pore polycarbonate filters. A concentration of 1×10^4 cells was suspended in DMEM containing 2% FBS and added to the upper chamber of the transwells. 750 μL of culture medium containing 10% FBS was added into the lower chamber. A cotton swab was used to remove the cells that remained on the upper surface of the membrane after 48 h. The cells that penetrated across the polycarbonate membrane were fixed with 100% methanol for 5 min at −20 °C and stained with hematoxylin and eosin. The membrane was removed from the plastic chamber with a scalpel and the slides were produced with Permount Mounting Medium (Fischer Chemical, Leicestershire, UK) and covered with coverslips. Ten random microscopic fields (100× magnification) were photographed, and cells were counted.

2.8. Sulforhodamine B viability assay

To analyze cell viability after exposure to the acid condition, chemo- and radiotherapy of the SCC4 and SCC25 cell lines the Sulforhodamine B (SRB) assay was performed as described by Vichai and Kirtikara (2006) [40]. The cells were seeded at a concentration of 5×10^3 cells per well in 96-well plates and fixed with 10% trichloroacetic acid 24 h after the ending time point. For the chemoresistance assay the drug Cisplatin (Bedford Lab, Bedford, Ohio, US) was added at different concentrations for 72 h. Later all the cells resulting from the different treatments were stained with 0.4% SRB (Sigma-Aldrich) and washed with 1% acetic acid. The plates were read in the microplate reader at 560 nm. The results were normalized against the density of cells seeded in the initial and treatment-free control.

2.9. Western blot

Whole cell protein lysate was extracted using RIPA (Radioimmunoprecipitation assay) buffer and protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The lysates were centrifuged at 4 °C and the supernatant was collected. Protein concentration of RIPA lysates was measured utilizing BCA protein assay kit (Thermo Scientific, Germany). A volume of homogenate containing 20 μg of denatured protein was separated by 10% Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Burlington, Massachusetts, US). After blocking with 5% milk (Nestlé, Brazil), membranes were incubated with primary and horseradish peroxidase coupled-secondary antibodies for the EMT marker E-cadherin and GAPDH as a standard control (9782T, Cell Signaling, Danvers, Massachusetts, US). Membranes were incubated in an enhanced chemiluminescence solution (Thermo Scientific) and developed.

2.10. Immunofluorescence staining

To evaluate the expression and location of EMT marker Vimentin at a single cell level, SCC4 and SCC25 cells were seeded on coverslips in 12-well plates (Greiner Bio-One), were kept for 48 h under normal growth conditions and then treated according to their experimental groups. Cells were fixed with 4% paraformaldehyde for 15 min at 4 °C, incubated for 30 min in 0.5% Triton-X100 and blocked for 30 min with T-buffer (0.2% Tween 20, 1% BSA in PBS). Cells were incubated for 1 h at room temperature with the first antibody, followed by incubation for 30–60 min with the secondary antibody. Nuclei were visualized with DAPI (Sigma, dilution 1:1000). Coverslips were mounted with Vecta-Mount (Vector Laboratories) and analyzed by fluorescence microscopy (Olympus BX-50F). Pictures were taken from at least five individual fields using the Olympus XC30 camera.

2.11. Chemo- and radiotherapy treatment

The chemotherapy treatment was performed using different dilutions of the drug Cisplatin as described by Sharma et al., 2018 and cell viability was assessed by SRB. Cell groups received fractionated irradiation with a daily dose of 2 Gy using the Faxitron MultiRad 225 irradiator for 5 days.

2.12. Statistical analysis

An initial data distribution analysis was performed. For the group's statistical comparison, when the distribution was normal, the two-way ANOVA and ANOVA tests were used, followed by the Bonferroni test, using the GraphPad Prism 5.0 program (La Jolla, CA). The level of significance considered was $p < 0.05$.

3. Results

3.1. Extracellular acidity induces a mesenchymal-like morphology and alters viability and survival capacity of OSCC cells

Previous studies have shown that the pH of OSCC ranges between 6.56 and 6.97 [34]. To mimic the influence of an acidic tumor microenvironment *in vitro*, we cultured OSCC cell lines, SCC4 and SCC25, in an acidified cell culture media at pH 6.8 and analyzed the effect of this condition on cell's adaptation and survival. Also, to mimic dynamic pH fluctuation of tumor microenvironment we analyzed cells that were reconditioned to neutral pH after experimental acidosis [24].

CPD findings concerning cell growth at the different experimental conditions revealed a compromised proliferation when cells were exposed to pH 6.8. On the other hand, when cells were reconditioned at physiological pH the proliferation capacity was recovered (Fig. 2A). The cell cycle phase distribution analysis revealed a slight decrease in proportion of G1 cells and an increase of the S phase cells for all groups exposed to acidic stress, however no statistical difference between the groups was observed. For all groups most of the cells were at the G1 and G2 phases (Fig. 2B). Viability assay indicated statistically significant decrease ($p < 0.0001$) in survival potential after exposition to pH 6.8

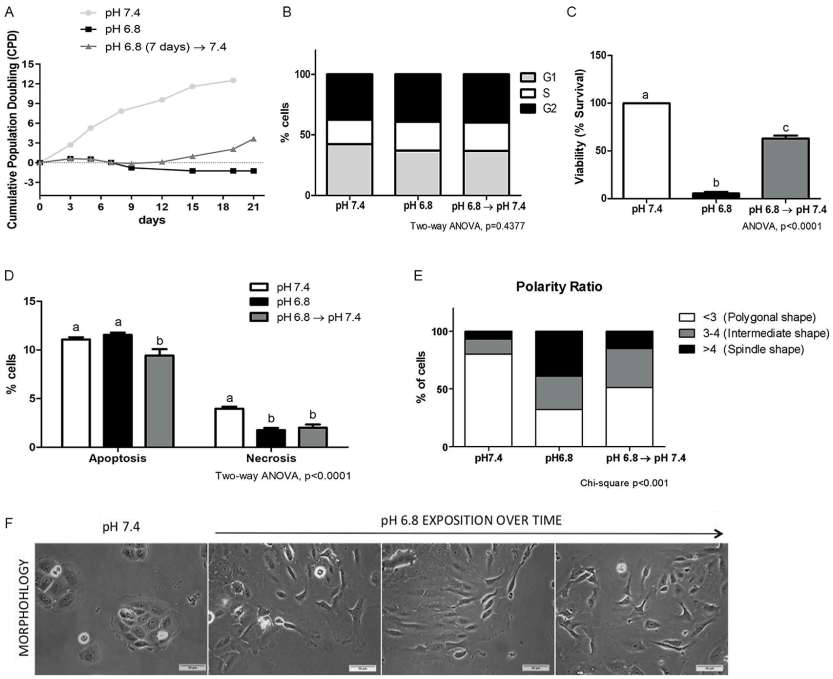


Fig. 2. Extracellular acidity alters cell proliferation, viability, survival, and morphology of SCC4 cell line. A – Cell multiplication analysis through cumulative population doubling (CPD) method. B – Cell cycle phase distribution analysis obtained by flow cytometry (Two-way ANOVA, $p = 0.4377$). C - SRB viability assay (ANOVA, $p < 0.0001$). D – Apoptosis and necrosis rates obtained by flow cytometry (Two-way ANOVA, $p < 0.0001$). E – Polarity ratio analysis (Chi-square, $p < 0.001$). F – Representative pictures indicating morphological changes, from an epithelial to a spindle-like cell shape, after exposition to acidic cell culture medium (pH 6.8) over time.

when compared to pH 7.4 group. After reconditioning the cells to pH 7.4 the viability level was partially restored (Fig. 2C). These findings were confirmed with another oral cancer cell line, the SCC25 cells, that also showed reduced viability after acidic stress, and recovered proliferation potential after reconditioning to pH 7.4 ($p < 0.0001$) (Fig. S1). Survival assay by flow cytometry showed decreased necrosis rates of the groups exposed to pH 6.8 ($p < 0.0001$), and decreased apoptosis of acidic cells reconditioned to pH 7.4 (Fig. 2D). In order to confirm if these findings are stable in the course of time, a long-term acidosis was performed extending pH 6.8 exposition. Long-term acidosis also resulted in decreased viability after pH 6.8 treatment which was recovered after reconditioning at neutral pH (Fig. S2A), with no significant alterations in cell cycle (Fig. S2B). However, prolonged acidosis resulted in increased apoptosis caused by acidosis (Fig. S2C).

Microenvironmental acidity induced a spindle shaped morphology after exposure to the acidic condition in SCC4 and SCC25 cells, the cells became significantly more elongated at pH 6.8 in comparison to cells grown at standard pH cell culture medium (Fig. 2F, S1). Polarity ratio analysis confirmed changes in cell morphology after exposition to pH 6.8. This polarity alteration was partially reversed when cells were subsequently reconditioned to pH 7.4 (Fig. 2E).

3.2. Microenvironmental acidity promotes EMT markers expression in OSCC cell lines

To further investigate the role of the acidic microenvironment in the induction of a mesenchymal-like phenotype, EMT markers were analyzed at protein and mRNA level. The expression of E-cadherin and N-cadherin of SCC4 and SCC25 cell lines was quantified through quantitative PCR (qPCR). We observed a significant reduction of E-cadherin expression accompanied by an increased N-cadherin expression at short-term experimental groups ($p < 0.05$) in comparison to the standard pH condition (Fig. 3A and Fig. S3A). When cells are exposed to long-term acidosis, E-cadherin expression is reverted in the reconditioned group, whereas N-cadherin levels remain high (Fig. 3B and Fig. S3B). However, E-cadherin protein levels are reduced in the experimental acidosis groups for short- and long-term acidosis compared with the pH 7.4 group (Fig. 3C). The expression and location of the EMT protein marker Vimentin at a single cell level was assessed by immunofluorescence. An increased expression of Vimentin was observed after pH 6.8 exposure in comparison to the pH 7.4 standard conditions for both SCC4 and SCC25 cell lines (Fig. 3D).

3.3. A low extracellular pH favors cell migration and motility

One of the known key events involved in metastasis is the migration capacity acquired by cancer cells. To determine whether extracellular acidity plays a role in OSCC cell migration *in vitro*, the wound healing assay was performed. A higher wound closure percentual was observed for the pH 6.8 → pH 7.4 group at the 10-hours' time point accompanied by a complete wound closure in 24 h when compared to pH 7.4 group of SCC4 cell line ($p < 0.0001$) (Fig. 4A, B). The same wound closure pattern was seen after long-term acidosis treatments (Fig. S4A, B). This phenomenon seems to be specific for tumor cells, since when non-tumoral epithelial cells (HaCaT) were challenged with acidic stress and recovered to neutral pH no alteration in cell migration and motility was detected (Fig. 4C). It was not possible to establish the pH 6.8 group with HaCat cells due to its high cell death rates, which also confirms that only cancer cells can survive and adapt to acidic stress. This assay was also performed with the SCC25 cell lines to which no wound closure difference was observed (Fig. S4C).

To further investigate this process *in vitro*, transwell migration assay was performed. According to the numerical scoring, our results strongly suggest that an acidic treatment followed by reconditioning the cells in standard pH cell culture medium (pH 6.8 → pH 7.4 group) resulted in an increased invasive capacity ($p = 0.022$) when compared to pH 7.4 group (Fig. 4D). This difference was not observed for the long-term acidosis group (Fig. S4D).

We also used the *in vitro* time lapse assays to assess the overtime motility of cells in an individual level. In accordance with collective migration findings, these results showed an increased motility potential ($p < 0.05$) of pH 6.8 → pH 7.4 group measured by mean distance and velocity (Fig. 4E). This finding was confirmed in the long-term acidosis, pH 6.8 group has covered a greater average distance ($p < 0.05$) in comparison to the other groups (Fig. S4E). In accordance with collective migration findings, the SCC25 cell line showed increased migration, however, did not reach a statistically significant difference regarding average distance and velocity after acidosis treatment (Fig. S4E). Also similar to what was observed in collective migration results, the keratinocyte cell line (HaCat) results showed no significant difference between the groups regarding individual cell motility (Fig. 4E).

3.4. The acidic extracellular pH increases stemness properties of SCC4 cell line

In order to evaluate the pluripotency properties after exposure to acidic pH, the relative expression of stemness markers CD44, NANOG and BMI-1 was quantified through quantitative PCR (qPCR). An increased CD44 expression was detected for pH 6.8 groups at short and long-term acidosis for both cell lines (Fig. 5A). The transcription factor NANOG related to cell self-renewal also revealed higher gene expression in pH 6.8 groups at a long-term acidosis basis for both cell lines studied, and also for the short-term acidosis in SCC25 cells (Fig. 5B). Although there was no statistically significant difference for SCC4 cells, a BMI-1 expression increase trend was observed in the test groups (pH 6.8 and pH 6.8 → 7.4) when compared to the pH 7.4 group (Fig. 5C).

3.5. The acidic microenvironment fosters Cisplatin chemoresistance and alters radioresistance of OSCC cell lines

To study the effect of the acidic microenvironment on cell's sensitivity to anti-cancer agents, cell viability was assessed using SRB assays after treatment with increasing concentrations of the chemotherapeutic agent cisplatin. For both SCC4 and SCC25 cell lines an increased ($p < 0.05$) chemoresistance was observed at higher drug concentrations during exposure to pH 6.8 (Fig. 6A and Fig. S5A). Moreover, when the OSCC cells were reconditioned to pH 7.4 (pH 6.8 → pH 7.4 group) the sensitivity to cisplatin was completely restored (Fig. 6B and Fig. S5B). For both OSCC cell lines these previously described results were also observed after long-term acidosis groups (Fig. 6C, D and Fig. S5C, D).

To investigate the effect of the pH 6.8 on OSCC cell lines response to fractionated radiation, cell viability was assessed using SRB assays. An increased radioresistance ($p < 0.0001$) of pH 6.8 group was observed for SCC4 cell line (Fig. 6E).

4. Discussion

The establishment of an acidic microenvironment through the deregulation of cellular energetics fosters tumor growth and progression. Cancer cells adaptation to this microenvironmental acidosis translates into a phenotypic shift that leads to increased migration and invasion capacity [41,42]. Our findings indicate that the acidic microenvironment initially reduced OSCC cells viability followed by an adaptation process. Those cells that adapted to acidic pH presented a mesenchymal-like phenotype in conjunction with increased migration and motility rates. Additionally, extracellular acidity was correlated with the induction of stemness properties and drove oral cancer cells to increased chemo- and radioresistance.

In line with previous findings obtained with melanoma cells, a decreased cell viability caused by impaired proliferation status (Fig. 2A) was also observed in the present study [24,43,44]. The decreased proliferation occurs during the acclimation to acidosis that acts by selecting

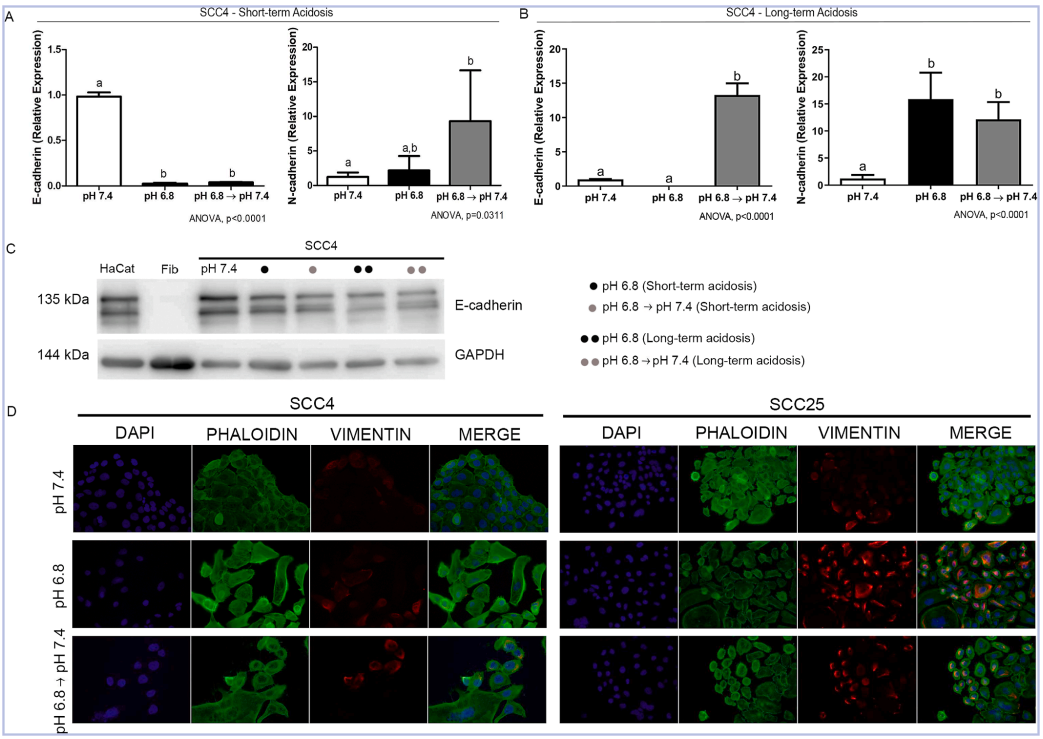


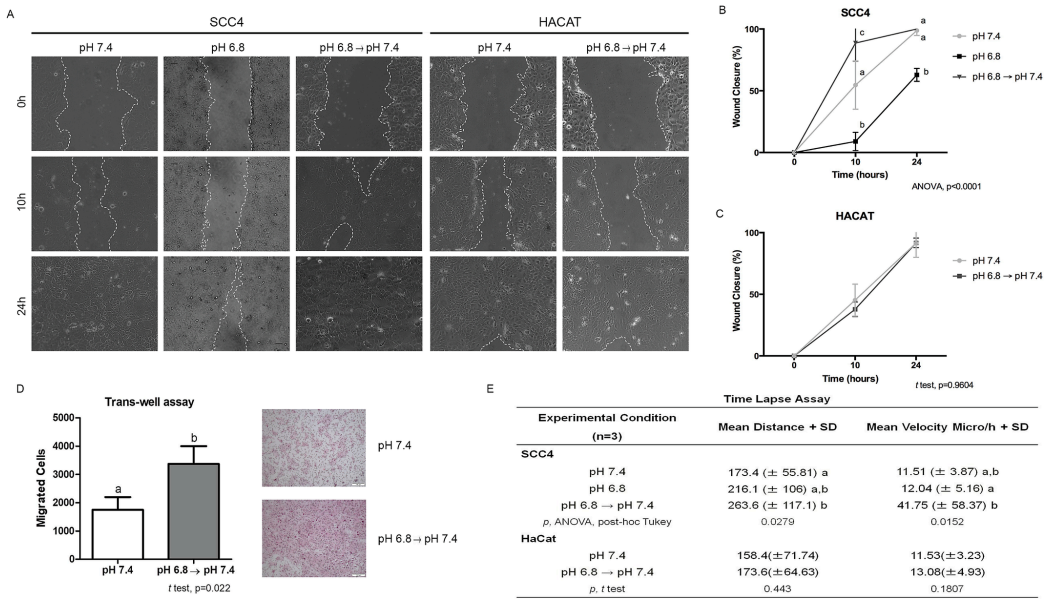
Fig. 3. Extracellular acidity alters EMT markers expression. A – Real-time PCR quantification of the EMT markers *E*-cadherin and N-cadherin from SCC4 cell line exposed to short-term acidosis. B – Real-time PCR quantification of the EMT markers E-cadherin and N-cadherin from SCC4 cell line exposed to long-term acidosis. Expression levels were standardized for GAPDH (ANOVA, $p < 0.05$). C – Western Blot analysis for the EMT marker E-Cadherin in fibroblasts (Fib), human keratinocytes (HaCat) and SCC4 cell line. D – Vimentin (red), Phalloidin (green) and DAPI (4′,6-diamidino-2-phenylindole) (blue) immunofluorescence staining of SCC4 and SCC25 cell lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adapted OSCC cells that are able to survive in this adverse condition. Accordingly, a lower concentration of cells was observed at the end of the acidic treatment (Fig. 2C and Fig. S1). No alterations in cell cycle phase distribution and apoptosis rates were detected in comparison to cells maintained at neutral pH for the same period of time (7 days). However, CPD analysis reveals that the decline in cumulative population doubling takes around 14 days to reach a plateau. Although long-term acidosis resulted in higher apoptosis levels (Fig. S2C), cell viability (Fig. S2C) is improved in comparison to short-term acidosis, suggesting that cells that survived are adapting. Another possible explanation would be the induction of cell senescence in a fraction of the cells, which would require further investigation [44]. Moreover, as previously described in the literature for melanoma, breast, and lung cancers, we observed that microenvironmental acidosis induced a phenotype shift in OSCC cells from an epithelial to a spindle-shaped morphology (Fig. 2E and F) [24,42,45,46].

Cancer cells' ability to spread into the adjacent stroma is highly dependent on phenomena related to epithelial-mesenchymal transition (EMT) [47]. During this process, epithelial tumor cells suffer diverse bio and morphological alterations acquiring migratory features [48]. In the past years, it became clear that EMT is far from being a static process, meaning that cell plasticity allows its reversal or even a partial EMT state under specific conditions [41,49,50]. In accordance with previous findings for breast and lung cancers [41,47], the acid adaptation induced an increased expression of the mesenchymal markers N-cadherin and Vimentin concomitantly with a decreased but not null expression of the epithelial marker E-cadherin in the OSCC cells (Fig. 3). Since OSCC cells maintained both mesenchymal and epithelial markers, the adaptation to low extracellular pH is a partial and not complete EMT state, indicating cell plasticity potential strongly influenced by the microenvironment [41,50].

The findings related to the induction of EMT have translated, as expected, into an increased migratory capacity of OSCC cells. It has already been described that extracellular acidity increased cell migration and invasion abilities of melanoma, lung, and breast cancer cells [42,43,46,51,52]. We also aimed to assess whether tumor cells exposed to transient acidity could sustain their metastatic phenotype under physiological growth conditions (pH 7.4) mimicking pH fluctuations as observed during tumor growth, when the tumor cells reach the bloodstream, or even during the formation of secondary tumors. This switch to neutral extracellular pH increased cell motility measured by wound healing, transwell and time-lapse assays. Such a change indicates that the cells acquired an undifferentiated and metastatic phenotype when growth conditions were normalized (Fig. 4). The switch to neutral pH is notably relevant for metastasis establishment as mentioned above.

In contrast, when exposed to acid pH, the human keratinocyte cell line (HaCat) not only did not show a difference in migration rates when compared to the control but also did not resist treatment with acidified



E

Time Lapse Assay

Experimental Condition (n=3)	Mean Distance + SD	Mean Velocity Micro/h + SD
SCC4		
pH 7.4	173.4 (± 55.81) a	11.51 (± 3.87) a,b
pH 6.8	216.1 (± 106) a,b	12.04 (± 5.16) a
pH 6.8 → pH 7.4	263.6 (± 117.1) b	41.75 (± 58.37) b
p, ANOVA, post-hoc Tukey	0.0279	0.0152
HaCat		
pH 7.4	158.4(±71.74)	11.53(±3.23)
pH 6.8 → pH 7.4	173.6(±64.63)	13.08(±4.93)
p, *t* test	0.443	0.1807

Fig. 4. Extracellular acidity favors cell migration. A, B – SCC4 cell line wound healing assay (ANOVA, $p < 0.0001$). A, C – HaCat cell line wound healing assay (*t*-test, $p = 0.9604$). D – SCC4 Trans-well assay (*t*-test, $p = 0.022$). E – SCC4 and HaCat cell lines time lapse assay (ANOVA $p < 0.05$ and *t*-test $p > 0.05$, respectively).

medium without recovering at standard extracellular conditions (pH 7.4) (Fig. 4C, E). Similar results were confirmed by Federici and colleagues (2014), which showed that normal human cells present high cell death rates under low pH conditions [53]. Thus, we can conclude that tumor cells are more likely to adapt to this challenging microenvironmental feature, while continuous acidity is not tolerated by non-neoplastic cells. We believe that this phenomenon may help to eliminate neighboring stromal cell populations allowing tumor growth.

Recently, the existence of molecular connections between EMT and the establishment of a cancer stem cell (CSC) status were suggested in the literature, indicating that a stem cell-like phenotype can be unleashed through EMT induction [48,54]. We observed that OSCC cells grown under microenvironmental acidity presented not only a partial EMT phenotype but also a pronouncedly increased expression of known stemness markers CD44 and NANOG accompanied by a tendency to increase in BMI-1 levels (Fig. 5A, B, C) [25,55]. This link between exposure to extracellular acidosis and cancer-associated stemness was previously described for breast and glioma cancer cells [25,52].

Similarly to our findings regarding an increased resistance to cisplatin treatment, acidosis was also previously correlated in the literature with the development of resistance to anticancer chemotherapy for melanoma, breast, prostate and colon carcinomas as well as osteosarcoma and OSCC cell lines among others [44,51,53,56–60]. The main mechanisms enrolled in acidity-driven chemoresistance can be categorized as activation of drug efflux transporters; increased expression of proton pumps; activation of the unfolded protein response pathway (UPR) [61]. Those mechanisms support our findings and help us to better understand how both OSCC cell lines presented chemoresistance at higher drug concentrations during short (Fig. 6A and Fig. S5A) and long-term (Fig. 6B, and Fig. S5B) exposure to pH 6.8. Extracellular acidity is known to select a subset of cells higher expressing drug efflux transporters that compromise cellular influx of drugs, thus impairing its chemotherapeutic capacity [62,63].

An unbalanced pH is also linked to the so-called ion trapping phenomenon, referring to the effect of microenvironmental ions on the drug distribution gradient, through which cellular intake of alkaline drugs is impaired in acidic tumors. Thus, basic chemotherapeutic agents remain accumulated at the lowest pH regions of the tumor [63,64]. The overexpression of proton pumps (particularly V-ATPases) that directly affect the establishment and maintenance of extracellular acidity, consequently, contributes to ion trapping events [63,65,66]. Based on these previous findings, we can assume that cisplatin is trapped in the extracellular acidic environment explaining OSCC cells' decreased sensitivity when cultured at pH 6.8. A study designed with melanoma, breast and colon cancer cell lines has proven that cisplatin absorption and sensitivity was decreased at acidic pH, in accordance with our results [53].

Another important factor to be considered is the mechanism of action on chemotherapeutic agents, including that of the widely used drug Cisplatin. Knowing that most of these agents interfere with DNA replication, targeting rapidly proliferating cancer cells [44,67,68], the reduced OSCC cell line proliferation under acidic conditions (Fig. 2A) could also explain the markedly low sensitivity to the chemotherapeutic agent observed in the present study (Fig. 6 and Fig. S5). Therefore, the resistant cells are able to survive the chemotherapeutic approach potentially leading to disease recurrence.

Both hypoxia and the presence of a lactate-rich microenvironment link with radioresistance development has been widely studied over the years [69–73]. On the other hand, the same is not observed regarding OSCC tumor acidity and its correlation with reduced radiosensitivity, which remains less explored [74]. Concerning the mechanisms that lead to lactate induced radioresistance, the main hypothesis relies on its known reactive oxygen species (ROS) scavenging capacity [75,76]. Since ROS are necessary for radiation-induced DNA damage [77–79], lactate antioxidant activity is believed to have a protective effect on cancer cells during radiotherapy [80]. Our study, interestingly, shows that not only the presence of high microenvironmental lactate but also tumor acidity itself contributes to the establishment of OSCC cell

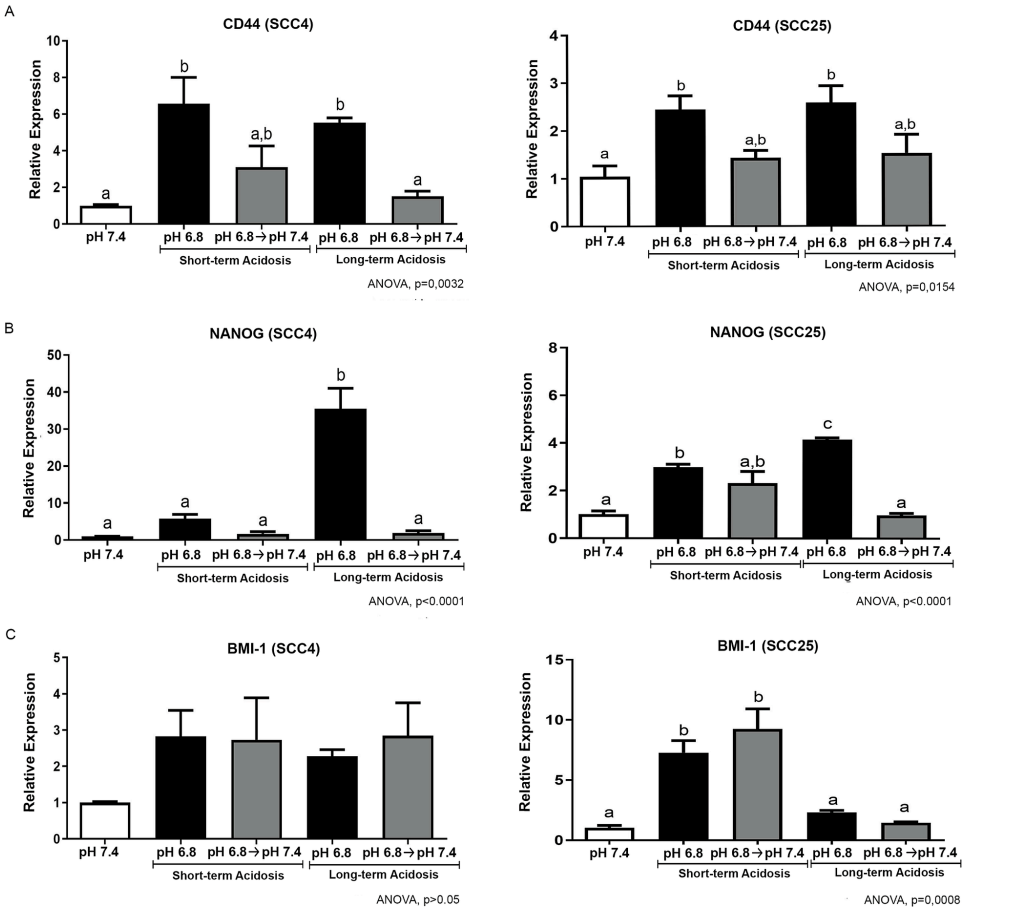


Fig. 5. Extracellular acidity favors the stemness properties of SCC4 and SCC25 cell lines. A- quantitative real time PCR gene expression assay for SCC4 and SCC25 cell lines with CD44 marker (ANOVA, $p = 0.0276$), B - NANOG (ANOVA, $p < 0.0081$) and C -BMI-1 (ANOVA, $p = 0.2592$).

populations resistant to fractionated irradiation. Poor radiotherapy response has been pointed out as a consequence of several factors. Since it is well accepted that fast cancer cell division is linked to a greater DNA damage response caused by ionizing radiation, the reduced cell proliferation under microenvironmental acidosis may explain the increased radioresistance observed in our findings [81,82]. Also the presence of quiescent or senescent dormant cell subpopulations such as cancer stem cells, that are insensitive to radiation is associated with radioresistance and subsequent tumor recurrence [83–88]. Another feature that has been related to treatment failure, including radiation resistance, is the existence of an EMT phenotype with decreased E-cadherin levels and an accelerated DNA repair [89–92]. Thus, it seems providential to further investigate the possibilities of intervening in tumoral acidosis through proton pump inhibitors in combination with radiotherapy treatment to sensitize tumors, improving clinical outcomes.

An important finding of this study is that whereas acidic pH increases treatment resistance, when the pH is neutralized treatment resistance is reversed however migration increases. These antagonistic results may be the consequence of different signaling pathways activated in response to acidic exposition and later to pH buffering. The acidic environment is very stressful to cells and probably activates strong survival signaling pathways, as those related to cell undifferentiation and stemness, which in turn, result in increased treatment resistance. However, cell migration may be impaired in such a hostile environment. It has been shown before that acidosis results in high oxidative stress levels in different contexts: hypertension [93], and cancer [94]. Moreover, when acidic pH was buffered to pH 7.4, the increased expression of important genes related to stemness, self-renewal and chemoresistance CD44 and NANOG was reverted reaching similar levels of control cells. Interestingly, Bmi-1, in contrast, remained increased in short-term acidosis. Besides its role in stemness maintenance, Bmi-1 has been implicated in oral cancer cells migration [95,96].

5. Conclusion

Taken together, our findings reveal that extracellular acidosis fosters important cellular alterations that can enable survival to chemotherapy and fractionated irradiation, which might lead to tumor recurrence.

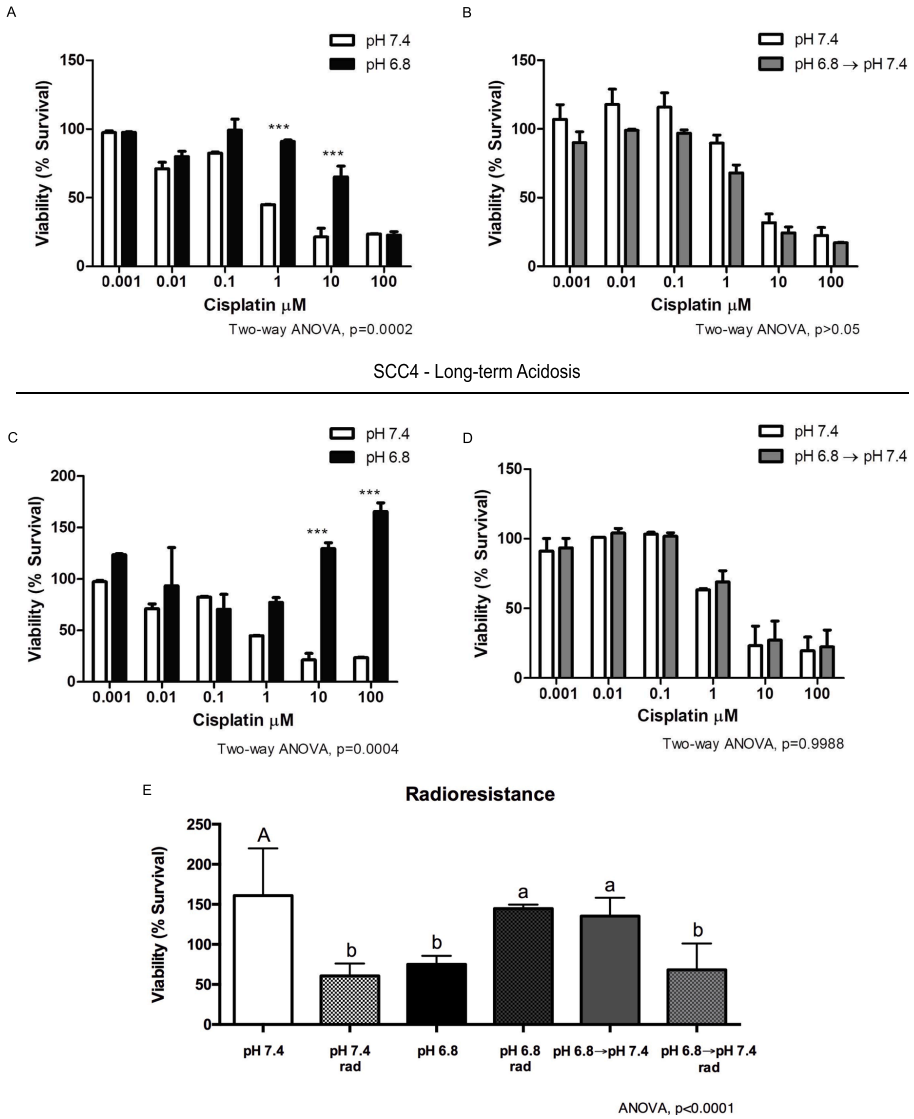


Fig. 6. The acidic microenvironment fosters Cisplatin chemoresistance and promotes radioresistance of SCC4 cell line. A Short-term acidosis SRB viability assay (ANOVA, $p = 0.0002$) after treatment with increasing concentrations of Cisplatin. B – Short-term acidosis after reconditioning to pH 6.4 SRB viability assay (ANOVA, $p > 0.05$) after treatment with increasing concentrations of Cisplatin. C - Long-term acidosis SRB viability assay (ANOVA, $p = 0.0004$) after treatment with increasing concentrations of Cisplatin. D - Long-term acidosis after reconditioning to pH 6.4 SRB viability assay (ANOVA, $p = 0.9988$). E – SRB viability assay (ANOVA, $p < 0.0001$) after 5 doses of 2Gy irradiation.

Besides, it was demonstrated that tumor microenvironmental acidosis has an important role in OSCC progression inducing an aggressive cell phenotype which includes the acquisition (or selection) of stemness and partial EMT features (Fig. 7). OSCC acidosis manipulation strategies must be considered as a very promising neoadjuvant therapeutic strategy that might increase oral cancer treatment success rates. However, tumor pH neutralization should be carefully assessed, while it can improve treatment response to chemo- and radiotherapy, it is important to consider that pH reconditioning may foster tumor cell's migration, invasion and spread. Therefore, tumor pH manipulation should be time-dependently planned.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.120163.

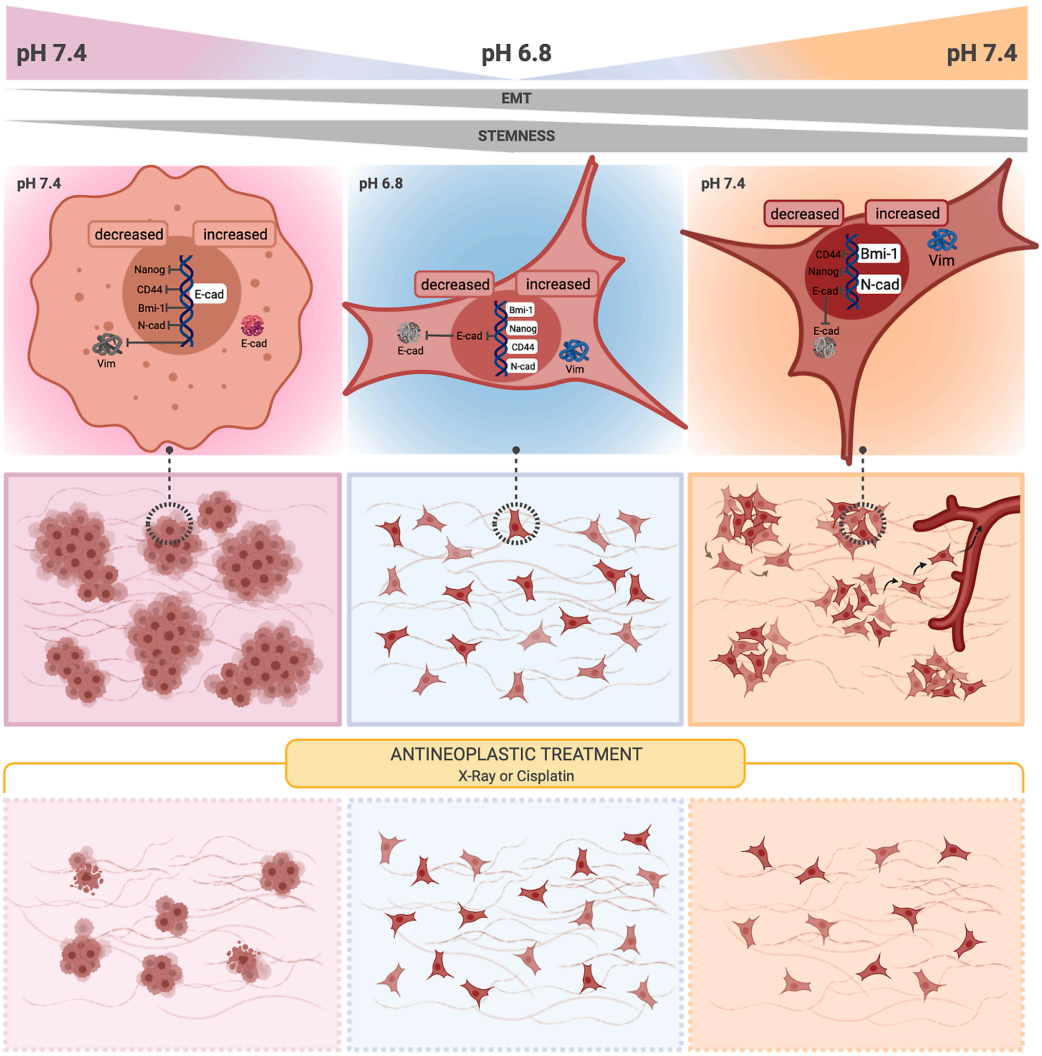


Fig. 7. Graphical Abstract: Oral squamous cell carcinoma molecular pattern and fate according to extracellular pH microenvironment before and after antineoplastic treatment. The acidic microenvironment caused an initial reduction of OSCC cells viability, followed by phenotypic, molecular, and behavioral changes suggesting an adaptation process. Cells acquired a mesenchymal-like phenotype along with increased migration and motility indexes. The induction of cellular stemness and increased chemo- and radioresistance was observed.

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CRediT authorship contribution statement

Bianca de Bem Prunes: Conceptualization, Investigation, Validation, Writing – original draft. **Júlia Silveira Nunes:** Conceptualization, Investigation, Validation, Writing – original draft. **Viviane Palmeira da Silva:** Conceptualization, Investigation, Writing – original draft. **Natalia Koerich Laureano:** Investigation, Writing – original draft. **Douglas Rodrigues Gonçalves:** Investigation, Writing – review & editing. **Ian Santana Machado:** Investigation, Writing – review & editing. **Silvia Barbosa:** Investigation, Writing – review & editing. **Marcelo Lazzaron**

Lamers: Methodology, Writing – review & editing. **Pantelis Varvaki Rados:** Conceptualization, Writing – review & editing. **Ina Kurth:** Methodology, Writing – review & editing. **Jochen Hess:** Conceptualization, Methodology, Writing – review & editing. **Adriana Jou:** Conceptualization, Methodology, Writing – review & editing. **Fernanda Visioli:** Conceptualization, Formal analysis, Resources, Writing – original draft, Supervision, Project administration.

Declaration of competing interest

The authors declare no conflict of interest.

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Figura 7 – Supplementary Figure 1

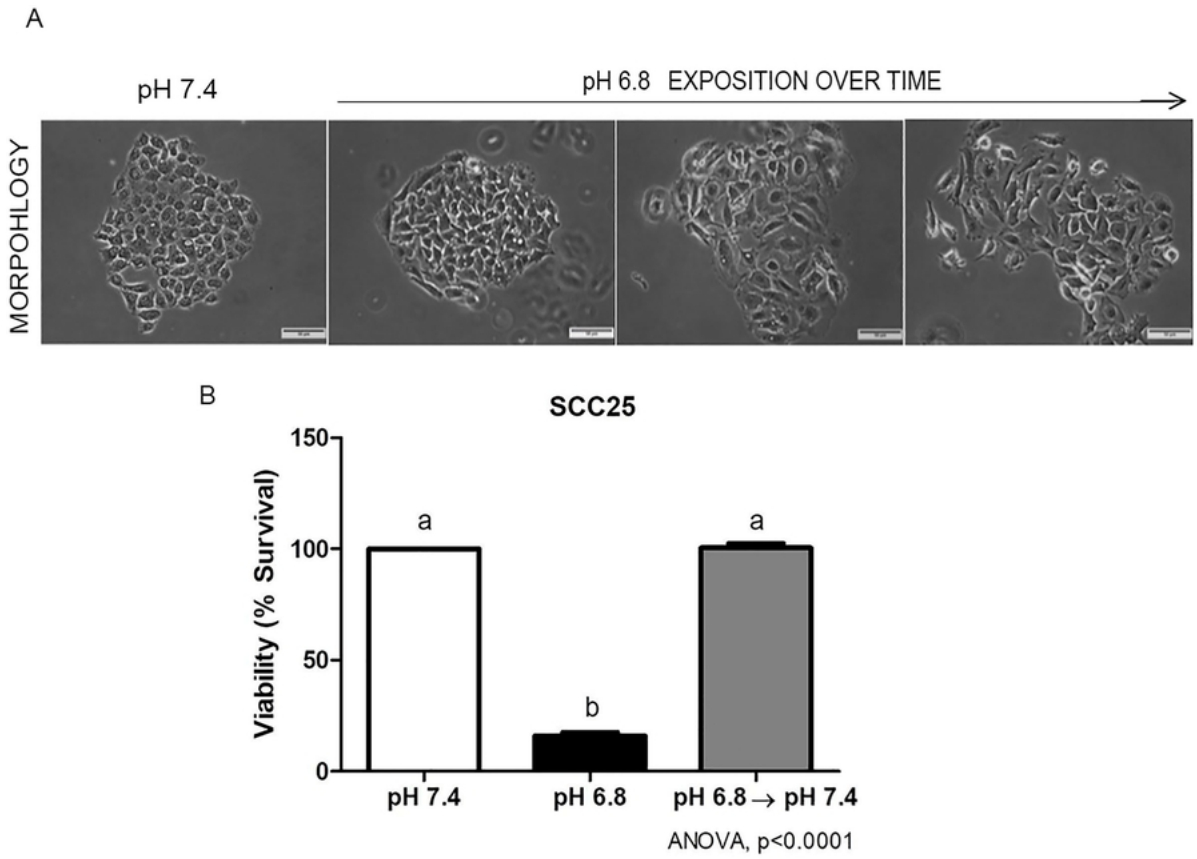


Figura 8 – Supplementary Figure 2

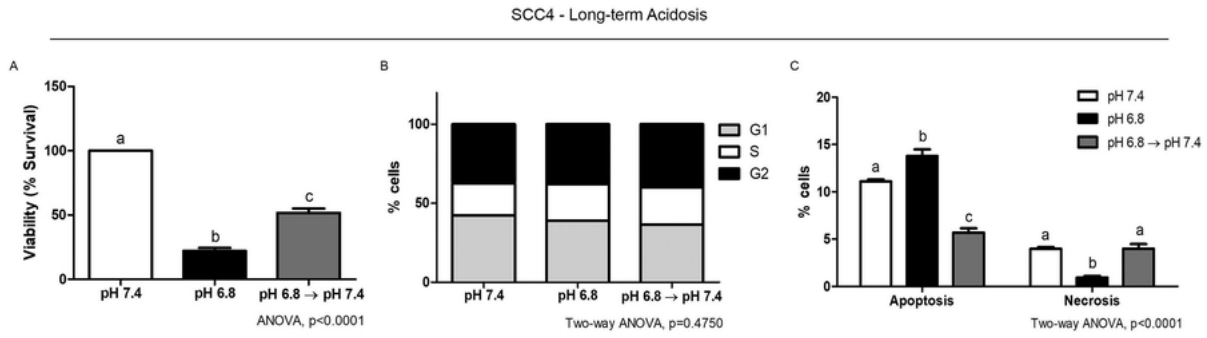


Figura 9 – Supplementary Figure 3

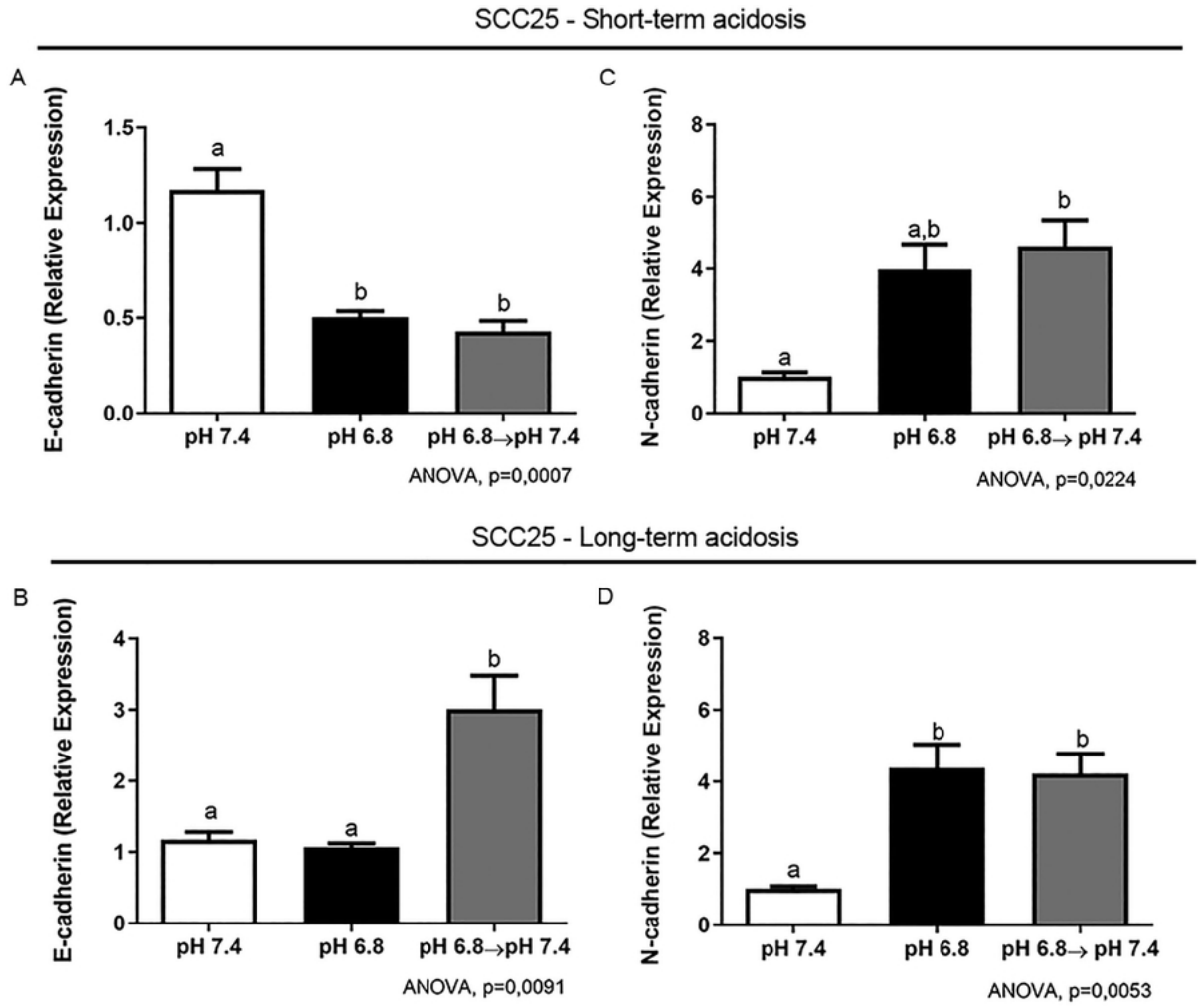
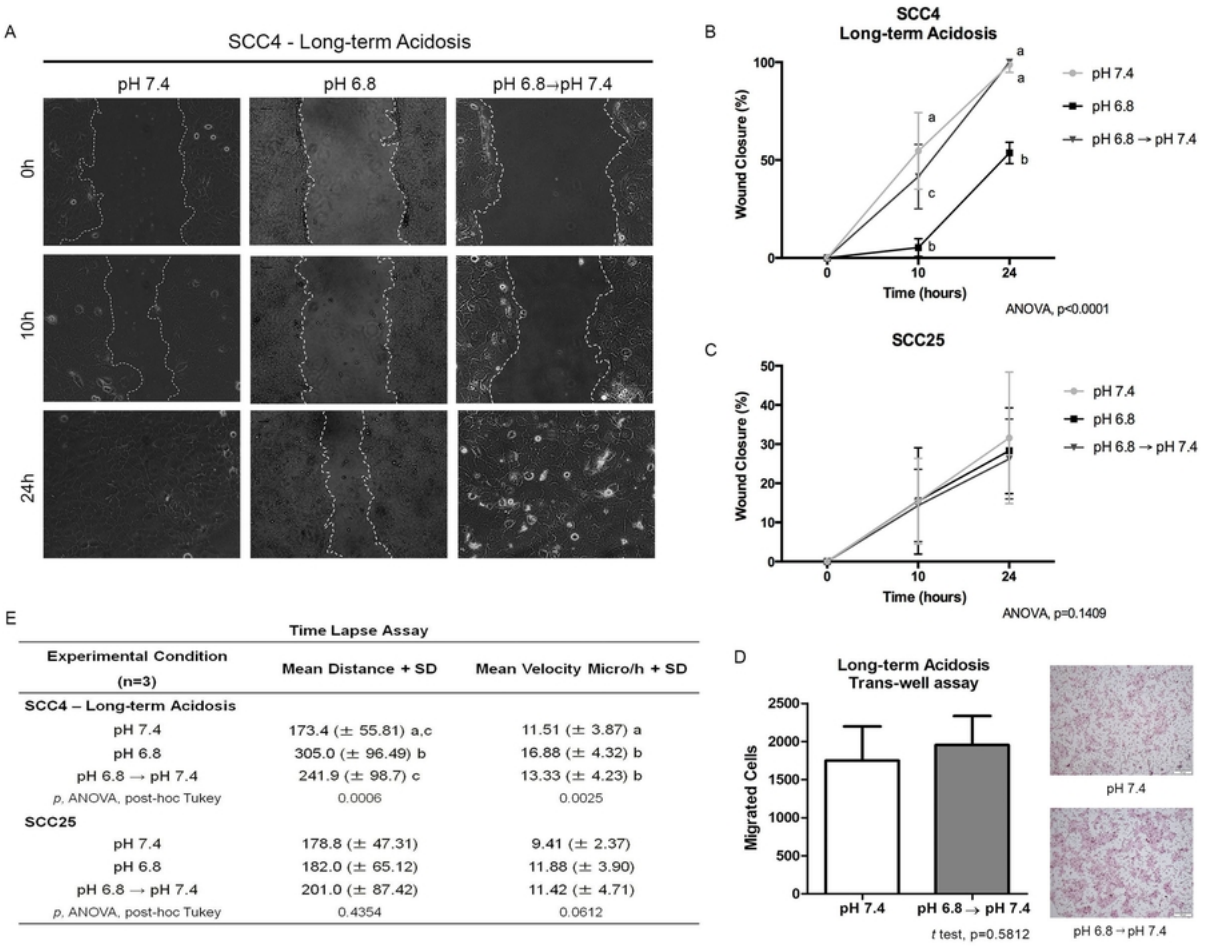


Figura 10 – Supplementary Figure 4

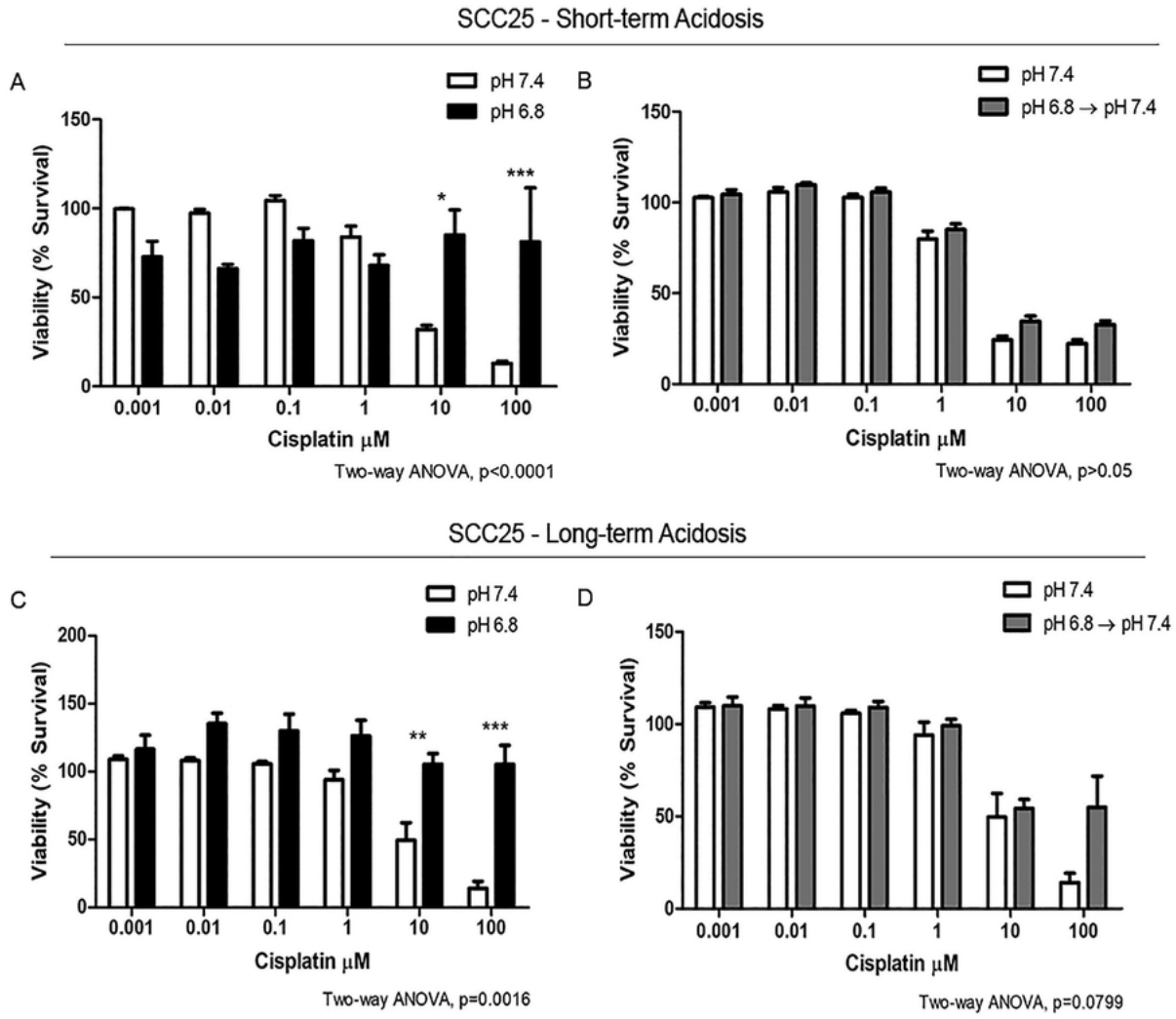


E

Time Lapse Assay

Experimental Condition (n=3)	Mean Distance + SD	Mean Velocity Micro/h + SD
SCC4 – Long-term Acidosis		
pH 7.4	173.4 (± 55.81) a,c	11.51 (± 3.87) a
pH 6.8	305.0 (± 96.49) b	16.88 (± 4.32) b
pH 6.8 → pH 7.4	241.9 (± 98.7) c	13.33 (± 4.23) b
p, ANOVA, post-hoc Tukey	0.0006	0.0025
SCC25		
pH 7.4	178.8 (± 47.31)	9.41 (± 2.37)
pH 6.8	182.0 (± 65.12)	11.88 (± 3.90)
pH 6.8 → pH 7.4	201.0 (± 87.42)	11.42 (± 4.71)
p, ANOVA, post-hoc Tukey	0.4354	0.0612

Figura 11 – Supplementary Figure 5



3.2 ARTIGO CIENTÍFICO II

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EFFECTS OF TUMOR-DERIVED ACIDOSIS AND MICROENVIRONMENTAL LACTATE ON IMMUNE ANTITUMOR RESPONSE: A SYSTEMATIC SCOPING REVIEW

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ABSTRACT

The microenvironment of solid tumors was proven to benefit neoplastic cells and to be immunosuppressive. In this context, the different types of cells are surrounded by elevated lactate levels and low oxygen and nutrient availability. In the present Review, we explore the effects of lactate and acidity present in the tumor microenvironment on immune cells. To that end, we developed a systematic scoping review of the literature in which ninety-six studies were included. A clear link between tumor-derived extracellular acidity/lactate abundance and the impairment of various immune cellular functions was observed.

1. INTRODUCTION

Inhibition of a cell's malignant transformation relies amongst others on an effective immune response. The theory that cancer cell populations are constantly eliminated by the host's lymphocytes hampering tumor formation dates back to the late 1960s (Burnet, 1971, 1970, 1957; Lawrence, 1959). A variety of mechanisms performed by both innate and adaptive immune system components has been lately unveiled outlining the so-called immunosurveillance. From this perspective, when a tumor is formed we can assume that the neoplastic cell clones acquired the ability to evade constant surveillance (Hanahan, 2022). It is now clear that an exiguous control of critically mutated cells by the different immunologic constituent parts can arise as a

result of a strongly suppressive environment (Bohn et al., 2018; Calcinotto et al., 2012; Fischer et al., 2007; Mendler et al., 2012).

The tumor microenvironment (TME) displays a set of metabolic conditions that are known to be affected by multiple variables such as cell-to-cell interplays, distinct oxygen and nutrient availability, and a changing vascular supply (Kaymak et al., 2021). In this context, a switched energy metabolism characterized by a highly glycolytic activity is commonly observed in tumor cells (Koppenol et al., 2011; Martinez-Outschoorn et al., 2017; Zhang and Li, 2020). Those malignant cells catalyze pyruvic acid into lactic acid through a process termed aerobic glycolysis, which culminates in overabundant extracellular concentrations of lactic acid and hydrogen ions establishing a markedly acidic TME (Corbet and Feron, 2017; Gatenby and Gillies, 2004; Huber et al., 2017; Koppenol et al., 2011; Vander Heiden et al., 2009; Zhang and Li, 2020). A number of adaptive advantages arise from this harsh microenvironment, including functional impairment of effector immune cells and activation of immunosuppressive cells that benefit neoplastic cells to evade antitumor immunity (Li et al., 2018; Pollizzi and Powell, 2014; Zhang and Li, 2020). Aggressive tumors were proven to be highly glycolytic and markedly acidic (Giatromanolaki et al., 2020, 2017).

Despite the consensus regarding the existence of a well-established acidosis-driven immunosuppression effect, a broad range of newly discovered mechanisms taking part in this process has been described over the last years (Damaghi et al., 2013; Giatromanolaki et al., 2020, 2017; Huber et al., 2017; Pilon-Thomas et al., 2016). Clinically those features convert to tumor malignancy and worse prognosis (Bohn et al., 2018; Ho and Liu, 2016; Liu et al., 2019; Zhang and Li, 2020). In many cases the response to onco-immunotherapeutic agents does not occur as expected, being either short-lived or ineffective. Therefore, the elucidation of how the biological factors inherent to the microenvironment of each tumor influence the efficacy of immunotherapy is decisive in altering the success rates of this type of treatment.

2. METHODOLOGY

A scoping review was conducted to compile and discuss the mechanisms underlying how the presence of lactic acid and/or tumor acidity results in antitumor

immune response inefficiency. This review was performed according to the frameworks proposed by Peters et al. (2015) and Munn et al. (2018), and data was compiled according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Extension for Scoping Reviews guidelines (Munn et al., 2018; Peters et al., 2015; Tricco et al., 2018).

The search was carried out on PubMed and EMBASE databases. We designed a search strategy with the aim of identifying all studies containing the combination of the following keywords for PubMed: Cellular Microenvironment[mh] OR Cellular Microenvironment[mh] OR Cell Niche*[tw] OR surround*[tw] OR microenvironment*[tw] OR environment*[tw] OR milieu*[tw] OR Extracellular Space*[tw] OR Intercellular Space*[tw] AND Hydrogen-Ion Concentration[mh] OR Lactates[mh] OR acid*[tw] OR pH[tw] OR Hydrogen Ion*[tw] OR Lactate*[tw] OR Lactic*[tw] AND Adaptive Immunity[mh] OR immun*[tw] OR Clonal Select*[tw] OR Antigen*[tw] OR Lymphocyte*[tw] OR t cell*[tw] OR CD4[tw] OR CD8[tw] OR natural killer*[tw] OR neutrophils*[tw] OR Dendritic*[tw] OR antigen-presenting*[tw] OR macrophages*[tw] OR b cell*[tw] OR interferon*[tw] OR major histocompatibility complex*[tw] OR perforin*[tw] OR granzyme*[tw] OR interleukin*. For EMBASE database the terms Neoplasm/exp OR Neoplas*:ti,ab,kw OR Tumor*:ti,ab,kw OR Cancer*:ti,ab,kw AND "tumor microenvironment"/exp OR "Cell Niche*":ti,ab,kw OR surround*:ti,ab,kw OR microenvironment*:ti,ab,kw OR environment*:ti,ab,kw OR milieu*:ti,ab,kw OR "Extracellular Space*":ti,ab,kw OR "Intercellular Space*":ti,ab,kw AND 'pH'/exp OR 'lactic acid derivative'/exp OR acid*:ti,ab,kw OR pH:ti,ab,kw OR "Hydrogen Ion*":ti,ab,kw OR "Lactate*":ti,ab,kw OR Lactic*:ti,ab,kw AND 'adaptive immunity'/exp OR immun*:ti,ab,kw OR "Clonal Select*":ti,ab,kw OR Antigen*:ti,ab,kw OR "Lymphocyte Activat*":ti,ab,kw OR "Lymphocyte Stimulat*":ti,ab,kw OR "Lymphocyte Transform*":ti,ab,kw OR "Lymphocyte*":ti,ab,kw OR "t cell*:ti,ab,kw OR "CD4":ti,ab,kw OR "CD8":ti,ab,kw OR "natural killer*:ti,ab,kw OR "neutrophils*":ti,ab,kw OR "Dendritic*":ti,ab,kw OR "antigen-presenting*":ti,ab,kw OR "macrophages*":ti,ab,kw OR "b cell*":ti,ab,kw OR "interferon*":ti,ab,kw OR "major histocompatibility complex*":ti,ab,kw OR "perforin*":ti,ab,kw OR "granzyme*":ti,ab,kw OR "interleukin*":ti,ab,kw were applied.

The selection process was performed by two independent reviewers (BBP and NKL). Duplicate publications were tracked and excluded. Initially, papers were selected considering their titles and abstracts. Next, full texts were retrieved for the second stage of selection. Subsequently, through this screening process, the articles were subjected to inclusion and exclusion criteria and reviewed by four authors (BBP, NKL, JSN, LV). Scientific articles that generated disagreement were reviewed by an independent researcher (FV) until consensus was reached (Figure 1).

As inclusion criteria, studies evaluating the effects of tumor-generated acidosis and/or acid lactic on immune response were selected. Reviews of the literature, conference abstracts, and scientific articles not written in English were excluded.

Data extraction was conducted by four reviewers (BBP, NKL, JSN, LV), and the extracted study information included: study design, type of cancer, acidification method or acidosis analysis method, the immune cells studied, as well as the immune receptor, and immune cytokines, and main results. The data and the compiled evidence were qualitatively summarized and interpreted and it is available in supplemental table 1.

3. RESULTS

The survey resulted in a total of 9451 references and from that 646 duplicates were removed. Afterward, 145 articles were analyzed by sequentially reading through their titles, abstracts, and complete articles. A flowchart illustrating the selection process steps is displayed in Figure 1. According to the inclusion and exclusion criteria previously described, a total of 96 were selected and categorized into the following topics.

3.1. VIABILITY, PROLIFERATION, CONCENTRATION, AND PROPORTION OF IMMUNOCYTES

Among the articles selected for the present review, 38 of them evaluated the effect of acidity or lactate on the viability, proliferation, or quantity of immunocytes. The most studied cell regarding this outcome was T lymphocytes with twenty-two articles exploring this effect. Most studies observed that decreases in pH values or increased lactate concentration lead to decreased proliferation and viability of T Lymphocytes

(Angelin et al., 2017; Calcinotto et al., 2012; Daneshmandi et al., 2019; Fischer et al., 2007; Gottfried et al., 2006; Johnston et al., 2019; Kim et al., 2017; Nakagawa et al., 2015; Wang et al., 2020; Yabu et al., 2011; Y.-X. Zhang et al., 2019). Similarly, increases in pH values or decreases in lactate concentrations resulted in higher T Lymphocytes quantification (Abumanhal-Masarweh et al., 2019; Comito et al., 2019; Jin et al., 2019; Ping et al., 2018; Wagner et al., 2020). Furthermore, when lactate extrusion was blocked, higher intracellular lactate concentration leads to higher T Lymphocytes viability (Renner et al., 2019). On the other hand, when a higher extracellular lactate concentration (20mM) was present the literature showed an increased T Lymphocytes quantification (Li et al., 2020; Yu et al., 2020). No influence of acid pH (Müller et al., 2000; Pilon-Thomas et al., 2016) or lactate (Mendler et al., 2012) on T Lymphocytes viability or quantification were found in three articles.

Seven articles explore the acid effect on NK cells and it was observed that decreases in pH values reduced the quantity (Lv et al., 2012; Renner et al., 2019) and viability (Müller et al., 2000) of these cells. In addition, an increase in extracellular lactate concentration decreases the viability (Harmon et al., 2019) and quantity (Seth et al., 2017) of NK. On the other hand, when intracellular lactate concentration increases the quantity of NK cells also increases (Beloueche-Babari et al., 2020). Only one article found no influence of acid pH on NK cells (Xie et al., 2016). Regarding B Lymphocytes, two articles observed increased quantification in decreased lactate concentration (Wagner et al., 2020) or increased pH (Abumanhal-Masarweh et al., 2019). However, one study found no effect of acid pH on B Lymphocytes quantification (Müller et al., 2000). Importantly, the proliferation and function of Tregs are not affected, and their maturation is favored in L-lactate presence (Angelin et al., 2017).

Two studies assessed the effect of acid on monocytes and one of them observed no interaction (Dietl et al., 2010), whereas the other one revealed a decreased viability of these cells under acid pH (Feng et al., 2015). The viability of DCs cells also decreased with higher extracellular lactate concentration (Gottfried et al., 2006), and increased with higher intracellular lactate levels (Beloueche-Babari et al., 2020). However, one study observed higher DC quantification in mild acid pH conditions varying between 6.5 and 7.0 pH, although under lower than 6.0 pH conditions, a

progressive decline in cell viability was observed (Erra Díaz et al., 2020). Similarly, for macrophage's acidification effects, the viability decreases under acid pH (Bidani et al., 1998; He et al., 2011) as well as the reverse is observed, the quantification is higher under higher pH (Kumar et al., 2013). Although the literature also observed no pH viability interaction (Thomas A. Heming et al., 2001). Regarding lactate concentration, elevated lactate levels also resulted in lower viability (Kumar et al., 2013) and lower macrophage quantification (Carmona-Fontaine et al., 2013). Although one article observed higher macrophage viability under higher lactate concentrations (Chen et al., 2019).

3.2. IMMUNOCYTE MORPHOLOGY AND MOTILITY

Motility is directly related to the capacity of several immunocytes to perform their cellular functions because they must be able to infiltrate the extracellular matrix reaching the site of action. Moreover, the migratory capacity is often related to cell morphology, since a cell with a high migratory capacity may have a greater amount of cellular projections. In this sense, the relationship between immune cell morphology and motility with pH acidification of the tumor microenvironment was evaluated in seven studies.

Initially, Ratner et al. (1985) observed that the locomotion of lymphocytes in collagen matrices was lower at pH 6.7 than at pH 7.2 when medium pH was directly acidified. Later the same research group defined that the acidification of a collagen matrix increased the locomotion activity of motile lymphocytes by 1.4 times, but weakly interfered with immobile lymphocytes (Ratner, 1992). Similar results were obtained in experiments with Matrigel in which the motility of lymphocytes was 1.6 times higher at pH 6.7 than at pH 7.1. Additionally, pre-incubation of lymphocytes at pH 6.7 was tested for subsequent analysis on pH 7.1 gels, and pre-incubation did not alter motility on collagen or matrigel. Thus, it was observed that acidification of the extracellular matrix increases locomotion activity in migrating lymphocytes, but does not increase recruitment of immobile lymphocytes. Despite the changes in locomotion only Wagner et al. (2020) studied the morphology of lymphoid cells under such conditions and observed that there was a decrease in the size of group 2 innate lymphoid cells (ILC2s)

when subjected to pH 6.0 (using both lactate and HCl) compared to cells cultured at pH 7.4 (Wagner et al., 2020).

Three studies examined the changes in morphology and motility of monocytic cells and macrophages when subjected to an acidic pH. Ye and colleagues (2018) observed that THP-1 macrophages of M2 phenotype lactate stimulation induced a morphological change from a polyhedral to a spindle-shaped aspect (Ye et al., 2018). Riemann et al. (2016), performed tests on macrophage strains (RAW264.7, differentiated THP-1) and monocytic cells (Mono Mac6, THP-1), as well as on primary human monocytes, and observed that the motility of these cells was not influenced by acidosis (Riemann et al., 2016b). Zhao et al. (2015) explored macrophage (RAW264.7) migration in a co-culture microfluidic chip to understand interactions in the bladder cancer acidic tumor microenvironment. The addition of lactate increased macrophage migration. When this phenomenon was separately evaluated, according to cell subtype, it was observed that M1 but not M2 macrophages had increased migration suggesting that these are more easily recruited by transitional cell carcinoma cells of the bladder (Zhao et al., 2015).

Finally, low microenvironmental pH influence on neutrophils was observed by Cao and colleagues (2015). Those cells, when exposed to an acid environment, showed defective chemotaxis directionality. Neutrophils in different test conditions had very similar euclidean velocities. On the other hand, cells in an acidic culture medium covered a longer path during the same period of time indicating that these cells presented a higher migration velocity (Cao et al., 2015a).

3.3.CELLULAR ACTIVATION AND FUNCTION

Besides the effects on viability and proliferation, immune cells usually require activation in order to properly exert their functions. T cells were found to be the most frequently reported immune cells - eleven publications - to have their activation and function altered by extracellular lactate abundance/acidity exposure. Zhang and colleagues (2019) observed a T cell activation marker CD25 inhibition along with a dramatic function suppression of T cells cultured at pH 6.5. Memory CD8+ T cells displayed an impaired immune response under extracellular acidosis caused by the

upregulation of co-inhibitory receptors and mTOR signaling pathway inhibition (Y.-X. Zhang et al., 2019). Besides tumor acidosis, lactate content in the extracellular compartment resulted in hampered T cell activation and reduction in the lactate content resulted, in turn, in slight immune cell recovery, demonstrating the causality link between lactate and the amplitude of suppression 72 (Caronni et al., 2018). As demonstrated by Daneshmandi et al., (2019), a combined LDHA inhibition and anti-PD-L1 treatment led to augmented CD8+ cytotoxic cell infiltration and activity (Daneshmandi et al., 2019). According to Walton et al., (2018) T cells require mTORC1 signaling for differentiation and activation of effector cells, which is hampered by acidic media-driven mTORC1 diminished activation in both CD4+ and CD8+ primary T cells (Walton et al., 2018). Two key CD8+ T cells cytolytic/apoptosis mediators, that act against cancer cells, perforin and granzyme B (Barth et al., 1991; Lieberman, 2003) were found to be diminished in the presence of acidosis/lactic acid. Granzyme B was markedly reduced under pH 6.5 (Jin et al., 2019) and pH 6.0 (Nakagawa et al., 2015). Calcinotto et al., 2012 observed a markedly impaired degranulation of the cytotoxic T lymphocyte pore-forming protein perforin in response to autologous tumor cells at pH 6.5 (Calcinotto et al., 2012). Interestingly, GrzB (Nakagawa et al., 2015) and perforin (Calcinotto et al., 2012; Nakagawa et al., 2015) degranulation by T cells were completely restored at neutral pH. NaHCO3 introduction was able to alleviate the CD8+ T cells effector responses from low pH-driven inhibition as illustrated by increased cell proliferation and IFN-γ production (Jin et al., 2019). Authors stated that the disruptive impact of extracellular acidity on T cell activity wasn't a consequence of pH on tumor target cells, as evidenced by the fact that those cells did display no changes in their HLA-I surface expression (Calcinotto et al., 2012). After both carbonic anhydrase IX (CAIX) inhibition followed by decreased extracellular acidification (Chafe et al., 2019) and experimental lactic acid exhaustion (Gao et al., 2019) T cell activity was found to be increased (Gao et al., 2019) as evidenced by higher T cell-mediated cancer cell killing (Chafe et al., 2019). Transcriptomics of tumor tissues revealed, “positive regulation of innate immune response”, “positive regulation of T cell activation”, and “innate immune response activating signal transduction” (Gao et al., 2019). Also, an increased proportion of both CD8+GranzymeB+ and CD8+IFNγ+ T cells demonstrated the

activation ability of intra and extracellular lactic acid removal for antitumor cellular immunity (Gao et al., 2019). More indirectly, the acidification-driven ARG1, IL-23p19, and IL-17A expression inhibited macrophage CD8+ T cell activation (Ohashi et al., 2013). Besides that, macrophages treated with oxamate - thereby at decreased lactate concentrations - were able to more effectively activate T cells suggesting the acquisition of an increased antigen presentation potential (Stone et al., 2019).

The second most cited immunocytes to have their activation and/or function affected by tumor microenvironmental lactate/acidosis were macrophages, investigated in five studies. Ohashi et al., (2013) observed that lactic acid-driven acidification increased ARG1, IL-23p19, and IL-17A expression in macrophages which led to T cell proliferation and activation inhibition (Ohashi et al., 2013). Kumar et al., (2013) found that dichloroacetate (DCA) treatment resulting in higher pH and declined lactate production *in vivo* led to tumor-associated macrophages with augmented tumoricidal activity, NO production, and increased IL1, IL6, and TNFα levels in mice tumor ascitic fluid compared to control groups (Kumar et al., 2013). Tumor tissue transcriptomics after intra- and extracellular lactic acid exhaustion demonstrated the acquisition of an immunocompetent TME (Gao et al., 2019). As indicated by the simultaneous activation of the innate and cellular immunity mainly through “toll-like receptor and NFkB signaling pathway, macrophage would then be activated (Gao et al., 2019). Interestingly, macrophage phagocytosis was observed to be increased under extracellular acidosis (Steinkühler et al., 2018). Exposure of macrophages to pH ranging from 5.0 to 6.5 increased cell binding and peptide presentation suggesting that acidic pH enhances peptide dissociation, consequently increasing the number of peptide-receptive MHC-I molecules (Chefalo and Harding, 2001; Steinkühler et al., 2018).

Natural Killer (NK) cells were found to develop activation and functional changes under acidosis or lactate abundance in four studies. Daneshmandi and colleagues (2019) observed augmented pro-inflammatory anti-tumor activity of NK cells after a combination of LDHA blockade and anti-PD-1 treatment in an *in vivo* melanoma model (Daneshmandi et al., 2019). Long et al., (2018) in turn, verified that NK cell functional activity and cytotoxicity were increased after lactate flux blockade and pH buffering through MCT4 inhibition in breast cancer 4T1 cells (Long et al., 2018). Two markers of

NK cell functionality, perforin 1 and LAMP-1, were found to be increased in NK cells after lactate levels were diminished and pH was reestablished (Long et al., 2018). In accordance, Müller et al., (2000) noted hampered NK cell activity against K562 cells under acidic culture conditions (Müller et al., 2000). Another cell activity indicator, the number of cytolytic granules, was reduced in NK when the pH was decreased from 7.2 to 5.6 (Lv et al., 2012).

DCs in the same context was assessed in three studies. In this sense, extracellular acidity was correlated with diminished expression of DC activation markers CD69, CD80, CD86, and HLA-DR (Jin et al., 2019). Complementary to that, Caronni and colleagues (2018) stated that DC conditioning by lactate impacted adaptive immune function by speeding up antigen degradation and impairing epitopes maintenance for MHC-I loading in phagosomes, consequently undermining antigen cross-presentation (Caronni et al., 2018). Furthermore, the same authors observed that DCs exposed to lactate *in vivo* failed to ignite antitumor reactions indicating that lactic acid reprograms innate immune response and antigen operation in these cells (Caronni et al., 2018). On the other hand, Yu and colleagues (2020) observed that lactic acid could significantly enhance the phagocytic function of DCs (Yu et al., 2020).

Two studies approached monocyte activation and functional alterations correlated with the TME metabolites and conditions of interest. Reduced lactate concentration (by oxamate treatment) was associated with altered monocyte activity. Monocytes treated with oxamate were able to activate T cells, suggesting that the lower lactate concentrations along with cell phenotype alterations drove an increased antigen presentation potential of these cells (Stone et al., 2019). However, THP-1 monocyte's phagocytic activity was observed to be increased in a time-dependent manner under reduced extracellular pH (Riemann et al., 2016b).

Two papers highlighted the correlation between tumor-derived acidosis/lactate presence in the TME and neutrophil abnormal activation and function. Although neutrophils in low pH conditions displayed faulty chemotaxis directionality and a more than 50% decrease in intracellular killing ability, a dramatic increase in phagocytic activity was observed (Cao et al., 2015b). In addition to that, Fischer et al., (2000)

observed that tumor cell killing via the cytolytic perforin/granzyme pathway was decreased at low pH (B. Fischer et al., 2000a).

One study assessed mast cell activation and function changes in low pH or lactate abundance. Lactate was found to suppress the secretion of TNF, IL-6, and MCP-1 and to inhibit IgE-mediated degranulation in mouse peritoneal and human skin mast cells (Abebayehu et al., 2019). Besides that, lactate can suppress the early and late stages of mast cell function and activation in a pH-dependent manner (Abebayehu et al., 2019). One study evaluated the influence of extracellular acidity over lymphokine-activated killer cells (LAK) and noted that this culture condition markedly affected LAK cell-mediated lysis of K562, Daudi, and Raji cells indicating that IL-2 mediated LAK cell activity against different target cell lines was abrogated after in pH 6.5 (Müller et al., 2000). Cytokine-induced killer cells (CIK) were also reported to have their activity against HepG2 cells remarkably suppressed at pH 6.5, strongly suggesting that its antitumor activities were negatively disturbed by tumoral acidity *in vivo* (Yuan et al., 2016). Besides, the authors pointed out that neutralizing the pH (NaHCO3) could reduce or even completely abolish this influence, amongst other effects by increasing perforin levels which translated as dramatic tumor growth suppression (Yuan et al., 2016). Furthermore, Meng and colleagues (2020) observed that reducing lactate release by DCA treatment *in vivo* enhanced antitumor immune response in mouse models of ascitic and subcutaneous HCC (Meng et al., 2020).

3.4.CYTOTOXICITY

Initially, the studies were developed with heterogeneous cytokine-activated lymphocyte cell populations and the relationship between cytotoxic capacity and microenvironment pH was evaluated. As early as 1994, Severin and colleagues found that the cytotoxicity of LAK (lymphokine-activated killer cells) against erythroleukemia cells decreased by up to 70% at acidic pH (6.8, 6.3, 5.8), stating that cytotoxic activity in human LAK cells is pH-dependent (Severin et al., 1994). These findings were later confirmed by Fischer et al., (2000) who described that LAK cells activity against tumor cells (K562, Daudi, Raji) in suspension was significantly reduced at extracellular pH values between 5.8 to 5.3 (B. Fischer et al., 2000b). The same was observed in the

cytolytic activity of LAK cells against six adherent tumor cell lines (HeLa, HepG2, LS174T, LS174Te, MCF-7, and RT112), reiterating the decline in the cytotoxic activity of LAK against both adherent and nonadherent erythroleukemia and histiocytic lymphoma cells under acidic conditions (B. Fischer et al., 2000a). In agreement with the previously mentioned studies, it was observed that cells called CIK (cytokine-induced killer cells) had their cytotoxicity against hepatocellular carcinoma cells decreased by about 35% in an acidic environment when lactic acid was added to the medium (Yuan et al., 2016). This effect was reversed when the medium had its pH adjusted with NaHCO3 to 7.4 (Yuan et al., 2016).

Looking more specifically at the effect on T lymphocytes, similar results have been observed. Fischer et al., 2007 reported that human CD8+ T lymphocytes had their cytotoxic activity decreased by about 50% against Melan-A-loaded T2 target cells in the presence of lactic acid, and consequently, with the reduction of pH. Similarly, (Nakagawa et al., 2015) saw a CD8+ T cell pH-dependent cytotoxic activity decrease, furthermore, when cells were incubated at normal pH for 4 hours, cytotoxic activity was almost completely restored. (Feng et al., 2017) reported that lactate has the ability to suppress T-cell cytotoxic activity against cells from different types of lung cancer (human lung adenocarcinoma A549 and H1299), NCI-460 (human large-cell lung cancer) through direct activation of PD-L1 expression. Further describing that lactate contributes to the protection of tumor cells from targeting cytotoxic T cells, establishing a direct connection between metabolic reprogramming of tumor cells and tumor immune response evasion. In 2019, Chafe and colleagues showed that carbonic anhydrase (CA9) inhibitors and hypoxia - which has as an ultimate consequence the pH raise and neutralization - alone or together doubled the cytotoxicity of CD3+ T cells against melanoma cells, at both in a 10:1 or at a 20:1 immune to tumor cells ratio (Chafe et al., 2019).

Four studies evaluating the cytotoxic capacity of NK cells were found. Loeffler et al., (1991) and Muller et al., (2000) reported a 5 to 35% reduction in the cytotoxic activity of NK cells against human erythroleukemia cells (K562, Daudi, and Raj cells) in situations where the pH was lowered from a maximum of 7.4 to a minimum of 6.4 (Loeffler et al., 1991; Müller et al., 2000). It was demonstrated that spleen and liver NK

cell's cytotoxicity against Yac-1 lymphoma cells progressively decreased as pH was dropped from 7.2 to 5.6 (Lv et al., 2012). Long and colleagues (2018) showed that Monocarboxylate Transporter 4 (MCT4) inhibition increased around 6% to 46% NK cell cytotoxicity against breast cancer cells (4TQ1) by blocking lactate efflux, reversing the acidity of the tumor microenvironment (Long et al., 2018). Mahaweni et al., (2018) demonstrated that hypoxia and lactate, responsible for lowering tumor microenvironment pH, reduced by 2 to 35% the cytotoxicity of NK cells against myeloma target cells (K562, RPMI8226/s, OPM-2, L363, JJN-3, UM-9) (Mahaweni et al., 2018).

In contrast, opposite results were found with cells of the monocytic lineage. Steinküler and colleagues (2018) evaluated the phagocytosis ability performed by macrophages in relation to the acidity of the tumor microenvironment (Steinkühler et al., 2018). When A549 tumor cells were cultured for a long time at pH 6.0 the levels of CD47 (a *don't eat me* signal) increased, but the interaction with the signal-regulatory protein alpha (SIRPα) present on macrophages was impaired, and increased phagocytosis by TH1 macrophages was seen. Yu et al., (2020) demonstrated that pretreatment of mouse lymphoma cells with lactic acid at different concentrations significantly increased phagocytosis by dendritic cells (Yu et al., 2020). Additionally, neutrophils in an acid medium presented a dramatic increase in the phagocytosis index. Approximately 250 bioparticles were engulfed by 100 neutrophils compared to 180 in pH 7.4 group. However, the intracellular killing ability decreased by more than 50% under an acidic environment compared to pH 7.4 condition (Cao et al., 2015b).

3.5.RECEPTORS, LIGANDS, AND ADHESION MOLECULES

Immune checkpoints

Immune checkpoints are molecules that modulate the amplitude of T-cell response in a T-cell receptor (TCR)-dependent manner in order to control cell activation and prevent aberrant inflammation reducing tissue damage during infectious disease outbreaks (Kubli et al., 2021; Okazaki and Honjo, 2006). This regulation happens through specific lock-and-key interactions between ligands and transmembrane receptors present in leukocytes such as the programmed cell death 1 (PD-1), cluster of

differentiation 28 (CD28), cytotoxic T lymphocyte antigen 4 (CTLA-4), and V-domain Ig suppressor of T cell activation (VISTA) (Lines et al., 2014). PD-1 is a receptor that binds to endogenous programmed cell death 1 ligand 1 (PD-L1), and 2 (PD-L2) shutting off T cell responses by tyrosine phosphatase activation (Keir et al., 2008). CTLA-4 is a receptor expressed by regulatory T cells (Tregs) that modulates CD28 activity in opposition to its triggering ligands, clusters of differentiation 80 (CD80) and 86 (CD86) both expressed on the surface of B-cells, Langerhans cells, monocytes and antigen-presenting cells (APCs) like dendritic cells (DCs) (O'Day et al., 2007). CD28 is a transmembrane protein present in T cells that when switched on by its ligands CD80 and CD86 exerts a costimulatory function, providing cell signals needed for T cell activation and survival (Dyck and Mills, 2017; Esensten et al., 2016; Lenschow et al., 1996; Linsley and Ledbetter, 1993; Mir, 2015; Peach et al., 1994). Although both CD80 and CD86 are competitive ligands of CTLA-4 and CD28, CTLA-4 binding affinity is 20-100 times greater than CD28 shaping a negative immune response fate. Therefore it plays an inhibitory role in the NF-κB pathway, compromising IL-2 production (Calvo and Rafii-El-Idrissi, 1997; Martins et al., 2008; Mir, 2015). The B7 family multimer VISTA, also known as B7-H5, PD-1H, Gi24, Dies1, SISP1, and DD1α is a transmembrane protein expressed on myelocytes and activated lymphocyte cells that functions as an immune checkpoint co-inhibitor (Huang et al., 2020; Verma et al., 2020).

During carcinogenesis, depending if those axes are activated or deactivated T cell response can be restrained therefore establishing an immunotolerant microenvironment and enabling tumor growth (Freeman et al., 2000). Immunotherapeutic antibodies capable of blocking CTLA4 and PD1 and allowing effector lymphocytes to mount an efficient response against malignant cells were the first successful oncology agents developed for clinical use and stood out over the past decade (Curran et al., 2010; Kubli et al., 2021; Martins et al., 2008; Postow et al., 2015; Wolchok et al., 2013).

Extracellular lactate has been shown to induce an increased expression of the immune checkpoint co-inhibitors PD-L1 and PD-L2 in different oropharyngeal squamous cell carcinoma cell lines (Verma et al., 2020) and PD-L1 on B16 melanoma and lung cancer cells (Daneshmandi et al., 2019; Feng et al., 2015; Seth et al., 2017). Also,

CD8+ T cells cultured under lactate acidified medium (pH 6.6) had an increased expression of co-inhibitory immune checkpoint PD-1 followed by a reduction in CD226 co-stimulatory receptor indicating a state of T cell dysfunction. This was accompanied by decreased expression of activation markers CD80 and CD86 in immature Mo-DCs (Jin et al., 2019). Moreover, PD-L1 activation by lactate in lung cancer cells compromised interferon-γ production and fostered apoptosis of cocultured Jurkat T-cell leukemia cells, revealing that lactate contributes to tumor cell preservation from cytotoxic T-cell activity (Feng et al., 2017). Evidence indicates that lactate-induced PD-L1 activation is mediated by its receptor GPR81 since the silencing of this molecule in lung cancer cells resulted in the inactivation of the PD-L1 promoter and lower PD-L1 protein levels (Feng et al., 2017).

In silico projections estimated that even small pH reductions were correlated with increased binding of the PD-1/PD-L1 complex. This higher affinity may be caused by the protonation of His68 amino acid, a component of the binding pocket between PD1 and PD-L1 (Klyukin and Alexandrov, 2020). *In vivo* blockage of Monocarboxylate transporter 1 (MCT1), which decreases extracellular lactate in TME, resulted in the rise of DCs and NK cell infiltration within the tumor. Nevertheless, it led to the upregulation of PD-L1, but not CD80, on NK cells and tumor-infiltrating DCs, suggesting the acquisition of a regulatory phenotype (Beloueche-Babari et al., 2020). Stone et al. (2019) observed that cervical cancer cell spheroids' lactate secretion inhibition by oxamate downregulated CD86 expression on macrophages (Stone et al., 2019). Concomitantly, PD-L1 overexpression in lung cancer cells was correlated with increased lactate production and extracellular acidification rates (ECAR) and this loop might impair effector T-cells activity (Kim et al., 2019). Moreover, Johnston and colleagues (2019) observed that VISTA multimers were able to bind to leukocytes at pH6.0, but not at physiological pH 7.4, and that this link led to suppressed T cell and Jurkat cells proliferation, IFN-γ production, and NF-κB phosphorylation (Johnston et al., 2019; Kim et al., 2019).

On the other hand, inhibition of NA/H exchanger, which drives H+ efflux in exchange for Na+ influx to maintain a neutral intracellular pH, has not resulted in significant changes in the expression of immune checkpoint blockers, PD-1 and

CTLA-4, in either CD4+ or CD8+ T cells (Guan et al., 2018). Also, Fischer and colleagues (2000) observed no changes in CD28 expression in human PBMCs under acidic cell culture conditions (Bianca Fischer et al., 2000).

Cell surface antigen profile

The Major Histocompatibility Complex (MHC), in humans known as Human Leukocyte Antigen proteins (HLA), is a group of membrane glycosylated proteins present at the cell's surface that are crucial for immune recognition of “self” and “non-self” antigens, directly impacting T-cell and NK cell responses (Dendrou et al., 2018; Hudson and Allen, 2016; Miles et al., 2015; Sim et al., 2017). The HLA classic subtypes consist of class Ia (-A, -B, -C), class Ib (-E, -F, -G, -H), and class II (-DR, -DQ, -DM, and -DP), that respectively carry out antigen presentation to CD8+ T cells, NK cells (NK cells), and CD4+ T cells (Allard et al., 2014; Crux and Elahi, 2017; Leddon and Sant, 2010). Large antigens go through a cytosolic degradation process resulting in smaller peptides being loaded onto HLA and shuttled to the cell surface (Miles et al., 2015; Neefjes et al., 2011). Recognition of HLA-peptide complexes occurs through heterodimer αβ T-cell receptor (TCR) expressed on the surface of T cells (Dendrou et al., 2018; Miles et al., 2015). Upon HLA and TCR interaction malignant cells can be identified and killed by CD8+ T cells *via* FAS or perforin pathways or even by evoking other inflammation components. However, tumors frequently present genetic or epigenetic instability leading to HLA gene critical alterations, loss, or silencing. Besides that, malignant tumors can hijack immune responses by expressing non-classical class Ib HLA molecules (Bukur et al., 2012; Dhatchinamoorthy et al., 2021).

A set of studies points out the fact that lactate and/or tumor acidosis compromise MHC/HLA and TCR expression. The presence of tumor cell-derived lactate *in vivo* drove the reduction of MHC II levels in dendritic cells (DCs) mediated by the G-protein-coupled receptor 81 for lactate (Gpr81) (Brown et al., 2020). Besides that, mouse mammary carcinoma (AT3) isolated from Gpr81−/− RNA-seq analysis suggested upregulation of T-cell receptor (TCR) signaling and antigen presentation pathways (Brown et al., 2020). The *in vitro* incubation of immature monocyte-derived DCs (Mo-DCs) in acidified culture medium resulted in a decreased HLA-DR (MHC II)

expression (Jin et al., 2019). *In vitro*, lactate secretion reduction and pH elevation after diclofenac treatment caused a slight increase in MHC I expression in M579 melanoma cells, and both MHC I and II were upregulated in 4T1 breast cancer cell's surface (Renner et al., 2019). CD8+ T cells cultured under pH6.5 had a significantly downregulated expression of TCR that was fully recovered after establishing a physiological 7.4 pH (Calcinotto et al., 2012). In contrast, there's evidence indicating that the presence of lactate and/or lower pH values do not alter or even foster an increase in the expression of such molecules. Stone et al. (2019) observed that decreasing extracellular lactate levels in cervical cancer spheroids with oxamate was followed by a reduction in HLA II expression in monocytes (Stone et al., 2019). Gupta and colleagues (2016) correlated lactate with elevated expression of MHC I (HLA-B) in monocytes (THP1 cells) *in vitro (Gupta et al., 2016)*. According to Fischer et al., 2007, lactic acid exerted no influence over both TCR and HLA-DR expression in primary cytotoxic T lymphocytes (CTLs) (Fischer et al., 2007). Altogether, the reported data suggests that the influence of the TME conditions on HLA/MHC is dependent on the cell type and lactate concentration studied.

G-protein-coupled receptors

With more than 800 subtypes, the G-protein-coupled receptor (GPCR) is a markedly diverse protein family that operates by detecting a variety of extracellular signals ranging from small organic molecules, photons, and ions to whole proteins. Following ligand coupling, the transmembrane structure suffers conformational changes, therefore, unleashing intracellular signaling networks leading to cellular response (Dorsam and Gutkind, 2007; Venkatakrishnan et al., 2013). GPCRs are enrolled in essential physiological activities such as muscle contraction, blood pressure adjustment, glandular hormone and enzyme release, and immune response. In the past decades, a correlation between GPCRs and tumor growth and metastasis was established and its role started to be investigated. In this sense, evading the immune system through the misappropriation of GPCRs function by malignant cells contributes to tumor progression (Dorsam and Gutkind, 2007).

Multiple studies correlate lactate produced by malignant cells with the lactate receptor GPR81 activation in an autocrine manner (Feng et al., 2017; Lee et al., 2016; Roland et al., 2014). According to Brown and colleagues (2020), tumor-derived lactate activation of GPR81 expressed by antigen-presenting dendritic cells suppressed cell-surface presentation of MHCII *in vitro (Brown et al., 2020)*. Also, GPR81 was depicted to mediate lactate-induced PD-L1 expression in lung cancer cells (Feng et al., 2017). Besides that the paracrine activation of GPR81 in dendritic cells was associated with lower levels of cAMP, IL-6, and IL-12 (Brown et al., 2020). This evidence indicates that GPR81 present in dendritic cells forestalls tumor-specific antigen presentation to other immune cells interfering with immune function and providing a favorable scenario for tumor cells to evade the immune reaction (Brown et al., 2020). Raychaudhuri et al., (2019) pointed out that lactate upregulated GPR81 expression in plasmacytoid dendritic cells (pDCs) and diminished IFNα induction by pDCs, which is mediated by GPR81 triggered intracellular Ca2+ deployment *in vitro (Raychaudhuri et al., 2019)*. Additionally, GPR81-deficient pDCs recovered IFNα induction even in the presence of lactate. Besides lactate-driven GPR altered function and expression, acidity (pH 5.9) promoted GPR4 - a sensor activated by acidic extracellular pH - overexpression in SCCHN cells resulting in increased secretion of IL6, IL8, and VEGFA (Jing et al., 2016).

Other adhesion molecules

Physiologically speaking, adhesion molecules such as differentiation clusters are present on the surface of different cell types acting in different ways. Whether as a source of cell phenotype identification, cell-cell adhesion components, receptors, or ligands, their action usually has as a consequence the initiation of signaling cascades that ultimately determines cell behavior (Chan et al., 1988). In this sense, the understanding of how tumor microenvironmental features like acidosis and/or lactate alter this molecule's function and expression is of major interest.

The cluster of differentiation 25 (CD25) is a transmembrane protein making up the interleukin 2 (IL-2) receptor. Present on multiple immune cells and a marker of T cell activity, it is vital for T cell proliferation and operation in a regulatory and effector manner. A set of studies revealed that CD25 expression was suppressed under acidic

conditions (Calcinotto et al., 2012; Y.-X. Zhang et al., 2019) and a decreased secretion of IFN-γ indicating compromised T cell function was also detected (Y.-X. Zhang et al., 2019). On the other hand, lactic acid wasn't able to induce changes in CD25 expression at an mRNA level (Fischer et al., 2007). Therefore, suppression of CD25 is caused directly by the acidic extracellular pH.

Stone and colleagues (2019) evaluated the expression of CD14, a monocyte protein and co-receptor to TLR, CD64, an Fcγ receptor, and CD206, a receptor associated with monocyte alternative phenotype (Stone et al., 2019). They found that after LDHA inhibition, and consequently lactate decrease, monocytes were able to activate T cells, indicating that at lower lactate concentrations the alteration in monocytes phenotype increased the Ag presentation capacity of these cells. Besides that, acidosis was found to increase the anti-inflammatory marker CD206 expression (Stone et al., 2019).

The T-cell immunoreceptor with Ig and ITIM domains (TIGIT), known to restrict T cell responses (Yu et al., 2009), was found to be highly expressed on CD8+ T cells under acidic conditions. Not only that but also its ligand CD155 present on melanoma cells, had an increased expression when cultured at a low pH. Besides the increased expression of both TIGIT and CD155, extracellular acidity fostered the conjugation between these two molecules suggesting that it plays a role in the loss of tumor immunogenicity (Jin et al., 2019). The same authors found a decreased expression of the activation marker CD69 in immature Mo-DCs under a lactic acid-acidified medium (Jin et al., 2019) which was not observed for cytotoxic T lymphocytes under similar culture conditions (Fischer et al., 2007).

Another molecule that has been found to be affected by a lactate-rich TME is the toll-like receptor 9 (TLR9), which is an important transmembrane receptor expressed in DCs, amongst others. This receptor ideally binds to DNA components from external pathogens like viruses and bacteria unleashing inflammatory cytokine feedback (Martínez-Campos et al., 2017). Raychaudhuri et al., (2019) pointed out that lactate derived from tumor cells hindered human plasmacytoid DCs (pDCs) TLR9-dependent activation and consequently attenuated IFNα induction, thereby bypassing an important arm of the anti-tumor immune response (Raychaudhuri et al., 2019). A set of studies

evaluated the influence of acidosis over the T cell receptor (TCR), a protein complex present on the T cell's surface that identifies antigen peptides attached to MHC/HLA molecules. Calcinotto and colleagues (2012) found that TCR and its co-receptor CD3 expression were downregulated in OTI CD8+ T blasts exposed to pH 6.5, and since suboptimal TCR activity in tumor-infiltrating lymphocytes (TILs) is linked to a state of cell anergy, this is believed to be an important correlation (Calcinotto et al., 2012). On the other hand, strong activation of TCR by a limited degree of CD3 stimuli and increased TCR signaling via ZAP-70 and p38 were seen under acidic conditions (Calcinotto et al., 2012; Hirata et al., 2008). Besides that, Fischer et al. (2007) found no changes in TCR expression under the same low pH 6.5 melanoma patients derived CTLs, which can be explained by inherent variations present on different cell subtypes (Fischer et al., 2007).

When considering macrophage activity, the interaction between the signal regulatory membrane protein α (SIRPα) and the transmembrane protein CD47 displays a *don't eat me* signal in a phagocytosis inhibitory manner (Barclay, 2009). When A549 lung carcinoma cells were cultured at pH 6.0, CD47 levels were elevated, referring to a cellular signaling sequence against the reduction of “self” signaling (Steinkühler et al., 2018). Lastly, no changes in the adhesion and antigen profile markers CD71, ICAM-1, CD45RO, CD95, CD2, CD18, CD44, CD54, CD56, and CD58 were observed when analyzing cytotoxic T lymphocytes, mononuclear cells, lymphokine-activated killer (LAK) cells, and natural killer cell clones under acidic pHe in two different studies (Bianca Fischer et al., 2000; Fischer et al., 2007).

3.6.CYTOKINE PRODUCTION AND SECRETION

Many cellular phenomena are influenced or even determined by the action of chemokines and cytokines. This broad category of small peptides is produced and secreted by various cell types including endothelial cells, epithelial cells, various stromal cells such as fibroblasts, and immunocytes in general. These molecules act specifically on plasma membrane receptors and are responsible for cell-cell crosstalk triggering different cell signaling pathways (Briukhovetska et al., 2021). Among the possible outcomes triggered by cytokines, we can mention steps of embryonic development; regulation of proliferation, maturation, and activity of certain cell populations;

up-regulation or down-regulation of genes and transcription factors; immune response balance, and angiogenesis (Dranoff, 2004). Many studies have shown that metabolic alterations resulting in acidity/increased lactate concentration in the tumor microenvironment can directly influence the production, secretion, and activity of these important molecules leading to immune-surveillance disturbance (Choi et al., 2013; Corbet and Feron, 2017; Fischer et al., 2007; Nenu et al., 2017). In the present review, we found 49 studies regarding this theme.

Twenty-one articles correlated acidosis or a lactate-rich microenvironment with suppressed or even abolished IFN production, indicating a strong interrelation that results in impaired immune cell function. Lactic acid and extracellular acidosis were found to cause strong IFN production impairment by diverse effector immune cells, peripheral blood mononuclear cells (PBMCs), LAK cells, CD8+ T cells, natural killer cells (NK), dendritic cells (DCs), and tumor-infiltrating lymphocytes (TILs) (Calcinotto et al., 2012; Caronni et al., 2018; Feng et al., 2017; B. Fischer et al., 2000a; Fischer et al., 2007; Gao et al., 2019; Jin et al., 2019; Johnston et al., 2019; Lv et al., 2012; Mendler et al., 2012; Müller et al., 2000; Nakagawa et al., 2015; Pilon-Thomas et al., 2016; Pötzl et al., 2017; Raychaudhuri et al., 2019; Seth et al., 2017; Vishvakarma and Singh, 2011; Xie et al., 2016; Y.-X. Zhang et al., 2019). Besides that, treating CD11b+CD163+ myeloid cells and CD3C T cells from human hepatocellular carcinoma suspension with the proton pump inhibitor omeprazole, blocking acidification, was associated with increased IFN-γ (Kuchuk et al., 2018). However, pH 6.5 markedly promoted the differentiation of monocytes into DCs and these cells when cultured at pH 6.5 showed a high ability to induce the production of IFN-g by allogeneic CD4+ T lymphocytes (Erra Díaz et al., 2020).

Nine studies observed that lactate and/or acidosis culminates with the downregulation of immune activation and the pro-inflammatory marker IL-6. CD8+ T cells, macrophages, human peripheral blood monocytes, and monocytic cell lines decreased their IL-6 production due to low pH/microenvironmental lactate suggesting this metabolic feature compromises the levels of this typical immune activation cytokine (Abebayehu et al., 2019, 2016; Dietl et al., 2010; El-Kenawi et al., 2019; Gao et al., 2019; Ratter et al., 2018; Riemann et al., 2016b). *In vivo* administration of the proton

pump inhibitor pantoprazole (PPZ) was correlated with a significant increase in pH and decreased lactate concentrations which led to augmented IL-6 expression in the ascitic fluid of PPZ tumor-bearing mice (Liu et al., 2019). On the other hand, seven papers pointed to IL-6 upregulation under lactate/acidosis exposure. The presence of both low pH and lactate in the extracellular milieu was found to contribute to IL-6 upregulation in monocytes, primary human monocytes, and macrophages indicating a shift to M2-like phenotype and, along with IL-8 and VEGF, induction of angiogenesis (Arts et al., 2016; Liu et al., 2019; Riemann et al., 2016b; Sloot et al., 2019; Stone et al., 2019). Besides immune cells, extracellular acidosis was also correlated with IL-6 upregulation in head and neck, prostate, breast, and cervix cancer cell lines (Jing et al., 2016; Riemann et al., 2017). Interestingly Riemann and colleagues (2017) observed that IL-6 expression by tumor cells was increased at a short-term acidosis (3 to 6 hours) although in a long-term low pH exposure (24 h) its expression was found to decrease (Riemann et al., 2017). These contradictory results suggest that IL6 regulation by tumor microenvironment pH depends on the cell type and total period of acidification.

Nineteen studies observed that lactic acid and/or extracellular acidosis were inversely correlated with TNF expression, in some cases leading even to its complete suppression. The authors reported lactate/low pH derived TNF suppression on macrophages, mast cells, monocytes, monocytic cell lines, primary human monocytes, primary human monocyte-derived macrophages, TILs, CTLs, PBMCs, LAK cells, CD4+ T helper cells, CD8+ T cells, B lymphocytes, myeloid cells, and CD3C T cells - those last two obtained from HCC human suspensions (Abebayehu et al., 2019, 2016; Bidani et al., 1998; Calcinotto et al., 2012; Dietl et al., 2010; El-Kenawi et al., 2019; B. Fischer et al., 2000a; Thomas A. Heming et al., 2001; He et al., 2011; Jin et al., 2019; Kuchuk et al., 2018; Mendler et al., 2012; Müller et al., 2000; Peter et al., 2015; Ratter et al., 2018; Riemann et al., 2016b; Seth et al., 2017). Riemann and colleagues (2017) observed a temporary increase in TNF-α expression upon 3-6 hours of acidosis followed by a reduced TNF-α expression under 24-hour incubation of Walker-256 breast carcinoma cell lines (Riemann et al., 2017). Interestingly, TNF-α primary human monocyte-derived macrophage downregulation by acidosis happened independently of the cell activation state (Riemann et al., 2016b). Heming et al., (2001) observed a progressive decrease in

TNF-α secretion by macrophages resulting from decrements in cell culture medium pH. Moreover, reduced TNF-α secretion was accompanied by increased cytosolic TNF-α content, suggesting that acidity led to cytokine intracellular retention (T. A. Heming et al., 2001a). In accordance, Hemming and colleagues (2001) stated that although TNF-α mRNA concentration was unaffected by low pH, its release to the extracellular compartment was in fact inhibited at lower pH values (T. A. Heming et al., 2001b). Monocytes cocultured with melanoma cells demonstrated that tumor-derived lactate is a critical inhibitor of monocyte TNF secretion. Also, the authors observed that the acidification to pH 6.6 decreased TNF levels significantly while pH 7.1 had only a minimal effect on the TNF secretion (Dietl et al., 2010). Altogether those findings demonstrate that extracellular lactate/acidosis: may hamper the recruitment of immune cells (Dietl et al., 2010; Riemann et al., 2016b); drive changes in macrophage activation patterns towards a phenotype reminiscent of tumor-infiltrating macrophages (El-Kenawi et al., 2019); targets genes encoding important monocyte effector cytokines such as TNF (Peter et al., 2015); can modulate and disrupt monocyte cytokine production (Dietl et al., 2010). On the other hand, two studies claimed that TNF levels were significantly increased in TC-induced macrophages (Arts et al., 2016) and that a 1.8-fold increase in TNF-α released in response to a decrease in environmental pH from 7.4 to 7.0 was observed in peritoneal macrophages (Bellocq et al., 1998). No significant effect was found on TNF-α production after co-culture of macrophages with thyroid cancer cell lines upon MCT-1 lactate transporter blocker treatment (Sloot et al., 2019).

IL-10 is an immune-suppressive cytokine and four studies observed IL-10 increase correlated with extracellular lactate and/or low pH. Stone and colleagues (2019) observed IL-10 reduction in the supernatants of SW756 cervical cancer cell line and macrophage co-cultures upon lactate production inhibition indicating that lactate mediated part of the crosstalk between tumor cells and macrophages, promoting secretion of IL-10 (Stone et al., 2019). Authors suggest that lactate-derived IL-10 up-regulation, among other findings, is an indicator that lactate contributes to the expression of characteristics present in M2 pro-tumoral macrophages (Stone et al., 2019). Co-culturing macrophages with thyroid cancer cell lines under MCT-1 lactate transporter blockade led to decreased IL-10 levels (Sloot et al., 2019). Caronni et al.,

(2018) observed DCs isolated from lactate-rich lung tumors (Caronni et al., 2018). Authors indicated that those cells showed little or no pro-inflammatory gene induction but importantly upregulated the production of immune-suppressive cytokines such as IL-10 (Caronni et al., 2018). In accordance with that, PPZ treatment in tumor-bearing mice led to a significant tumor ascitic fluid reduction in IL-10 expression (Vishvakarma and Singh, 2011). On the other hand, five studies observed lactate/acidosis-related IL-10 decrease. In this sense, IL-10 production was impaired in monocyte-derived DCs (mo-DCs) (Erra Díaz et al., 2020), human PBMCs (B. Fischer et al., 2000a; Müller et al., 2000; Ratter et al., 2018), LAK cells (B. Fischer et al., 2000a), macrophages, and monocytes (Arts et al., 2016) upon incubation with lactate or HCl medium acidification. Ohashi and colleagues (2013) found no difference in macrophage IL-10 expression under lactic acid incubation (Ohashi et al., 2013).

Three papers established a correlation between lactate or HCl-adjusted cell culture medium and the downregulation of the cytokine IL-12. PBMC and LAK cells, CD4+ T helper cells, B lymphocytes, monocytes, CTLs, and DCs cells had a reduction or even a complete suppression in IL-12 and its variations alpha and beta under acidic or lactate-rich microenvironments indicating inhibition of innate functions (Caronni et al., 2018; Mendler et al., 2012; Müller et al., 2000). Interestingly, the subtype IL-12p40 which have as a widely praised function, the establishment of a negative feedback loop through competitively binding to the IL-12 receptor, was found to be overexpressed in macrophages upon lactate incubation (Cooper and Khader, 2007).

Considered an important TAM-related cytokine, Interleukin 1β (IL-1β) was overexpressed under acidosis in four studies. Its *in vitro (El-Kenawi et al., 2019),* and *in vivo* (Riemann et al., 2016b) gene expression and production were augmented in macrophages and mo-DCs (Erra Díaz et al., 2020; Liu et al., 2019). Conversely, IL-1β was downregulated in the presence of low pH or lactate in 3 studies. Monocytic cell lines, macrophages, and human PBMCs presented lower levels of IL-1β after cell incubation with lactic acid or extracellular acidosis (Arts et al., 2016; Ratter et al., 2018; Riemann et al., 2016b). Another cytokine that was found to present an inversely correlated expression with the culture medium pH/lactate was IL-8, which is described along with other cytokines to play a role in angiogenesis. Four studies evidenced that,

not only immune but mainly tumor cells raised their IL-8 mRNA expression and protein secretion under the influence of low pH and/or lactate abundance. This finding was observed in head and neck squamous cell carcinoma FaDu cell line, human COLO357 and FG pancreatic adenocarcinoma cells, and also in human papillary thyroid carcinoma cells-induced macrophages (Arts et al., 2016; Jing et al., 2016; Shi et al., 2000, 1999). In opposition to that, Sloot and colleagues (2019) observed no significant effect of MCT-1 lactate transporter blocker treatment in IL-8 production after co-culture of macrophages with thyroid cancer cell lines (Sloot et al., 2019).

Less frequently the studies approached the influence of extracellular acidity or lactate over a myriad of other interleukins such as IL-2, IL-4, IL-5, IL-13, IL-17, IL-23. Two papers related the presence of lactic acid and low pH with the upregulation of IL-17 (Ohashi et al., 2013; Yabu et al., 2011) and IL23p19 (Ohashi et al., 2013) in macrophages, BMDMs, and BMDCs attributing to those cytokines the involvement in T cell proliferation and activation inhibition. One study observed an increase in IL17 after myeloid-specific deletion of LDH-A in a K-Ras murine model of lung carcinoma, although the authors highlighted that this cytokine has a dual effect on tumor growth since it acts facilitating angiogenesis through fibroblasts VEGF production activation but also IL-17 producing T cells (Th17) were demonstrated to be effective in seems to promote cytotoxic T cells responses (Muranski et al., 2008; Murugaiyan and Saha, 2009; Numasaki et al., 2003). Importantly, Vishvakarma et al., (2011) observed that PPZ administration to tumor-bearing mice lead to lower concentrations of IL-4 in tumor ascitic fluid, indicating that microenvironmental conditions after proton pump inhibition became favorable for expansion of cell-mediated immune responses (Cortes et al., 2009; Klausen et al., 2000; Shanker et al., 2000). Besides, IL-4 has also been demonstrated to promote tumor cell survival in certain types of tumors (Contasta et al., 2003). On the other hand, the co-culture with B16F10 cells releasing high amounts of lactic acid or the direct lactic acid addition to the cell culture medium markedly inhibited IL-4 production by NK cells (Xie et al., 2016). Two studies showed that IL-2 production and secretion by TILs and CTLs, a feature (along with CD25 expression) frequently tested for rating T-cell anergy, was decreased or even completely suppressed at low pH and lactic acid-rich environments (Calcinotto et al., 2012; Fischer et al., 2007). One

study revealed a decrease in IL33-mediated IL-13 secretion by mast cells after lactic acid incubation, thus suggesting that lactic acid antagonizes the proinflammatory effects of IL-33 (Abebayehu et al., 2016). Peter and colleagues (2015) found out that genes encoding for relevant monocyte effector proteins, such as IL-23, are lactic acid targets (Peter et al., 2015). They observed an up-regulated expression of this cytokine, related to pro-tumoral cell fates, in the presence of lactic acid and corresponding acidification (Peter et al., 2015). Lastly, melanoma cells-derived lactate caused a reduction in IL-5 cytokine production which jeopardized the survival and activity of group 2 innate lymphoid cells (ILC2s) followed by compromised eosinophil antitumor response and enhanced tumor growth (Wagner et al., 2020).

A set of monocyte-chemotactic proteins (MCPs) also referred to as chemokine (C-C motif) ligands (CCLs) that critically act as a chemoattractant for several leukocytes were found to be affected by the microenvironmental conditions described above. Seven studies observed an inverse correlation between lactate/acidosis and the four types of MCP of chemokines: MCP-1 (CCL-2), MCP-3 (CCL-7), MCP-5 (CCL-12), and MiP-1a (CCL-3) (Abebayehu et al., 2019, 2016; El-Kenawi et al., 2019; Peter et al., 2015; Riemann et al., 2017, 2016b). The most frequently studied molecule, approached by six studies, was MCP-1 which was found to be downregulated by acidosis or lactate abundance in monocytes, macrophages, mast cells, monocytic cell lines, normal fibroblasts, AT1 prostate carcinoma cells, and Walker-256 rat breast carcinoma (Abebayehu et al., 2019; El-Kenawi et al., 2019; Peter et al., 2015; Riemann et al., 2017, 2016b). Peter and colleagues (2015) found out that genes encoding for relevant monocyte effector chemokines like MCP-1 and MCP-3, are lactic acid targets (Peter et al., 2015). The genes coding for those two molecules were markedly suppressed by lactic acid/acidification and also a strong suppression in MCP-1 secretion by monocytes was observed under pH 6.6 and lactic acid incubation (Peter et al., 2015). El-Kenawi et al., (2019) observed through culture medium multi-analyte profiling the presence of significant alterations in the release of many inflammatory chemokines such as MCP-5 by macrophages, demonstrating that extracellular acidosis changes macrophage activation patterns (El-Kenawi et al., 2019). Finally, one paper reported that lactic acid reduced IL-33–mediated MIP-1a secretion by mast cells (Abebayehu et al., 2016). In

opposition to the above findings, a set of three studies indicated that MCP-1 presented a positive correlation with lactate/acidosis being suppressed in macrophages incubated with an acidic culture medium or when lactate production/low pH were experimentally controlled suggesting that this cytokine has a role in tumor progression (El-Kenawi et al., 2019; Kuchuk et al., 2018; Sloot et al., 2019). No impact on MCP-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression by macrophages exposed to lactate were found (Arts et al., 2016). Besides, no changes in MCP-1 levels in AT1 R-3327 prostate cancer cells under pH 6.6 was observed (Riemann et al., 2016a).

Five papers addressed the influence of metabolism products on the vascular endothelial growth factor (VEGF-A) which is a signal protein that stimulates the formation of blood vessels and may also affect the function of immune cells (Senger et al., 1983). Four of them related low pH or extracellular lactate to increased levels of this molecule in macrophages (Liu et al., 2019; Seth et al., 2017) and head and neck squamous cell carcinoma FaDu cell line (Jing et al., 2016). Besides, its expression was found to significantly decrease in the tumor ascitic fluid of PPZ-treated tumor-bearing mice when compared to controls (Vishvakarma and Singh, 2011). In opposition to that, only one study correlated low pH (6.6 and 6.8) with decreased secretion of both VEGF and VEGF-C by THP1 macrophages (Feng et al., 2015).

Five papers reported a correlation between the enzyme responsible for synthesizing nitric oxide (NO) from L-arginine, inducible nitric oxide synthase (iNOS), and lactate and/or low pH. iNOS is produced by diverse cells triggered by cytokines and participates in the immune defense and cardiovascular system (Green et al., 1994; Knowles and Moncada, 1994). Acidosis was found to decrease the gene expression of iNOS (Nos2) in macrophages which were pointed out by the authors as a proinflammatory marker (El-Kenawi et al., 2019). Harhaji and colleagues (2006) observed reduced tumor cell NO production in a pH-dependent manner (Harhaji et al., 2006). The authors reported two different effects depending on the cell lines assessed: the inhibitory effect of acidosis on NO production in C6 fibroblasts was related to decreased in viability; L929 fibroblasts were rescued from NO-dependent apoptotic and necrotic death. Moreover, acidity downregulated IFN-g+ IL-1-induced expression of iNOS mRNA and

protein, and completely abolished the activation of iNOS transcription factor IRF-1 in L929 fibroblasts. Finally, pH 6.8 was augmented, while pH 6.0 was reduced, IFN-g- induced iNOS activation/NO release and NO-dependent anticancer activity of rat and mouse macrophages. Those effects were observed in the presence of lactic acid acidified culture medium instead of HCl (Harhaji et al., 2006). Riemann et al., 2017 found acidosis exposure time-dependent differences: iNOS expression in tumor cells was significantly induced for 3 to 6 hours, which may affect tumor cell survival and proliferation followed by a recovery to basal levels after 24 hours of acidosis (Riemann et al., 2017). Amongst other inflammatory mediators, iNOS was significantly decreased in vivo by combined hypoxia + acidosis. Knowing that this cytokine plays a role in the immune response, its alterations may be crucial to mount a response against tumor cells or let them evade the immune response and grow. On the other hand, the RAW264.7 macrophage cell line showed increased iNOS expression upon extracellular acidosis (Riemann et al., 2016b). The authors then state that acidosis may hamper immune cell recruitment, but it fosters inflammation when macrophages are present by increasing iNOS levels (Riemann et al., 2016b). Also, acidity was found to significantly increase iNOS expression in AT1 R-3327 prostate cancer cells, which was accompanied by a 30% elevation in nitrate/nitrite formation (Riemann et al., 2016a).

Osteopontin (OPN), a multifunctional protein that interacts with diverse receptors related to signaling pathways enrolled in cancer processes, is known to play a vital role in immune response and was found to be correlated with the metabolic products of interest in the present review (Zhao et al., 2018). OPN was found to be augmented in AT1 prostate carcinoma cells, Walker-256 breast carcinoma cells (Riemann et al., 2017), and AT1 R-3327 prostate cancer cells (Riemann et al., 2016a) upon extracellular acidosis. Opposing that, acidosis led to a reduction in OPN expression in monocytic cell lines which was translated by the authors as a reduction in the recruitment of immunocytes (Riemann et al., 2016b).

Transforming Growth Factor-β (TGF-β) has a critical role in cell division, differentiation, and death, so aberrant TGF-β signaling produces consequences in cancer fate (Kubiczkova et al., 2012). Among the main functions held by TGF-β the regulation of inflammatory processes, stem cell and T cell regulation, and differentiation are described as key activities (Letterio and Roberts, 1998; Massagué and Xi, 2012).

Two papers related diminished TGF-β expression and release by effector cells like PBMC and LAK cells (B. Fischer et al., 2000a; Müller et al., 2000) besides CD4+ T cells, and B lymphocytes and monocytes (Müller et al., 2000). In opposition to that, the expression of TGF-β was found to significantly decrease in the tumor ascitic fluid of tumor-bearing mice treated with PPZ (Vishvakarma and Singh, 2011).

Finally, one study revealed that the incubation of THP-1 monocytes with 5mM lactate was able to induce prostaglandin E 2 (PGE2) secretion (Wei et al., 2015). A molecule that, in physiological processes, is involved in crucial processes such as fever modulation, pain, and inflammation. In cancer disease, PGE2 produced by stromal cells in the tumoral site enhances cancer cell division, and survival, fostering angiogenesis and inducing metastasis (Finetti et al., 2020).

Collectively, the above findings highlight, in one way or another, an undeniable correlation between cyto/chemokines expression, production, secretion, and microenvironmental lactate abundance, and/or acidosis. Cytokines expression, production, and secretion in both immune and cancer cells can be directly affected by these metabolic alterations of TME.

3.7.DIFFERENTIATION, POLARIZATION, AND MATURATION OF MONOCYTES

Monocytes are part of the mononuclear phagocyte system and are directly related to cancer pathogenesis and progression (Olingy et al., 2019). Once developed in bone marrow progenitors, monocytes circulate in peripheral blood and enter tissues turning into different types of cells: macrophages and dendritic cells (Ginhoux and Jung, 2014). Macrophages are the most common immune cells in tumor tissue, often termed tumor-associated macrophages (TAMs). The tumor microenvironment produces different stimuli resulting in macrophage polarization to a tumor-suppressive M1-like state or a tumor-promoting M2-like state (Mantovani et al., 2017). M1 macrophages express high levels of iNOS, nitric oxide, and inflammatory cytokines such as TNF-α and IL-1 (Zhao et al., 2015) while M2 macrophages express arginase 1, CD206, IL-4 receptor α-chain, among others (Murray and Wynn, 2011) and may favor angiogenesis, epithelial-mesenchymal transition, migration and invasion in the primary tumor and

suppress the antitumor immune responses due to their high production of Arg-1 and VEGF (Lin et al., 2019; Zhao et al., 2015).

Reprogrammed energy metabolism induces a tumor-suppressive immune microenvironment in cancers (El-Kenawi et al., 2019). This review detected eighteen articles that investigated monocyte polarization in relation to the acidic environment. Two of them observed that acid lactic is able to skew macrophages toward an M2-like phenotype (Ohashi et al., 2017; Zhang and Li, 2020). Higher levels of M2 macrophage markers (CSF1R and CD163) were seen in HNSCC tumors with a higher concentration of lactic acid (Ohashi et al., 2017). In the same way, treatment of THP-1 cells or human monocytes with gastric cancer cell-derived conditioned media or lactic acid leads to an M2-like state, and knockdown of LDHA reverted this phenotype as revealed by reduced expression of M2-related markers and cytokines (Zhang and Li, 2020).

Ten articles investigated that lactate may favor M2 macrophage polarization. The analyses of bone marrow-derived macrophages (BMDMs) and the THP-1 cell line showed that lactate increased M2 macrophage marker expression (Arg-1 and MRC-1) (Mu et al., 2018). In breast cancer, lactate activation of ERK/STAT3 signaling (Mu et al., 2018) and G protein-coupled receptor 132 (Gpr132) (Chen et al., 2017) promoted M2 macrophage polarization. Gpr132 is a key sensor in mediating the reciprocal interaction between cancer cells and macrophages during breast cancer metastasis (Chen et al., 2017). In pancreatic cancer cells, TAMS secreted CCL18 inducing a glycolytic phenotype partially due to VCAM-1 paracrine production, which induces lactate secretion from pancreatic cancer cells and drives alternative activated M2-like polarization of macrophages in a dose-dependent manner (Ye et al., 2018). Lactate in transitional cell carcinoma of the bladder (CBCT) cells inhibited M1 polarization (reduced expression of iNOS and p-NF-κB-p65) and induced macrophage M2 polarization (increased the expression of Arg-1 and HIF-1α) (Zhao et al., 2015). In cervical cancer, lactate up-regulated IL-6, IL-10, and HIF-1α expression and down-regulated p65 NF-κB activity, which contributes toward M2 polarization (Stone et al., 2019).

Macrophage-expressed lactate dehydrogenase-A (LDH-A) and lactate are major drivers of T cell immunosuppression. Deletion of LDH-A specifically on myeloid cells promoted the accumulation of macrophages with a CD86 high and MCP-1high M1-like phenotype. Furthermore, it was associated with increased antitumor T cell activity by induction of IL-17 and IFNγ-producing CD8+ T (Tc17 and Tc1) cells (Seth et al., 2017).

The role of microenvironment acidification on M2 like phenotype polarization was confirmed by studies where neutralization of intratumoral acidity reduced the pro-tumoral phenotype of macrophages and decreased tumor incidence and invasion of prostate cancer (Balza et al., 2017; Bohn et al., 2018; El-Kenawi et al., 2019). In melanomas with high glycolytic activity, tumor microenvironment acidification induced the GProtein–coupled-receptor–dependent expression of the transcriptional repressor ICER in TAMs. This leads to non-inflammatory phenotype macrophage polarization and promotes tumor growth (Bohn et al., 2018). Aiming to elucidate therapeutic targets of tumor cell responses to stress, Balza et al. (2017) exposed murine bone marrow-derived macrophages (BMDM) to IL-4 for 48 hours with or without esomeprazole and/or sulfasalazine. In the absence of drugs, 60-70% of the cells displayed the M2 marker CD206 and the expression of M1 marker CD86 was irrelevant (Balza et al., 2017; El-Kenawi et al., 2019). This suggests that IL-4 is an important driver in macrophage M2 phenotype at low pH (Balza et al., 2017; El-Kenawi et al., 2019).

Lactate in conditioned media from pancreatic cells induced differentiation of THP-1 cells (a monocytic undifferentiated cell line), since when MCT4 (lactate exporter) was inhibited THP-1 differentiation decreased. No polarization was detected since both CCR7 (M1) and CD206 (M2) markers were up-regulated (Hutcheson et al., 2016).

Dendritic cells (DCs) link innate and adaptive immunity playing a critical role in the initiation of the adaptive immune response and the maintenance of self-tolerance (Hawiger et al., 2001). The tumor milieu can influence DCs differentiation (Harhaji et al., 2006) and acidosis and lactic acid promote differentiation of monocytes into DCs (mo-DCs) (Erra Díaz et al., 2020) and modulation of DC phenotype (Gottfried et al., 2006). Low pH (pH 6.5) promoted mo-DCs differentiation in the absence of TNF-a although was unable to induce the differentiation in the absence of IL-4 (Erra Díaz et al.,

2020) Gottfried et al. (2015) analyzed this in a 3-dimensional tumor model through the exposure of a monocytes culture to IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) within multicellular tumor spheroids (MCTSs) (Gottfried et al., 2006). Monocytes invaded the MCTSs and differentiated into tumor-associated dendritic cells (TADCs). It was detected that both melanoma and prostate carcinoma MCTS co-cultures produced high levels of lactic acid, which when blocked, the TADC phenotype reverted to normal. In conclusion, tumor-derived lactic acid is an important factor modulating the DC phenotype in the tumor environment, which may critically contribute to tumor escape mechanisms (Gottfried et al., 2006).

On the other hand, tumor growth can be inhibited by elevated concentrations of lactic acid promoting antitumor immunity. Developing a tumor vaccine, Yu et al. (2019) tested this concept in mouse xenograft models. The effects on DCs of lactic acid-stimulated tumor cells included enhancing phagocytic function and stimulated maturation and aggregation. Moreover, lactic acid increases the immunogenicity of irradiated whole-tumor cell vaccines mediated by CD8+ T cells and thus inhibits tumor growth (Yu et al., 2020).

4. DISCUSSION

The tumor microenvironment is constituted by multiple components that are known to be constantly changing. Namely malignant cells develop complex and pivotal interactions with endothelial cells, diverse stromal components, and fibroblasts. In the last decades, game-changing interplays among cancer cells and the immune component have gradually been elucidated (Hiam-Galvez et al., 2021; Hinshaw and Shevde, 2019). The understanding that adaptive and innate immune system fitness is vital in blocking a cell's malignant transformation mounting the so-called immunosurveillance strategy is already a consensus (Bohn et al., 2018; Calcinotto et al., 2012; Fischer et al., 2007; Hanahan, 2022; Mendler et al., 2012). Then, neoplastic development and progression require a set of alterations in the surrounding milieu. We now know that tumor cells have the ability to carve the environment through the discharge of metabolites, chemical factors, cyto- and chemokines leading to protumoral

critical immune reprogramming (Hanahan, 2022). In this sense, emerged the understanding that tumor microenvironmental acidosis/lactate-driven immunosuppression markedly affects malignancy, clinical prognosis, and onco-immunotherapeutic agents' effectiveness (Bohn et al., 2018; Ho and Liu, 2016; Liu et al., 2019; Zhang and Li, 2020). Both extracellular acidity and lactate richness produce a harsh microenvironment that results mainly in the selection of cells with specific mutations and acquired mechanisms that are able to survive (Liu et al., 2019; Pollizzi and Powell, 2014; Zhang and Li, 2020).

In this sense, one of the main findings of the present review is the direct correlation between the presence of the above-mentioned environment and decreased concentration, viability, and proliferative capacity of immunocytes frequently with a straight influence on cell death triggering (Abumanhal-Masarweh et al., 2019; Comito et al., 2019; Daneshmandi et al., 2019; Gottfried et al., 2006; Jin et al., 2019; Johnston et al., 2019; Kim et al., 2017; Nakagawa et al., 2015; Ping et al., 2018; Wagner et al., 2020; Wang et al., 2020). Exploring this correlation we observed that the pH gradient can affect not only concentration but also the localization of immune cells. Therefore, we hypothesize that, in this context, a tumor progression favorable niche may be established. Although a reduction in immunocyte viability and survival is most often observed in the presence of this hostile microenvironment, this effect seems to be cell-type dependent. For example, while most immune cells become non-viable, T regulatory (Tregs) cells are unaffected, keeping their concentrations and function unchanged and contributing to a scenario of immune tolerance creating room for tumor progression. Tregs are known to have a well-established immune suppression activity, hampering the immunological response against tumor cells through cytotoxic CD8+ T cells functions and proliferation suppression in many described ways (Campbell and Koch, 2011; Sakaguchi et al., 2008; Y.-X. Zhang et al., 2019).

In addition to hampered immune cell proliferation in more acidic tumor regions, the locomotion capacity of these cells seems to also be affected. Since migration skills are vital for immunocytes to be able to reach critical sites and perform their required immune defense, locomotion impairment configures a valuable strategy for cancer cell maintenance and proliferation. Again, tumor acidity/lactate effect on immune cells

migration capacity was revealed to be cell type dependent. For example, neutrophil locomotion was found to be increased in the above-mentioned environmental conditions. Interestingly, neutrophils play a well-established pro-tumor role, fostering angiogenesis and metastatic potential of tumor cells (Wu et al., 2020). In accordance, four out of five papers observed increased VGFA levels resulting from acidosis/lactate abundance.

Like VEGF, several cytokines had their expression and concentration altered according to tumor microenvironment conditions. Cytokines are vital molecules establishing crosstalk between tumor cells and immunocytes, however, this regulation process seems to be complex and dose and time-dependent. No relationship was observed between outcome and specific cell types, supporting the hypothesis that fluctuations related to lactate concentrations and pH exposure are what possibly influence the findings. The findings of the present review were contradictory in this regard, indicating that even small changes in pH levels or lactic acid concentration can deeply affect results. A significant example of that is the contradictory findings regarding lactic acid and/or extracellular acidosis correlation with TNF expression. This cytokine was found to be suppressed in most papers (Calcinotto et al., 2012; Caronni et al., 2018; Feng et al., 2017; B. Fischer et al., 2000a; Fischer et al., 2007; Gao et al., 2019; Jin et al., 2019; Johnston et al., 2019; Lv et al., 2012; Mendler et al., 2012; Müller et al., 2000; Nakagawa et al., 2015; Pilon-Thomas et al., 2016; Pötzl et al., 2017; Raychaudhuri et al., 2019; Riemann et al., 2016b; Seth et al., 2017; Vishvakarma and Singh, 2011; Xie et al., 2016; Y.-X. Zhang et al., 2019) although, interestingly, Riemann and colleagues (2017) observed a temporary increase in Walker-256 breast carcinoma cell TNF-α expression upon short-term acidosis (Riemann et al., 2017). Besides that, two studies showed increased TNF levels in Thyroid Cancer conditioned medium-induced macrophages (Arts et al., 2016), and TNF-α release in response to pH7.0 in peritoneal macrophages (Bellocq et al., 1998). Importantly, slightly more acidic extracellular conditions, on the other hand, were correlated with reduced TNF-α secretion accompanied by increased cytosolic TNF-α concentrations in macrophages (T. A. Heming et al., 2001a), despite that, TNF-α mRNA levels remained unchanged in

monocytes (Dietl et al., 2010; T. A. Heming et al., 2001b) suggesting acidity-driven TNF intracellular retention.

Another cytokine showing contradictory findings was IL-10, for which four studies observed its increase correlated with lactate or acidity. Contrary to that, five studies IL-10 decrease in monocyte-derived DCs (mo-DCs) (Erra Díaz et al., 2020)), human PBMCs (B. Fischer et al., 2000a; Müller et al., 2000; Ratter et al., 2018), LAK cells (B. Fischer et al., 2000a), macrophages, and monocytes (Arts et al., 2016) upon incubation with lactate or HCl medium acidification. Again suggesting that cytokine production and release can be strongly affected even by slight changes in experimental conditions. Altogether, the above results demonstrate that highly dynamic interactions are established between tumor metabolic alterations - TME molecular characteristics - and cellular responses.

Besides direct immune cellular effects and alterations regarding cytokines, important findings were observed from the assessment of cell surface molecules. Although more homogeneous, this chapter of the present review also reveals the complex molecular changes driven by tumor-generated lactate and low pH. For example, Steinkühler and colleagues (2018), when evaluating the macrophage checkpoint CD47, a “marker of self” molecule that interacts with SIRPα, leading to a protecting signal for 'self' cells, preventing them to be phagocytized, observed an increased expression of CD47 in tumor cells. Interestingly, despite its increased levels at pH 6.0, the interaction between CD47 and SIRPα present on macrophages, seems to be jeopardized since increased phagocytosis of A549 cancer cells by THP1 monocytes was seen (Steinkühler et al., 2018). A possible explanation for such a finding could be the weak interaction between CD47 and SIRPα under acidosis (pH6.0). The in vitro condition proposed by the authors was a very acidic condition of pH 6.0, it would be very important to check if a wider range of acidic pH values, closer to physiologic conditions would also inhibit the ligation of CD47 e SIRPα. This finding, although contradictory, is in line with recent results attesting that, in colorectal cancer, high CD47 expression was correlated with poor prognosis, whereas increased SIRPα on macrophages was correlated with favorable outcomes (Sugimura-Nagata et al., 2021) revealing that the interaction between CD47 and SIRPα is paradoxical for different types

of tumors and remains as an open issue yet to be explored (Arrieta et al., 2020; Barrera et al., 2017; Giatromanolaki et al., 2022; Kazama et al., 2020; Xu et al., 2020; Yanagida et al., 2020; Yang et al., 2019).

Due to cancer cells' ability of hijacking immune checkpoint pathways, such as PD-1/PD-L1, disrupting cytotoxic T Cell responses, approaches aiming CD47/SIRPα axis are currently gaining strength as promising targets in immunotherapy protocols - alone or in combination with PD-1/PD-L1 blockade - in clinical and preclinical trials (Giatromanolaki et al., 2022; Sikic et al., 2019; Voets et al., 2019). Importantly, the present review demonstrates that the most studied immune checkpoint - PD-1/PD-L1 - is under the influence of tumor-generated acidity and lactate abundance. Lactate was able to increase the expression of PD-L1 in oropharyngeal squamous cell carcinoma cell lines (Angelin et al., 2017), melanoma and lung cancer cells (Trabold et al., 2003; Yang et al., 2013; D. Zhang et al., 2019). Besides that, lactate acidified medium (pH 6.6) drove CD8+ T cells to increase their PD-1 expression entering a state of T cell dysfunction (Berod et al., 2014).

Considering this remarkable background, we understand that strategies focusing on TME lactate abundance and acidosis could be possible therapeutic game changers, especially when facing the fact that, despite its encouraging results, immunotherapy works not in all but in a set of patients. Tumor-generated acidosis drastically impairs immune cell functions, and several *in vitro* and *in vivo* studies in recent years have highlighted that buffering extracellular acidity not only improves antitumor immune responses but also CTLA-4 and PD-1 checkpoint blockers responses (Balgi et al., 2011; Berod et al., 2014; Calcinotto et al., 2012; Certo et al., 2021; Colegio et al., 2014; Day et al., 2012; Gottfried et al., 2006; Guak et al., 2018; Kang et al., 2013; Liang et al., 2017; Magistretti, 2011; Tang et al., 2015; Tian et al., 2022; Voena and Chiarle, 2016; Xie et al., 2016; Yang et al., 2013; Zarrouk et al., 2014). We emphasize that pH control strategies may broaden the range of patients who benefit from immunotherapeutic agents, then the development of clinical trials assessing the adjuvant/neoadjuvant buffering agents is an open field that could contribute to improved cure scores.

5. CONCLUSION

Extracellular acidity and lactate, both features of most solid tumor microenvironments, compromise various immunocytes functionalities. Multiple evidences demonstrate that decreased immune cell concentration, proliferation, and viability, along with higher cell death were observed under acidosis/lactate. Additionally, several cytokines had their parameters altered along with tumor microenvironment conditions pointing to the existence of flexible interplays between cellular immune responses and tumor-derived metabolites. Cell surface receptors and ligands also display sophisticated changes when exposed to lactate and acidosis determining immune response fate in a pro or antitumor manner.

In this sense, strategies such as targeting lactate transporters, inactivating lactate dehydrogenase A (LDHA) - responsible for converting pyruvate into lactate - approaching pH regulation through antibodies and molecules aiming monocarboxylate transporters (MCTs), Na+/H+ and Na+/HCO3 proton pumps, and carbonic anhydrases and therefore managing critical metabolic features, may represent an extraordinary opportunity to boost cancer immunotherapy outcomes by improving patient antineoplastic immunity. Despite all that, further knowledge regarding how adjuvant strategies interact and contribute to contemporary immunotherapies is still frankly unexplored. The development in this area can disclose new insights into cancer treatment possibilities in the foreseeable future. Foremost we conclude that throwing light on cancer metabolic particularities is certainly a worthwhile strategy for future research to provide new adjuvant drugs in order to improve anticancer immune activity.

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Credit authorship contribution statement

Conceptualization: BBP, NKL, PVR, FV; Data curation: BBP, NKL, JSN, LVR, FV; Formal analysis: BBP, NKL, JSN, LVR, FV; Funding acquisition: FV; Investigation: BBP,

NKL, JSN, LVR, PVR, FV; Methodology: BBP, NKL, JSN, LVR; Project administration: FV; Resources: FV; Software: not applicable; Supervision: FV; Validation: BBP and NKL; Visualization: BBP, NKL, JSN, LVR; Writing - original draft: BBP, NKL, JSN, LVR, PVR, FV; Writing - review & editing: BBP, NKL, JSN, LVR, PVR, FV.

Declaration of Competing Interest

All authors declare no conflict of interest.

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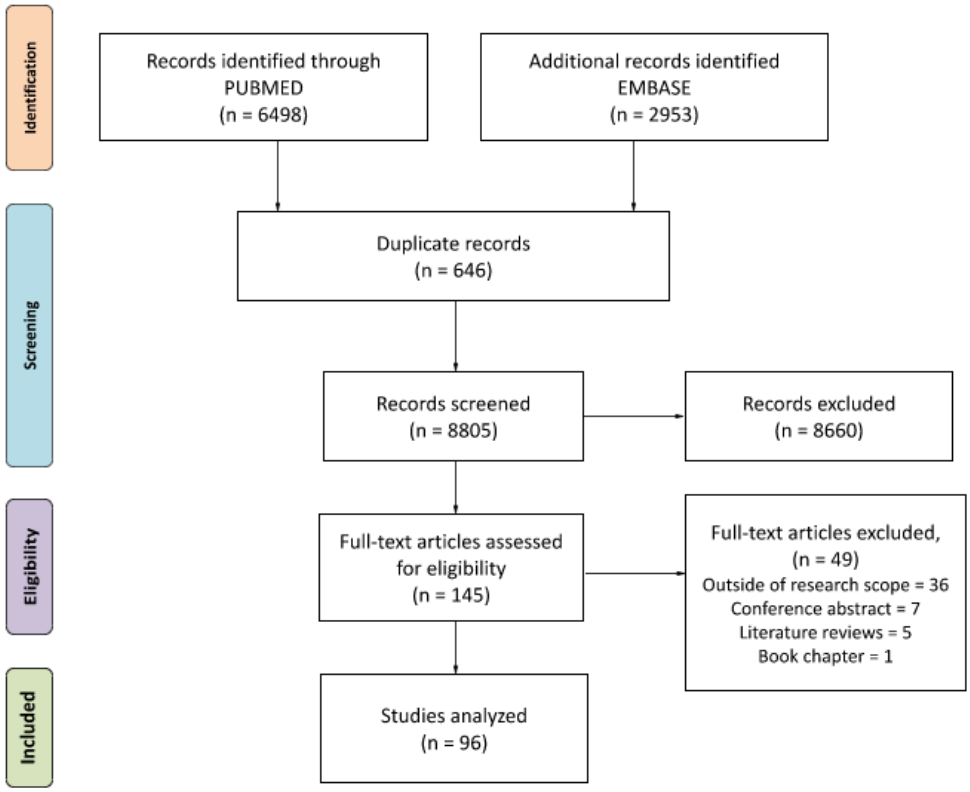
Figure 1. Screening process flowchart



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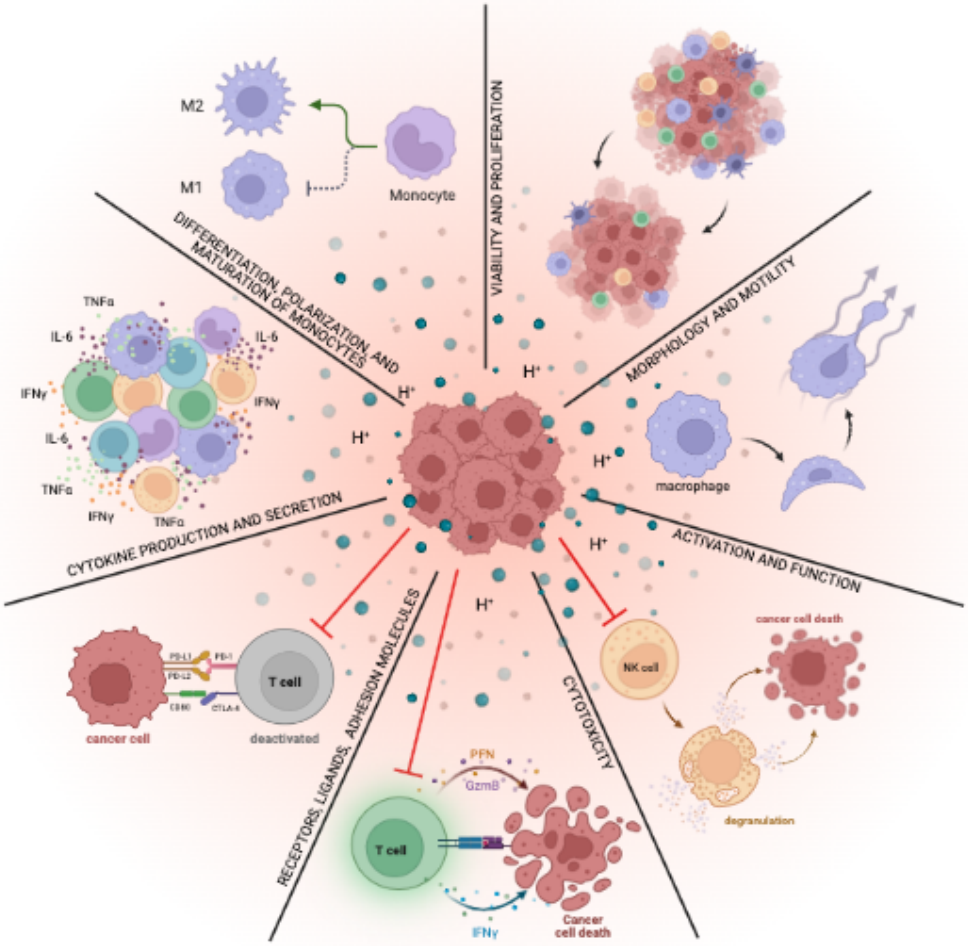
M2
M1
Monocyte
DIFFERENTIATION, POLARIZATION, AND MATURATION OF MONOCYTES
VIABILITY AND PROLIFERATION
MORPHOLOGY AND MOTILITY
TNFα
IL-6
IFNγ
macrophage
CYTOKINE PRODUCTION AND SECRETION
ACTIVATION AND FUNCTION
H+
T cell
cancer cell
deactivated
RECEPTORS, LIGANDS, ADHESION MOLECULES
CYTOTOXICITY
NK cell
cancer cell death
degranulation
PFN
GzmB
Cancer cell death

Study / year	Study design	Type(s) of cancer(s): site and histologic type - (for cell lines identify the name)	Acidification method or acidosis analysis method	Immune cell (s) studied	Immune receptor/marker (s) on cell or MHC	Immune cytokine (s) produced by...	Other mechanism
Balza et al. 2017	*in vitro/ in vivo/ ex vivo*	human melanoma and sarcoma tissue samples. murine sarcoma cell line - MCA. murine melanoma cell line - B16-F10. human melanoma cell line - MePa	LysoSensor dye in human samples, colorimetric L-Lactate assay kit, pHe was measured at 25C using a Jenco 6230N pHmeter, pHi was estimated as the ratio of the fluorescence signal obtained at 490 nm (pH sensitive)	macrophages, Murine bone marrow derived macrophages (BMDM)	F4/80+, CD206+ M2, CD86+ M1	N.A.	v-ATPase
Abebayehu et al. 2019	*in vitro/ in vivo/ ex vivo*	N.A.	L-(+)-lactic acid (5mM-12.5 mM) and Sodium L-lactate (12.5 mM). pH was measured for media alone, lactic acid conditioned media, and lactate-conditioned media using a Beckman Phi 45 pH meter	Mouse peritoneal mast cells (PMC) from C57BL/6J mice. Skin mast cells (SkMC) from 5 human donors.	CD63 and CD107a (degranulation markers)	TNF, IL6, MCP1	MCT1
Abebayehu et al. 2016	*in vitro/ in vivo*	N.A.	Between 6 and 12.5 mM lactic acid (media pH decreased to 6.5 immediately after adding LA, and returned to 7.2 within 6–8 h), sodium lactate	mouse bone marrow–derived mast cells and primary human mast cells	N.A.	IL-33, IL-6, TNF, MCP-1, MIP-1a, and IL-13	TGFb–activated kinase-1, JNK, ERK, NF-kB, p38, MCT
Abumanhal-Masarweh et al. 2019	*in vitro/ in vivo/ ex vivo*	breast cancer cell line - 4T1 (triple negative)	the extracellular in vivo pH is measured by microelectrode using pH meter (cybersacn pH11, Thermo Scientific).	CD45+ immune cells, CD19+ (B cells), CD3+ (T-cells), CDF/48+ (macrophages)	CD45-FITC, F4/80-BV510, CD19-BV421, CD3-APC and CD-31- BV421	N.A.	N.A.
Angelin et al. 2017	*in vitro/ in vivo*	N.A.	in vitro - L-lactate (LGHL, 20 mg 3 dL1 [12C6]-D-glucose, 20 mM Na [13C3]-L-lactate) or 40mM Na L-lactate and in vivo - 150mM Na L-lactate	T cells (Treg, Tconv, Th17)	Foxp3 on Tregs	N.A.	NAD:NADH ratio, Myc gene expression, LDH
Arts et al. 2016	*in vitro/ in silico/ ex vivo*	nonmedullary thyroid cancer cell lines - TPC1; FTC133; BC-PAP	1 mM of lactate, thyroid cancer conditiones medium (Lactate Fluorometric Assay Kit), cellular acidification rate (ECAR)	TC-induced macrophages	CD68	TNF, IL-6, IL-8, IL-10, IL-1b, granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP1)	AKT1/mTOR, lactate receptor GRP81
Bellocq et al. 1998	*in vitro*	N.A.	MEM supplemented with 1% FCS and	peritoneal macrophages	N.A.	iNOS and TNF-alpha produced	Activation of NFKB pathway

			adjusted by addition of HCl to pH ranging between 7.4 and 6.8.			by macrophages	
Beloueche-Babari et al. 2020	*in vivo/ in vitro*	B cell lymphoma cell line - Raji	tumor intracellular lactate build-up measured by in vitro high-resolution 1 H NMR analyses	proportion of monocyte-derived and conventional dendritic cells (DCs), natural killer (NK) cells, monocytes, macrophages and neutrophils	PDL1 in NK cells and DC, CD80 in DCs	N.A.	MCT1
Bidani et al. 1998	*in vitro*	N.A.	RPMI 1640 medium with NaHCO3 (0.1–25 mM depending on the final desired pH, see below), 25 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin. The pH of the incubation medium was titrated to 5.0–7.4 (37°C, 5% CO2) with 5 N HCl.	alveolar macrophages	N.A.	TNF-alpha	N.A.
Bohn et al. 2018	*in vitro/ in vivo*	melanoma cell line - B16. colon adenocarcinoma cell line - MC38	Extracellular acidification rate, Culture of BMDMs for 8 h in the presence of concentrations organic acid lactic acid or the inorganic acid HCl that result in a pHe characteristic of melanomas (pH 6.1)	TAMS, bone marrow–derived macrophages (BMDMs)	TAMs - F4/80 protein, CD11b and major histocompatibility complex class II		ICER, LAMP2, Gpr65, Gpr132, Gpr68, Gpr4 and Gpbar1. M2 markers - Arg1, Clec10a, Vegfa and Hif1a. M1 markers - iNOS, TNF
Brown et al. 2020	*in vitro/ in vivo*	mouse mammary cancer cell line - AT-3	L-lactate 2mM	dendritc cells (derived from femurs/tibias bone marrow of WT and Gpr81−/− mice) - in vitro generated DCs - Culture of murine bone marrow precursor cells with GM-CSF and IL-4 produces mature DCs referred to as “GM-DCs”. In vitro converted FoxP3+ CD4+ Treg cells. Tcells (CD4+ naïve T cells were isolated from spleen and mesen- teric lymph nodes of OT-II-Tg)	MHCII, TCR, CD80, CD86, CD40	cAMP, IL-6, IL-10, IL-12, TNF-alpha	GPR81
Calcinotto et al. 2012	*in vitro/ in vivo*	murine melanoma cell line - B16	TILs were cultured at pH ranging from 7.4 to 6.5 for 3 days	CD8+ T lymphocytes.	IL-2Ra (CD25), surface expression	IL-2, IFNg, TNF-a produced by	STAT5 and and

					of human leukocyte antigen (HLA) class I T cell receptor (TCR)	T-cells	extracellular signal–regulated kinase (ERK) pathways
Cao et al. 2015	*in vitro*	N.A.	RPMI 1640 Medium was added with 10% FBS, and then adjusted separately to pH 6.0, 6.5, 7.0, 7.4 by the addition of 70% HCl. The pH value was detected using pH meter.	Human neutrophils	N.A.	N.A.	ROS production by neutrophils. PI3K/ AKT pathway
Carmona-Fontaine et al. 2017	*in vitro*	N.A.	10, 20, 30, 40 mM lactate	macrophages	ARG1, MRC1	N.A.	VEGFA
Carmona-Fontaine et al. 2013	*in vitro*	aggressive metastatic breast adenocarcinoma cell line - MTLn3. glioma cell line - C6-HRE-GFP that expresses GFP under hypoxic conditions	Adding lactic acid to the growth media	primary bone marrow-derived macrophages (BMDMs)	N.A.	N.A.	N.A.
Caronni et al. 2018	*in vitro/ in vivo/ ex vivo*	lewis lung carcinoma cell lines - 3LL; LL2; LLC/1. lung adenocarcinoma cell line - LG1233	The concentration of lactic acid in the obtained TCM was measured by using the Lactate Colorimetric Assay Kit II (BioVision). 10 mmol/L of lactic acid, 5mmol/L lactate	Mouse FL-DCs from bone marrow of C57BL/6, Lung tumor DC, OT-I T cells	MHCI, MHCII	IL12alpha, IL12beta, IFNgamma, IFNbeta, IL10	N.A.
Chafe et al. 2019	*in vitro/ in vivo*	murine skin melanoma cell line - B16F10	Extracellular pH - Accumet pH meter and Accumet pH electrode for B16F10 supernatant. Intracellular pH - Fluorometric Intracellular pH Assay Kit.	T cells	N.A.	N.A.	CAIX
Chefalo et al. 2001	*in vitro*	N.A.	Cell medium was replaced with citrate-buffered saline at varying pH levels for 20 min at room temperature and subsequently washed three times in standard medium.	C57BL/6 isolated macrophages	MHC-I processing peptide ability	N.A.	(MHC-I) pathway involved in processing of protein Ags expressed within the cell (endogenous Ags)
Chen et al. 2019	*in vitro/ in vivo/ ex vivo*	breast cancer cell lines - MDA-MB-231; MDA-MB-468; BT-474; MCF-7	lactate production was performed using kits from Biovision, XF24 Extracellular Flux Analyzer was used to determine the ECAR	Peripheral blood monocytes from patients with breast cancer or from healthy donors. TAMs were isolated from fresh breast cancer	CD68+ TAMs	N.A.	TAM extracellular vesicles (EVs),

				samples (pri-TAMs) or polarized TAMs by treating human monocyte-derived macrophages (MDMs) with the conditioned medium of MDA-MB-231 cells (pol-TAMs).			
Chen et al. 2016	*in vitro/ in vivo*	breast cancer cell lines - EO771; 4T1.2; PY8119; PY230; SCP-4; SCP-6; SCP-28; MDA-1883; MDA-2287. melanoma cell line - B16F10	Lactate levels in the CM of distinct cancer cell lines measured using a Vitros 250 chemistry analyzer, 100 µL of 2× lactate or HCl	RAW264.7 macrophage cell line and bone marrow-derived macrophages (BMDMs)	CD206 (M2 macrophage marker)	N.A.	Gpr132, arginase 1 (Arg-1)
Comito et al. 2019	*in vitro*	prostate cancer cell line - PC3. human cancer associated fibroblasts isolated from surgical explants - CAFs	Lactate	PBMC, CD4+ T helper cells, Th1 effector cells, CD25+/FOXP3+ Treg cells, CD4+/IL17+ Th17 cells, CD4+/IL4+ Th2 cells	N.A.	IFN-gamma, TGFβ, IL-17, IL-4, IL-1β, IL-12, IL-6, CXCL10	MCT1, SIRT1-mediated T-bet deacetylation, NF-kB
Daneshmandi et al. 2019	*in vitro/ in vivo*	murine melanoma cell lines - B16-F10; B16-shLDH	L-lactate (1 mmol/L and 10 mmol/L). Lactate release from the cultured cells was determined by Lactate Colorimetric Assay Kit II.	CD8+ T cells from spleen and lymph nodes of C57BL/6 mice	PD-L1	IFN-gamma, granzyme, perforine from CD8+ T cells	N.A.
Dietl et al. 2010	*in vitro*	melanoma cell line - MelIm	Monocytes were incubated in the presence of 2, 5, 10, and 20 mM L-LA or sodium L-lactate (NaL). Control samples were cultured with LPS with or without 1% hydrochloric acid (HCl) added to titrate the pH of the medium to ~7.1 and ~6.6, corresponding to the pH of media containing 10 and 20 mM LA, respectively.	human LPS-stimulated monocytes	N.A.	TNF, IL-6 produced by monocytes	ROS production by monocytes
Dıaz et al. 2020	*in vitro*	N.A.	Isotonic HCl - pH 6.5 remained at pH 6.5-6.8 along the culture	human monocytes, peripheral blood mononuclear cells (PBMCs), DCs differentiated from monocytes, macrophages differentiated from monocytes	HLA-DR, CD86, CD1a+CD16, aryl hydrocarbon receptor (AHR), CD1a+ CD14, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN),	IFN-g from allogeneic Tcells. IL-1b and IL-10 from mo-DCs	mTORC1

					and CD11c		
El-Kenawi et al. 2019	*in vitro/ in silico/ in vivo*	murine prostate cancer cell lines - TRAMP-C2; TRAMP-C3	Supplementation of media with the zwitterionic organic buffers PIPES and HEPES (25mM each) and adjustment of the pH to either 7.4 or 6.8. Intratumoral pH measured in vivo by a microelectrode.	macrophages (BMDM from mice)	N.A.	Il-6, OPN, Serpin E1, CCL2, GFBP–6, MMP–2, CCL12, WISP–1, OSF–2, Proliferin, Chemerin, CCL17, Pentraxin 3, CXCL16, Coagulation factor III, CCL22, VEGF, Cystatin C, CD40, CD14, IL–1a, IL–3, M–CSF, IL–1ra, LIX, Osteoprotegerin, CCL5, MMP	pro-inflammatory markers Nos2, Ccl2, iNOS protein / anti-inflammatory markers Cd206, Arg1 and Reltna
Feng et al. 2017	*in vitro*	human lung adenocarcinoma cell line - A549. human large-cell lung cancer cell line - NCI-460. human lung adenocarcinoma - H1299	10, 20 mM lactate	Jurkat cell line	N.A.	IFNgamma	GP81, TAZ, PD-L1
Feng et al. 2014	*in vitro*	N.A.	RPMI-1640 complete media containing 2 % FBS and pH was adjusted to 6.6, 6.8 and 7.2 with 5.5 % NaHCO3 and 1 N HCl	Monocytes/macrophages: THP-1	N.A.	VEGF and VEGF-C produced by macrophages	N.A.
Fischer et al. 2007	*in vitro*	serum lactic acid levels measurement: lymphoid malignancies - 58 patients. myeloid malignancies - 20 patients. breast cancer: 19 patients. gastrointestinal cancer - 15 patients. urogenital cancer - 15 patients. sarcoma: 4 patients. lung cancer: 3 patients melanoma: 2	Lactic acid levels were measured in the serum of patients with malignant disease. CTLs were incubated for the indicated time periods with lactic acid, HCl, sodium lactate . The administration of 20 mM lactic acid led to a pH decrease from pH 7.4 to approximately pH 6.5 to 6.8.	CD8 T cells (CTLs)	N.A.	IL-2 and IFN- produced by CTLs	N.A.

		patients. other types of cancer: 4 patients					
Fischer et al. 2000	*in vitro*	target cells: erythroleukemia cell line - K562. B lymphoblastoid cell lines - Daudi; Raji. histiocytic lymphoma cell line - U937.	RPMI culture media were adjusted to pH 5.8, 6.3, 6.8, or7.2 by addition of aqueous HCl or NaOH solution (1 N) . For media with pH below 7.2, RPMI 1640 without bicarbonate was used	effector cells: PBMC and LAK cells	N.A.	TNF-alpha, IFN-gama, IL-2,IL-7, IL-12, IL-10 and TGF-b produced by effector cells	N.A.
Fischer et al. 2000	*in vitro*	erythroleukemia cell line - K562. B lymphoblastoid cell lines - Daudi; Raji. cervix carcinoma cell line - HeLa. hepatocellular carcinoma cell line - HepG2. breast adenocarcinoma cell line - MCF-7. colon adenocarcinoma cell line - LS174T. carcinoembryonic antigen (CEA) nonproducing subline - LS174Te. human bladder epithelial carcinoma - RT112	Aliquots of standard culture medium were adjusted to pH 5.3, 5.8, 6.3, 6.8, or 7.2 by adding aqueous hydrogen chloride or sodium hydroxide solution (1 N).	Unstimulated peripheral blood mononuclear cells, lymphokine-activated killer (LAK) cells, and natural killer cell clones	N.A.	N.A.	N.A.
Gao et al. 2019	*in vitro/ in silico/ in vivo*	murine melanoma cell line - B16F10. human cervical carcinoma - Hela, - lewis lung carcinoma cell line - LLC	Lactic Acid Assay Kit	Raw 267.4 macrophages, T cells (CD8+GranzymeB+ and CD8+IFNγ+), macrophages (CD11b, F4/80, CD206)	T cells (CD8+GranzymeB+ and CD8+IFNγ+), macrophages (CD11b, F4/80, CD206)	IFNγ and IL6 as immune activation markers of Tcells	NFkB (induces the transcription of proinflammatory cytokines) and NFAT (is required for T cell activation)
Gottfried et al. 2006	*in vitro*	urothelial carcinoma cell lines - J82; 28 UMUC3. melanoma cell lines - MelIm; Mel108. prostate carcinoma cell line - PC3	In order to evaluate the impact of lactic acid on the dendritic cell differentiation, L-lactic acid was added to the cultures at various concentrations. Retitration of the pH to physiologic 7.4 in the culture medium was performed with NaOH	Monocytes were obtained by leukapheresis of healthy donors were isolated and then differentiated in Dendritic cells or Langerhans cells	N.A.	N.A.	N.A.
Guan et al. 2018	*in vitro/ in vivo*	glioma cell lines - GL26-cit; SB28-GFP	NHE1 inhibition by HOE642 to impair extracellular acidification pH of tumors was not measured	CD11b+/CD45low-med = microglia CD11b+CD45hi cell population = infiltrating myeloid cells CD4+CD25+FoxP3+ =	PD-1, CTLA-4	N.A.	N.A.

				regulatory T (Treg) CD4+IFNγ+ = T helper type 1 (Th1) CD4+ Lymphocytes CD8+ Lymphocytes			
Gupta et al. 2016	*in vitro*	N.A.	20mM Sodium D-Lactate	Human monocytic THP1 cells	MHCI	N.A.	PRMT1 and HIF-1
Harhaji et al. 2006	*in vitro*	astrocytoma cell line - C6L929. fibrosarcoma cells	The pH of the control culture medium was adjusted for the experiments by addition of 1 M HCl (pH <7.2) or NaOH (pH>7.2). The use of lactic acid instead of HCl for the acidification of cell culture medium was specifically indicated.	macrophages	N.A.	Nitric oxide produced by tumor cells	N.A.
Harmon et al. 2018	*in vitro/ in vivo*	colorectal liver metastasis	Lactate concentrations were analyzed using a colorimetric enzymatic assay. Intracellular pH was measured using the pH sensitive dye pHrodo. Acidification with sodium lactate and lactic acid at 10 and 20 mmol/L.	NK cells from hepatic mononuclear cells from donor liver perfusate, K562 NK cell line, SW620 colon adenocarcinoma	N.A.	N.A.	N.A.
He et al. 2011	*in vitro*	N.A.	The pH of the medium was adjusted by HCl or NaOH.	Macrophages wild-type and macrophages TDAG8 deficient.	N.A.	TNF-a	N.A.
Heming et al. 2001	*in vitro*	N.A.	The pH of the incubation medium (RPMI 1640 containing NaHCO3 and 25mM Hepes) was titrated with HCl to 5.5, 6.5 or 7.4 at 37 °C	activated macrophages by lipopolysaccharide (LPS) were obtained by bronchoalveolar lavage of rabbits	N.A.	TNF-a	N.A.
Heming et al. 2001	*in vitro*	N.A.	Acidified cell culture medium RPMI 1640 (pH titrated to 5.5, 6.5 or 7.4 with HCl) containing 25 mM Hepes, NaHCO3 (concentration dictated by CO2/HCO3−/H+ equilibrium at desired pH in the presence of 5% CO2), 250 units/ml penicillin, 50 lg/ml gentamicin and 10 lg/ml LPS.	macrophage-like cell lines RAW 246.7 and J774 A.1	N.A.	TNF-a	N.A.
Hirata et al. 2008	*in vitro*	N.A.	Cells were transferred to RPMI-1640 supplemented with 10% FBS containing 10mM PIPES for pH 6.3 or 10mM HEPES for pH 7.4 and 8.0. Cells were grown without CO2 gas supply. The culture medium pH was adjusted by addition of NaOH.	Jurkat T cell	N.A.	N.A.	activation of TCR signaling by p38 MAPK pathway

Hutcheson et al. 2016	*in vitro/ ex vivo*	pancreatic cancer cell lines - BXPC3; Capan-2; Hs766t; MIA PaCa-2; Panc-1; PL5; PL45	8mM Lactic acid, Lactate readings acquired on electrochemical analyzer	macrophages (THP-1 human monocytic cell line)	M1 marker: CCR7 and M2 marker: CD206	N.A.	MCT4
Jiayun Yu et al. 2019	*in vitro/ in vivo*	T cell lymphoma cell line - E.G7. murine breast cancer cell line - 4T1	lactic acid at 0 mmol/L, 5 mmol/L, 10 mmol/L, 20 mmol/L, and 30 mmol/L for 24 hours	Bone marrow-derived dendritic cells (DCs), CD4+ T cells, CD8+ T cells, NK cells, OVA-specific CTL	B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules on the surface of DCs; CD11c and PHK67; CD11b and CD11c levels - agregation of DCs, CD11b+Gr1+ cells (myeloid suppressor cells), Treg cells	INF–γ from lymphocytes	N.A.
Jin et al. 2019	*in vitro/ in vivo*	colorectal adenocarcinoma cell line - MC39	L-lactic acid (20mM) to reach pH 6.6, in vivo tumoral pH measurement with pH electrode	CD8+ T cells, monocytes and dendritic cells from peripheral blood mononuclear cells from healthy donors, EL4 Tcell line; JE6.1 Jurkat cell line	TIGIT receptor (T-cell immunoreceptor with Ig and ITIM domains), PD-1, MHC-II, Lag-3, Tim3, CD226, CD69, CD80, CD86, HLA-DR, CD155 on melanoma cells	IFN-γ secreted by T cells, TNF, Granzyme B on T cells	N.A.
Jing et al. 2016	*in vitro*	head and neck squamous cell carcinoma cell lines - FaDu; Tca8113	extracelullar acidosis pH 5.9, 6.5	N.A.	N.A.	IL6, IL8, and VEGFA secreted by tumor cells	GPR4
Johnston et al. 2019	*in vitro/ in vivo*	colorectal adenocarcinoma cell line - MC38	2-(N-morpholino)ethanesulfonic acid (MES)	lymphoblasts (Raji cells); T cells (Jurkat); NK cells; B cells	VISTA on leukocytes; PD-1, LAG-3 and TIM-3 on Tcells;	IFN-γ by CD4+ T cells	NFkB
Kim et al. 2019	*in vitro/ in silico*	NSCLC cell lines - A549; H460	Glycolysis was evaluated using lactate production, hexokinase activity, and ECAR assays	effector Tcells (Jurkat)	PD-L1 on non-small-cell lung cancer	IFN-γ secreted by T cells	N.A.
Kim et al. 2016	*in vitro*	N.A.	titrating buffer with 3.7% HCl - pH 3.3, pH 5.7 (STAP condition) and pH 7.1 (Ctrl)	Jurkat T lymphocyte cell line, SUP-T1 and A301	N.A.	N.A.	N.A.
Klyukin et al. 2020	*in silico/ in vitro*	N.A.	representative pH levels corresponding to circumneutral physiological conditions of blood	N.A.	PD-1/PD-L1	N.A.	N.A.

			(pH 7.4) and acidic tumor microenvironment (pH 5.5) are considered				
Kuchuk et al. 2018	*in vitro/ ex vivo*	tumor and adjacent non-tumor snap-frozen tissues from patients with hepatocellular carcinoma undergoing curative resection (n=57). hepatocellular carcinoma cell lines - C3A; PLC/PRF/5; SNU-449. breast cancer cell line - T-47D	Omeprazole (a selective inhibitor of proton pumps that is broadly used to block acid secretion by cells through inhibition of the HC/KC ATPase system) was used to modulate V-ATPase	M2 macrophages (CD163+ and CD209 +) CD3+ CD11b+ CD163+ myeloid cells	N.A.	CCL22, IFNG, TNF	N.A.
Kumar et al. 2013	*in vivo*	T cell lymphoma of spontaneous origin - dalton lymphoma DL	Ascitic pH was regulated using DCA drug (Dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK) - it causes a shift of glycolysis to oxidative metabolism of glucose)	Tumor-associated macrophages (TAM)	N.A.	IL1, IL6 and TNFa produced by TAMs	N.A.
Liao et al. 2019	*in vitro*	fibrosarcoma cell line - HT-1080	1M of L-lactate (pHs - 7.2, 7.0, 6.7, 6.5)	mouse macrophage RAW 264.7 (BMDM/Raw 264.7 cells, TAMs from mice)	F4/80 (M0), iNOS (M1), CD206 (M2)	N.A.	N.A.
Loeffl et al. 1991	*in vitro*	murine lymphoma cell line - YAC-1	SRPMI medium was adjusted to physiologic PH (7.4), neutral PH (7.0), and acidic PH (6.7 or 6.4) by addition of 1 N HCl or 1 N NaOH	Natural Killer	N.A.	N.A.	N.A.
Long et al. 2018	*in vitro/ in vivo*	murine breast cancer cell line - 4T1	Treatment with lactate (0.625, 1.25, 2.5, 10 mmol/L). The pH value of the culture medium supernatant was measured by PP-15-P11 Sartorius pH-Meters, 4T1 cell supernatant was taken to measure with the Mouse Lactate ELISA Kit.	mice spleen NK cell	N.A.	perforin 1 (PRF1), LAMP-1 (CD107a) in NK cells	MCT4
Lv et al. 2012	*in vitro*	mouse lymphoma cell line - Yac-1	cells were exposed to low pH (5.6 or 6.5) or 7.2 as control for different times. The medium and cells were harvested at 4, 8, 12 and 16 hours after exposure to different cultural conditions.	NK cells from liver and spleen	N.A.	IFN-γ from liver and spleen NK cells	N.A.
Mahaweni et al. 2018	*in vitro*	multiple myeloma cell lines - OPM-2, UM-9 RPMI8226/s, L363, JJN-3.	50 mM sodium l-lactate	NK cells were isolated from fresh blood from healthy donors	N.A.	N.A.	Degranulation levels - CD107a

		myelogenous leukemia cell line - K562 (HLA class I deficient)					expression on NK cells
Mendler et al. 2011	*in vitro*	renal cell carcinoma cell lines - RCC-26; KT195	Tumor lactic acidosis milieu (L) was created by adding 20 mM lactic acid (Sigma) to M resulting in pH values of 6.3 6 0.07.	cytotoxic T effector cell clone, CTL-JB4	N.A.	IFNgamma, TNFa, and IL12 produced y CTLs cells	activation of MAPK pathway (p38, JNK, c-Jun), MEK1, ERK
Meng et al. 2019	*in vitro/ in vivo*	hepatocellular carcinoma cell lines - HCCLM3 (human); H22 (mouse); Hepa1–6 (mouse)	Lactate generation was measured using a lactate colorimetric assay kit	ascitic cells	N.A.	IFN-γ, IFN-β, CXCL10, TNF	IL-6/STAT3 pathway
Mu et al. 2018	*in vitro*	breast cancer cells - primary (p)HUVECs cells; MDA-MB-231; MCF7	lactate acid stimulation for 12 h	macrophages (THP1 - human monocytic cell line) and BMDM	N.A.	N.A.	N.A.
Müller et al. 2000	*in vitro*	erythroblastoid cell line - K562 (used as NK-sensitive target cells). B cell lymphoma cell lines - Daudi; Raji (used as NK-resistant target cell lines)	Culture media were adjusted by addition of 1 M HCl to pH 7.2, 7.0, 6.8 and 6.5, respectively	effector cells: PBMC and LAK cells. CD4+ T helper cells, CD19+ B lymphocytes and CD14+ monocytes	adhesion molecules CD2, CD18, CD28, CD44, CD80, CD54, CD56 and CD58	TNF-a, IFN-c, IL-7, IL-10, IL-12 and TGF-b1 produced by effector cells	N.A.
Na Liu et al. 2019	*in vitro/ in vivo*	lewis lung carcinoma cell line - LLC; murine melanoma cell line - B16-F10	lactate (2, 5, 20, 40, 50mM), lactate concentration - Lactate Assay Kit	macrophages (BMDM/Raw 264.7 cells, TAMs from mice)	Mrc1, Arg1, Fizz1 (protumoral macrophage–associated factors), CD11C–CD206+ (protumoral cells)	IL-1b, and IL-6	ATP6V0d2 - TFEB - HIF-2alpha
Nakagawa et al. 2015	*in vitro*	mouse thymoma cell line - EL4. mastocytoma cell line - P815	The lactic acidosis milieu was created by directly adding lactic acid solution to CTM (complete T-cell medium) and was adjusted to an objective pH immediately before usage.	CD8+cytotoxic T lymphocytes (CTLs)	N.A.	IFN-gamma produced by CTLs	N.A.
Ohashi et al. 2017	*in vitro/ ex vivo*	head and neck squamous cell carcinoma cell lines - HSC-2; HSC-4	0, 20, or 30 mM lactic acid and concentrations of lactate in the obtained supernatants of human tissue samples were measured using a Determiner LA Kit	macrophages	CD68, CD163, ARG1, CSF1R	N.A.	LDHA, GLUT1
Ohashi et al. 2013	*in vitro/ in vivo*	murine melanoma cell line - B16. ovalbumin-expressing EL4 thymoma cell line - EG7	addition of lactic acid at 15mM concentration or HCl at 10mM	macrophages, TCD8+ cells	N.A.	IFN-γ or mouse IL-17A	N.A.
Peter et al. 2015	*in vitro*	N.A.	RPMI-1640 cell medium in the presence of 10 and 20 mM L-lactic	Monocytes were obtained from healthy donors	N.A.	TNF, IL23, CCL2, CCL7	N.A.

			acid (LA) or 20 mM sodium Llactate (NaL). Furthermore, monocytes were incubated with LPS in combination with 1% hydrochloric acid (HCl) to titrate the pH of the medium to ~7.1 or ~6.6, corresponding to the pH of media containing 10 or 20 mM LA, respectively.			produced by monocytes stimulated by LPS	
Pilon-Thomas et al. 2016	*in vitro/ in vivo*	murine melanoma cell lines - B16; Yumm 1.1. pancreatic cancer cell line - Panc02	acidification of cell medium not specified	CD8+ T cells	N.A.	IFN-gamma produced by T cells	immunotherapy with anti-CTL4 and anti-PD1
Ping et al. 2018	*in vitro/ ex vivo*	human gastric cancer cells	Lactate (5mM), lactate sample levels by metabolomics determination from UPLC Ultimate 3000 system	Jurkat cell line, TILs	CD4, CD8	IFN-γ, GZMB	LDHB
Pötzl et al. 2017	*in vitro/ in vivo*	B-cell lymphoma	pH values and lactate concentrations were determined in tumor supernatants. Acidosis was also found when the tissue pH in the spleens of mice that developed tumors was measured using a pH microsensor. For increasing the tissue pH in vivo, λ-myc mice received drinking water supplemented with 200mM sodium bicarbonate as of day 40 after birth	Natural Killer cells	N.A.	IFN-γ produced and released by NK cells	N.A.
Ratner et al. 1992	*in vitro*	murine mammary adenocarcinoma cell line - 410.4. renal adenocarcinoma murine cell line - Renca	The ECM gels were equilibrated in 10% CO2 at 37°C for 24 hr with at least six changes of DMEM adjusted to either pH 7.1 or 6.7.	lymphocytes	CD3	N.A.	N.A.
Ratner et al. 1985	*in vitro*	mammary adenocarcinoma of a strain BALB/cfC3H mouse derived subpopulations - 68H; 168; 410	In experiments involving variation of pH, phosphate-buffered RPMI 1630 (GIBCO) was substituted for bicarbonate-buffered RPMI 1640, NaHC3 was omitted, and pH was adjusted with 0.2 M dibasic sodium phosphate to pH 6.7	Lymphocytes were obtained by teasing the inguinal, brachial. cervical, and mesenteric lymph node	N.A.	N.A.	N.A.
Ratter et al. 2018	*in vitro/ in vivo/ ex vivo*	N.A.	3.5 or 15 mM Na-L-lactate, Lactate infusion in patients	PBMCs and CD14+ monocytes were isolated from fresh blood donated by healthy volunteers or type 1 diabetes patients	TLR4, TLR2 on PBMCs	(IL)-1β, IL-6, TNF-α, and IL-10 from PBMCs	N.A.

Raychaudhuri et al. 2019	*in vitro/ in vivo*	murine breast cancer cell line - 4T1	potassium lactate (K+-Lac) solution was added directly to the pDC cultures, glycolysis stress test was done to measure the Extracellular Acidification Rate, 0.5g/kg i.p. lactate injection in mice	Plasmacytoid dendritic cells from healthy donors, naïve CD4+ T cells isolated from PBMCs by magnetic immunoselection, intratumoral pDCs as well as pDCs from peripheral blood from patients with breast cancer	CD25, CD45, CD3, CD19, BDCA4, CD123, FoxP3	IFNα induction by pDCs	intracellular Ca2+ mobilization, cell surface GPR81 receptor, MCT1
Renner et al. 2019	*in vitro/ in vivo*	melanoma cell line - M579. pancreatic cancer cell line - PANC-1. murine melanoma cell line - B16. murine breast cancer cell line - 4T1	pH changes in culture medium was determined non-invasively by the PreSens technology; Tumor pH by a micro fiber optic pH meter with needle-type housed pH microsensors (20/0.4) using a manual micromanipulator; Lactate 10mM and 15mM.	(Flu)-specific CD8+ T (FluT) cells derived from PBMCs from healthy donors; NK cells; T CD8+ cells	MHCI, MHCII	IFN-gamma, IL-2, TNF	MCT1 and MCT4
Riemann et al. 2017	*in vitro/ in vivo*	alveolar epithelium cell line - AT1. dunning prostate carcinoma - R-3327. rat breast carcinoma cell line - Walker-256	In vitro - bicarbonate morpholino ethane sulfonic acid–buffered Ringer solution with pH adjusted to 6.6. In vivo - to induce a pronounced extracellular acidosis in the solid growing tumors, animals were treated with a combination of inspiratory hypoxia and meta-iodobenzylguanidine (MIBG) in order to force anaerobic glycolysis in the tumor cells (the extracellular pH in these tumors decreased from 7.05 to 6.65).	N.A.	N.A.	MCP-1, IL-6, TNF-α, and iNOS tumor cells	N.A.
Riemann et al. 2016	*in vitro*	N.A.	Extracellular acidosis (pH 6.6) was applied using isoosmotic bicarbonate MES-buffered Ringer solution) 20 mM, for incubation periods longer than 6 h the pH 6.6 was achieved with 1 M HCl, (pHe) was checked with a blood gas analyzer	monocytic cell lines (Mono Mac 6, THP-1); macrophages (RAW264.7, PMA-differentiated THP-1); RAW264.7 murine macrophages, human monocytes were isolated from peripheral blood	N.A.	IL-1ß, IL-6, TNF-α, MCP-1, iNOS, COX-2 and osteopontin from monocytic cells	N.A.
Riemann et al. 2016	*in vitro*	prostate cancer cells - AT1 R-3327	RPMI medium was supplemented with MES- (pH 6.6) buffered Ringer solutions in room air supplemented with 5 % CO 2	N.A.	N.A.	MCP-1 (monocyte chemotactic protein 1), iNOS and osteopontin	N.A.

Seth et al. 2017	*In vitro/ in vivo*	lewis lung carcinoma cell line - LLC. melanoma cell line - B16-F10	1, and 10 mM lactate	Mø	PDL-1 on LLC cells, CD8, MCP-1	IFNg	LDH-A, VEGF
Severin et al. 1994	*in vitro*	target cells: human erythroleukemia cell line - K-562	RPMI 1640 supplemented with 2 mM L-glutamine, 10% human AB serum, 100 U/ml penicillin and 50 pg/ml streptomycin and 25 mW HEPES buffer (LM medium). LM containing 100 U/ml IL-2 was adjusted to pH 5.8, 6.3, 6.8 or 7.4 by addition of aquous HCl or NaOH solutions (1N).	lymphokine-activated killer cells (LAK cells)	N.A.	N.A.	N.A.
Shi et al. 2000	*in vitro*	human pancreatic adenocarcinoma cell line - COLO357. human colon adenocarcinoma cell line - SW620. human prostate adenocarcinoma cell line - PC3	Cells were treated with NaHCO3-deficient RPMI 1640 or MEM medium having different pH levels	N.A.	N.A.	IL8 produced by tumor cells	N.A.
Shi et al. 1999	*in vitro*	human pancreatic carcinoma cell lines - SG, FG, and L3.3 variants (originally established from COLO 357)	For acidosis treatment, tumor cells were incubated in media of different pH levels	N.A.	N.A.	IL-8	N.A.
Sloot et al. 2018	*in vitro/ ex vivo*	Thyroid carcinoma cell lines - TPC-1 (papillary, RET/ PTC rearrangement); FTC133 (follicular, PTEN-deficient)	Lactate fluorometric assay kit	human monocytes (PBMCs) from PHTS patients and healthy volunteers (controls)	N.A.	IL-6, IL-8, IL-10, TNF-alpha, IL-1beta, MCP-1, IL-1	MCT-1
Steinkühler et al. 2018	*in vitro*	human embryonic kidney cell line - HEK 293. adenocarcinomic human alveolar basal epithelial cell line - A549	Not clear - pH 6	macrophages (THP1 - human monocytic cell line)	SIRPα	N.A.	"marker of self" protein CD47
Stone et al. 2019	*in silico/ in vitro*	low-grade or high-grade cervical intraepithelial neoplasia and invasive carcinoma. cervical cancer cell lines - SiHa, CaSki, HeLa, SW756, C33A. human skin cell line - HaCat	YSI 2700 Select 3 (YSI Life Sciences, Yellow Springs, OH) analyzer to determine the lactate concentration in supernatants of tumor spheroids or plasma from patients	Mφs (Human primary monocytes), T lymphocytes from healthy donors	CD3, CD4, CD8, CD14 APC, CD16, CD25, CD45, CD64, CD69, CD86, CD206, HLA-DR	IL-1β, IL-10, IL-6	p65-NFκB and HIF-1 expression in M s
Verma et al. 2020	*in vitro*	oropharyngeal epithelial cell lines - MEER (transformed with HPV16	sodium lactate (10mM); lactic acid (10mM)	N.A.	PD-L1, PD-L2, CD80	N.A.	N.A.

		E6, E7, and HRASG12V); MOE E6E7 (transfected with HPV E6/E7 but lacking the HRASG12V mutation); MOE LXSN (transfected with the LXSN vector), human embryonic kidney cell line - HEK 293T (expressing SV40 large T antigen), human cervical cancer cell line - HeLa. head and neck squamous carcinoma cell lines - UM-SCC1 (negative for HPV-16 and for HLA-A2); UM-SCC47 (HPV-16+); UM-SCC84; UPCI:SCC90 (HPV+), breast cancer cell line - 4T1. melanoma cell line - B16-F10						
Vishvakarma et al. 2011	*in vivo*	murine T cell lymphoma	Proton pump inhibitor pantoprazole (PPZ) was administered in a dose of 0.4, 4.0 or 40 mg/kg body weight in 0.2 ml PBS at every 24, 48 or 72h interval till day 20 following tumor transplantation. A significant increase was observed in the level of pH in tumor ascitic fluid obtained from tumor-bearing mice administered with PPZ compared to the control. The level of lactate, however, showed a decline in the ascitic fluid of PPZ-administered group.	N.A.	CD62L	IL-4, IL-10, TGF-b and VEGF	N.A.		
Wagner et al. 2020	*in vitro/ in vivo*	melanoma cell lines - B16F0 or B16F10	HCl (pH 6.0), lactic acid (LA) and sodium lactate (NaLA) at 20mM concentration. Lactic acid concentration was measured in medium using L-Lactate Assay Kit	group 2 innate lymphoid cells (ILC2s)	CD3+, CD8+ cells, F4/80+ macrophages	IL-5	LDHA	
Walton et al. 2018	*in vitro/ in vivo*	primary T cell cultures were sourced from C57BL/6 mice (loxP-flanked Tsc2 alleles) and Cd4-Cre (Tsc2fl/fl Cd4-	Supplementation of media with the zwitterionic organic buffers PIPES and HEPES (25mM each) and adjustment of the pH. Extracellular pH was determined by measuring	T cells	N.A.	N.A.	mTORC1	

		Cre, resulting in TSC2 selectively deleted in T cells, “TSC2 −/−”) or without Cre (Tsc2fl/fl, “TSC2 +/+”) or with OVA-specific CD8+ T cells (OT-1). Splenocytes from mice with TSC2 −/− and TSC2 +/+ T cells.	the pH of a sample of culture media using the Mettler Toledo SevenGo pH meter SG2 with either the InLab micro probe or the InLab 413 SG/2 m probe with automatic temperature compensation.				
Wang et al. 2020	*in vitro/ in vivo*	melanoma cell lines - YUMM1.7 and B16-ova / colon adenocarcinoma cell line - MC38	lactic acid (2mM, 5mM, 10mM)	Treg	CD36	N.A.	N.A.
Wei et al. 2015	*in vitro/ in vivo*	colorectal cancer cell line - HCT116. human colon cancer cell line - HT29 cells, colorectal adenocarcinoma cell line - Caco2. human hepatoma cell line - HepG2. human breast cancer cell line - MDA-MB-231 cells	Lactate addition at 5mM to cell culture medium	human THP-1 monocytes	N.A.	PGE2 secreted by THP-1 monocytes	COX2 enzyme levels
Xiaofeng Li et al. 2020	*in vitro/ in vivo*	SMMC-7721 and HepG2 HCC cell lines with different levels of CD147 expression were established	20 mM sodium l-lactate; Lactate production by lentivirally transduced hepatoma cells was measured using a lactate assay kit; Extracellular pH was measured with a pH meter, and all pH measurements were taken within 2 min of samples collection.	Proportion of lymphocites	Cd147	N.A.	GLUT-1, MCT1, MCT4
Xie et al. 2016	*in vitro/ in vivo*	breast cancer cell lines - BT474, HCC1954; MDAMB231. murine melanoma cell line - B16F10		NKT cells are CD1d-restricted T lymphocytes, which possess semi-invariant TCR	N.A.	IFNγ and IL4 producted from NKT cells	mTOR pathway signaling
Yabu et al. 2010	*in vitro*	N.A.	addition of 15 mM L-lactic acid to cell medium	Bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells (BMDCs) were obtained from C57BL/6J	N.A.	IL-17A produced by splenocytes	N.A.
Ye et al. 2018	*in vitro/ in vivo/ ex vivo*	human pancreatic ductal adenocarcinoma cell lines - PANC-1; Capan-2; SW1990; BxPC-3; MIA	M0 macrophages treated with 0, 5, 15, 25mmol/l of lactate, ECAR using an Extracellular Flux Analyzer, acidification of the	THP- 1 monocytes, M2 macrophages	CD68, M2-macrophage markers (CD163, CD206, fibronectin)	CCL22, CCL18, and interleukin (IL)-10 from M2 macrphages	VCAM-1, PITPNM3 (CCL18 potential

		PaCa-2	medium was evaluated by visually inspecting the color of the medium, lactate production measured by lactate oxidase-based colorimetric assay				receptor) in PDAC cell lines
Youan et al. 2016	*in vitro/ in vivo*	hepatocellular carcinoma cell line - HepG2	acidic (pH 6.5) adjusted with lactic acid, NaHCO3 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Peripheral blood mononuclear cells (PBMCs) were isolated from patients with HCC and cytokine-induced killer (CIK) cells were developed	CD3+, CD3+ CD56+, CD4+ and CD8+	perforin	N.A.
Zhang et al. 2019	*in vitro/ in vivo*	murine breast cancer cell lines - 4T1; EMT-6. murine hepatoma cell line - Hepa 1-6. melanoma cell line - B16-F10. murine colorectal cell line - CT26.	Intratumoral pH measurement using the fluorescent probe SNARF-4F. Cell culture acidification pH 6.5. Concentrations of lactate in culture supernatants of cells transfected for 72 h were determined enzymatically using a Lactate Colorimetric/Fluorometric Assay Kit. The tumoral pH values were measured with an MI-407 needle-type pH microelectrode and a MI 402 reference microelectrode.	CD8+ T cells (from spleen of C57BL/6 mice)	CD25	IFN-γ from Tcells	LDHA
Zhang et al. 2020	*in vitro*	human gastric cancer cell lines - MGC803, SGC-7901, NCI-N87, HGC-27, AGS, SNU-1	media produced by gastric cell lines - lactate level in the culture media was detected by a Lactate Assay Kit (Biovision, Milpitas, CA, USA)	THP-1 (monocytes) and human monocytes	M1 (CCR7) M2 (CD163, FIZZ1 and ARG1).	M1 (IL-1, TNFA, IL-12 and INOS) , M2 (IL-10, YM1, FIZZ1) macrophages	LDHA
Zhao et al. 2015	*in vitro*	transitional cell carcinoma of the bladder cell line - T24	13 mM lactate	macrophages (RAW264.7 - generally recognized as M1 macrophages)		iNOS	Arg-1 protein, HIF-1α, p-NF-κB-p65

4 CONSIDERAÇÕES FINAIS

O microambiente tumoral é caracterizado por um complexo sistema no qual há constante interação entre diferentes componentes - células tumorais, imunócitos, células do estroma e vasos sanguíneos - sob a influência de subprodutos metabólicos, flutuação de oxigênio e nutrientes, bem como impermanente aporte vascular (KAYMAK et al., 2021). Ademais, em decorrência do efeito Warburg, as células tumorais passam a secretar altas concentrações de lactato no compartimento extracelular, levando ao acúmulo desse metabólito e à acidificação do microambiente, ambos considerados características comuns a diversos tipos de tumores sólidos (CORBET; FERON, 2017; GATENBY; GILLIES, 2004; HANAHAN; WEINBERG, 2011; HUBER et al., 2017; KOPPENOL; BOUNDS; DANG, 2011; MARTINEZ-OUTSCHOORN et al., 2016; RACKER, 1972; VANDER HEIDEN; CANTLEY; THOMPSON, 2009; ZHANG; LI, 2020). Notavelmente, os tumores com alta agressividade demonstram-se excessivamente glicolíticos e significativamente ácidos(GIATROMANOLAKI et al., 2017, 2019). As células neoplásicas, inseridas nesse ambiente hostil, utilizam boa parcela dos nutrientes disponíveis, comprometendo e alterando funcionalmente os demais tipos celulares com os quais coexistem. Nesse cenário, configura-se um nicho no qual diversas vantagens adaptativas às células neoplásicas malignas se consolidam, favorecendo a patogênese, a agressividade e a progressão do tumor. É crescente o número de evidências na literatura indicando que a produção e liberação de prótons e ácido láctico por células tumorais causa profundas alterações celulares, influenciando diretamente a manutenção de fenótipos celulares resistentes e agressivos. A modulação do microambiente tumoral estabelecida e retroalimentada por esses fenótipos favorece processos vitais para a progressão tumoral tais como migração e invasão, ativação de vias de sinalização relacionadas à sobrevivência, estabelecimento de metástases e escape imunológico (IPPOLITO et al., 2019).

Considerando a relevância do tema, o presente estudo se propôs a investigar o papel da acidificação tumoral sobre a modulação do comportamento celular de linhagens de carcinoma espinocelular bucal3.1. Para esse fim, desenvolvemos um modelo in vitro mimetizando as flutuações do pH extracelular, para aproximar-se e reproduzir as condições dinâmicas observadas no microambiente de tumores sólidos. Observamos, em um primeiro momento, que a diminuição do pH é um evento altamente estressor às células tumorais, impondo um importante desafio à sua capacidade proliferativa, demonstrado pela dificuldade e até mesmo falha na recuperação da proliferação. Dessa forma, sugere-se que a acidez extracelular aplica uma pressão seletiva, desencadeando a morte de diversas células e, em contrapartida, selecionando subgrupos celulares altamente resistentes, capazes de se dividir e resistir ao tratamento com agente quimioterápico.

Quando recondicionadas em meio de cultivo de pH neutro, observou-se não somente a retomada, mas a potencialização de funções celulares dependentes de energia, como

a migração celular. Tais alterações de comportamento e fenótipo das células neoplásicas foram acompanhadas, como esperado, pela ativação de vias e aumento da expressão de marcadores associados a esses fenômenos.

Como demonstrado, exposições contínuas e intermitentes à acidose do microambiente desencadeiam diferentes efeitos celulares indicando que as flutuações de pH, mesmo que sutis, são capazes de alterar os desfechos biológicos. Esse conhecimento auxilia na elucidação e entendimento do caráter contraditório dos achados analisados ao longo do artigo 2 da presente tese 3.2. Apesar de heterogêneos, os resultados obtidos a partir da revisão de escopo evidenciam os importantes efeitos, tanto da abundância de lactato, quanto do baixo pH sobre a resposta imunológica, alterando-a de forma muito significativa. As evidências analisadas demonstraram diminuição da capacidade proliferativa, viabilidade e concentração, de diferentes células do sistema imune, acompanhadas de altas taxas de morte celular. Ademais, importantes alterações na expressão de citocinas inflamatórias, receptores e ligantes de superfície celular, bem como nas interações célula-célula, foram observadas durante ou após exposição à acidez e/ou lactato.

Observamos que, mesmo na atualidade, grande parte dos estudos in vitro não levam em consideração esse importante aspecto do microambiente tumoral, contribuindo para o estabelecimento de um viés de pesquisa. Em decorrência disso, aprofundam-se discrepâncias entre achados observados in vitro e quando em comparação a testes in vivo. A exemplo, podemos citar tratamentos antineoplásicos, os quais, muitas vezes, demonstram-se eficazes frente à testagem in vitro, no entanto, quando transacionados para modelos in vivo, não entregam a mesma eficácia, possivelmente por terem sido testados em modelos experimentais desconsiderando importantes condições do do microambiente tumoral. Idealmente, experimentos de cultivo celular, tanto em 2D quanto 3D, deveriam mimetizar as principais condições metabólicas do microambiente tumoral, como privação de glicose e nutrientes, altas concentrações de lactato e pH ácido.

Estratégias que permitam a modulação dessas condições específicas do microambiente podem representar valiosos coadjuvantes terapêuticos, aumentando a resposta aos diferentes modelos de tratamento antitumoral. Contudo, é importante destacar que, como observado no artigo 1 3.1 da presente tese, a neutralização do pH, para determinadas subpopulações de células tumorais, pode potencializar comportamentos migratórios, induzindo invasão e metástase. Esses achados são importantes indicativos da necessidade de aprofundamento dos estudos nessa área, já que a indicação de moduladores de pH/lactato possivelmente irá requerer cautela.

Da mesma forma, a modulação do pH tumoral pode potencializar a resposta ao tratamento imunoterápico. Apesar de representar uma importante arma no tratamento anticâncer, atualmente, sabe-se que as diferentes modalidades de imunoterapia beneficiam apenas um subgrupo de pacientes. Para predizer a resposta ao tratamento realiza-se, em alguns pacientes, a avaliação de parâmetros biológicos, tais como a expressão de

PD-1/PD-L1 previamente à administração de agentes imunoterápicos. Em consonância, a análise de marcadores metabólicos, como a presença de ácido lático ou mesmo de regiões tumorais acídicas por meio de exames séricos e utilização sondas marcadores, podem ser recursos valiosos na predição da eficácia dos tratamento imunoterapeuticos.

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