UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

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Programa de Pós-Graduação em Biociências

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"All sorts of things can happen when you're open to new ideas and playing around with things"

Stephanie Kwolek

Chemist who invented Kevlar and winner of the Lavoisier Medal for technical achievements.

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De maneira geral é um simples obrigado do tamanho do mundo.

SUMÁRIO

1. RESUMO

Células imunes apresentam plasticidade funcional influenciada por fatores externos capazes de se alterar fenotipicamente, favorecendo a progressão do tumor. Os neutrófilos possuem um espectro de ativação comumente denominado antitumoral/pró-inflamatório (N1) e imunossupressor/pró-tumoral (N2). A presença dessas células no microambiente tumoral é essencial para a formação de um sítio propício para o desenvolvimento das células tumorais. No Glioblastoma, o nicho tumoral formado impacta diretamente na malignidade da doença. Este tipo de tumor cerebral é o mais frequente diagnosticado e possui alta mortalidade, com sobrevida média de 15 meses após a descoberta da doença. Considerando os fatos apresentados, esse estudo visa observar a participação dos neutrófilos na progressão dos glioblastomas. Para isso, foram realizados testes experimentais *in vitro* com linhagem celular de glioma humano (U87MG) e neutrófilos de voluntários saudáveis, e avaliados a capacidade de ativação das células imunes em diferentes condições de cocultivo, bem como a influencia na viabilidade tumoral. Para os neutrófilos, a migração é um fator importante e bem descrito na literatura a participação da sinalização purinérgica; foi realizada uma revisão de literatura sobre o assunto utilizando sobre os estudos com células humanas. De forma geral, foi observado mudanças morfológicas importantes entre os fenótipos induzidos e em cocultivo, bem como maior ativação imune na presença de células tumorais, confirmando a importância da comunicação intercelular.

1.1 ABSTRACT

Immune cells have functional plasticity influenced by external factors capable of altering phenotypically itself, favoring tumor progression. Neutrophils have an activation spectrum commonly called anti-tumor/pro-inflammatory (N1) and immunosuppressive/pro-tumor (N2). The presence of these cells in the tumor microenvironment is essential for the formation of a favorable site for the development of tumor cells. In Glioblastoma, the tumor niche formed directly impacts the malignancy of the disease. This type of brain tumor is the most frequently diagnosed and has high mortality, with an average survival of 15 months after the discovery of the disease. Considering the facts presented, this study aims to observe the participation of neutrophils in the progression of glioblastoma. Thus, experimental tests were performed *in vitro* with human glioma cell line (U87MG) and neutrophils from healthy volunteers, and the ability of immune cells to be activated in different coculture conditions was evaluated, as well as the influence on tumor viability. For neutrophils, migration is an important factor and well described in the literature, the participation of purinergic signaling; a literature review on the subject was carried out using studies on human cells. In general, important morphological changes were observed between the induced and co-cultured phenotypes, as well as greater immune activation in the presence of tumor cells, confirming the importance of intercellular communication.

2. LISTAS

2.1 Lista de abreviaturas

AMP: Peptídeo antimicrobiano

ATP: Trifosfato de adenosina

BHE: Barreira hemato-encefálica

C3: Componente 3 do complemento

CCL3: Ligante 3 da quimiciona

DAMP: Padroes moleculares endógenos

GBM: Glioblastoma

ICAM-1:Molécula de adesão intercelular 1

IFN-Y: Interferon gama

IL-1β: Interleucina 1 beta

IL-2: Interleucina 2

IL-6: Interleucina 6

IL-8: Interleucina 8

IL-12: Interleucina 12

IL-23: Interleucina 23

M1: Macrófago anti tumoral

M2: Macrófago pró tumoral

MMP9: metaloprotease 9

N1: Neutrófilo anti tumoral

N2: Neutrófilo pró tumoral

NE: Elastase

NET: Armadilhas extracelulares dos neutrófilos

OMS: Organização Mundial da Saúde

P1: Receptor purinérgico

P2: Receptor purinérgico

PAMP - Padrões Moleculares associados a patógenos

PDGFR: Receptor para fator de crescimento derivado de plaquetas

PI3K: Fosfoinositídeo 3-quinase

PMN: Leucócitos polimorfonucleases – Utilizado como sinônimo de Neutrófilo nesse texto

PPR - receptor de reconhecimento de padrão

ROS: Espécies reativas de oxigênio

RNS: Espécies reativas de nitrogênio

TAN: Neutrófilo associado ao tumor

TGF-β: Fator de crescimento transformante

Th17: Linfócito TCD4 “ajudante”

TNFα: Fator de necrose tumoral alfa

T-regs: Linfócitos T reguladores

VEGF-A: Fator de crescimento endotelial vascular

2.2 Lista de Figuras

3. AMBIENTE TECIDUAL NA SAÚDE E NA DOENÇA

3.1. HOMEOSTASE

Para discutirmos sobre doença, primeiro precisamos entender o estado fisiológico do corpo. Em todos os organismos existe um mecanismo muito importante para a manutenção da vida chamado de homeostase. De maneira geral, protege o organismo contra alterações provenientes do ambiente, como a falta ou o excesso de suprimentos. Contudo, o corpo promove ações no meio interno para manter o equilíbrio fisiológico independente das alterações externas [1,2].

O organismo humano é composto por múltiplos órgãos que agem unificando um único sistema. O tecido normal e saudável contém muitos tipos celulares, sinais moleculares, e diversos microambientes que trabalham em sintonia [2]. Os sinais autócrinos e parácrinos produzidos compõem formas de comunicação intercelular essenciais para as vias bioquímicas, resultando na troca de informações capazes de promover respostas que permitem a manutenção homeostática [2].

Mantendo o raciocínio e considerando a relevância das interações entre células, é razoável interpretar o câncer como um microssistema e ressaltar o comportamento de células não tumorais na manutenção do microambiente tumoral [3-5].

3.2 INFLAMAÇÃO

Usualmente, a inflamação é descrita como um processo fisiológico que responde a danos que perturbam a homeostase. Portanto, a finalidade consiste em eliminar aquilo que perturba o equilíbrio interno, mesmo que, por consequência, apresente alterações fisiológicas momentâneas à essa condição atípica para restaurar a funcionalidade e a homeostase [6,7]. Os sinais clássicos da inflamação aguda, também conhecidos como cardinais, são a manifestação de dor – por mediadores químicos, calor – pelo aumento da velocidade do fluxo sanguíneo, tumor – caracterizado por formação de edema e infiltração leucocitária, rubor – decorrência da vasodilatação, e comprometimento de função – por fenômenos alternativos (degeneração ou necrose), repercussões fisiopatológicas teciduais locais [6-8]. Perturbações que perdem a integridade tecidual também são capazes de acionar os mecanismos inflamatórios, por exemplo, células necróticas, morte celular, ou demais situações com ausência de patógenos, é denominada inflamação estéril [9].

A resposta aguda é ativada nos primeiros momentos em que há a perturbação do sistema, seja com invasão de patógenos ou dano tecidual, através da liberação de mediadores solúveis, primordialmente com citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNFα) e IL-1β, e quimiocinas, como IL-8 [8]. Em resumo, com a liberação desses e outros fatores, as células imunes inatas ativadas são atraídas até o local injuriado e iniciam o processo de reconhecimento, recrutamento de demais células imunes inatas e adaptativas e recuperação tecidual. A ação é montada a fim de zelar o tecido, a resposta imune exacerbada acarreta mais dano, contudo, a resolução da inflamação e consequente remoção do infiltrado imunológico restabelecem a homeostase [10, 11].

Em situações que ocorre remoção das células imunes, sustentando um microambiente anormal, o que leva à progressão para um perfil cônico de inflamação – a qual é considerada *hallmark* de diversas doenças, como o câncer [12,13]. O perfil inflamatório documentado em microambientes tumorais tem sido foco de muitas investigações [14]. Esses estudos indicam que além da presença de mutações e/ou deleções de genes que controlam a proliferação e morte celular, o perfil imunossupressor caracterizado pela secreção de citocinas através das células que compõem o microambiente tumoral, como o fator de crescimento tumoral- β (TGF-β), interleucina (IL) -6, IL-10 e prostaglandina E2, é essencial para perpetuação tumoral [15].

3.3 SISTEMA IMUNE INATO

O sistema imune inato é a primeira linha de defesa que o nosso corpo usa para se proteger de invasores e, curiosamente, seus mecanismos são evolutivamente conservados. Ao contrário da resposta adaptativa, se baseia em uma reação inespecífica, com amplo espectro de eficácia - inespecífica para antígenos, mas não para sinais de agressão -, orquestrado por componentes celulares e proteínas do sistema complemento [8,16].

O sistema complemento e os peptídeos antimicrobiais (AMPs) reconhecem estruturas externas presentes na circulação e desencadeiam uma resposta humoral [17]. Os AMPs são estruturas conservadas evolutivamente nas quais, em mamíferos, estão presentes nos grânulos de neutrófilos e em células epiteliais, constituindo cerca de 50 aminoácidos. São originados através de precursor inativo

via quebra proteolítica e liberados de acordo com a demanda, capazes de controlar a regulação de sua atividade [18]. Já o sistema complemento é composto por uma sequência de proteínas que são ativadas também por quebra proteolítica, nas quais apresentam funções de sinalização pró-inflamatórias e ativação de fagócitos [19]. Há três vias distintas – clássica, alternativa e lectina-, que convergem na ativação da proteína componente C3 e promovem o aumento da resposta imune e fagocítica [19, 20].

A porção celular da resposta inata consiste na presença de células hematopoiéticas, nomeadas de células mieloides. Essas células compartilham funções através de sensores de reconhecimento de epítopos não-próprios, os padrões moleculares associados a patógenos (PAMPs), ou padrões moleculares associados a danos endógenos (DAMPs), os quais determinam assinatura de potencial dano. Esses receptores são definidos como receptores de reconhecimento de padrões (PPRs) e regulam a transcrição e processamento de citocinas, apoptose, autofagia e fagocitose [21] (Fig. 1).

3.3.1 NEUTRÓFILOS

Dentre os componentes celulares da resposta inata estão os neutrófilos, que podem ser metaforicamente comparados com a “infantaria do exército”. A relação entre eles é simples: são, literalmente, as primeiras células recrutadas para o local de infecção ou injúria tecidual e atuam contra uma grande variedade de patógenos,

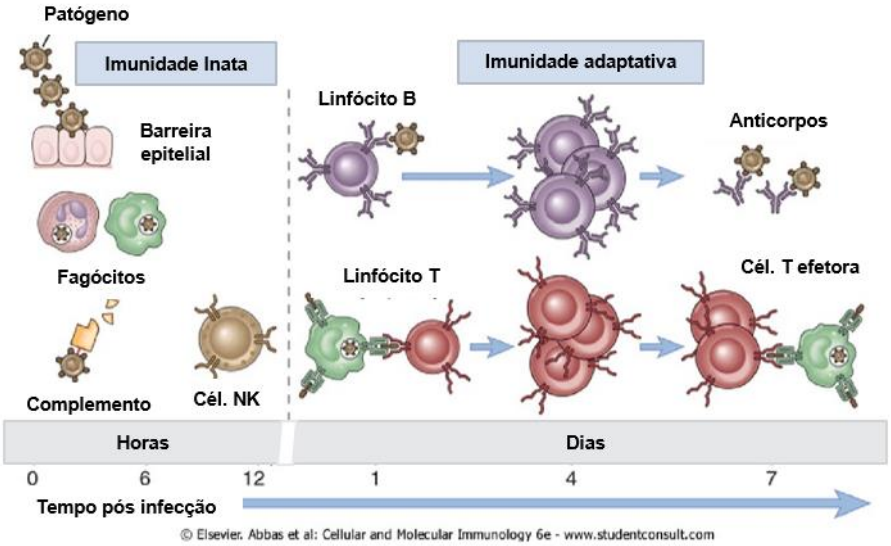


Figura 1: Exemplo dos componentes resposta inata e adaptativa (Adaptado Abbas, 2012)

incluindo bactérias e fungos [8, 22-29]. Os neutrófilos são conhecidos também como leucócitos polimorfonucleares (PMNs), produzidos na medula óssea junto às demais células imunes. Tanto neutrófilos quanto monócitos derivam do mesmo progenitor, entretanto, os PMNs sofrem um processo de maturação de seus grânulos de forma sequencial: grânulos primários (azurofílicos), secundários (específicos), terciários (gelatinase) e as vesículas secretórias [30, 31]. Entender o processo de maturação de seus grânulos e, por consequência, seus nomes, facilita na compreensão do processo de degranulação – função característica dos neutrófilos.

As células já maduras são liberadas para a corrente sanguínea em processo mediado pelo receptor CXCR4, o qual é essencial para reter os PMNs na medula óssea [32,33]. Os neutrófilos são a maior população de células imunes em

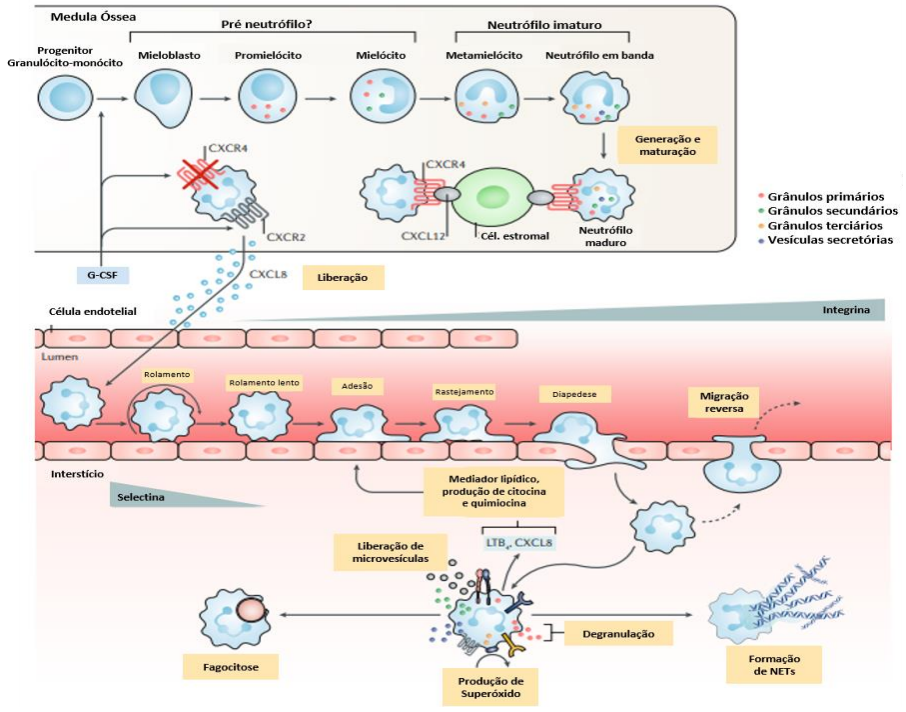


Figura 2: Visão geral do desenvolvimento e função dos neutrófilos

A geração e maturação de neutrófilos ocorrem na medula óssea. O fator estimulador de colônias de granulócitos (G-CSF) é um regulador crítico desses eventos e contribui para a liberação das células para a circulação. Ao detectar estímulos inflamatórios ambientais, os neutrófilos sofrem uma cascata de recrutamento especial e, eventualmente, migram para os tecidos. No local da inflamação, os neutrófilos exercem suas diferentes funções efetoras, levando à eliminação dos microrganismos invasores. (Adaptado Németh, 2020)

circulação, com aproximadamente 50-70% do total, mas com sobrevida inferior a 12h em condições fisiológicas e de 2-3 dias em locais de infecção [34-36] (Fig. 2).

Nos últimos anos a visão simplista sobre a atuação dos neutrófilos vem sendo contestada e o interesse na sua biologia está emergindo. Portanto, novos estudos revelaram um papel mais complexo e multifacetado dos PMNs [37-40]. É compreendido que os neutrófilos participam da resposta imune através da regulação e recrutamento de outras células imunes, como monócitos/macrófagos e células dendríticas, por exemplo [32], bem como da interação entre células B e T [22]. É imprescindível reconhecer que a comunicação é bidirecional e, dessa forma, os neutrófilos recebem sinais moduladores que auxiliam nos processos de iniciação, supressão ou regulação da reação imune [41].

A diversidade das funções dessas células tem relação com o conceito de heterogeneidade: da mesma forma que outras células imunes, como macrófagos, os neutrófilos apresentam diferentes fenótipos programados para exercer ou ser mais proficientes em funções mais discretas [42]. Como em M1 e M2, nos macrófagos, os PMNs possuem espectro de ativação ainda não elucidado, entretanto, podem ser agrupados seguindo a mesma lógica; N1 (polarização pró-inflamatória) e N2 (polarização anti-inflamatória) [43, 7]. Considerando a plasticidade celular recentemente descoberta, não é surpresa que os neutrófilos sejam considerados importantes no entendimento de patogenias, como autoimunidade, inflamação crônica e câncer [35, 38, 44, 45].

3.3.1.1 Subpopulações de Neutrófilos

As subpopulações dos neutrófilos (N1 e N2) são polarizadas em estados de ativação diferentes em resposta a sinais e citocinas vindas do microambiente [44, 46]. O fator de crescimento tumoral-β (TGF-β) é um dos mecanismos avaliados em estudos com neutrófilos por caracteristicamente estar bloqueado quando N1 está presente. Quimiocinas secretadas elevam o perfil inflamatório no microambiente e o neutrófilo age citotoxicamente contra as células tumorais, reduzindo a taxa de crescimento do câncer [44]. Esse fenótipo antitumoral aumenta a expressão de TNF-α, CCL3, molécula de adesão intercelular 1 (ICAM-1) e reduz a produção de arginase-1, inibe a angiogênese e promove resposta antitumoral de linfócitos T [47, 48]. Em contrapartida, estudos com animais indicam que o fenótipo N2 é adquirido

pela presença de TGF-β, favorecendo o infiltrado de neutrófilos com alta expressão do receptor de quimiocina CXC tipo 4 (CXCR4), fator de crescimento endotelial vascular A (VEGF-A) e metaloprotease 9 (MMP-9) [48]. A supressão da proliferação e da atividade das células T, além da produção de interferon-gama (IFN-γ), potencialmente resulta no aumento dos níveis de arginase-1 nos grânulos neutrofílicos de N2 e ROS [47].

A produção de espécies reativas de oxigênio (ROS) e nitrogênio (RNS), e consequente liberação de radicais livres pelos neutrófilos tem capacidade de dupla ação: em tumores bem estabelecidos e desenvolvidos, a ROS pode ter efeito antitumoral, com citotoxicidade direta e efeito pró-tumor pela inibição de demais células imunes. Além disso, quando associado ao infiltrado de neutrófilos durante a carcinogênese podem contribuir para instabilidade genética [44]. Neutrófilos são os principais produtores de VEGF-A e liberam altos níveis de MMP-9, o qual libera a forma ativa do VEGF-A da matriz extracelular. Porém, N2 é apto a liberar MMP-9 mesmo na ausência de inibidor de proteases e o aumento dos níveis colabora com a angiogênese e a invasão tecidual [48]. Os grânulos também podem favorecer os dois perfis anti- e pró-tumorais. A elastase (NE) granular inibe a fosfatidilinositol 3-quinase (PI3K), a ausência ativa a sinalização do receptor do fator de crescimento derivado de plaqueta (PDGFR) favorecendo a proliferação celular do tumor. Não obstante, a NE consegue conter a resposta antitumoral em alguns tipos de câncer, aumentando a resposta celular para a imunidade adaptativa [46].

Estudos observaram que o fenótipo N2 favorece o recrutamento de T-regs para a área tumoral, expressam TGF-β, IL-6 e IL-23. Sugere-se que essas citocinas em conjunto promove o perfil Th17. Apesar do Th17 estar bem consolidado no âmbito da inflamação, ainda há pouco conhecimento sobre seu papel no câncer. Já o fenótipo N1 expressa altos níveis de IL-12 e TNF-α, as quais são características de perfil inflamatório, atraindo linfócitos T, macrófagos e demais quimiocinas. A regulação positiva de IFNγR1 em neutrófilos N1, com o aumento de IFNγ pelas T

citotóxicas, contribui para a hipótese que o microambiente modula a polarização dos neutrófilos [44] (Fig. 3).

Foi observado através da técnica de imuno-histoquímica a presença de infiltrado de neutrófilos em pacientes com glioblastoma (GB), tumor cerebral de elevada agressividade. Ainda, correlacionaram positivamente a extensão do infiltrado com a classe tumoral, e o GB apresentando infiltração significativa. Estudos concluíram que há relação entre o aumento da quantidade de neutrófilos circulantes com o grau de infiltração tumoral. Curiosamente, apesar do GB apresentar alta vascularização e necrose limitada, os neutrófilos foram principalmente encontrados em capilares e frequentemente observados em alto número nas áreas necrosadas [38]. Ainda que existam evidências da importância do fenótipo N2 no desenvolvimento tumoral, o perfil de citocinas liberadas pelo microambiente tumoral e a resposta efetora dos neutrófilos ainda não está consolidada.

3.3.1.2 Papel da Sinalização Purinérgica na Ativação de Neutrófilos

O infiltrado imune no nicho tumoral é recrutado por diversos atrativos liberados pela própria célula tumoral. Os tumores são capazes de induzir e manter um ambiente inflamatório favorável rico em fatores solúveis que sustentam a sua

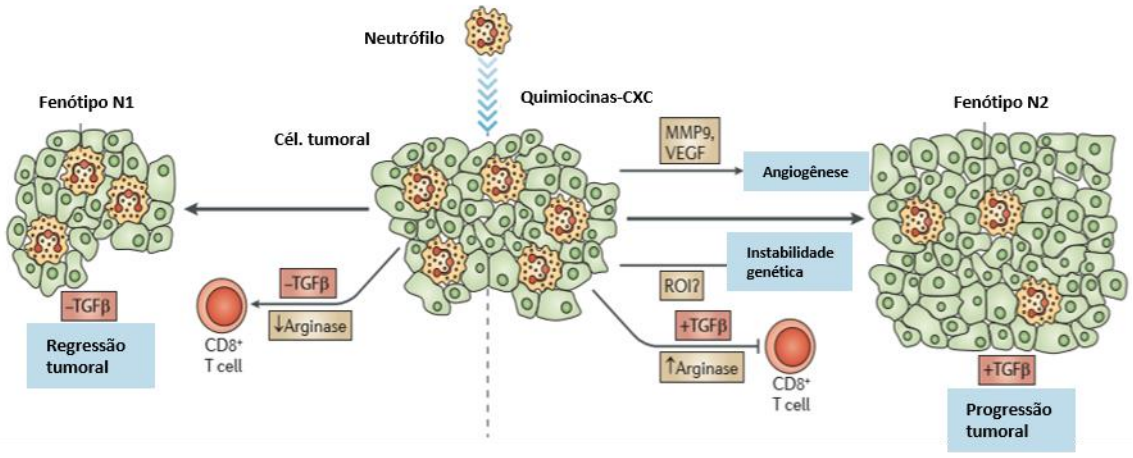


Figura 3: Neutrófilos associados ao tumor:

Quimiocinas produzidas pelo tumor auxiliam no recrutamento de neutrófilos. Esses, por sua vez, pode promover instabilidade genética (ROS), estimular a formação de novos vasos (MMP9 e VEGF). A modulação fenotípica está relacionada com a liberação de TGFβ pelos tumores. Em contrapartida, a inibição do TGFβ realiza uma reprogramação para o perfil N1, associado a maior atividade citotóxica, maior expressão de TNF e iCAM1, bem como menor expressão de arginase. Na presença de N1, a ativação dos linfócitos TCD8+ aumenta e, por consequência, tem melhor atividade antitumoral. (Adaptado Mantovani, 2011)

proliferação [49]. Para isso eles modulam as funções das células inflamatórias recrutadas para trabalharem a seu favor [7]. Nesse contexto, o trifosfato de adenosina (ATP) possui papel duplo na manutenção do microambiente, dependendo não apenas da sua concentração, mas da atividade das ectonucleotidases (CD39 e CD73) e das células imunes presentes. O papel do ATP extracelular na progressão tumoral é complexo. Baixos níveis de ATP podem promover o desenvolvimento tumoral e o perfil imunossupressor, enquanto em condições opostas podem ativar células apresentadoras de antígenos, as quais contribuem para a atividade antitumoral [50] ou, inversamente, estimular a quimiotaxia de células imunes as quais perpetuam o processo inflamatório associado ao câncer [91].

O ATP intracelular é usualmente descrito como fonte principal de energia que impulsiona as funções celulares; não obstante, a sua presença no meio extracelular é interpretada como sinal de "*find me*" ou "perigo" que serve como mensageiro intracelular ou mediador autócrino [51]. A ativação dos receptores purinérgicos pela comunicação parácrina e autócrina, o ATP e seus produtos de hidrólise modulam funções importantes nas células [52-54]. Os receptores purinérgicos são divididos em dois grandes grupos: P1 e P2, sendo receptores de adenosina e de nucleotídeos, respectivamente. A família P2 ainda é subdividida em receptores do tipo P2X e P2Y [55]. Embora não totalmente esclarecido, o ATP estimula o recrutamento de células imunes inflamatórias definindo o gradiente quimiotático – em conjunto com demais fatores [56] (Fig. 4).

A migração dos neutrófilos depende de um sinal excitatório frontal e um inibitório na superfície traseira da célula, apontando o sentido de migração em direção ao ATP intracelular. Esse é reconhecido pelos receptores P2 e ativa a liberação de ATP intracelular pela via Panexina-1 de estimulação autócrina. De mesmo modo, a via fornece os sinais inibitórios, como adenosina, na borda traseira. Por fim, a adenosina é reconhecida pelos receptores A2a (tipo P1) bloqueando a

sinalização do receptor. Esse ciclo de retroalimentação purinérgica promove o movimento dos neutrófilos em direção à lesão [51,57, 58].

A polarização dos neutrófilos pode ser dependente da sinalização purinérgica. A ativação dos neutrófilos e suas funções efetoras, como a degranulação, a formação de armadilhas extracelulares (NETs) e a produção de espécies reativas de oxigênio mostram-se relacionadas com a ativação purinérgica [49, 51, 56, 59]. Os níveis de ATP descritos no microambiente tumoral poderiam resultar em estimulação parácrina dessas células [60] e o fenômeno de dessensibilização dos receptores P2Y, nesse nicho, conseguiria diminuir a resposta imunológica pela exposição prolongada ao nucleotídeo [61], com isso é especulado se a hiporresponsividade do neutrófilo não estaria relacionada.

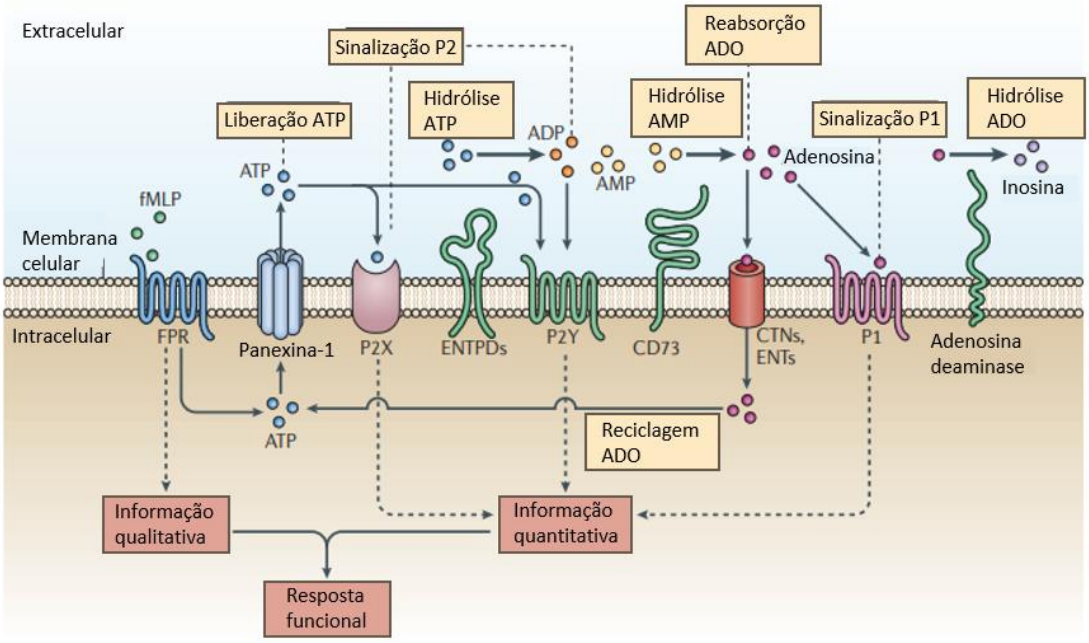


Figura 4: Componentes da sinalização purinérgica autócrina.

Desenho esquemático dos principais elementos envolvidos na sinalização purinérgica. Ativação de receptor, como a estimulação do receptor formil-peptídeo (FPRs) pelo fMLP promove a abertura dos canais de panexina-1 e a liberação de ATP intracelular. O ATP extracelular promove a ativação autócrina dos receptores P2. As ectonucleotidases – ENTPDs, CD39 – e ecto-5'-nucleotidase (CD73) promove a hidrólise do ATP em adenosina, a qual ativa os receptores P1. A adenosina é neutralizada pela adenosina deaminase, que converte em inosina ou recicla pelos transportadores CNTs. A sinalização autócrina dá informações quantitativas que definem a resposta qualitativa na percepção de patógenos. (Adaptado Junger, 2011)

3.4 MICROAMBIENTE TUMORAL

Apesar de tecidos saudáveis serem robustos contra diversas perturbações [62, 63], tumores são capazes de interferir na homeostase a ponto de o sistema ser incapaz de recuperar o equilíbrio interno [64, 65, 66], promovendo suporte para o crescimento de células alteradas e mudanças de microambiente. O nicho tumoral é formado por um complexo ambiente constituído de vasos sanguíneos e linfáticos, fibroblastos, células endoteliais e imunes, citocinas, matriz extracelular e vesículas [67, 68]. Mutações genéticas nas células tumorais vêm sendo descritas por explicarem o sucesso na perpetuação do câncer, contudo, as interações célula-célula dentro do microssistema atuam na seleção e, por consequência, na evolução [69].

O infiltrado imune é um componente primordial no microambiente tumoral e possuem papel importante na sua progressão [68, 70]. Os neutrófilos associados ao tumor (TANs) não são os componentes mais abundantes, mas evidências comprovam que a sua presença é primordial e pode ter sido subestimada [71], reforçando o diálogo entre célula tumoral e imune, modulando fenotipicamente o PMN e este, por sua vez, auxiliando na perpetuação da doença [72].

A plasticidade fenotípica de células imunes abre espaço para questionamentos quanto a suas funções previamente descritas e em que momento elas passam a proteger o tumor [7]. Sabe-se que as células inatas e adaptativas do sistema imunológico trabalham juntas no monitoramento e defesa do nosso corpo. Em estados iniciais de lesões malignas, entende-se que o nosso sistema imune consegue manter suas funções de ataque, entretanto, com o desenvolvimento e crescimento tumoral, esta passa a dominar o microambiente e se sobrepõe aos ataques. Assim, aprimora seus mecanismos de escape tumoral e se torna incontrolável [73], ou seja, a modulação das células imunes pelo câncer efetivamente converte a ação de ataque em proteção ao tumor [7, 47, 73, 74].

No âmbito do GB, este promove um nicho pró-angiogênico e um estado de inflamação, dessa forma, recruta as células imunes com a liberação de quimiocinas pelas próprias células tumorais e aumenta a expressão das moléculas de adesão nas células endoteliais e permite o afastamento dessas células tornando a barreira hematoencefálica (BHE) altamente permeável [73]. Ademais, neutrófilos ativados

liberam sinais químicos capazes de enfraquecer a BHE - os quais atuam diretamente no citoesqueleto, glicocálix das células endoteliais, como as espécies reativas de oxigênio (ROS), enzimas proteolíticas e citocinas [75].

3.4.1 GLIOBLASTOMA

O Glioblastoma (GB) é considerado a forma primária mais comum e mais agressiva dentre os tumores cerebrais, representando mais de 40% das neoplasias do sistema nervoso [76-78]. É caracterizado pela proliferação celular elevada, invasão tecidual e altas taxas de mortalidade [73, 79]. Clinicamente, os gliomas são divididos em quatro graus, sendo o I avaliado como benigno, baixa proliferação e bom prognóstico pós-remoção cirúrgica [77, 80]; as lesões de grau II são pouco mais intensas e capazes de evoluir para os demais graus. Os gliomas mais agressivos estão caracterizados nos graus III e IV, pois em ambos há alta taxa de proliferação celular, se espalhando rapidamente pelo tecido nervoso. Segundo a OMS, o GB é caracterizado como grau IV, ou seja, são tumores altamente infiltrativos, com proliferação celular, angiogênese e necrose intensas, com média de sobrevida em aproximadamente 15 meses [76, 81] (Fig. 5). O tratamento de primeira escolha consiste em remoção cirúrgica combinada com radioterapia e quimioterapia utilizando como fármaco o agente alquilante de DNA, a temozolomida (TMZ) [79, 81, 82]. Entretanto, a recorrência do tumor é quase sempre observada.

Uma das principais razões por que os gliomas não são totalmente removidos durante o procedimento cirúrgico é a natureza difusa tumoral. A capacidade das células GB em infiltrar os tecidos adjacentes associado à variabilidade do tumor propriamente dito e a localização das células tumorais dentro do cérebro, resultam na incapacidade de ressecar completamente o tumor [83, 84]. As características multiformes são observadas tanto na macro quanto na microscopia: apresenta regiões de necrose e hemorragia; com necrose em pseudopaliçada, núcleos e células pleomórficas e proliferação microvascular; e ainda geneticamente, com deleções, amplificações e mutações pontuais [85]. Devido ao alto grau de invasão e proliferação, os sintomas podem variar de dores de cabeça, confusão mental, déficit neurológico focal à perda de memória, alterações de personalidade e convulsões [76, 77].

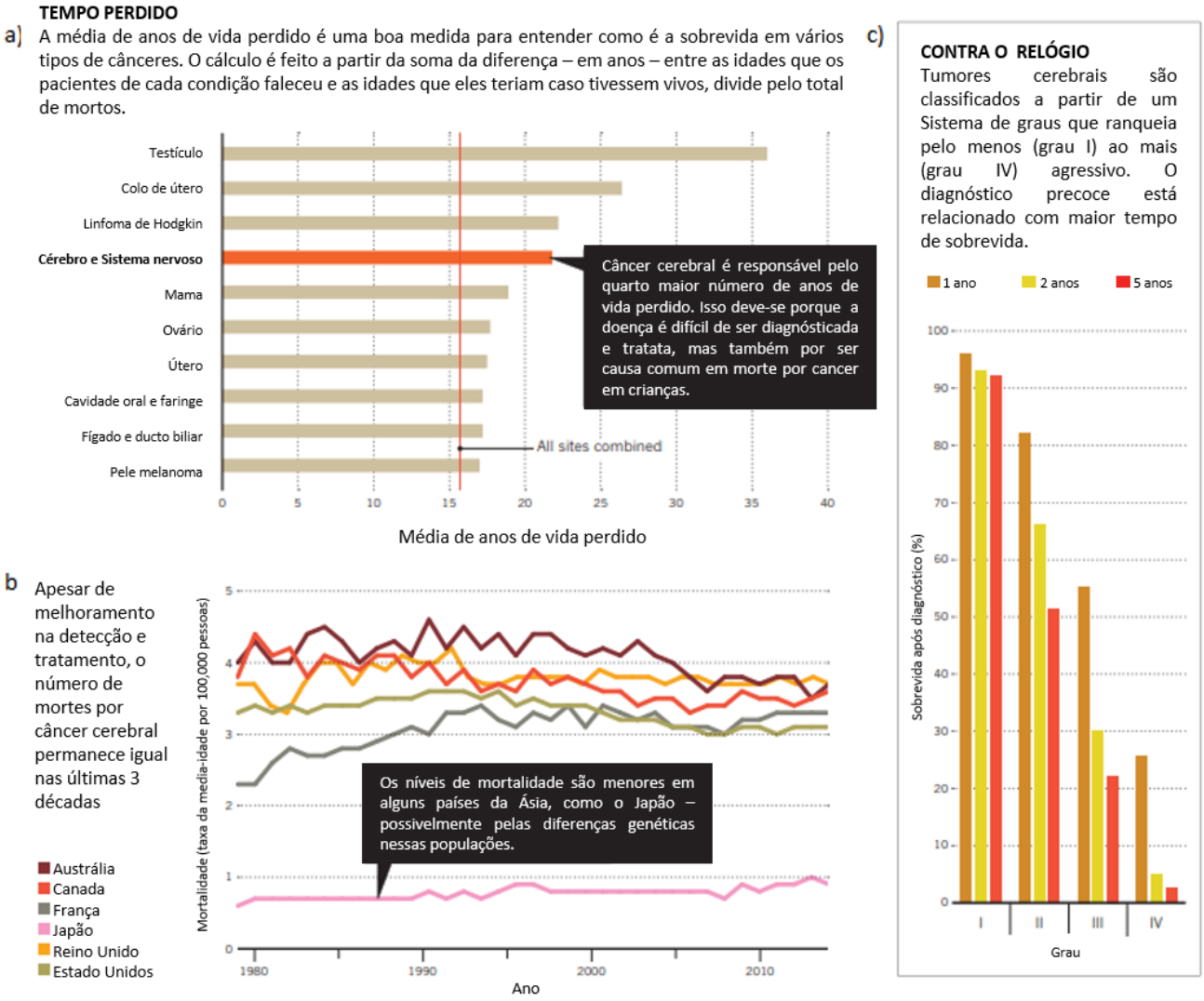


Figure 5: Dados epidemiológicos de câncer cerebral do ano de 2018

a) Considerando todos os tumores cerebrais, a média de "vida perdida" pela doença em comparação com os demais cânceres. O cálculo consiste na soma da diferença em anos entre a idade que o paciente faleceu e a idade provavelmente teria vivido, dividido pelo total de mortos. b) Mortalidade permanece alta nos últimos 30 anos independente dos avanços da tecnologia e de terapias. c) Sobrevida após diagnóstico para todos os graus de gliomas, pontuando o grau IV (Glioblastoma). (Nature, 2018)

Considerando um prognóstico favorável após o diagnóstico e as limitações terapêuticas encontradas, é de extrema importância que estudos sejam desenvolvidos. Compreender o microambiente do GB e como as células não-neoplásicas presentes influenciam na sua progressão pode auxiliar na formulação de novas terapias com diferentes alvos terapêuticos.

3.5 MODELO *in vitro*: cultivo 2D x 3D

Modelo de cultivo celular *in vitro* é o primeiro passo para entender comportamento celular, bem como morfologia, mecanismos, produção de proteínas e efeitos que drogas em desenvolvimento ou em uso possuem sobre essas células. Usualmente essa estratégia é utilizada em pesquisas pré-clínicas, sendo essencial para a compreensão da evolução do câncer [86].

Atualmente, o cultivo 3D vem ganhando mais espaço no meio acadêmico em estudos voltados a situações mais complexas. Isso deve-se ao fato de que a estrutura tridimensional consegue mimetizar as interações celulares, refletindo diretamente no comportamento de diferenciação, proliferação e vitalidade celular; da mesma forma que expressão gênicas e proteicas dependem diretamente desses estímulos [86-89].

Em cultivo celular de monocamada, as células expandem aderidas ao fundo de uma placa ou garrafa. O crescimento uniforme permite ter acesso ilimitado aos nutrientes presentes no meio de cultura, da mesma forma com o oxigênio e metabólitos moleculares [86]. Ademais, a interação homogênea com o ambiente permite que as células em cultivo se encontrem na mesma fase do ciclo celular, o que interfere em processos como a apoptose [86-89] (Fig. 6).

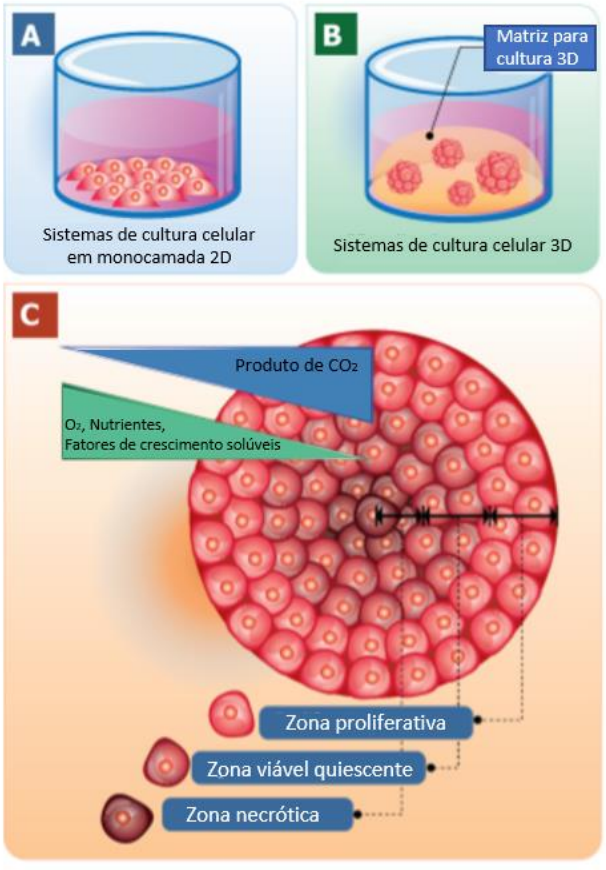
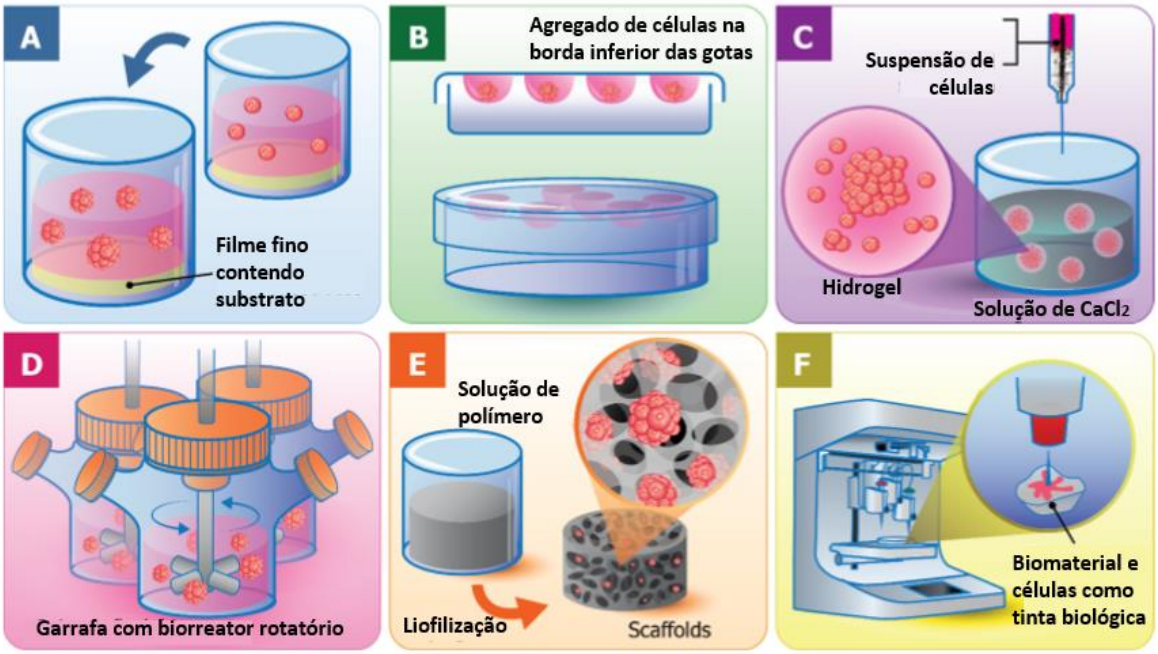


Figura 6: Diagrama esquemático de cultura 2D e 3D.

A: Cultura tradicional 2D; B: Cultura tridimensional; C: Estrutura da cultura 3D com as diferentes zonas de oxigenação, nutrição e CO_2. (Adaptado de Chaicharoenaudomrun, 2019)

Nas condições encontradas *in vivo*, as células tumorais não estão nas mesmas condições que um cultivo 2D proporciona. Dependendo da localização, a oferta de nutrientes pode ser escassa [87]. Com o propósito de reproduzir as circunstâncias da doença, modelos 3D foram elaborados (Fig. 7).

A técnica da gota é relativamente simples de reproduzir, aplicável a vários tipos celulares e baixo custo. O esferoide formado tende a ser altamente compactado e com pouca diferença de tamanho entre as esferas formadas, além disso, exigem relevância pato e fisiológica por produzirem seu próprio meio extracelular [87,89]. A



técnica se baseia na tendência das células em aderirem umas às outras, facilitada pela gravidade [89]. Esse modelo, entretanto, possui algumas desvantagens como tamanho limitado da gota, formato da esfera dependente da manipulação e apesar de produzirem o seu próprio meio extracelular, não é totalmente fiel ao que acontece *in vivo* [89, 90].

Figura 7: Modelos de criação cultura 3D (Adaptado de Chaicharoenaudomrun, 2019)

4. JUSTIFICATIVA

O GB é considerado a forma primária mais comum e mais agressiva de tumores cerebrais. Estudos avaliam a presença de células com perfil inflamatório no microambiente tumoral, dentre as principais células encontram-se os neutrófilos. Há evidências da presença de neutrófilos com fenótipos N1 e N2, porém não sendo bem compreendidos os mecanismos efetores que alteram o comportamento funcional do leucócito e seu papel na perpetuação do câncer. Foi observado que o fenótipo N2 favorece o recrutamento de T-regs para a área tumoral e expressa TGF-β, IL-6 e IL-23, sugerindo que essas citocinas em conjunto, promovem o perfil Th17. As novas evidências que surgem abrem espaço para novas perguntas. Essas incógnitas incluem qual o exato espectro de ativação dos neutrófilos, a base molecular para essa plasticidade e não a relevância na ativação, expressão e regulação dessas células.

Com os achados publicados em cultura *in vitro* e *in vivo*, hipotetizamos que o glioma humano é capaz de induzir a polarização imunossupressora dos neutrófilos e isso pode favorecer a progressão tumoral.

5. OBJETIVOS

5.1. Geral:

Avaliar a participação dos neutrófilos na progressão do glioblastoma.

5.2. Específicos:

a) Padronizar modelo de cocultivo 2D e 3D com linhagem U87MG de glioma e neutrófilos humanos de cultivo primário;
b) Avaliar o impacto da comunicação entre neutrófilos e células de GB sobre aspectos morfológicos e funcionais dos neutrófilos, incluindo potencial migratório, de degranulação e formação de NETs;
c) Investigar a contribuição dos neutrófilos sobre a viabilidade e proliferação das células de GB;
d) Determinar se as células tumorais interferem no *life-spam* dos neutrófilos;

e) Realizar uma pesquisa bibliográfica e escrever um artigo de revisão abordando o tema da participação da sinalização purinérgica na funcionalidade de neutrófilos associados ao tumor.

Capítulo 1: ATIVAÇÃO DE NEUTRÓFILOS POR ESTÍMULOS TUMORAIS

(manuscrito em preparação)

Bad influencer: Glioma-neutrophil crosstalk induces neutrophil pro-tumor activation

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ABSTRACT

INTRODUCTION: The permanence of immune cells in the tumor area supports an abnormal microenvironment of chronic inflammation, where neutrophils have an important role. The modulation into a pro-tumoral phenotype is still unknown. Due to the high mortality of glioblastoma and its frequency, we aim to evaluate the neutrophil response in the presence of glioma cells and whether cross-communication has a major influence on these behaviors.

MATERIALS AND METHODS: Neutrophils were isolated from healthy volunteers' peripheral blood and divided in three groups: inactive (N0), IFNγ stimulated neutrophil (N1) and TGFβ stimulated neutrophil (N2). Human U87MG glioma cells were cocultured with neutrophils following 1:30, 1:60, 1:120 proportions. Morphology was evaluated with panoptic staining and DAPI and phalloidin staining. U87MG viability and proliferation was determined after 24, 72 and 120 h of coculture by MTT and SRB assays. Neutrophil migration was done by transwell assay and by time lapse microscopy. Also, we evaluated NETs formation and neutrophil degranulation. Data was analyzed by ANOVA followed by Tukey-Kramer.

RESULTS: TGFβ stimulated cells had more irregular morphology than the other groups, but the average cell area between them had no significant difference. In the transwell assay, it showed higher response when glioma cells were seeded in the bottom chamber, the same was observed with degranulation and NETs assay. It was observed that neutrophils viability cultivated in contact with U87MG had a considerable increase after 120 h of culture when compared to the control group.

CONCLUSION: Cellular crosstalk between tumor and neutrophils has a very important role in tumor progression, but more studies are still needed.

Keywords: Neutrophil, PMN, Neutrophil Plasticity, TAN, Glioblastoma

1. Introduction

The immune system is composed of several innate and adaptive cells, including monocytes, neutrophils and lymphocytes. Among the innate ones, under physiological conditions, neutrophils are the most abundant circulating leukocytes in humans [1]. These cells have a characteristic lobular nucleus that supports and facilitates its migration, and, for that reason, they are also called polymorphonuclear cells (PMN) [2]. They are produced in the bone marrow and have a short life span, depending on constant bone marrow replenishment. They are involved in many immune and inflammatory processes [3, 4, 5], evidence suggests that neutrophils contribute to the resolving process of infectious diseases. However, its hyperresponsive function may help to the perpetuation of chronic diseases, such as sepsis and cancer [1, 6]. The response of PMNs is causally related to the stimulus received from microenvironment, thus, its modulation is impacted by conditions, for instance disease progression, circadian changes, aging and fluctuations in the microbiome [7].

The permanence of immune cells, especially in the tumor area, supports an abnormal microenvironment of chronic inflammation - considered a *hallmark* of cancer [8]. The inflammatory profile documented in tumor microenvironment has been the focus of many studies [9]. The excessive activation of neutrophils is a situation in which the normal function of these cells is remodeled to a pathogenic phenotype and this process of reinstatement is still unknown [10-12]. The neutrophil activation spectrum is under the spotlight for a better understanding of the pro-tumor (N2) and anti-tumor (N1) effects in different conditions.

Immune surveillance is constant. In an immunocompetent person, the body can eradicate mutated cells and promote the fight against cancer formation [111]. However, tumor cells adaptation is something that continues without complete understanding. Glioblastoma cells (GB) can prevent the immune response by having a limited number of antigens and a high capacity to recruit immunosuppressive cells [13]. This type of tumor represents 82% of the total cases of malignant gliomas and its histology shows high cellular and mitotic activity, angiogenesis and necrotic areas. It is highly invasive and infiltrative; however, it is confined to the central nervous system and rarely goes on metastasis. Malignant glioma cells undergo

multiple processes involving sequential and cumulative genetic changes, as a result of intrinsic and environmental factors [14].

The immune infiltrate in the tumor niche has an important role in the tumor perpetuation and progression. These cells are recruited by chemokines secreted by the tumor cell itself. Thus, cancer can induce and maintain a favorable inflammatory environment [15]. Neutrophils are present inside and around solid tumors and, together with macrophages and NK cells, constitute up to 80% of the immune infiltrate. However, it is not yet clear whether the neutrophils associated with the tumor represent an unfavorable factor for the patient [16].

The evidence makes room for questions about the exact spectrum of neutrophil activation and how it alters the main functions of migration, degranulation and NETs formation, and how this modulation affects tumor progression. Hence, our main objective was to evaluate the response of neutrophils in the presence of tumor stimuli and whether cross-communication has a major influence on these behaviors.

2. Materials and Methods

2.1. Material

Chemicals applied in the present study were described in the supplementary Table 1. All other chemicals and solvents were obtained from standard commercial suppliers with analytical grade standard and were used as received.

2.2. Glioma cell line culture

Human U87MG glioma cell line obtained from ATCC (American Type Cell Collection; Rockville, Maryland, USA) was grown in culture flasks with Dulbecco's modified Eagle's medium (DMEM) prepared with 8.4 mM HEPES, 23.8 mM sodium bicarbonate ($NaHCO_3$), 0.1% fungizone, 100 U/L penicillin/streptomycin 0.5 U/L and 10% of heat-inactivated Fetal Bovine Serum (FBS), at final pH of 7.4. Cells were grown at 37°C with 95% minimum relative humidity and 5% CO_2, until reach the confluence.

2.3. Human sample collection and neutrophil isolation

Peripheral blood was collected from healthy volunteers into EDTA or heparin blood collection tubes, within less than 15 min after the donation, the blood was processed as previously described [17]. Briefly, the total blood was diluted with an equal volume of 3% Dextran (Sigma-Aldrich) in saline solution and incubated for 20 min at RT. This process allows the red blood cells sedimentation in order to collect the upper layer with leukocytes. After that, the leukocyte liquid was gently loaded on top of Histopaque-1077 (Sigma-Aldrich) with 1:2 volume proportion, and centrifuge at 1103 g for 30 min, at room temperature, no break. This step is extremely important due to the effective separation of low-density leucocytes from high-density ones. The high-density fraction was further subjected to red blood cell lyses with 0.2% saline solution for 25 sec and neutralization with 1.6% saline solution, to remove the lyses product, the sample was centrifuged for 809 g, 10 min, at RT. The isolated neutrophil was kept in culture with DMEM 10% FBS for 24h, 48h, 72 and 120h. Individuals were informed and signed a written consensus for collecting blood samples. All experiments were performed in accordance with relevant guidelines and regulations (Approval number: CAAE 78664117.0.0000.5345).

2.4. Neutrophil stimulation

Isolated neutrophils were seeded in a 6-well plates (5×10^6 cells per well). They were further divided into three groups: inactive neutrophils (N0) which were cultured in DMEM/10% FBS, 20 ng/mL IFNγ stimulated neutrophils to induce proinflammatory phenotype (N1) [18], and 10 ng/mL TGFβ stimulated neutrophils to induce anti-inflammatory phenotype (N2) [19]. Cells were cultured for 48 h at 37°C with 95% minimum relative humidity and 5% CO_2.

2.5. Glioma-neutrophil 2D co-cultures

U87MG were seeded in 48-well plates (15×10^3 cells/well) and cells were allowed to attach during 90 min. Therefore, neutrophils were added following increasing glioma:neutrophil ratios (1:30, 1:60, 1:120). Cells were co-cultured in DMEM/ 10% FBS for 24, 72 and 120 h.

2.6. Analysis of neutrophil morphology

Immediately before any test protocol with neutrophils, each group was fixed on slides with panoptic staining [20]. Images were taken in an inverted microscope coupled

with a camera (magnification 100× EVOS microscope, Thermofisher Scientific - Waltham, Massachusetts, USA) and then analyzed with Fiji ImageJ software (Bethesda, Maryland, USA) [109]. Neutrophils were also stained with DAPI (1:10,000) and Phalloidin (Alexa 488) (1:500) according to previously described [110].

2.7. MTT assay

U87MG glioma cells and neutrophils were co-cultured as described above (item 2.5). Following 24, 72 and 120 h cell viability was determined by MTT assay [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] as previously described [21]. The absorbance was determined at 560 and 630 nm on the microplate reader (SpectraMax® M3-Molecular Devices, Silicon Valley, California, USA). Data were expressed as absorbance and calculated by the difference from 560 and 630 nm and the cell viability and growth inhibition were considered by 100% and 0%, respectively, of the control group (glioma cells cultured in absence of neutrophils).

2.8. SRB assay

U87MG glioma cells and neutrophils were co-cultured as described above (item 2.5). Following 24, 72 and 120 h cell viability was determined by sulforhodamine B (SRB) assay. Cell cultures were fixed with 10% trichloroacetic acid for 1 h and stained with SRB solution for 15 min, then the excess dye was completely removed by washing repeatedly with 1% acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution. The absorbance was determined at 515 nm using a microplate reader (SpectraMax® M3-Molecular Devices, Silicon Valley, California, USA) [22]. Data were expressed as percentage considering 100% of the control group (glioma cells cultured in absence of neutrophils).

2.9. Trypan-blue assay

Neutrophil viability was measured after the pre-established period (24, 72 and 120 h) per group using trypan-blue assay. Cell viability and death was counted in a Neubauer chamber as described previously [23]. Data were expressed as the percentage of living cells.

2.10. Neutrophil degranulation assay

Immediately after the neutrophil isolation, 4×10^6 cells were added into a microtube with the conditions as mentioned before in a final volume of 250 µL and the following the Turbidimetric determination of lysozyme protocol was assessed [24]. Neutrophils exposed to HBSS or 0.1% Triton™ X-100 (Sigma-Aldrich, St. Louis, Missouri, USA) were applied as negative or positive controls, respectively. Cells were incubated at 37°C with 95% minimum relative humidity and 5% CO_2, for 1 h and the incubation was stopped by putting the microtubes in ice. For the assay, it was added 900 µL of degranulation buffer and *Micrococcus lysodeikticus* ATCC® No. 4698™) (0.25 mg/mL) at a pH 7.0, in a cuvette, after that 100 µL of the supernatant. The absorbance was determined at 450 nm at 1 and 4 min after the mixing. Results were expressed by % of degranulation, using the Triton 100× value as 100% and calculated by the difference between the values at 4 minutes and 1 min.

2.10. Sytox Green: NETs assay

Aliquots of 1×10^6 neutrophils/mL HBSS/Ca^{+2} (1 mL was prepared in microtubes following the conditions - cell cultured with DMEM 10% FBS and another with 0.5% FBS; conditioned media prepared 24h after with U87MG in high confluence also with 10% and 0.5% FBS). The sytox green reagent (Invitrogen, Waltham, Massachusetts, USA) was added to each tube at a final concentration of 0.2 µM. HBSS and 0.1% Triton™ X-100 were used as negative and positive controls, respectively. The incubation was carried out for 3 h at 37°C with 95% minimum relative humidity and 5% CO_2. Then, it was transferred 200 µL of each condition to a separate well of a 96-well black plate and the fluorescence was quantified at excitation/emission wavelengths of 488/520 nm with a microplate reader with KC4™ Data Analysis Software (Winooski, Vermont, USA) [25]. The NETs formation was expressed as relative fold change by dividing the average of the experimental condition by the average of the untreated condition (negative control).

2.11. Transwell assay: Neutrophil transmigration

Neutrophil migration was evaluated using a 24-well transwell chamber (Thermofisher, Scientific - Waltham, Massachusetts, USA) according to Chen et al [26]. For this, 1×10^6/mL neutrophils were seeded in the upper chamber of an 3-µm pore size insert in HBSS/Ca^2 and allowed to migrate toward the lower chamber, which contained: U87MG cells with DMEM 10% and 0.5% FBS, and conditioned

media prepared previously with 10% and 0.5% FBS . Following 3 h of incubation 37°C with 5% CO_2, the cells in the lower chamber were collected, neutrophils were counted in a Neubauer's chamber and the results are expressed as the total amount of neutrophils and the percentage per group [26].

2.12. Time lapse microscopy of neutrophil migration

Neutrophil migration in the 2D cultures was tracked for 20 h obtaining serial images at 5 min intervals. From these images, the migration paths of individual cells were plotted using ImageJ software, and the total distance traveled toward the point source of the tumor cell was measured for each neutrophil. From these data we obtained migration speed and path deviation for each cell. Some neutrophils were pre-treated with 10 μM caffeine (non-selective adenosine receptor antagonist) [27] in order to evaluate if the migration is stimulated by the tumor.

2.13. Statistical analysis

Data were expressed as mean ± S.D. of at least 3 independent experiments and were subjected to one-way analysis of variance (ANOVA) followed by Tukey–Kramer *post-hoc* test (for multiple comparisons) or Bonferroni's test or Student's *t*-test when necessary. Differences between mean values were considered significant when $P < 0.05$.

3. Results

3.1. Neutrophil morphology

In order to characterize morphology alterations associated to neutrophil polarization, PMN cells were exposed to INFγ or TGFβ, N1-proinflammatory and N2-anti-inflammatory stimulators, respectively, or co-cultured with glioma cells and images of panoptic-stained neutrophils were collected from microscope. It was possible to comprehend that neutrophils exposed to TGFβ were more irregular and it was difficult to identify each nuclei lobule, which are characteristic of neutrophils (Fig. 1, panels a, b and c). However, the average cell area between groups had no significant difference, the same was visualized with the nuclei scope (Fig. 1, panels d, e and f). Although there was no statistical relevance, it is important to discuss the wide range of N2-*like* phenotype. The number of lobules was measured as well, and

its morphology contradicts the banana shape that other studies had shown [70-74] (Fig. 1 c). By the other hand, the treatment of neutrophils with INFγ did not induce significant morphological changes. All treated groups were compared with the respective untreated ones, respecting the same culture conditions. Considering the neutrophils cocultured with glioma cells, it was not possible to observe great difference between conditions, nonetheless there is a significant change in the area analysed between 24h and 72h in 1:125 proportion (figure 1 g). It is possible to visualize an increase in the nucleus area, confirming the hypothesis that the nucleus becomes bigger and might be the reason why it is difficult to identify the lobules. In order to exclude any bias, the samples were compared with their own controls.

3.2. Glioma-neutrophil crosstalk modulates neutrophil responses

To test whether glioma cells modulate the human neutrophil chemotaxis, the transwell apparatus was applied. Neutrophils were placed in the upper chamber and allowed to migrate toward to bottom chamber which contained U87MG or U87MG-conditioned medium (CM) in presence or in reduced-FBS conditions (DMEM/10% FBS and DMEM/0.5% FBS, respectively) (Fig. 2). The higher response of neutrophil migration is possible to observe when glioma cells were seeded in the bottom chamber in an optimal environment containing DMEM/10% FBS (3 times increase when compared to control). Glioma cells (DMEM/0.5% FBS) or glioma-CM prepared at both conditions (DMEM/10% or 0.5% FBS) did not promoted neutrophil chemotaxis when compared to the respective controls (1.4 and 1.5 times higher; and 1.75 times lower, respectively). In addition, the effect was smaller when compared to U87-DMEM/10% FBS group as could be observed in the Figure 2 (panel c) that shows the proportion of neutrophils migration in response to different stimulus applied. Accordingly, representative images exhibited on Figure 2 (panel d) indicate the direct neutrophil-glioma interaction, and neutrophils form a cord-like shape structure around glioma cells.

Next, the dynamic of neutrophil migration in presence of glioma cells was tested using time-lapse microscopy. In order to prove that the neutrophil migrates towards the tumor cell, we record glioma-neutrophil cocultured for 20 h, the images were taken in each 5 min and neutrophil was followed until it touches the glioma. Thus, we could evaluate the direction and distance. Caffeine, a non-selective P1 purinergic

antagonist was used to impair cell migration so we could compare the migration with a deficient one. Notably, it was detectable that the neutrophils could migrate long distances and had more accurate direction toward glioma cells, which was impaired by caffeine (Fig. 2, panels e and f; Supplementary video 1 and 2). Taken together, these data suggest that glioma-neutrophil crosstalk is important to stimuli secretion which in turn potentiates neutrophil migration toward cancer cells.

We further evaluated the impact of glioma cells on neutrophil degranulation and NETosis, important functional parameters of these cells [75, 78-80] (Fig. 3). Similarly to previous results, glioma cells cultured in DMEM/10% FBS induced 9 and 3.6 times of neutrophil degranulation when compared to negative and positive controls (cells exposed to HBSS or LPS, respectively) (Fig. 3, panel b). Neutrophils exposed to glioma-CM prepared in DMEM/0.5% FBS also exhibited increased degranulation (5.5 times) when compared to negative control. The Neutrophil Extracellular Traps (NETs) formation was determined only in PMN cells exposed to glioma-CM, as the glioma co-culture protocol may affect the efficiency of the method applied (Fig. 3, panel c). Glioma-CM (DMEM/0.5% or 10% FBS) impaired the NET formation when compared to PMA (positive control), suggesting that soluble factors produced by glioma cells induce a immunosuppressive neutrophil polarization.

3.3 Glioma-neutrophil crosstalk induces tumor cell proliferation and neutrophil survival

Given the results suggesting the impaired anti-tumor functions of glioma-associated neutrophils, we further analyze whether neutrophil-glioma crosstalk impacts glioma cell proliferation and neutrophil lifespan. Fresh neutrophils were cocultured with glioma cells at increasing glioma-neutrophil ratios and their viability were analyzed. Based on the data obtained, it was possible to infer that in the first 24 h of coculture, neutrophil acts as an attacker, decreasing glioma cell viability (Fig. 4, panel a). In the next 72 h it is probably to presume that the viability inhibition is indirectly associated with time of exposure and neutrophil amount. Regarding the 120 h of neutrophil-glioma co-culture, the tumor growth seemed to extrapolate inhibition pattern, which represents the potential modulation of neutrophil. Interestingly, the glioma cell proliferation was inversely proportional to cell viability inhibition (Fig. 4, panel b) and the glioma-neutrophil crosstalk also increased the neutrophil life-span when

compared to neutrophils cultured in absence of tumor cells (Fig. 4, panel c). These data suggest that glioma cells “educate” neutrophils to assume pro-tumor (or N2-like polarization) which in turn favor neutrophil survival.

4. Discussion

The main focus of studies in immunotherapy is concentrated on the behavior of T cells [30-32], however, there is another line of research that covers the other innate immune cells [33-35] and that demonstrate the importance of an effective antitumor response [32, 36-39]. Neutrophils, since they are the first to reach the injury site, are the main innate cells and, only now, have started to be studied as tumor helpers, losing the consolidated certainty of their antitumor role [12]. Notwithstanding, neither the factors that influence its modulation, nor how the functions are altered is well defined, requiring attentive looks and further studies regarding this ambiguous and hidden behavior.

4.1. Neutrophil Migration

The neutrophil activation process begins with stimuli that direct this cell from the bloodstream to the tissue. The chemotaxis migration of PMNs is very characteristic because they respond to a low stimulus concentration, which can be up to 1% over the entire length of the cell body. The migratory process requires signal amplification, autocrine communication and intracellular signal transduction, in the same way that it requires cellular flexibility, provided by the multilobular nucleus shape [26, 40].

The first migration step consists in adhesion to epithelial cells, rolling, firmer adhesion – which are essential to initiate the pre-activation state known as *priming*- and diapedesis. Activation of neutrophils trigger positive feedback loops to maintain cell response [41], operating both autocrine and paracrine [26, 41] with pro-inflammatory stimuli [41, 42].

For neutrophil effector functions to be carried out, it needs to get to the location. Usually, the inflammatory response is due to pathogens [43] and, perhaps because of this, the study of neutrophil modulation in cancer has been slow to happen. Probably due to the difficulty in characterizing the modulation and finding specific human antibodies for analysis. Migration studies using transwell relies on soluble factors previously secreted by the cell or pathogen of interest [44-48]. The absence

of cells adhered to the lower chamber in these experiments may omit an interesting behavior by neutrophils. As we can see in figure 2 a-d, the considerable increase in the number of neutrophils that migrated after 3 h of cultivation to the lower chamber may indicate the secretion of chemokines and cytokines from both cells, showing that crosstalk is relevant when studying cancer, being the best condition for studying behavior.

Tumors, in general, as well as other structural cells that make up the tumor niche, produce chemokines which maintain the pro-inflammatory environment. This two-way contribution provides a favorable situation for the tumor and the neutrophils influx [49]. Among the soluble factors are interleukin-8 (IL-8/CXCL8) [50, 51] - most effective form , extracellular ATP [52-55] - activation of the purinergic system which promotes guided movement, fMLP [56] and activation of CXCR1/2 receivers [57-59], for example. Thus, further studies are needed on which soluble factors are present in the culture medium and assess whether, in addition to these, there are other cytokines that could have this chemotactic role. Gliomas naturally secrete IL-8 [27], but the conditioned medium of these cells did not have a significant influence on the migration of neutrophils, these cells do not suffer external stimuli to react. The reasoning follows the same line when we observe the two conditions with different concentrations of FBS but with the adhered cell, it is possible to observe that U87MG receives stimuli from neutrophils that were activated in the same way that it activated them. The potentiation of the response in a paracrine way mimics, in a certain way, cell-cell communication and shouts its importance. Trying to match the conditions *in vitro* to the maximum with the clinics, even in a controlled environment, shows us that there are several factors, cytokines, metabolic pathways, which are interconnected and active. The complexity of the interaction confirms how little we know about the number of variables that should be considered when the purpose is to discover a key point in choosing a new therapeutic target.

Studies have shown the influence of purinergic signaling on neutrophil migration [55]. The extracellular adenosine molecule activates P1-type receptors, which are involved in the purinergic cascade [55]. In order to prevent the migration of neutrophils and evaluate whether the migration felt to the tumor is due to stimuli secreted in the medium or if it is random, we previously treated the neutrophils with caffeine - a non-selective P1 receptor antagonist - and observed the treated and

untreated migration for a period of 20 h. It was noticed that the distance and trajectory of untreated neutrophils is longer and more accurate, respectively, when compared to the treated ones. Roughly speaking, with the adenosine receptor blocked with an antagonist, the neutrophil is lost in the migration direction. Even receiving strong stimuli such as IL-8 and extracellular ATP, it is unable to complete its *push-and-pull* movement and, consequently, is bewildered (figure 2 e-f). The same behavior was detected in studies that blocked P2 type receptors [60-62], confirming it to be one of the important pathways in neutrophil migration and requiring further studies for its complete understanding and intracellular signaling.

4.2. Neutrophil activation and morphology

The PMN activation process is important for it to perform its functions, it is presented in, mainly, three states: inactive, *priming* and complete activation. *Priming*, despite being a pre-activation state, has direct effects on neutrophil morphology. The cellular flexibility, important for diapedesis, and the increase in adhesion proteins already indicate that the cell is in the process of activation. The deformability of the cytoplasm begins and impacts cell properties and survival [63], so when exposed to inflammation mediators, changes in cytoplasmic actin occur, allowing less deformability and, consequently, permanence in the injured tissue [64].

These cytoplasmic changes were observed in the cells of the groups treated with IFNγ and TGFβ, that is, after polarization for the N1 and N2 profiles, respectively. Deformation is due to the neutrophil activation and the more deformed, the greater is the activation. It is possible to evaluate both in panoptic stained slides and in cocultured cells, that the number of neutrophils with flower shape is directly proportional to the culture time with the glioma cells (figure 1 c/g - 3 a). This deformation influences the cytoplasm/nucleus ratio of each phenotype. We found the presence of a pattern in the cell and nuclear area in the untreated and IFNγ treated groups, however in the TGFβ treated group the cell area is very variable and, certainly, because the standard deviation is very large, there is no statistically significant difference (figure 1 a-e). It is worth mentioning that morphological changes in the immune cells related to their anti or pro-tumor phenotype, can be a low-cost resource to predict the patient's prognosis and response to treatment.

The segmented nucleus characteristic of PMNs, occupies almost a quarter of the total volume. The shape relates to the malleability and flexibility of these cells to leak blood vessels and migrate [65, 66]. In healthy humans, the number of lobules varies from 3 to 4, which can be changed according to activation [67 68]. It is verified that the count of the lobes of the treated and untreated groups varies in a similar way (figure 1 f), however in the inactive neutrophils the visualization of the segments and lobes is easier, while in the IFNγ treatment both the cell and the nucleus appear more rounded and with the nucleus filling more the cytoplasm. Impressively, the nucleus of cells modulated to the N2 profile proved to be irregular and the counting was difficult (figure 1 b/c). PMNs that were seeded with glioma cells present the same irregular morphology and the lobes were also challenging to count. It was possible to visualize the existence of lobes however the constrictions were hard to find, despite that the average number of lobules were about 2-3 (figure 1 h). Therefore, the main hypothesis relies on the fact that the nucleus becomes bigger, explaining why it is considered the lack of segmented lobes in neutrophils associated with the tumor, but it is not clear the absence of constrictions (figure 1 c/g). Besides, it seems that the nucleus volume becomes bigger within time cocultured (figure 1 g).

The comparative nuclear morphology of type N1 and N2 phenotypes has been discussed by many authors [70-74], however there are divergences in the conclusions of how neutrophils are physically faced with the modulation suffered by conventional or tumor inflammatory stimuli. It is commonly agreed, including our data, that there is a difference in the lobular pattern. The not significant difference between the morphologies contributes to this mismatched information. Ocular analysis depends on training and is bound to interpretation bias. That said, it is important to perform computational analysis with a greater number of cells through the *machine learning* technique, improving the fine analysis of these differences and establishing an accurate morphological design.

4.3. Neutrophil degranulation

The neutrophil degranulation process is a potent mechanism against pathogens, being especially harmful if released in an uncontrolled manner. Cell dynamics need to be evaluated in the right context so that the complex kinetic response can be observed [74]. Soon, mobilization begins with the release of vesicles followed by

tertiary granules - composed mainly of gelatinase. These degranulation steps start right after *priming* and this state can be reversed. Subsequently, the secondary granules, also called specific granules are overflowed [75]. They are enriched with metalloproteinase-9 (MMP-9), important in the tumor environment for providing means for angiogenesis [76, 77]. They play an important role in cell biology since they are involved with production of superoxide anion, cell adhesion and diapedesis [75]. The last granules to be released are the primary ones, or azurophilic, are rich in myeloperoxidase (MPO), proteolytic and bactericidal proteins, being directly involved with phagocytosis [75, 78].

It is important to note that in all granules the presence of lysozyme is constant. For this reason, the technique used for screening granulation has appropriated the ability of lysozyme to cause the lysis of bacteria (micrococci). The principle of methodology, although accurate, is limited. The absorbance is measured in its stabilization (time 1 minute) and after 4 min, the difference in absorbance is extrapolated to the amount of bacterial lysis it had. In this test we can see that the ideal condition for future tests is the same in relation to migration (incubation with seeded cells). This confirms, once again, that there are greater stimuli in the presence of the tumor cell than only its soluble factors (figure 3 b).

To understand the granules release kinetics in cocultivation, a flow cytometry assay would specify the granules involved in this situation [75, 78]. There are few studies observing degranulation behavior, mainly in the tumor field, nevertheless, reviews have been published relating the granular content to the ability to assist in tumor progression and metastasis [75]. Neutrophils have been neglected for a long time and now their entire contribution to the development of cancer is being tested. Its effector functions act directly, and it is essential that studies focus on understanding the behavior of neutrophils when challenged by tumor cells, and then assess which metabolic pathways may or may not be involved.

4.4. Neutrophil Extracellular Traps (NETs)

NETs are a nuclear network composed of granular proteins such as elastase (NE) and MPO [79]. This process is considered a mechanism of cell death different from apoptosis and necrosis, since it consists of first disintegrating the nuclear envelope, exposing the content in the cytosol. The detriment of the intercellular membrane

leads to the loss of organelles and, consequently, the integrity of the plasma membrane is impaired [69]. Briefly, the NE translocates to the nucleus and histones partially cleaved, providing the de-chromatin deconditioning [80, 81].

As observed in our results (figure 3 c), there is a small portion of neutrophils that undergo this process after stimulation. This low quantity agrees with other studies [82] and the increase in NETosis in the group treated with conditioned medium of U87MG corroborates with the physiological stimuli described as NET inducers: fMLP, IL-8, LPS [82, 83]. The most favorable situation for the release of the nuclear content is in the presence of PMA and Ionomycin, being considered the positive controls for this method [25].

In the same way that it is necessary to quantify the soluble factors in coculture to determine what is altered and influences the activation of neutrophils, this situation is no different. Phenotypic modulation by tumoral signals has recently been highlighted [12, 84] and, therefore, evaluating the presence of factors such as GM-CSF, IL-8, ATP, and components of oxidative stress, may contribute to the definition of neutrophil functional behavior in different conditions.

Under tumor conditions, NETs have been linked to the formation of metastases, because, theoretically, these bonds capture the circulating tumor cells and facilitate the disposition in other tissues [85, 86]. Nevertheless, the quantification of NETs in patients diagnosed with cancer remains challenging. The first study in this area, in 2013, related the presence of NETs in the tumor niche with a worse prognosis [87], from this other research indirectly links the NETosis process with greater tumor survival [88-90].

As can be inferred, IL-8 appears to be closely linked to several neutrophil functions. It is interesting to note that it has been described as an adjunct to the formation of new vessels, in addition to the innate cell recruitment [88-90]. Inter and intracellular communication leads to a pro-inflammatory environment favorable to the tumor by calling new circulating neutrophils, which can build a new communication network that secretes even more IL-8 into the environment and generates a vicious cycle [16, 84]. The quantification of IL-8 and its receptors in both cells studied is essential to continue the study. Preliminary data from our research group show high rates of IL-8 in the medium containing macrophages from healthy patients and serum from

patients diagnosed with glioma. This strongly suggests the close relationship of this interleukin with the formation of the tumor microenvironment.

4.5. Neutrophil life-spam

Neutrophils are formed from the common myeloid precursor in the bone marrow and circulate for a limited time, being able to be active for approximately 3 days when stimulated [5, 12, 91]. Considering the high rate of neutrophil loss per day, the bone marrow can maintain homeostasis and the total number of circulating neutrophils remains constant [92]. The aging process of the cell is marked by the phenotypic activation triggered by some disturbance [ref]. Thus, fresh cells are those within the bone marrow, while circulating cells that are called aged cells [91, 93].

In the presence of inflammatory inducers, such as bacterial lipopolysaccharides (LPS) and extracellular nucleotides, PMNs perform the function of phagocytosis or degranulation to neutralize these pathogens [94]. These innate cells enter in an apoptosis process soon after the problem is resolved, however, in chronic situations, it is described that neutrophils end up entering the path of cell necrosis [95, 96]. Nonetheless, there is evidence that survival changes when there is cellular hyperresponsiveness, increasing life span by mechanisms are still under study for different chronic inflammatory diseases [97-101].

A transcriptomic study revealed that the signaling pathways between young and aged cells are different both in the activation of effector functions against pathogens and in the processes of cell death [102]. It was also observed the high expression of activation markers, such as CD11b, causally related to cell adhesion [103]. This information allows us to infer that the apoptosis process delay is influenced by neutrophils adhesion, primarily in the epithelial tissue, but, considering tumor microenvironment, the adhesion in other cells. It was possible to observe, during coculture treatment, the difficulty in releasing neutrophils from tumor cells. PMNs are cells in suspension and this characteristic was an experimental surprise, allowing new questions to arise. To understand the behavior of neutrophils and how they are modulated, you need to look at the big picture, all functions and responses to activation appear to be linked to proteins and similar pathways. The complexity of PMNs is fascinating and intriguing. How can a single cell be able to act in different ways using the same mechanisms? We come back to the issue of cell-cell communication between neutrophils and glioma cells, one is stimulated by the other and this is apparently influencing the entire behavior of the PMN.

We observed that the neutrophils viability cultivated in contact with U87MG had a considerable increase after five days in relation to the control group. As shown in figure 4 c, after 72 h we can notice small differences, but not significant, which already indicate the pattern for the next period evaluated (120 hours). It is noticeable that neutrophil modulation indicates onset by the third day while the viability of U87MG in that same period suggests a break in the immune cell attack process against the tumor. In other words, a period of prolonged contact is necessary for the neutrophil to effectively assist in tumor growth (figure 4 a/b).

4.6. Glioma cell viability

The great importance in studying the tumor microenvironment is to understand the crucial role of the interactions that occur there [104, 105]. The tumor niche includes, besides tumor and stromal cells, fibroblasts, immune and endothelial cells [9, 106]. The immune infiltrate, mainly neutrophils, is a feature of chronic inflammation, contributing to tissue damage and this non-resolution of the wound can mediate tumor progression [12]. In order to test the hypothesis that the presence of neutrophils in direct contact with glioma cells can contribute to the increase in tumor viability, we performed the MTT (figure 4 a) and SRB (figure 4 b) assays under different conditions of neutrophil quantity and coculture time. It is possible to identify that in the first 24 hours, PMNs act as expected: they attack tumor cells and have great success in inhibiting the viability of gliomas. This action persists until the next hours and in 72 hours it is possible to observe a slight change in this behavior, suggesting that from that point on, the action of the tumor cell in the modulation of neutrophils seems to have an effect. The neutrophils plasticity happens according to the stimuli received and there are indications that time is also an important factor; other authors also present evidence to support this theory [12, 91, 107, 108]. In 120 hours, it is observed that the tumor reacts to the presence of the neutrophil and its viability increases. This behavior proves the "contact time" factor is a variable that must be considered when studying an immune response in tumor progression.

5. Conclusion and future perspectives

The neutrophil functional complexity goes far beyond the pathogen’s response, it permeates different acute and chronic pathological conditions with causally related

activity to the state of the disease. The functional plasticity of these innate cells, despite being a very consistent theory, there is still lacking evidence. Solid experimental data is needed to confirm the circumstantial indication found. The presence of anti and pro-tumoral phenotypes in tumor microenvironment and in blood circulation play a fundamental role in the disease. The N2-like profile with its immunosuppressive properties acts to assist tumor development, however, how these functions work and whether there are changes in the *modus operandi* is not yet elucidated. An important element about its functionality is that there is no characterization of specific behavior for each phenotype; and in this study it was observed that cell communication, direct contact and time were important study factors for neutrophils and tumor cells (Figure 05). To determine the phenotype and relate to neutrophil behavior, specific molecular markers are needed, and these are still being defined. Neutrophils, once so well understood, have become a lot of question marks. The scenario involving modulation of PMNs and contribution to the non-resolution of chronic diseases is confusing; it is mysterious; so, this subject is growing in academia and studies related to tumors are emerging quickly. The immune infiltrate in the tumor microenvironment brings evidence of the importance in cancer progression, and, very importantly, the tumor ability to modulate the immune response. Cellular crosstalk is the key point to evaluate behaviors and consequences.

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7. Conflicts of interest

The authors declare that there are no conflicts of interest.

8. References

1. Mócsai A. et al. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med. 2013. 210: 1283-1299
2. Manley HR, Keightley MC, Lieschke GJ. The neutrophil nucleus: an important influence on neutrophil migration and function. Frontiers in immunology. 2018 Dec. 4;9:2867.
3. Mantovani, A., Cassatella, M. A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. 2011. 11, 519–531
4. Lawrence SM, Corriden R, Nizet V. The ontogeny of a neutrophil: mechanisms of granulopoiesis and homeostasis. Microbiol Rev. 2012.
5. Görgens, A., Radtke, S., Möllmann, M., Cross, M., Dürig, J., Horn, P. A., et al. Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell Rep. 2013. 3, 1539–1552.
6. Coffelt, S. B., Wellenstein, M. D. & de Visser, K. E. Neutrophils in cancer: neutral no more. Nat. Rev. Cancer. 2016. 16, 431–446.
7. Nemeth T., Sperandio M., Mócsai A. Neutrophils as emerging therapeutic target. Nature Reviews Drug Discovery. 2020. 19, 253-275.
8. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016. 388: 2023-2038
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011 Mar 4;144(5):646-74.
10. Gustafson M. P., Lin Y., New K.C., Bulur P.A., O'Neill B.P., Gastineau D.A., Dietz A.B., Systemic immune suppression in glioblastoma: the interplay between CD14+ HLA-DR monocytes, tumor factors and dexamethasone. Neuro-oncology. 2010. 631-644.
11. Moses, K. & Brandau, S. Human neutrophils: their role in cancer and relation to myeloid- derived suppressor cells. Semin. Immunol. 2016. 28, 187–196.
12. Giese MA, Hind LE, Huttenlocher A. Neutrophil plasticity in the tumor microenvironment. Blood. 2019 May 16;133(20):2159-67.
13. Nørøxe D.S., Poulsen H.S., Lassen U. Hallmarks of glioblastoma: a systematic review. ESMO Open. 2016. 1(6).
14. Omuro, A et al. Glioblastoma and Other Malignant Gliomas. JAMA. 2013. 310(17), 1842.
15. Di Virgilio F. et al. Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. Nat Rev Cancer. 2018. 18 (10): 601-618.
16. Erpenbeck L., Schön MP. Neutrophil extracellular traps: protagonists of cancer progression? Oncogene. 2017. 4;36(18): 2483-2490.
17. Oh H., Siano B., Diamond S. Neutrophil Isolation Protocol. J Vis Exp. 2008.
18. Andzinski L., Kasnitz N., Stahnke S., Wu C.F., Gereke M., von KockritzBlickwede M., Schilling B., Brandau S., Weiss S., Jablonska J. Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. Int J Cancer. 2016; 138:1982-93.
19. Fridlender Z.G., Sun J., Kim S., Kapoor V., Cheng G., Ling L., Worthen G.S., Albelda S.M. Polarization of tumor-associated neutrophil phenotype by TGF-beta: “N1” versus “N2” TAN. Cancer Cell. 2009;16:18394.
20. Paulíková E., Kociková A., Sabol M. Modification of a panoptic method of staining isolated cells. Bratislavske lekarske listy. 1993 Dec; 94(12):638-40.
21. AZAMBUJA, J. et al. Inhibition of the Adenosinergic Pathway in Cancer Rejuvenates Innate and Adaptive Immunity. International journal of molecular sciences. 2019. v. 20, n. 22, p. 5698.
22. Vichai, V., Kirtikara, K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 2006. 1, 1112–1116.
23. Crowley, L. C., Marfell, B. J., Christensen, M. E., & Waterhouse, N. J. Measuring Cell Death by Trypan Blue Uptake and Light Microscopy. Cold Spring Harbor Protocols, 2016.
24. Mörsky P. Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: reexamination of reaction conditions. Analytical biochemistry. 1983 Jan 1;128(1):77-85.
25. Carmona-Rivera, C., Kaplan, M.J. Induction and quantification of NETosis. Curr. Protoc. Immunol. 2016. 115:14.41.1-14.41.14.
26. CHEN Y. et al. ATP Release Guides Neutrophil Chemotaxis via P2Y2 and A3 Receptors. Science. 2006. 15;314(5806):1792-5.
27. Braganhol E., Kukulski F., Lévesque S.A., Fausther M., Lavoie E.G., Zanotto-Filho A., Bergamin L.S., Pelletier J., Bahrami F., Yebdri F.B., Moreira J.C. Nucleotide receptors control IL-8/CXCL8 and MCP-1/CCL2 secretions as well as proliferation in human glioma cells. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2015 Jan 1;1852(1):120-30.

28. Gao B. et al. Fabrication of three dimensional islet models by the geometry controlled hanging drop method. Acta Mechanica Sinica. 2019. volume 35, pages329–337

29. Friedrich J., Seidel C., Ebner R., Kunz-Schughart L. Spheroid-based drug screen: considerations and practical approach. Nature Protocols. 2009; 4(3):309-324.

30. Blanc C., Hans S., Tran T., Granier C., Saldman A., Anson M., Oudard S. and Tartour E. Targeting Resident Memory T Cells for Cancer Immunotherapy. Front. Immunol. 2018. 9:1722.

31. Gajewski T.F., Corrales L., Williams J., Horton B., Sivan A., Spranger S. Cancer immunotherapy targets based on understanding the T cell-inflamed versus non-T cell-inflamed tumor microenvironment. InTumor Immune Microenvironment in Cancer Progression and Cancer Therapy. 2017 (pp. 19-31).

32. Hernandez C., Arasanz H., Chocarro L., Bocanegra A., Zuazo M., Fernandez-Hinojal G., Blanco E., Vera R., Escors D., Kochan G. Systemic Blood Immune Cell Populations as Biomarkers for the Outcome of Immune Checkpoint Inhibitor Therapies. International Journal of Molecular Sciences. 2020 Jan;21(7):2411.

33. Cheng G., Zhang N., Wang Y., Rui J., Yin X., Cui T. Antibodies of IgG, IgA and IgM against human bronchial epithelial cell in patients with chronic obstructive pulmonary disease. Clin Lab. 2016;62(6):11 01–1108.

34. Karayama M., Inui N., Suda T., Nakamura Y., Nakamura H., Chida K. Antiendothelial cell antibodies in patients with COPD. Chest. 2010;138(6):1303–1308.

35. Demaria O., Cornen S., Daëron M., Morel Y., Medzhitov R., Vivier E. Harnessing innate immunity in cancer therapy. Nature. 2019 Oct;574(7776):45-56.

36. Chen Z. and Hambardzumyan D. Immune Microenvironment in Glioblastoma Subtypes. Front. Immunol. 2018. 9:1004.

37. Villanueva M.T. et al. Macrophages get a CAR. Nature Reviews Immunology. 2020 Apr 6:1.

38. Cassetta L., Pollard J.W. Targeting macrophages: therapeutic approaches in cancer. Nature Reviews Drug Discovery. 2018 Dec;17(12):887-904.

39. Muntasell A., Ochoa M.C., Cordeiro L., Berraondo P., de Cerio A.L., Cabo M., López-Botet M., Melero I. Targeting NK-cell checkpoints for cancer immunotherapy. Current opinion in immunology. 2017 Apr 1;45:73-81.

40. Salvermoser M., Begandt D., Alon R. and Walzog B. Nuclear Deformation During Neutrophil Migration at Sites of Inflammation. Front. Immunol. 2018. 9:2680.

41. Németh, T. & Mócsai, A. Feedback amplification of neutrophil function. Trends Immunol. 2016. 37, 412–424.

42. Kovács, M. et al. The Src family kinases Hck, Fgr, and Lyn are critical for the generation of the in vivo inflammatory environment without a direct role in leukocyte recruitment. J. Exp. Med. 2014. 211, 1993–2011.

43. Parkos, C. A. Neutrophil-Epithelial Interactions: A Double-Edged Sword. Am J Pathol. 2016. 186, 1404–1416.

44. Deng Y. et al. An in vitro transepithelial migration assay to evaluate the role of neutrophils in Respiratory Syncytial Virus (RSV) induced epithelial damage. Scientific Reports. 2018. 8(1), 6777

45. Yamahashi, Y., Cavnar, P. J., Hind, L. E., Berthier, E., Bennin, D. A., Beebe, D., & Huttenlocher, A. Integrin associated proteins differentially regulate neutrophil polarity and directed migration in 2D and 3D. Biomedical microdevices. 2015. 17(5), 100.

46. Zaki S. S. O., Kanesan L., Leong M. Y. D., & Vidyadaran, S. The influence of serum-supplemented culture media in a transwell migration assay. Cell Biology International. 2019.

47. Yang K., Wu J., Xu G., Xie D., Peretz-Soroka H., Santos S., Alexander M., Zhu L., Zhang M., Liu Y., Lin F. A dual-docking microfluidic cell migration assay (D2-Chip) for testing neutrophil chemotaxis and the memory effect. Integrative Biology. 2017 Apr 1;9(4):303-12.

48. Chan E.C., Ren C., Xie Z., Jude J., Barker T., Koziol-White C.A., Ma M., Panettieri R.A., Wu D., Rosenberg H.F., Druey K.M. Regulator of G protein signaling 5 restricts neutrophil chemotaxis and trafficking. Journal of Biological Chemistry. 2018 Aug 17;293(33):12690-702.

49. Rapoport B.L., Steel H.C., Theron A.J., Smit T., Anderson R. Role of the Neutrophil in the Pathogenesis of Advanced Cancer and Impaired Responsiveness to Therapy. Molecules 2020, 25, 1618.

50. Verbeke H., Struyf. S., Laureys G., Van Damme J. The expression and role of CXC chemokines

in colorectal cancer. Cytokine Growth Factor Rev. 2011, 22, 345–358

51. Najjar Y.G., Rayman P., Jia X., Pavicic P.G., Jr., Rini B.I., Tannenbaum C., Ko J., Haywood S., Cohen P., Hamilton T.. Myeloid derived suppressor cell subset accumulation in renal cell carcinomaparenchyma is associated with intratumoral expression of IL1β, IL8, CXCL5, and Mip-1α. Clin. Cancer Res. 2017, 23, 2346–2355.

52. Fredholm BB et al. Purines and neutrophil leukocytes. General Pharmacology: The Vascular System. 1997 Mar 1;28(3):345-50.

53. Chen Y., Shukla A., Namiki S., Insel P.A., Junger W.G. A putative osmoreceptor system that controls neutrophil function through the release of ATP, its conversion to adenosine, and activation of A2 adenosine and P2 receptors. Journal of leukocyte biology. 2004 Jul;76(1):245-53.

54. Grassi F., et al. Purinergic control of neutrophil activation. Journal of molecular cell biology. 2010 Aug 1;2(4):176-7.

55. Junger W.G., et al. Immune cell regulation by autocrine purinergic signalling. Nature Reviews Immunology. 2011 Mar;11(3):201-12.

56. Lu Q., Yuan K., Li X., Jiang H., Huo G., Jia W., Huang G., Xu A. Detecting Migration and Infiltration of Neutrophils in Mice. Journal of visualized experiments: JoVE. 2020 Feb 6(156).

57. Jamieson T., Clarke M., Steele C.W., Samuel M.S., Neumann J., Jung A., Huels D., Olson M.F., Das S., Nibbs R.J. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. J. Clin. Investig. 2012, 122, 3127–3144.

58. Lu X., Horner J.W., Paul E., Shang X., Troncoso P., Deng P., Jiang S., Chan Q., Spring D.J., Sharma P. Effective combinatorial immunotherapy for castration-resistant prostate cancer. Nature 2017, 543, 728–732.

59. Rennard S.I., Dale D.C., Donohue J.F., Kanniess F., Magnussen H., Sutherland E.R., Watz H., Lu S., Stryszak P., Rosenberg E. CXCR2 antagonist MK-7123. A phase 2 proof-of-concept trial for chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 2015, 191, 1001–1011.

60. Ayata CK, Ganal SC, Hockenjos B, Willim K, Vieira RP, Grimm M, Robaye B, Boeynaems JM, Di Virgilio F, Pellegatti P, Diefenbach A. Purinergic P2Y2 receptors promote neutrophil infiltration and hepatocyte death in mice with acute liver injury. Gastroenterology. 2012 Dec 1;143(6):1620-9.

61. Ben Yebdri F., Kukulski F., Tremblay A., Sévigny J. Concomitant activation of P2Y2 and P2Y6 receptors on monocytes is required for TLR1/2-induced neutrophil migration by regulating IL-8 secretion. European journal of immunology. 2009 Oct;39 (10):2885-94.

62. Chen Y., Yao Y., Sumi Y., Li A., To U.K., Elkhal A., Inoue Y., Woehrle T., Zhang Q., Hauser C., Junger W.G. Purinergic signaling: a fundamental mechanism in neutrophil activation. Sci. Signal. 2010 Jun 8;3(125).

63. Vogt K. L., Summers C., Condliffe A. M. The clinical consequences of neutrophil priming. Current Opinion in Hematology. 2018.

64. Condliffe A. M., Kitchen E., Chilvers E. R. Neutrophil Priming: Pathophysiological Consequences and Underlying Mechanisms. Clinical Science. 1998. 94(5), 461–471.

65. Olins A.L., Zwergerb M., Herrmannb H., Zentgraf H., Simond A.J., Monestier M., Olins D.E. The human granulocyte nucleus: Unusual nuclear envelope and heterochromatin composition. European Journal of Cell Biology. 2008. 87, 279–290.

66. Olins A.L., Hoang T.V., Zwerger M., Herrmann H., Zentgraf H., Noegel A.A., Karakesisoglou I., Hodzic D., Olins D.E. The LINC-less granulocyte nucleus. Eur. J. Cell Biology. 2008.

67. Campbell MS, Lovell MA, Gorbsky GJ. Stability of nuclear segments in human neutrophils and evidence against a role for microfilaments or microtubules in their genesis during differentiation of HL6O myelocytes. Journal of Leukocyte Biology. 1995. Volume 58.

68. Hoffmann K, Sperling K, Olins AL and Olins DE. The granulocyte nucleus and lamin B receptor: avoiding the ovoid. Chromosoma. 2007. 116(3):227-35

69. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A. Novel cell death program leads to neutrophil extracellular traps. The Journal of Cell Biology. 2007. Vol. 176, No. 2, 231–241.

70. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, Damti P, Lumbroso D, Polyansky L, Sionov RV, Ariel A. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. Cell reports. 2015 Feb 3;10(4):562-73.

71. Granot Z, Jablonska J. Distinct functions of neutrophil in cancer and its regulation. Mediators of inflammation. 2015.

72. Saha S, Biswas SK. Tumor-associated neutrophils show phenotypic and functional divergence in human lung cancer. Cancer cell. 2016 Jul 11;30(1):11-3.

73. Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. Critical reviews in oncology/hematology. 2012 Jun 1;82(3):296-309.

74. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. Trends in immunology. 2007 Aug 1;28(8):340-5.

75. Mollinedo F. Neutrophil degranulation, plasticity, and cancer metastasis. Trends in immunology. 2019 Feb 15.

76. Deryugina EI, Zajac E, Juncker-Jensen A, Kupriyanova TA, Welter L, Quigley JP. Tissue-infiltrating neutrophils constitute the major in vivo source of angiogenesis-inducing MMP-9 in the tumor microenvironment. Neoplasia. 2014 Oct 1;16(10):771-88.

77. Gonzalez-Avila, G., Sommer, B., García-Hernández, A. A., & Ramos, C. Matrix Metalloproteinases' Role in Tumor Microenvironment. Tumor Microenvironment. 2020. 97–131.

78. Cassatella MA, Östberg NK, Tamassia N, Soehnlein O. Biological roles of neutrophil-derived granule proteins and cytokines. Trends in immunology. 2019 May 30.

79. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004. 303. 1532-1535

80. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018. 18(2): 134-147.

81. Metzler KD, Goosmann C, Lubojemska A, Zychlinsky A, Papayannopoulos V.. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. 2014. Cell Rep. 8: 883-896.

82. Arpinati L., et al. NETosis in cancer: a critical analysis of the impact of cancer on neutrophil extracellular trap (NET) release in lung cancer patients vs. mice. Cancer Immunol Immunother. 2020 Feb;69(2):199-213.

83. Phillipson M, Kubes P. The neutrophil in vascular inflammation. Nat Med. 2011. 17:1381–1390

84. Mugaku B., et al. Neutrophil Extracellular Trap Formation Correlates with Favorable Overall Survival in High Grade Ovarian Cancer. Cancers (Basel). 2020 Feb 21;12(2).

85. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, Bourdeau F, Kubes P, Ferri L. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest. 2013. 123:3446–3458.

86. Park J., et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med. 2016 Oct 19;8(361).

87. Berger-Achituv S, Brinkmann V, Abed UA, Kuhn LI, Ben-Ezra J, Elhasid R et al. A proposed role for neutrophil extracellular traps in cancer immunoediting. Front Immunol 2013; 4: 48.

88. Zhao JJ, Pan K, Wang W, Chen JG, Wu YH, Lv L et al. The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. PloS One 2012; 7: e33655.

89. Perisanidis C, Kornek G, Poschl PW, Holzinger D, Pirklbauer K, Schopper C et al. High neutrophil-to-lymphocyte ratio is an independent marker of poor diseasespecific survival in patients with oral cancer. Med Oncol 2013; 30: 334.

90. Xiao WK, Chen D, Li SQ, Fu SJ, Peng BG, Liang LJ. Prognostic significance of neutrophil-lymphocyte ratio in hepatocellular carcinoma: a meta-analysis. BMC Cancer 2014; 14: 117

91. Rosales C. et al, Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Front. Physiol. 2018. 9:113.

92. Von Vietinghoff S., Ley K. Homeostatic regulation of blood neutrophil counts. J. Immunol. 2008. 181, 5183–5188.

93. Adrover J.M., Nicolás-Ávila J. A., and Hidalgo, A. Aging: a temporal dimension for neutrophils. Trends Immunol. 2016. 37, 334–345.

94. Ulevitch R.J., Tobias P.S. Recognition of gram-negative bacteria and endotoxin by the innate immune system. Curr Opin Immunol. 1999;11:19–22.

95. Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. J Innate Immun. 2010;2:216–27.

96. Presbitero A., et al. Game of neutrophils: modeling the balance between apoptosis and necrosis. BMC Bioinformatics 2019, 20(Suppl 6):475.

97. Li KX, Zheng LX, Guo HQ, Hong FF, Yang SL. LTB4-induced anti-apoptosis and infiltration of neutrophils in rheumatoid arthritis. Clinical and Experimental Rheumatology. 2020 Feb 14.

98. Ibrahim SA, Kulshrestha A, Katara GK, Riehl V, Sahoo M, Beaman KD. Cancer associated V-ATPase induces delayed apoptosis of pro-tumorigenic neutrophils. Molecular Oncology. 2020 Jan 10.

99. Aga E, Mukherjee A, Rane D, More V, Patil T, van Zandbergen G, Solbach W, Dandapat J, Tackenberg H, Ohms M, Sarkar A. Type-1 interferons prolong the lifespan of neutrophils by interfering with members of the apoptotic cascade. Cytokine. 2018 Dec 1;112:21-6.

100. Nagaoka I, Suzuki K, Murakami T, Niyonsaba F, Tamura H, Hirata M. Evaluation of the effect of α-defensin human neutrophil peptides on neutrophil apoptosis. International journal of molecular medicine. 2010 Dec 1;26(6):925-34.

101. Ley K, Hoffman HM, Kubes P, Cassatella MA, Zychlinsky A, Hedrick CC, Catz SD. Neutrophils: New insights and open questions. Science immunology. 2018 Dec 7;3(30).

102. Zhang, D., Chen, G., Manwani, D., Mortha, A., Xu, C., Faith, J. J., et al. Neutrophil ageing is regulated by the microbiome. Nature. 2015. 525, 528–532.

103. Jaiswal, S., Jamieson, C. H., Pang, W. W., Park, C. Y., Chao, M. P., Majeti, R., et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 2009. 138, 271–285.

104. Domingues PC, Gonza´lez-Tablas M, Otero A´, et al. Tumor infiltrating immune cells in gliomas and meningiomas. Brain Behav Immun. 2016; 53(C):1-15.

105. Wu S. et al. The Prognostic Landscape of TumorInfiltrating Immune Cells and Immune Checkpoints in Glioblastoma. Technol Cancer Res Treat. 2019; 18: 1533033819869949.

106. Uribe-Querol E., et al. Neutrophils in Cancer: Two Sides of the Same Coin. Journal of Immunology Research. 2015.

107. Buckley CD, Ross EA, McGettrick HM, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. J Leukoc Biol. 2006;79(2):303-311.

108. Patel S, Fu S, Mastio J, et al. Unique pattern of neutrophil migration and function during tumor progression. Nat Immunol. 2018;19(11):1236-1247.

109. Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", Nature methods 9(7): 676-682, PMID 22743772, doi:10.1038/nmeth.2019

110. Jonkman J, Brown CM. Any way you slice it—a comparison of confocal microscopy techniques. Journal of biomolecular techniques: JBT. 2015 Jul;26(2):54.

111. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. Annual Review of Pathology: Mechanisms of Disease. 2014 Jan 24;9:181-218.

9. List supplementary of tables

Reagent	Composition
DMEM	2g DMEMlow glucose, 0.74g sodium bicarbonate, 0.625g HEPES, 2ml antibiotic (penicillin + streptomycin), 200ul fungizone (Amphotericin B)/200ml milliQ water
Dextran 3%	3g Dextran/0.9g NaCl/100ml milliQ water
PBS 1x	100ml milliQ water / 0,8g NaCl, 0,02g KCl, 0,115g Na_2HPO_4 e 0,02g KH_2PO_4 pH 7.4
0.5% BSA	0.5g BSA/100ml PBS 1x
Lyse solution NaCl 0,2%	0.2g NaCl / 100ml milliQ water
Lyse stop solution NaCl 1,6%	1.6g NaCl / 100ml milliQ water
Solução de Sulforodamina B (SRB) 0,4%	0.04g SRB / 10ml acetic acid 1%
Acetic acid 1%	1ml glacial acetic acid / 100ml water
Tris base 10mmol/L pH 10,5	0.12g Trisma / 100ml distilled water
Degranulation Buffer (phosphate buffer)	30.5ml K2HPO4 0.1M (1.74g/100ml) + 18.5ml KH2PO4 0.1M (1.36g/100ml)
Micrococcus solution	125mg Micrococcus/5ml phosphate buffer

Table 1: List of reagents prepared *in house*

Video hyperlink
Neutrophil-glioma migration without caffeine
Neutrophil-glioma migration with caffeine

Table 2: List of hyperlinks to the migration videos

10. List of figures

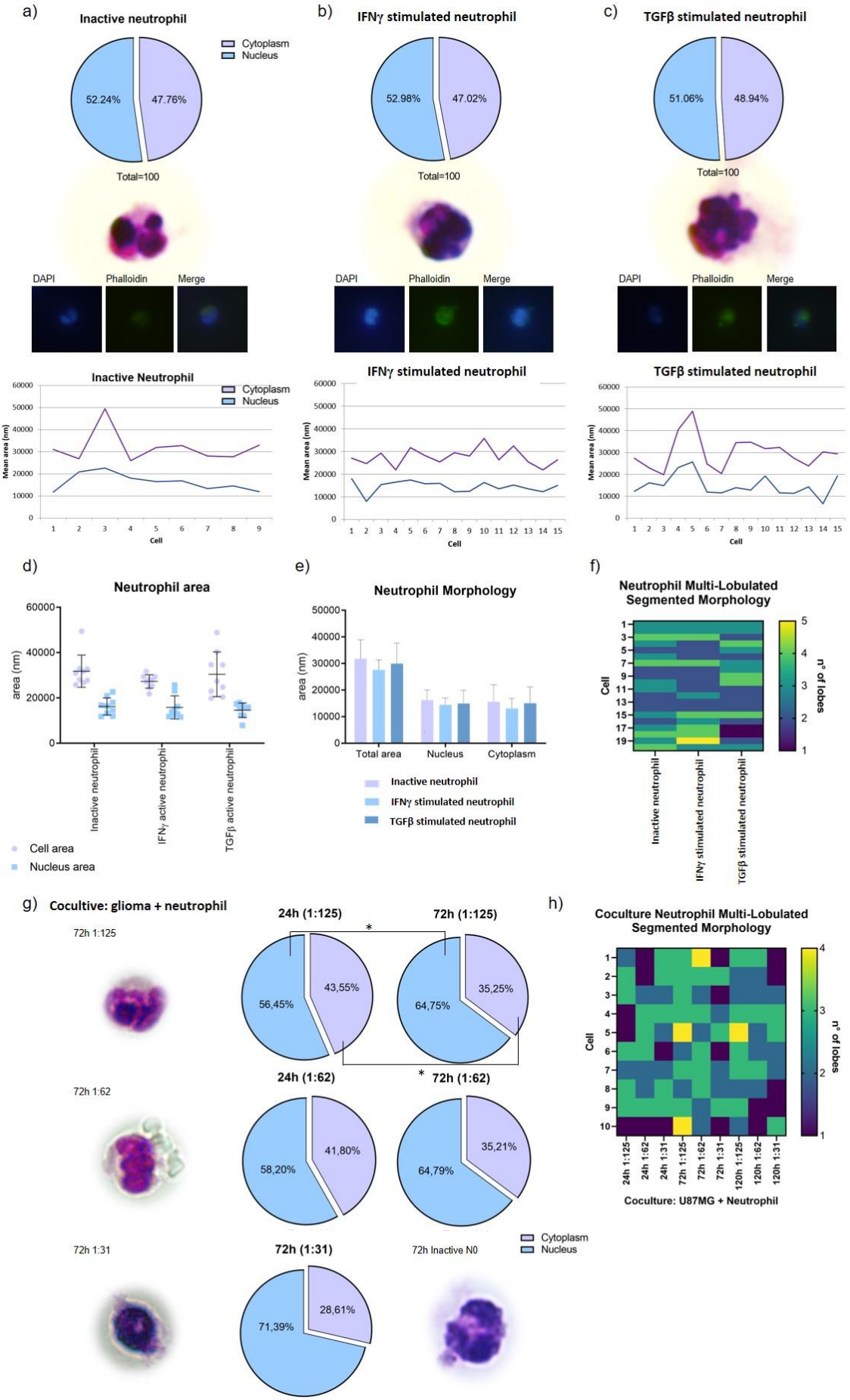
a)
Inactive neutrophil
Cytoplasm
Nucleus
52.24%
47.76%
Total=100
DAPI
Phalloidin
Merge
b)
IFNγ stimulated neutrophil
52.98%
47.02%
Total=100
DAPI
Phalloidin
Merge
c)
TGFβ stimulated neutrophil
51.06%
48.94%
Total=100
DAPI
Phalloidin
Merge
Inactive Neutrophil
IFNγ stimulated neutrophil
TGFβ stimulated neutrophil
Mean area (nm)
Cell
d)
Neutrophil area
area (nm)
Inactive neutrophil
IFNγ active neutrophil
TGFβ active neutrophil
Cell area
Nucleus area
e)
Neutrophil Morphology
area (nm)
Total area
Nucleus
Cytoplasm
Inactive neutrophil
IFNγ stimulated neutrophil
TGFβ stimulated neutrophil
f)
Neutrophil Multi-Lobulated Segmented Morphology
Cell
n° of lobes
Inactive neutrophil
IFNγ stimulated neutrophil
TGFβ stimulated neutrophil
g)
Cocultive: glioma + neutrophil
72h 1:125
24h (1:125)
72h (1:125)
56,45%
43,55%
64,75%
35,25%
72h 1:62
24h (1:62)
72h (1:62)
58,20%
41,80%
64,79%
35,21%
72h 1:31
72h (1:31)
71,39%
28,61%
72h Inactive N0
Cytoplasm
Nucleus
h)
Coculture Neutrophil Multi-Lobulated Segmented Morphology
Cell
n° of lobes
24h 1:125
24h 1:62
24h 1:31
72h 1:125
72h 1:62
72h 1:31
120h 1:125
120h 1:62
120h 1:31
Coculture: U87MG + Neutrophil

Figure 1: Neutrophil morphology. a) Inactive neutrophil. Average size of inactive neutrophils and its nucleus. Neutrophil image by panoptic coloration. Neutrophils stained with DAPI, phalloidin and both, highlighting the nucleus and the cytoskeleton, respectively. Size of each cell compared to its nucleus. b) IFN active neutrophil. Average size of IFN g active neutrophils and its nucleus. Neutrophil image by panoptic coloration. Neutrophils stained with DAPI, phalloidin and both, highlighting the nucleus and the cytoskeleton, respectively. Size of each cell compared to its nucleus. c) TGF active neutrophil. Average size of TGF B active neutrophils and its nucleus. Neutrophil image by panoptic coloration. Neutrophils stained with DAPI, phalloidin and both, highlighting the nucleus and the cytoskeleton, respectively. Size of each cell compared to its nucleus. d) Neutrophil area. Cell and nucleus area of inactive neutrophil, IFNg active neutrophil and TGF B active neutrophil. e) Neutrophil morphology. Morphology comparing total area, nucleus and cytoplasm of inactive neutrophil, IFN g active neutrophil and TGF B active neutrophil. f) Neutrophil multi-lobulated segmented morphology. Number of lobes in the nucleus of inactive neutrophil, IFNg active neutrophil and TGFB active neutrophil. g) Cocultive of U87MG and neutrophil: Panoptic coloration of neutrophil in contact with U87MG for 72h in the proportions of 1 (U87MG) : 125 (Neutrophil) and 1:62, respectively. Graphics representing the total cell area and its nucleus in 24h and 72h in the proportion of 1:125 1:62 and 1:31 (* = $p<0.05$) h) Coculture neutrophil multi-lobulated segmented morphology. Number of lobes in the neutrophil nucleus according to time and concentration in the cocultive (U87MG and neutrophils).

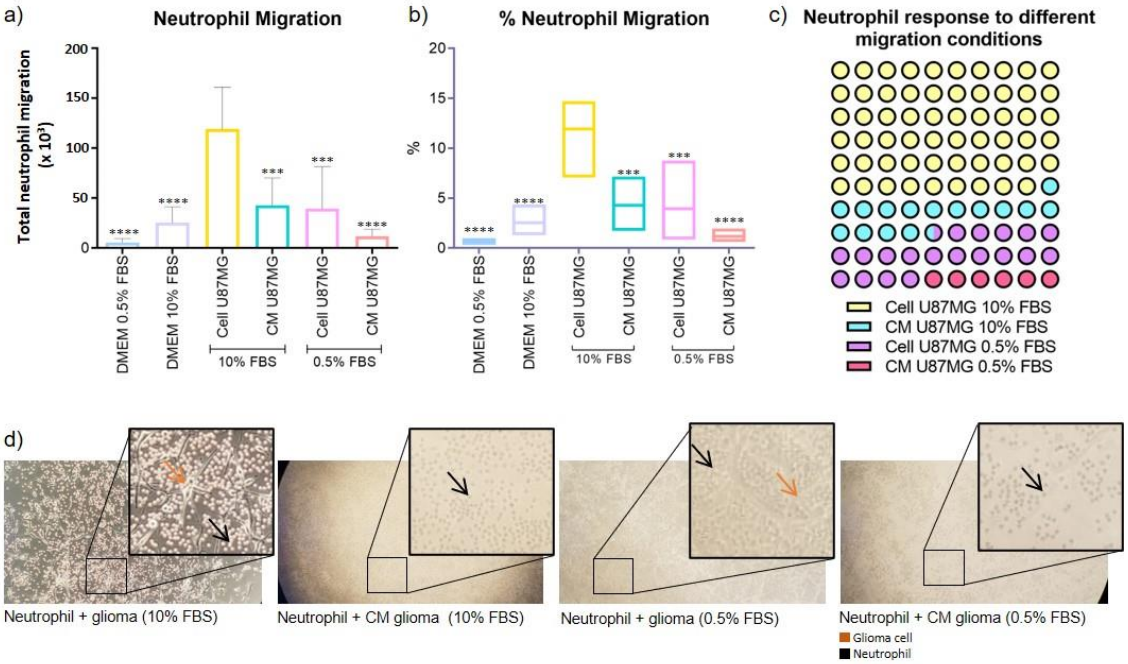

Figure 2: Neutrophil migration. a) total of neutrophil migrated to the bottom chamber using Transwell assay. Initial 1,000,000 neutrophils in the upper chamber. b) percentage of neutrophil migration. c) conditions compared to visualize the better situation for neutrophil-glioma cells response. d) neutrophil disposed of in the bottom chamber after 3h, optical microscope, 40x. e) neutrophil migration towards tumor cells, neutrophil were followed through frames after 20h exposure. f) neutrophil pre-treated with caffeine demonstrates irregular migration. (*** = p<0.001 **** = p< 0.0001 compared to Cell U87MG 10%FBS)

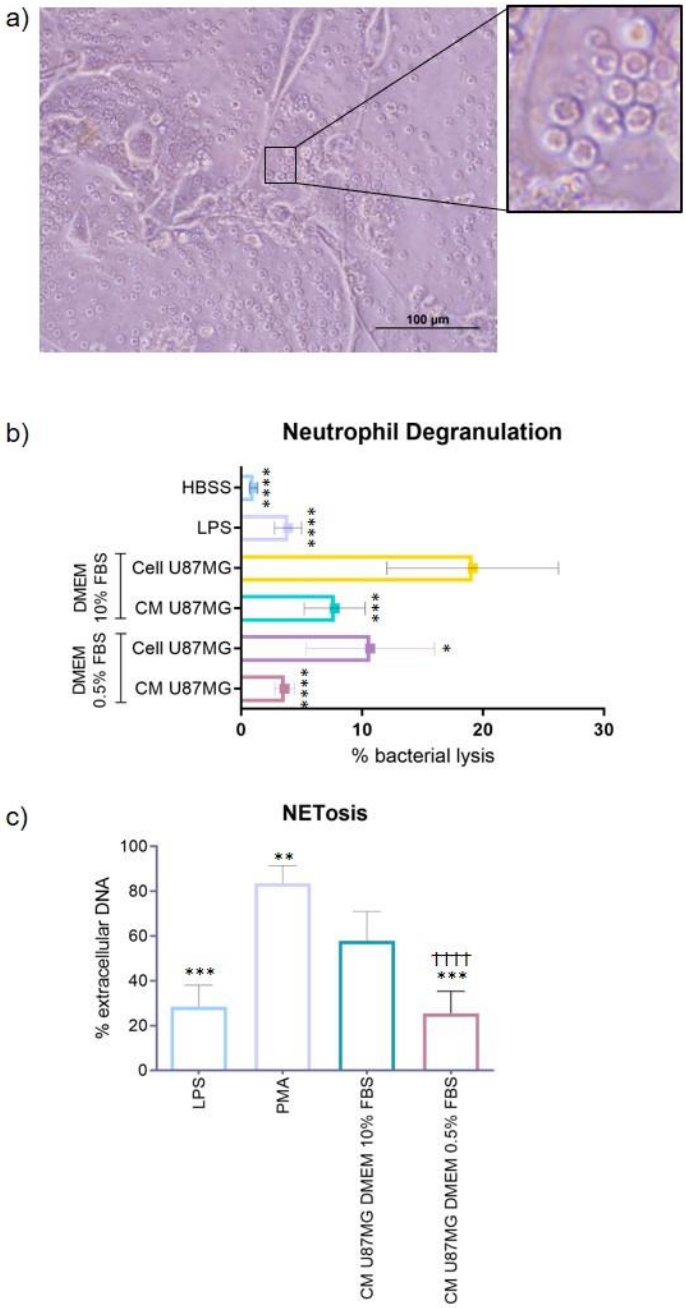

Figure 3: Neutrophil functions. a) cocultured neutrophil and glioma cells (U87MG) after 72h demonstrates neutrophil irregular morphology (flower shape). b) neutrophil degranulation observed after 1h incubation. The amount of lysozyme release is directly related to bacterial lysis, which means that more bacterial lysis, more lysozyme in the media and more neutrophil granules. The total percentage is calculated using Triton 100x as 100%. (* = p<0.05 *** = p<0.001 **** = p< 0.0001 compared to Cell U87MG 10%FBS) c) NETs formation observed after 3h incubation with SytoxGreen reagent. (** = p<0.01 *** = p<0.001 compared to CM U87MG 10%FBS and † compared to PMA)

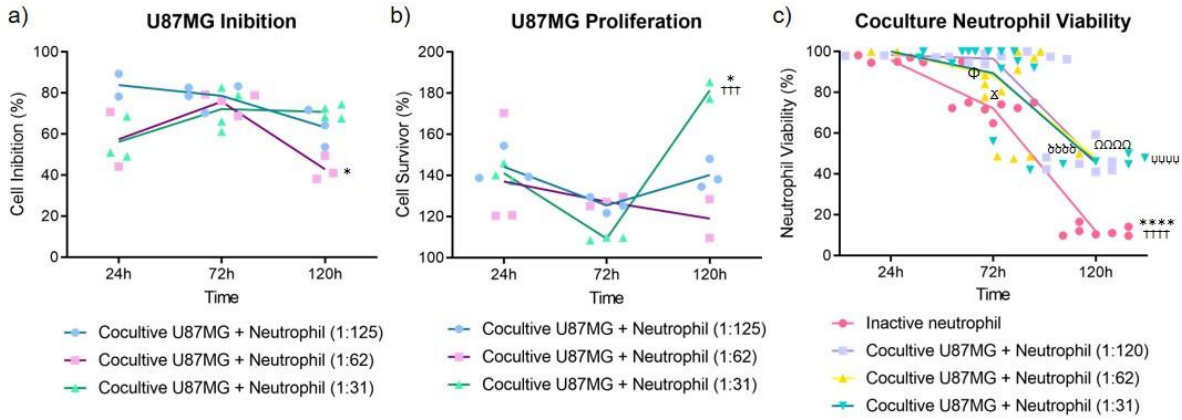

Figure 4: Tumor progression and neutrophil modulation. a) U87MG inhibition after 1h 30min MTT assay, using 0% inhibition of the untreated condition, which means U87MG without neutrophils. (* = p<0.05 referred to 1:62/72h) b) U87MG proliferation using SRB assay, considering 100% proliferation of untreated tumor cells, which is U87MG without neutrophils. (* = p< 0.05 compared to 1:31/24h and ϯϯϯ = p< 0.001 compared to 1:31/72h c) neutrophil viability in coculture with U87MG and without using Trypan-blue protocol. (*compared to inactive/24h and ϯ to inactive/72h; Ω to 1:120/24h/72h; Ϫ 1:62/24h and μ 1:62/72h ф 1:31/24h and ϭ 1:31/72h)

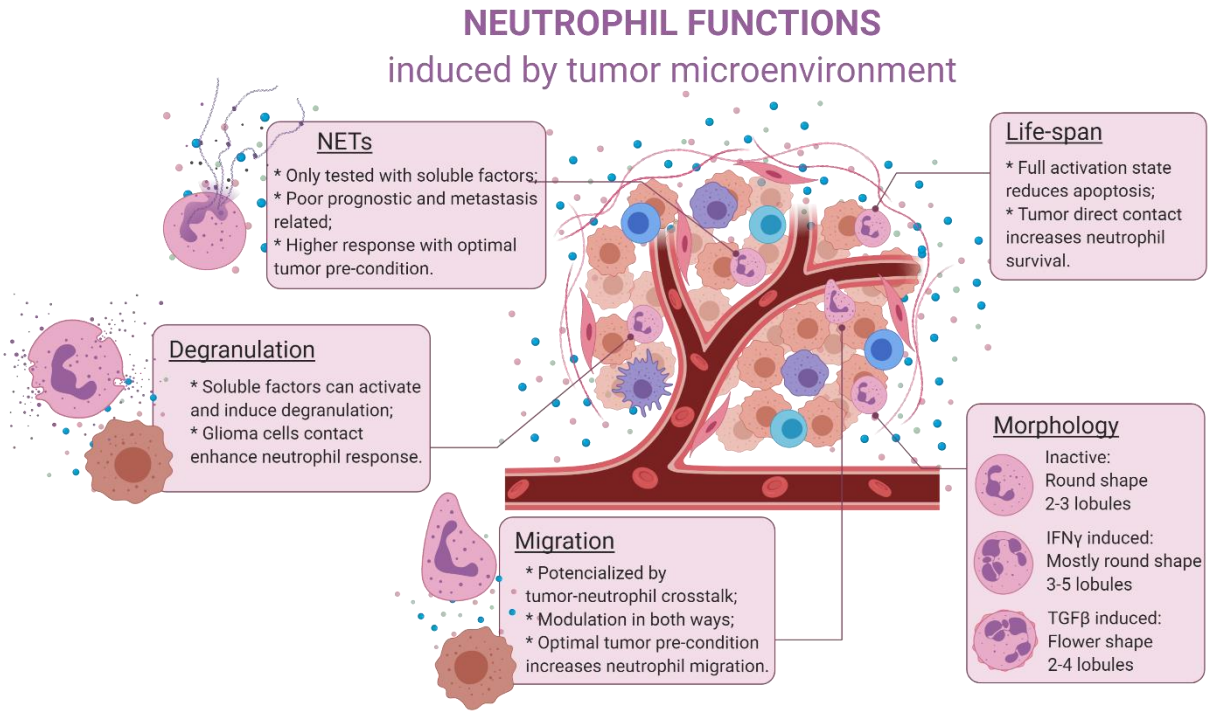

Figure 5: Neutrophil functions induced by tumor microenvironment

11. Abbreviations

ATCC: American Type Cell Collection; Rockville, Maryland, USA

ATP: Adenosine triphosphate

DMEM: Dulbeccos's modified Eagle's medium

FBS: Fetal bovine serum

fMLP: N-formyl-methyonyl-leucyl-phenylalanine

GBM: Glioblastoma

HBSS: Hank's Balanced Salt Solution

IL-8: Interleukin 8

IFNγ: Interferon gamma

LPS: Lipopolysaccharide

MMP-9: Metalloproteinase-9

MPO: Myeloperoxidase

N0: Inactive neutrophil

N1: pro-inflammatory neutrophil

N2: anti-inflammatory neutrophil

NaHCO 3: Sodium bicarbonate

NE: Elastase

NET: Neutrophil extracellular traps

NK cells: Natural Killers

P1: Purinergic receptor 1

P2: Purinergic receptor 2

PBS: Phosphate-buffered saline

PMN: Polymorphonuclear cells

RPM: Revolution per minute

SRB: Sulforhodamine B

TGFβ: Transforming growth factor beta

U87MG: Glioblastoma cel

Capítulo 2: SINALIZAÇÃO PURINÉRGICA

O extravasamento de nucleotídeos usualmente intracelulares, como o ATP, é causado por mecanismos de estresse celular. Dano e morte das células promovem a liberação em forma de sinal de perigo [ref]. Entretanto, outras situações também levam a essa resposta: a estimulação mecânica – alongamento e tensão, hipóxia ou presença de patógenos [1]. A presença do ATP e seus produtos de hidrólise (ADP, AMP e ADO), além da presença de pirimidinas como o UTP e seus produtos, desencadeiam a cascata de sinalização purinérgica por meio da sensibilização dos purinoceptores, os quais estão amplamente distribuídos nas células imunes. A amplitude dos efeitos sinalizados pelos agonistas púricos e pirimidínicos é mediada pelas ectonucleotidases, as quais são enzimas responsáveis pelo controle do nível extracelular desses sinalizadores. Essa ativação tem papel importante na manutenção do microambiente tumoral e na ativação de células inflamatórias.

PAPEL NO CÂNCER

O ATP é uma moeda energética que apresenta características excitatórias quando em ambiente extracelular. Esse nucleotídeo desempenha função dupla no microambiente tumoral, dependendo, não somente de sua concentração, mas da expressão combinada da CD39 e da CD73 e de propriedades do infiltrado imune. Esse conjunto implica na formação de adenosina, a qual é uma molécula que causa imunossupressão [2]. Então, considerando que as células tumorais estão sempre desenvolvendo métodos de escape tumoral, a inibição da resposta imune pelo excesso de ATP e ADO é um mecanismo muito efetivo [3]. O nicho tumoral apresenta pontos de hipóxia causados pela alta taxa de proliferação celular não acompanhado pela taxa formação de novos vasos. Como apresentado anteriormente, essa condição contribui para o aumento do ATP extracelular e, consequentemente, seleciona a população de células imunossupressoras presentes, como, por exemplo, maior quantidade de células T regulatórias facilitando o perfil Th17 em oposição aos linfócitos T-$CD4^+$ efetores [4-6]. A relação direta da sinalização purinérgica tanto na progressão e perpetuação do câncer e na ativação dos neutrófilos, instiga a hipótese de que a modulação fenotípica N1/N2 poderia ter auxílio dessa via. A regulação purinérgica dos neutrófilos chama a atenção, sendo considerada elemento importante, e, com isso, escrevemos uma revisão bibliográfica com foco em estudos *in vitro* com células humanas.

Referências

[1] Burnstock G, Boeynaems JM. Purinergic signaling and immune cells. Purinergic signaling. 2014 Dec 1;10(4):529-64.

[2] Allard, B., Beavis, P. A., Darcy, P. K. & Stagg, J. Immunosuppressive activities of adenosine in cancer. Curr. Opin. Pharmacol. 29, 7–16 (2016).

[3] Allard, B., Longhi, M. S., Robson, S. C. & Stagg, J. The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. Immunol. Rev. 276, 121–144 (2017).

[4] Trabanelli, S. et al. Extracellular ATP exerts opposite effects on activated and regulatory CD4+ T cells via purinergic P2 receptor activation. J. Immunol. 189, 1303–1310 (2012)

[5] Pellegatti, P., Falzoni, S., Pinton, P., Rizzuto, R. & Di Virgilio, F. A. Novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. Mol. Biol. Cell 16, 3659–3665 (2005).

[6] Suadicani, S. O., Brosnan, C. F. & Scemes, E. P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J. Neurosci. 26, 1378–1385 (2006).

REVIEW: Neutrófilos humanos e ativação de receptors purinérgicos

Neutrophils fast and furious: the purinergic pathway

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Abstract

The purinergic system is an important signaling pathway, which has called attention to cancer studies. Its activation and upregulation on the cell tumor membrane are already being discussed and considering that the tumor microenvironment has a close relationship with infiltrated immune cells, neutrophil modulation into an anti-inflammatory profile might be expected. The N1 and N2 phenotypes are entering the scope of tumor progression understanding. The need for more data about that condition opens the hypothesis that the modulation could be due to the involvement of the purinergic pathway. Neutrophil migration is guided by purinergic receptors activation, mostly because of the excess of extracellular ATP as an injury signal. The nucleotide hydrolysis activates P2Y receptors, which are linked to metabolic routes, and interacts with many other biochemical paths. The main issue is that the data available in the literature refers mainly to the N1 profile, therefore, the purinergic response can be extrapolated into the cancer context. Furthermore, most studies evaluate conditions in *in vivo* protocols, and the focus of this review is to analyze the human neutrophil response and extrapolate to a cancer point of view. Understanding neutrophil purinergic activation under tumor conditions can provide knowledge to create new therapeutic strategies with purine targets.

Introduction

The normal tissues are composed by different types of cellular, molecular and microenvironmental signals that operate in accordance to ensure the usefulness of the tissue and, consequently, the homeostasis. This ensures a balance between cell proliferation and death, organ structure, and function [1]. In non-physiological conditions, like infection, tissue damage, or inflammatory process by innate immune cells, initiating, triggering, or recruiting of proinflammatory cells and plasma proteins occur in the sensitized site [2]. Although sensitive tissues are resistant to many disorders, the formation of tumor mass is capable of damaging the tissue homeostasis to the point of not being restored [1].

The origin of cancer is not completely elucidated, however, the functional relationship with inflammation is widely discussed [3]. Chronic inflammation is considered to contribute to tumor stabilization based on the release of cytokines and chemokines into the microenvironment that increases cell proliferation. This growth is only sustained due to the presence of immune cells, growth and angiogenesis factors, and DNA damage promoting agents [4–6]. Cell proliferation caused by tissue regeneration after disturbance increases until tissue repair [7]. In contrast, cell growth, which perpetuates gene mutations, continues its development in microenvironments rich in pro-inflammatory factors, establishing

irreparable lesions. The power of inflammatory cells in tumor progression is undeniable, promoting the neoplastic process and providing an attractive microenvironment [4]. The immune system is a regulated and integrated cellular network that preserves and restores homeostasis and purinergic signaling help to adjust the functions of immune cells [8]**.**

Neutrophils, also known as one of the polymorphonuclear leukocytes, are a major agent during the early stages of the inflammatory response. They are the first leukocytes to be recruited, migrating within a few hours. Also, they eliminate pathogens through multiple mechanisms [9–11]. Tissue tips that initiate neutrophil recruitment to injured sites have common mechanisms, such as the involvement of specific chemokines and exogenous ligands [12]. However, promoters of early migration of polymorphonuclear cells to distant sites are not defined [13].

Stressed cells release adenosine triphosphate (ATP) as a danger and '*find me*' signal, guiding the phagocytes' migration, such as neutrophils [14]. The purinergic pathway is a transduction system with receptors expressed in manly all tissues and cells and interacts with physiological and pathophysiological responses [8]. Extracellular nucleotides and nucleosides, such as ATP and adenosine (ADO) are potent signaling molecules that, through activation of purinergic receptors (P2 and P1, respectively); modulate proliferation, differentiation, cell death and control of inflammatory response events. The P2 receptors are further classified as ionotropic P2X (P2X1-7) which is sensitized exclusively by ATP and metabotropic P2Y ($P2Y_{1,2,4,6,11\text{-}14}$) which are G-protein coupled-receptors stimulated by nucleotides such as ATP, ADP, UTP, UDP and UDP-glucose [15]. The metabotropic P1 receptors are sensitized by ADO. To date, four P1 receptors have been cloned in humans A1, A2A, A2B and A3 which exhibit differential affinities by ADO [16]; A1 and A3 are negatively coupled to adenylate cyclase, while A2A and A2B are positively coupled to adenylate cyclase [17] (Table 1).

In general, ATP is a pro-inflammatory signaling, which is recognized as a DAMP (damage-associated molecular pattern) while ADO, one of its degradation products, acts by counteracting ATP actions, triggering an immunosuppressive and immunomodulatory response [18]. An important factor controlling the ATP/ADO-mediated response is their extracellular metabolism by ectonucleotidases, such as CD39 and CD73. Such enzymes are present on both tumor and immune cells surface, and they act integrated into extracellular ATP hydrolysis and ADO generation [19] (Figure 1).

Neutrophil migration depends on a frontal excitatory and a back inhibitory signal on the cell surface. ATP release via the Pannexin-1 (PNX-1) pathway intercedes the activation of these granulocytes at the front edge by autocrine stimulation of the $P2Y_2$ receptor. At the same

time, the pathway provides the necessary inhibitory signals, releasing ATP that will be degraded to ADO by CD39 and CD73 coupled activity. After that, ADO is recognized by P1 type receptors, localized on the neutrophil tail, such as A2A, which blocks chemoattractant signaling and, alternatively binds to A3 receptors, which stimulates immune migration. This purinergic feedback loop promotes neutrophil movement towards the chemoattractant source [8, 14]. The rapid conversion of extracellular ATP into ADO by neutrophils allows the binding of ADO to A2A receptors, providing an important counterpoint to stimulation of $P2Y_2$ and A3 receptors. Suppressive actions of A2A receptors provide a limiting mechanism for the main functions of neutrophils [20]. In summary, $P2Y_2$ and A2A receptors mainly provide excitatory and inhibitory responses, respectively, producing a push-pull movement, allowing neutrophil migration [21]. As neutrophils are recruited to several contexts, including bacterial products, complement proteins (C5a), immunocomplexes (ICs), chemokines, and cytokines, a phenotypic adaptation to these different microenvironments is inevitable [12, 22].

There is evidence of the presence of antitumor (N1) and pro-tumor (N2) phenotypes, but investigations are initial, and the effector mechanisms that modulate the leucocyte functional behavior, as well as its role in the perpetuation of the disease, are not well understood [5, 17]. Studies consider the existence of these two opposing N1-proinflammatory and N2-antiinflammatory neutrophil subpopulations with different activation states, exhibiting an activation spectrum similar to which has been proposed to macrophage polarization [7, 12]. Although they have unclear distinctive markers, a recent study point to Arginase-1 and LOX-1 as hallmarks of tumor-associated neutrophils [23].

In this regard, neutrophil polarization to an inflammatory phenotype may also be dependent on purinergic signaling. CD11b expression, a neutrophil activation marker, bacterial elimination, and oxidative burst also depends on the autocrine mechanism described before, on the other hand, the extracellular ATP levels of non-self-source, such as microenvironment or inflammation site, could result in P2X and P2Y stimulation in a paracrine way [20, 24]**.** Under desensitization conditions, such as prolonged high ATP levels, the $P2Y_2$ receptors seem to be useful for maintaining the signaling, due to the lower response, it keeps cells viable, which was already demonstrated in macrophages [25]. It is speculated that this may contribute to the neutrophil hyporresponsiveness observed in the tumor sites [26]. In this review, the focus will rely on studies using human neutrophils to highlight the need for further progress in the area.

The review aimed to evaluate the relationship between the purinergic signaling with neutrophil activation and migration. It shows that there are few studies with human cells and

there are no reports on patients. The search consisted of using keywords following the PICOT methodology, with the coming filters: human; experimental article; up to 10 years; English. Duplicated articles and those that, due to the abstract, did not address the subject in question were excluded. For those selected, a ranking-questionnaire was applied in order to assess the quality of the information obtained in the study. To eliminate bias attributed to the high impact journal or author, the articles were blinded and their information extracted by another member. In short, the number of final articles was reduced, proving that most studies are in vivo and, although the purinergic system already plays an important role in migration, few portray its action in PMN functions and associating it with the N2 phenotype.

Driving License: Inflammation and $P2Y_2$ receptors activation response

P2Y-type receptors have shown to be major influencers of neutrophil activation. Kukulski demonstrated *in vitro,* using transwell apparatus, that $P2Y_2$ receptor agonism is fundamental for TLR-4 induced neutrophil migration. In addition, it showed potentiation of neutrophil migration with UTP, one of the ligands of this receptor. They discussed the possibility of mediation by IL-8 secretion induced by TLR-4 monocytes activation. Cross-communication between monocytes and neutrophils is very important for both functional understandings. On the one hand, IL-8 secreted by monocytes potentiates $P2Y_2$ receptor activation, on the other hand, $P2Y_2$ activation promotes the recruitment of monocytes, macrophages, and lymphocytes by increasing vascular cell adhesion molecule-1 (VCAM-1) expression on epithelial cells [27] (Figure 2)**.**

Interestingly, the study published by Gabl in 2015 argues that possibly $P2Y_2$ downregulation comes from the neutrophil cytoskeleton. It demonstrates that receptors not occupied by their ligands undergo an agonist-induced conformational change, which elevates intracellular calcium levels by coupled G-protein signaling. It is proposed that the $P2Y_2$ receptor blockade also blocks the NADPH-oxidation signaling pathway [28].

In different circumstances, $P2Y_2$ overstimulation is unfavorable for neutrophil migration. A study performed by Li and colleagues has shown that increased levels of systemic ATP may interfere with endogenous ATP production. This mechanism is essential for maintaining the immune response to damage, as the exacerbated response triggers severe associated diseases, including autoimmune diseases [21].

CD39 acts as a regulatory enzyme of the extracellular ATP and ADO levels available for purinergic signaling. CD39 hydrolyzes the first nucleotides until AMP, however, it is necessary to have a second enzyme to complete the hydrolysis of ATP to ADO, CD73, and

regulate the immune function [29]. Hence, both enzymes determine the amount of extracellular ADO is feasible. Its production by the sequential activity of CD39/CD73 presents an immunosuppressive environment completely convenient for cancer cells to develop. Meaning that an increase in the expression of both CD39 and CD73 inhibits anti-tumor response by immune cells [30]. The necessity to keep the harmony between inflammatory and anti-inflammatory responses is on behalf of escaping exacerbated immunosuppression or uncontrolled inflammation, nonetheless, the CD39/CD73 axis can depress the self-tolerance mechanism. On the other hand, the outcome of the activity escalation of these enzymes generates elevated levels of extracellular immunosuppressive ADO [29, 30].

Survival of the Dead: neutrophil's life modulation through purinergic activation

Regarding the half-life of neutrophils, it is commonly agreed that they become viable in circulation for a few days, approximately four, and then undergo apoptosis [12, 31]. Understanding the mechanisms that affect the lifespan of these immune cells may help in finding new therapeutic targets. Neutrophils have few mitochondria in their cytosol and therefore prefer to produce their energy through glycolysis. Thus, mitochondria rarely participate in ATP formation by neutrophils [23, 32]**.** However, for a higher level of intracellular ATP production, the cell complements ATP through to the Krebs Cycle probably by the activation of the mTOR pathway, an important metabolic pathway that regulates biological and physiological processes such as proliferation, growth, cell survival, and autophagy. This pathway allows the flow of calcium into the neutrophil mitochondria, related to the activation of the $P2Y_2$ purinergic receptor, leading to the production of intracellular ATP. The ATP produced is externalized by PNX-1, which acts, in an autocrine way, in the $P2Y_2$ receptors, potentiating neutrophil migration [24] (Figure 3).

One study observed that increased NAD^+ levels are directly related to neutrophil aging, probably because of increased energy demand. Besides that, intracellular ATP levels are not keeping up with expectations. It is argued that there may be an increase in ATP formation but also an increase in consumption, and therefore the final balance is smaller [33]. Thus, ATP levels are decreased in aged neutrophils compared to their controls and activate neutrophils. Considering that intracellular ATP is produced by activating purinergic signaling and generated by the few existing mitochondria, the decrease in ATP may be caused by increased energy demand or decreased production [33].

Another P2Y receptor has been drawing attention for its relationship with neutrophil apoptosis inhibition, it is the $P2Y_6$. The study conducted by Nakaoka evaluated the interaction between the $P2Y_6$ antagonist (MRS2578) and apoptotic behavior. It was found

that in the presence of the antagonist, apoptosis was reactivated. It turns out that $P2Y_6$ ligand, UDP, induces suppression of programmed cell death when bound to the receptor. Besides that, preventing the binding ligand-$P2Y_6$ allows neutrophil apoptosis, suggesting that the induction by HNP-1 down-regulates pro-apoptotic action and up-regulates anti-apoptotic profile by Bcl-xl, which in turn inhibits apoptosis. The mitochondrial membrane potential and caspase-3 activity result in decreased pro-apoptotic signals through the $P2Y_6$ signaling pathway [34] (Figure 4).

In cancer, increased survival of tumor-associated neutrophils (TAN) has been found, an important factor for the development and growth of tumor mass [35]. Unfortunately, there are just a few studies in the area and the understanding of the mechanisms involved in TAN lifespan in cancer is still unclear. The importance of the purinergic pathway in neutrophil activation and migration is known, but a better understanding of the connection between this pathway and cellular death may arise new molecular targets for cancer therapeutics.

Suicide squad: extracellular nucleotide levels in inflammatory and cancer diseases

Injured tissues, whether inflamed or infected, secrete neutrophil-recruitment chemokines that inform the attack site to peripheral blood circulating neutrophils. Neutrophils are the first immune cells to reach the damaged tissue. So roughly, these cells are the infantry of our immune system. Upon arrival at the inflamed site, neutrophils begin the process of receptor-mediated respiratory burst and degranulation [36].

The purinergic signaling pathway consists of the ectonucleotidases expressed at the neutrophil membrane and hydrolyzes ATP into ADP, ADP into AMP, and finally ADP into ADO. Each of these nucleotides/nucleoside is capable of signaling by distinct purinergic receptors. ATP has been reported to bind to P2X1, $P2Y_{1,}$ and $P2Y_2$. ADO amplifies the chemotactic signal by binding to A3 receptors. Patel and colleagues observed the chemotactic activity of N2-type immunosuppressive neutrophils compared to inactive neutrophils and found that there was less chemotactic activity in N2-type, probably due to the lack of extracellular ADO, suggesting that ATP hydrolysis might be slow down in this situation [13].

Cancer can be characterized as a process of chronic inflammation, and by its nature, the release of ATP in the tumor microenvironment becomes constant, unlike acute inflammation, where the peak release of ATP requires greater neutrophil activity. Consequently, the way that ATP is secreted in the extracellular medium is crucial for the P2-mediated responses [14].

Studies discuss the need for extracellular ATP for direct migration, as well as its modulation for the release of proteolytic enzymes. On one hand, neutrophil recruitment is appropriate and necessary for maintaining homeostasis. On the other, neutrophil enzymes lack specificity for necrosis cells, damaging adjacent tissues. Although neutrophil recruitment is appropriate and necessary to maintain necrotic debris removal, neutrophil enzymes damage adjacent tissues due to a lack of specificity for necrotic cells [37, 38]**.** In cancer, this nonspecific behavior may be one of the factors influencing its progression.

The purinergic cascade produces a very important metabolite in neutrophil activation and migration control: ADO. Its signaling occurs by activation of A1 receptors, promoting migration and push and pull movement while signaling by A2A receptor activation inhibits adhesion [39]. Another study showed that ADO has a unique feature of promoting chemotaxis while inhibiting the activation and the consequent release of reactive oxygen species (ROS). Hence, neutrophils migrate to the site of infection without damaging healthy tissues along their path [40]**.**

An ADO feature that deserves attention is its ability to inhibit proinflammatory mediator's production by monocytes and dendritic cells, such as ROS and tumor necrosis factor (TNF), besides the immunosuppressive function A2A-mediated in T regulatory cells [38, 39]. Under physiological conditions, ADO plays an essential role in regulating inflammation while signaling cellular damage [39]**.** These characteristics may be involved in the maintenance of the tumor microenvironment, considering that extracellular ADO has immunosuppressive action while recruiting more leukocytes (Figure 5).

Neutrophil and monocyte modulation may also be related to purinergic signaling itself. In the case of N1 profile activation by gram-positive pathogens, it was observed in *in vitro* studies that the presence of extracellular ADO down-regulates trans-endothelial migration; and inhibition of CD73, nucleotidase responsible for the conversion of AMP to ADO, increased the number of neutrophils at the injured site. That is, in a situation of acute inflammation by bacteria, the high amount of extracellular ADO is not interesting for resolution. It was found that ADO promoted better basolateral-apical migration by endothelial monolayers but did not regulate migration through these layers [41]. Another feature regulated by extracellular ADO is the power of neutrophil phagocytosis and degranulation, important mechanisms of bactericidal activity. They also showed that the formation of extracellular neutrophil traps (NETs) was more efficient against *S. pneumoniae* when the neutrophil was A2B deficient [41]**.** Therefore, adenosine not only reduces neutrophil migration, but also affects its killing weapons. Changes in the amount and time of action of NETs are related to the promotion of autoimmunity, the presence of vascular diseases, and thrombosis, besides contributing

to tumor progression and metastasis. Studies found a relation between elevated NETs production and poor prognosis in both human and mouse tumors. Moreover, these neutrophils traps are capable of catching circulating tumor cells and favoring metastatic implants formation [38, 42, 43].

Concluding remarks:

To summarize, as several studies have shown, the purinergic pathway and neutrophils' features are slightly interconnected. The driving license of neutrophils has a $P2Y_2$ stamp. In fact, monocyte IL-8 activates $P2Y_2$ receptors and regulates neutrophil migration. In addition, free ligand $P2Y_2$ increases intracellular calcium levels and blocks NADPH-oxidation, inducing a conformational change in the cytoskeleton. Nevertheless, very high ATP levels and $P2Y_2$ overstimulation can interpose in physiological response [21, 27, 28].

Although neutrophils are not zombies that survive after death, their aging process has subtle characteristics. It was found to have higher NAD+ levels and smaller ATP levels, due to increased energy demand in aging cells. Meanwhile, neutrophils can act like zombies, containing programmed cell death, by $P2Y_6$ pathway stimulation that blocks neutrophil apoptosis via mitochondrial membrane potential, caspase-3 activity, and Bcl-xl up-regulation [32–35].

Above all, neutrophils are the immune suicide squad. In this particular case, a small change can impair the immune response and allow tumor progression. As shown before, the purinergic system has a crucial role in neutrophil's actions. For instance, the neutrophil phenotype can inflect extracellular ATP hydrolysis and, consequently chemotaxis regulated by P1 receptors. ADO receptors also modulate migration, adhesion, and ROS release, allowing neutrophils to migrate without shattering other tissues [13, 14, 36, 37, 39, 40].

In cancer, the constant and average ATP release induces differential neutrophil responses. Also, the lack of specificity in neutrophil enzymes may contribute to cancer progression that is especially associated with purinergic pathway activation. ADO is a key molecule that has already established its role as an immunosuppressive agent, regulating immune cell recruitment, modulating neutrophil killing features, and promoting cancer progression.

N1 and N2 represent extremes of different molecular phenotypes, which depend upon the microenvironment [7]. Considering that purinergic signaling has great influence in

neutrophil's activation and migration, our hypothesis is that the neutrophil activation spectrum (N1/N2) is related to activation of purine receptors and its signaling. Few studies show the relationship between these phenotypes and the purinergic pathway, with this review it was possible to speculate association among them, as shown in Table 2.Therefore, it is known that the immune system has a fundamental role in the tumor progression, although all the mechanisms are not well elucidated. There is evidence, not well explored, about two neutrophil phenotypes: proinflammatory (N1) and anti-inflammatory (N2), having extreme importance in the tumor microenvironment. *In vivo* and *in vitro* studies have found that these polymorphonuclear leukocytes modulate the tumor's microenvironment [5, 6]. This antitumor phenotype enhances the expression of TNF-α, CCL3, ICAM-1, and reduces arginase-1 production, inhibits angiogenesis, and promotes antitumor response of T lymphocytes [44, 45]. In contrast, it is discussed that the N2 phenotype is acquired by the presence of TGF-β, favoring the infiltration of neutrophils with high expression of CXCR4, VEGF-A, and metalloprotease 9 (MMP-9) [45]. Neutrophils are the major producers of VEGF-A and release high levels of MMP-9, which releases the active form of VEGF-A from the extracellular matrix. However, N2 is able to release MMP-9 even in the absence of a protease inhibitor and increased levels contribute to angiogenesis and tissue invasion [46] (Figure 6).

Neutrophil chemoattraction involves chemokines, lipids, anaphylatoxins from the complement system (C5a-C3a), and platelet activation factor (PAF), but IL-8 promotes a more potent binding with CXCR1 and CXCR2. There are also additional mediators that can work as recruiters of neutrophils [6]. Thus, the purinergic system has also an important role in activating neutrophil's migration. Besides, this pathway has signaling molecules that modulate differentiation, proliferation, and are able to control inflammatory events, which might be the main responsible for neutrophil activation.

Immune migration responds mainly to chemokines, the CXCR1 and CXCR2 receptors are the main involved in neutrophil recruitment, their activation occurs by CXC chemokines, including the most active one IL-8 [31, 47]. The microenvironment chemokine release plays an essential role in tumor progression. Considering that in glioblastoma cells, the purinergic signaling is active, a study from our group showed that the presence of ATP and ADO delivered by tumor cells, increased IL-8 production, acting directly on the neutrophil recruitment. The amount of IL-8 at the site regulates the magnitude of immune infiltration [48]. Also, the neutrophil expression of NTPDase1, known as CD39, may operate in mold the immune response as the eATP take the role of a potent stimulus of IL-8 discharged by neutrophil upon NTPDase1 inhibition implying that inactive neutrophils may be unresponsive

to stimulus on the grounds that intrinsic NTPDase1 activity [48]. Neutrophil participation in tumor progression has been investigated in preclinical studies with animals. However, few studies correlate tumors and neutrophils in humans. The scientific literature regarding human neutrophils in a cancer context is interfered with by the unclear identification of different subsets and with functions that might be misinterpreted. Understanding how the purine receptors activation generates intracellular responses might be helpful to focus on different potential drug targets. The tumor behavior in the presence of immune cells has called attention due to the closest communication between them. To sum up, more studies regarding neutrophil purinergic activation in human tumor sites are needed, because understanding neutrophil purinergic activation under tumor conditions can provide knowledge to create new therapeutic strategies with purine targets.

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Glossary:

A2: Alpha-2 adrenergic receptor. It is a G protein-coupled receptor.

A2a/A3: P1 purinergic receptors sensitized by adenosine.

ADO: Adenosine. It is a purine nucleoside that participates in the purinergic system as a form of extracellular signaling, modulating proliferation, differentiation, cell death, and control of inflammatory response events, acting mainly as immunossupressive/immunomodulatory molecule via P1 receptors sensitization.

ADP: Adenosine diphosphate. It is an organic compound that also participates in the purinergic system as a form of extracellular signaling, inducing platelet aggregation and microglial migration via $P2Y_{12}$ sensitization.

AMP: Adenosine monophosphate. It is a nucleotide formed in the extracellular environment mainly via ATP hydrolysis mediated by CD39 enzyme activity. Until now, there is no purinoceptor described to be activated by this nucleotide.

ATP: Adenosine triphosphate. It is a purine nucleotide involved in complex signaling pathways, including driving energy to the cells, being a precursor to DNA and RNA, and, in this case, participating in the purinergic system, a form of extracellular signaling via P2 receptor agonism.

CD39: Ectonucleoside triphosphate diphosphohydrolase-1. It is located at the cell surface of immune and cancer cells and its enzyme activity hydrolyzes P2 receptor ligands, like ATP, ADP, UTP, and UDP to the respective monophosphate-nucleosides.

CD73: Ecto-5′-nucleotidase**.** An enzyme that converts AMP to ADO and it also acts as cell-cell and cell-matrix protein, important for cell communication and migration.

HNP-1: A human protein that belongs to the alpha defensin family of antimicrobial peptides.

IL-8: Interleukin/chemokine released by macrophages and other cells of the innate immune response.

LPS: Lipopolysaccharide. Large molecule made of a lipid and a polysaccharide that occur in the membrane of Gram-negative bacteria, acting as triggers for the innate immune system.

N1: Antitumor neutrophils that have proinflammatory mechanisms.

N2: Pro-tumor neutrophils that have anti-inflammatory mechanisms.

NAD^+: It is a cofactor involved in redox reactions, carrying electrons from one reaction to another.

NADH: It is the reduced form of NAD^+, cofactor involved in redox reactions.

$NADP^+$: A coenzyme called nicotinamide adenine dinucleotide phosphate, acting as a cofactor in anabolic metabolism.

NETs: Extracellular neutrophil traps. It is a defense mechanism, where the neutrophils release chromatin to form an extracellular fibril matrix, which binds pathogens.

P1/P2 ($P2Y_1$, $P2Y_2$, $P2Y_6$, P2X1): purinergic receptors that, when activated by purine or pyrimidine allow the purinergic response.

PNX-1: Pannexin-1. It is a large transmembrane channel in the plasmatic membrane, allowing the passage of ions and small molecules, such as ATP.

TLR-4: Toll-like receptor 4. Receptor activated by LPS derived from Gram negative bacteria or by endogenous ligands, as HMGB1, that function in the innate immune system, being located at cell membrane of the immune cells, like macrophages, dendritic cells and neutrophils.

UDP-glucose: Uridine diphosphate glucose. Known as a nucleotide sugar involved in glycosyltransferase reactions in metabolism.

VCAM-1: Vascular cell adhesion molecule-1. It is a protein that functions as a cell adhesion molecule.

References:

[1] Basanta D, Anderson A. Homeostasis back and forth: An eco-evolutionary perspective of cancer. *Cold Spring Harb Perspect Med* 2017; a028332.

[2] Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008; 454: 428.

[3] Lecot P, Sarabi M, Pereira Abrantes M, et al. Neutrophil Heterogeneity in Cancer: From Biology to Therapies. *Front Immunol* 2019; 10: 1–19.

[4] Coussens L, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860.

[5] Dumitru C, Lang S, Brandau S. Modulation of neutrophil granulocytes in the tumor microenvironment: mechanisms and consequences for tumor progression. *InSeminars cancer Biol* 2013; 23: 141–148.

[6] Donskov F. Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *InSeminars cancer Biol* 2013; 23: 200–207.

[7] Grecian R, Whyte MKB, Walmsley SR. The role of neutrophils in cancer. *Br Med Bull* 2018; 128: 5–14.

[8] Wang X, Deyu C. Purinergic regulation of neutrophil function. *Front Immunol* 2018; 9: 399.

[9] Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends Immunol* 2011; 32: 452–460.

[10] Amulic B, Cazalet C, Hayes GL, et al. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol* 2012; 30: 459–489.

[11] Mantovani A, Cassatella MA, Costantini C, et al. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; 11: 519–531.

[12] Giese MA, Hind LE, Huttenlocher A. Neutrophil plasticity in the tumor microenvironment. *Blood* 2019; 133: 2159–2167.

[13] Patel S, Fu S, Mastio J, et al. Unique pattern of neutrophil migration and function during tumor progression. *Nat Immunol* 2018; 19: 1236–1247.

[14] Dosch M, Gerber J, Jebbawi F, et al. Mechanisms of ATP release by inflammatory cells. *Int J Mol Sci* 2018; 19: 1222.

[15] Burnstock G. Introduction to Purinergic Signaling. pp. 1–15.

[16] Peleli M, Fredholm BB, Sobrevia L, et al. Pharmacological targeting of adenosine receptor signaling. *Mol Aspects Med* 2017; 55: 4–8.

[17] Klotz K-N, Kachler S. Inhibitors of membranous adenylyl cyclases with affinity for adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 2016; 389: 349–352.

[18] Allard D, Chrobak P, Allard B, et al. Targeting the CD73-adenosine axis in immuno-oncology. *Immunol Lett* 2019; 205: 31–39.

[19] Chen Y, Corriden R, Inoue Y, et al. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 2006; 314: 1792–5.

[20] Chen Y, Yao Y, Sumi Y, et al. Purinergic signaling: A fundamental mechanism in neutrophil activation. *Sci Signal*; 3. Epub ahead of print 2010. DOI:

10.1126/scisignal.2000549.

[21] Li X, Kondo Y, Bao Y, et al. Systemic Adenosine Triphosphate Impairs Neutrophil Chemotaxis and Host Defense in Sepsis. *Crit Care Med* 2017; 45: e97–e104.

[22] Cruvinel MW, Mesquita JD, Araújo J, et al. Immune system-part I. Fundamentals of innate immunity with emphasis on molecular and cellular mechanisms of inflammatory response. *Rev Bras Reumatol* 2010; 50: 434–461.

[23] Si Y, Merz SF, Jansen P, et al. Multidimensional imaging provides evidence for down-regulation of T cell effector function by MDSC in human cancer tissue. *Sci Immunol*; 4. Epub ahead of print 18 October 2019. DOI: 10.1126/sciimmunol.aaw9159.

[24] Bao Y, Ledderose C, Seier T, et al. Mitochondria regulate neutrophil activation by generating ATP for autocrine purinergic signaling. *J Biol Chem* 2014; 289: 26794–803.

[25] del Rey A, Renigunta V, Dalpke AH, et al. Knock-out mice reveal the contributions of P2Y and P2X receptors to nucleotide-induced Ca2+ signaling in macrophages. *J Biol Chem* 2006; 281: 35147–55.

[26] Giuliani P, Zuccarini M, Carluccio M, et al. A New Investigational Perspective for Purines Against Glioblastoma Invasiveness. *Curr Drug Targets* 2018; 19: 1871–1881.

[27] Kukulski F, Yebdri F Ben, Bahrami F, et al. Endothelial P2Y2 receptor regulates LPS-induced neutrophil transendothelial migration in vitro. *Mol Immunol* 2010; 47: 991–999.

[28] Gabl M, Winther M, Welin A, et al. P2Y2 receptor signaling in neutrophils is regulated from inside by a novel cytoskeleton-dependent mechanism. *Exp Cell Res* 2015; 336: 242–252.

[29] Corriden R, Chen Y, Inoue Y, et al. Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. *J Biol Chem* 2008; 283: 28480–28486.

[30] Perrot I, Michaud H-A, Giraudon-Paoli M, et al. Blocking Antibodies Targeting the CD39/CD73 Immunosuppressive Pathway Unleash Immune Responses in Combination Cancer Therapies. *Cell Rep* 2019; 27: 2411-2425.e9.

[31] Németh T, Sperandio M, Mócsai A. Neutrophils as emerging therapeutic targets. *Nat Rev Drug Discov*. Epub ahead of print 22 January 2020. DOI: 10.1038/s41573-019-0054-z.

[32] Iula L, Keitelman IA, Sabbione F, et al. Autophagy mediates interleukin-1β secretion in human neutrophils. *Front Immunol* 2018; 9: 1–14.

[33] Richer BC, Salei N, Laskay T, et al. Changes in Neutrophil Metabolism upon Activation and Aging. *Inflammation* 2018; 41: 710–721.

[34] Nagaoka. Evaluation of the effect of α-defensin human neutrophil peptides on neutrophil apoptosis. *Int J Mol Med*; 26. Epub ahead of print 21 October 2010. DOI: 10.3892/ijmm_00000544.

[35] Shaul M, Levy L, Sun J, et al. Tumor-associated neutrophils display a distinct N1

profile following TGFβ modulation: A transcriptomics analysis of pro-vs. antitumor TANs. *Oncoimmunology* 2016; 5: e1232221.

[36] Grassi F. Purinergic control of neutrophil activation. *J Mol Cell Biol* 2010; 2: 176–177.

[37] Bayliss J, Delarosa S, Wu J, et al. Adenosine triphosphate hydrolysis reduces neutrophil infiltration and necrosis in partial-thickness scald burns in mice. *J Burn Care Res*; 35: 54–61.

[38] Lecot P, Sarabi M, Pereira Abrantes M, et al. Neutrophil Heterogeneity in Cancer: From Biology to Therapies. *Front Immunol* 2019; 10: 2155.

[39] Mahamed DA, Toussaint LE, Bynoe MS. CD73-generated adenosine is critical for immune regulation during Toxoplasma gondii infection. *Infect Immun* 2015; 83: 721–729.

[40] Rose FR, Hirschhorn R, Weissmann G, et al. Adenosine Promotes Neutrophil Chemotaxis. 167.

[41] Bou Ghanem EN, Clark S, Roggensack SE, et al. Extracellular Adenosine Protects against Streptococcus pneumoniae Lung Infection by Regulating Pulmonary Neutrophil Recruitment. *PLoS Pathog* 2015; 11: 1–23.

[42] Amulic B, Sollberger G. Why Immune Cells Extrude Webs of DNA and Protein. NETS: two faced players in Immunity. *Sci* 2018; 33: 44–51.

[43] Sorvillo N, Cherpokova D, Martinod K, et al. Extracellular DNA NET-works with dire consequences for health. *Circ Res* 2019; 125: 470–488.

[44] Hurt B, Schulick R, Edil B, et al. Cancer-promoting mechanisms of tumor-associated neutrophils. *Am J Surg* 2017; 214: 938–944.

[45] Galdiero MR, Varricchi G, Loffredo S, et al. Roles of neutrophils in cancer growth and progression. *J Leukoc Biol* 2018; 103: 457–464.

[46] Loffredo S, Borriello F, Iannone R, et al. Group V secreted phospholipase A2 induces the release of proangiogenic and antiangiogenic factors by human neutrophils. *Front Immunol* 2017; 8: 1–10.

[47] Kukulski F, Ben Yebdri F, Bahrami F, et al. The P2 receptor antagonist PPADS abrogates LPS-induced neutrophil migration in the murine air pouch via inhibition of MIP-2 and KC production. *Mol Immunol* 2010; 47: 833–9.

[48] Kukulski F, Bahrami F, Ben Yebdri F, et al. NTPDase1 Controls IL-8 Production by Human Neutrophils. *J Immunol* 2011; 187: 644–653.

List of Figures:

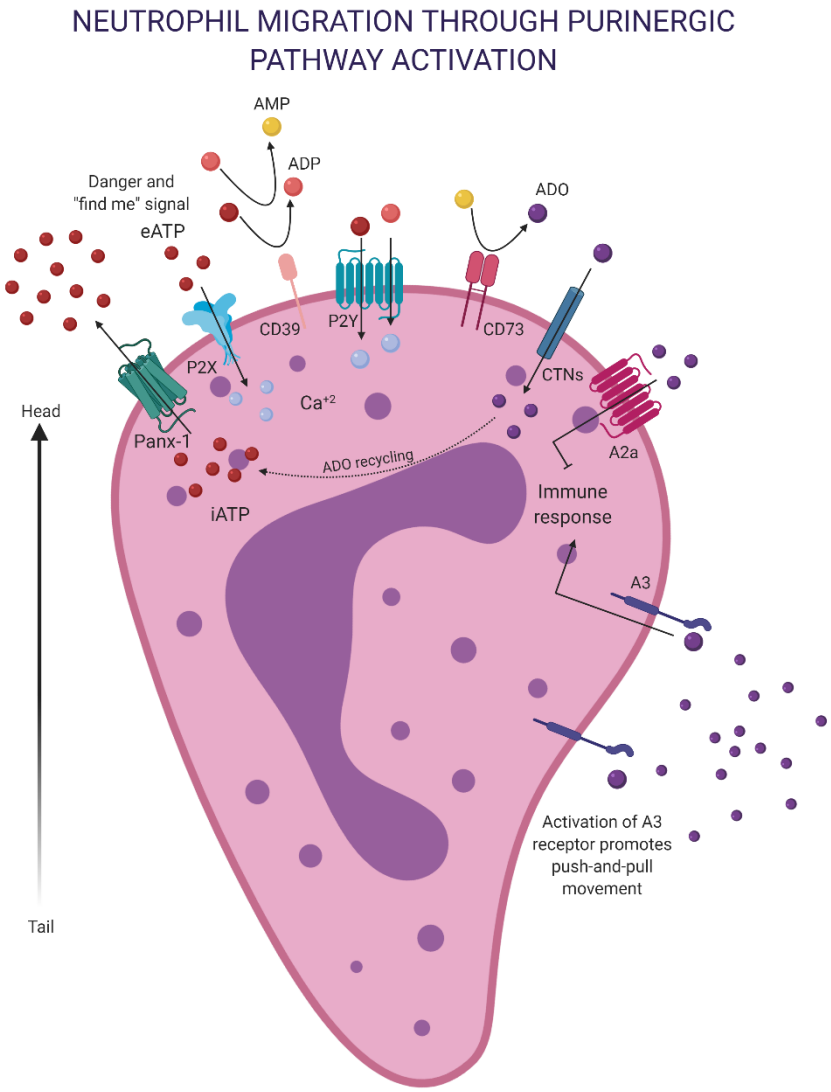

Figure 1: **NEUTROPHIL MIGRATION THROUGH PURINERGIC PATHWAY ACTIVATION.** Pannexin-1 (PNX-1) releases ATP (red balls), a sign of danger and “find me”. The increase of extracellular ATP potentiates neutrophil migration. ATP is hydrolyzed to ADP (pink balls) and ADP to AMP (yellow balls) by the CD39. CD73 hydrolyzes AMP to adenosine (ADO) (purple balls). Receptors P2X recognize ATP and receptors P2Y recognize ATP and ADP. This recognition increases the calcium flow to the neutrophil interior, helping in its activation. Besides, extracellular adenosine binds in two main receptors: 1) A2A, which inhibits the immune response, limiting important functions of the neutrophil, and 2) A3, excitatory for immune response. These bindings alternate, generating a movement of “push and pull” that changes neutrophil phenotype and allows its migration.

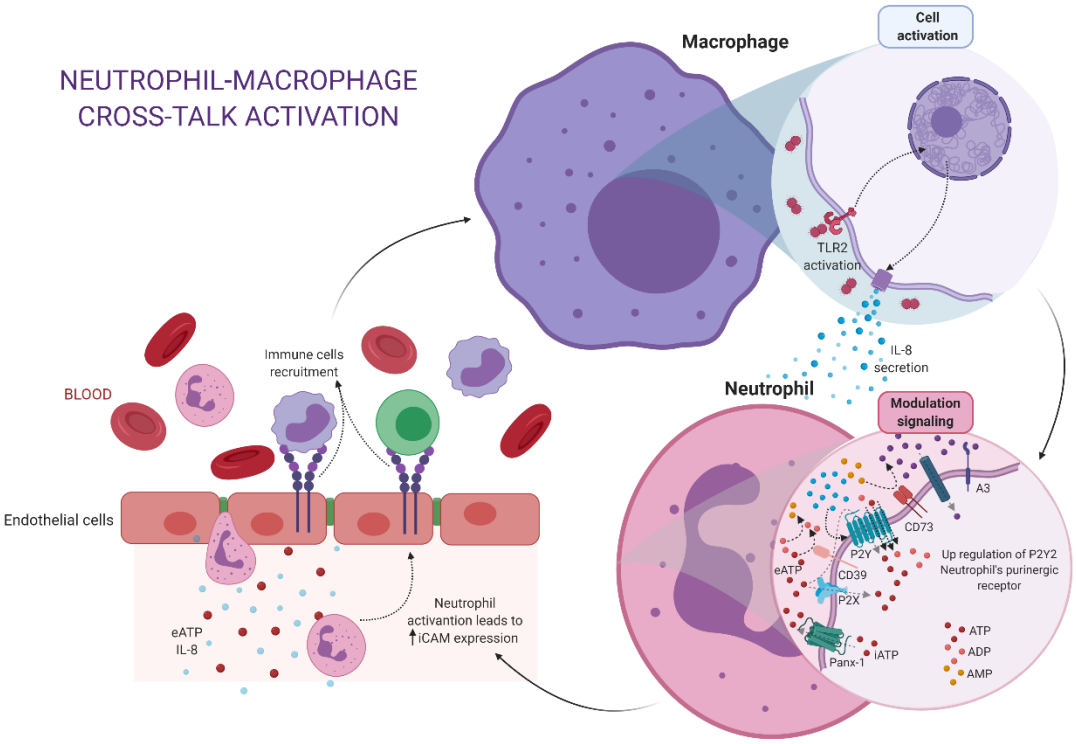

Figure 2: **NEUTROPHIL-MACROPHAGE CROSS-TALK ACTIVATION.** When monocyte Toll-like receptors are activated, they secrete Interleukin 8 (IL-8) (blue balls). This chemokine increases the $P2Y_2$ regulation, recognizing extracellular ATP (red balls) and ADP (pink balls) and allowing calcium flow that leads to neutrophil activation. The activation causes an increase of adherence receptors iCAM expressed on the surface of blood vessels epithelial cells. Thereby, immune cells from the bloodstream adhere and pass, by diapedesis, to the referred spot.

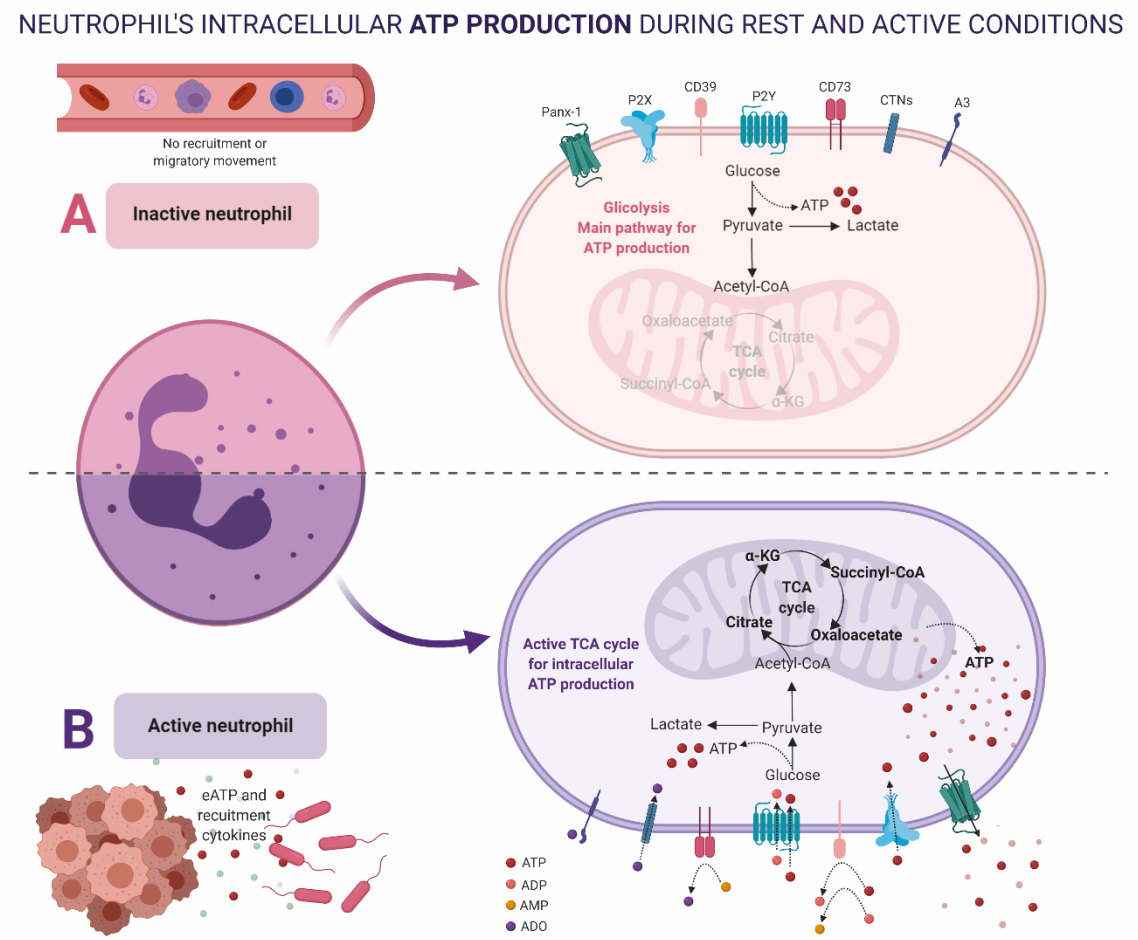

Figure 3: **NEUTROPHIL'S INTRACELLULAR ATP PRODUCTION DURING REST AND ACTIVE CONDITIONS.** When neutrophil is inactive, the ATP (red balls) production is made by glycolysis, due to the few mitochondria in the cytosol. This pathway does not use mitochondria and leads to a minimal quantity of ATP. However, when activated, neutrophil requires another pathway: the citric acid cycle. Here, NADH produced is oxidized in the respiratory chain reaction and synthesizes ATP and returns NAD^+ to the chain. Thus, the amount of ATP produced in this cycle is bigger than in the glycolysis, attending the necessities of activated neutrophil.

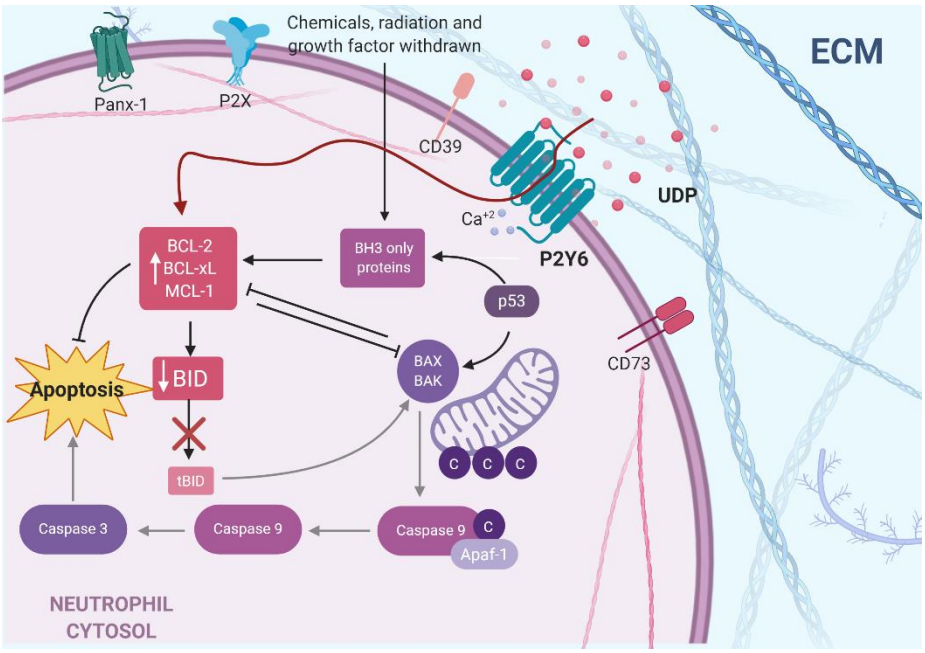

Figure 4: **APOPTOSIS INHIBITION THROUGH $P2Y_6$ ACTIVATION.** Apoptosis suppression: UDP (red balls), when bound to its receptor, $P2Y_6$, changes the mitochondrial membrane potential, activating the proteins BCL-xL, BCL-2, MCL-1, and inhibiting BID. That leads to incapacity of cytochrome C release, a molecule involved in the caspase cascade. Since Caspase 3 is responsible to activate apoptosis, its inhibition also inhibits apoptosis.

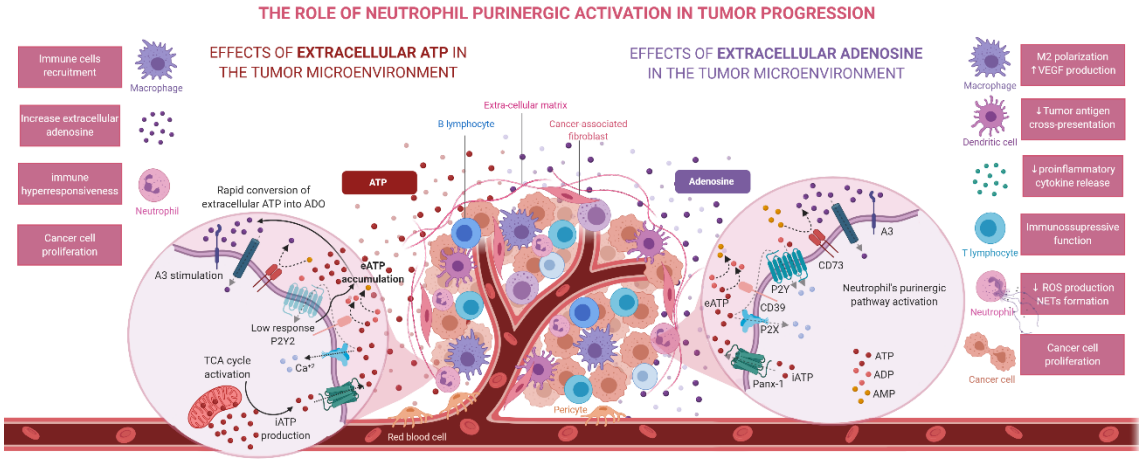

Figure 5: Hypothesis of **tHE ROLE OF NEUTROPHIL PURINERGIC ACTIVATION IN TUMOR PROGRESSION.** The release of ATP (red balls) in the microenvironment is constant, and its overflow activates the neutrophil purinergic system, causing immune cell recruitment, immune hyperresponsiveness, increasing extracellular adenosine, and causing cancer cell proliferation. Adenosine (purple balls), on the other hand, is produced due to the excess of ATP, increasing CD73 and CD39 activities. Extracellular adenosine overflow causes macrophages polarization to M2 (pro-tumor), increases vascular endothelial growth factor (VEGF), decreases tumor antigen cross-presentation, decreases proinflammatory cytokine release, increases immunosuppressive functions by lymphocytes, decreases ROS formation, allows NET's formation and helps cancer proliferation.

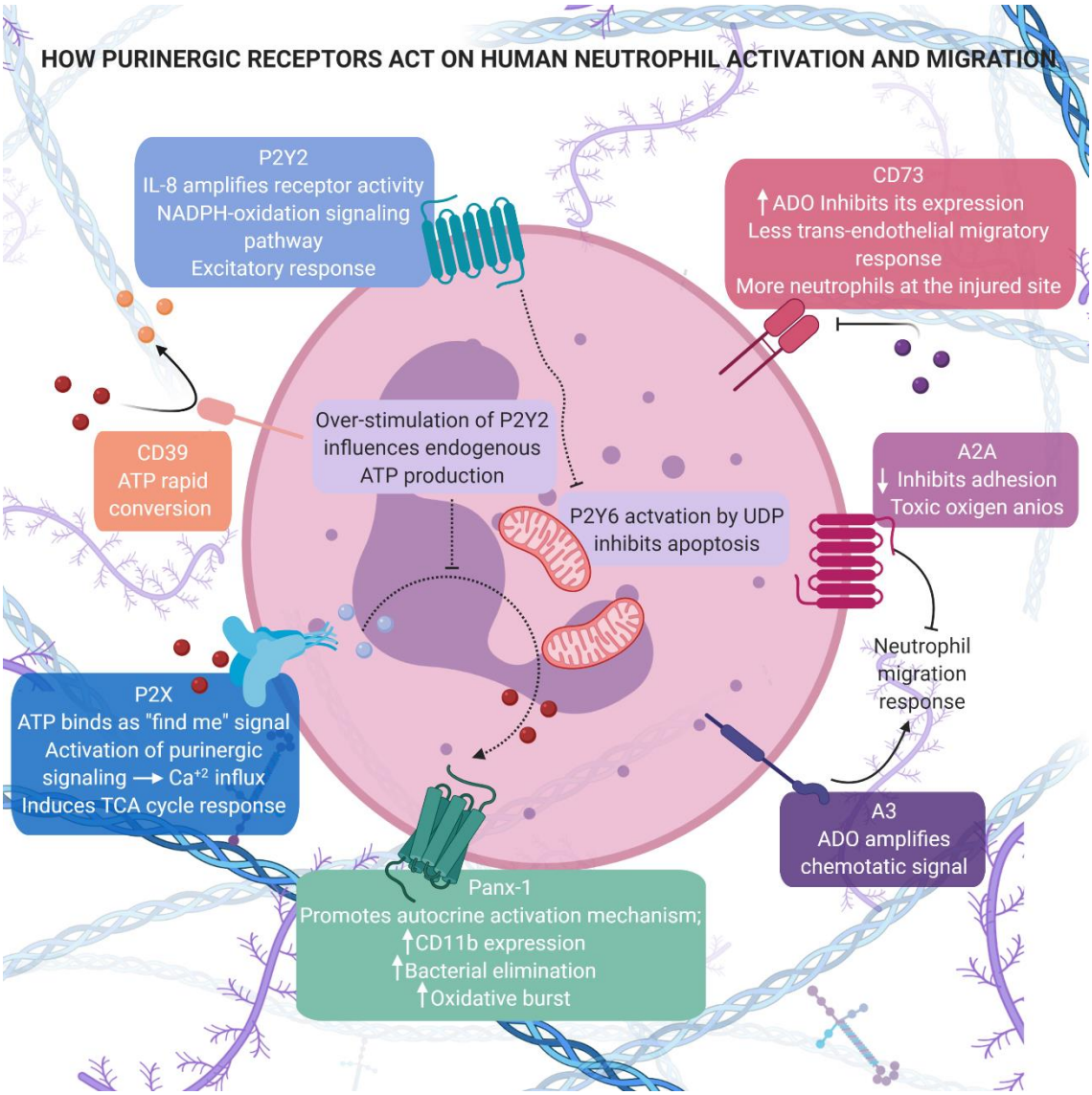

Image 6: **OVERVIEW OF NEUTROPHIL PURINERGIC ACTIVATION.** Purinergic receptors acting on human neutrophils: 1) $P2Y_2$: It allows NADPH-oxidation signaling pathway, it is affected by Interleukin 8 (IL-8), which has the ability to amplify its activity, and has an excitatory response, related to neutrophil activation. Besides, over-stimulation of the receptor influences endogenous ATP production; 2) CD73: It has its expression increased with high amounts of adenosine and decreased response to trans-endothelial migration. It also allows more neutrophils to the injured site; 3) A2A: is capable of decreasing adhesion inhibition and decreases toxic oxygen anions; 4) A3: Adenosine amplifies chemotactic signal; 5) PANX-1: Promotes autocrine activation mechanism, increases CD11b expression, as well as increases bacterial elimination and oxidative burst; 6) P2X: It is the receptor for “find me” ATP, activates purinergic signaling, leading to calcium influx to the cell and induces citric acid cycle responsible for the production of ATP; 7) CD39: Hydrolyses ATP to ADP and ADP to AMP, very quickly.

List of tables:

Table 1

Purinergic receptors involved in neutrophil's physiology

Receptor	Agonist	Mechanism	Action on Human Neutrophils	References
A1	ADO	Gi protein	↑ Adhesion ↑ Chemotaxis Inhibits neutrophils microvascular permeability Promotes A2 activation	Dianzani et. al.,1994 Abbracchio et. al, 2009 Corriden et. al, 2010
A2a	ADO	Gs protein	↓ Chemotaxis ↓ Adhesion ↓ ROS production ↓ Activation ↓ Degranulation	Dianzani et al. 1994 Abbracchio et. al, 2009 Corriden et. al, 2010 Mahamed et. al. 2015
A3	ADO	Gi protein	Inhibits migration	Abbracchio et. al, 2009 Corriden et. al, 2010
$P2Y_1$	ADP	Gq protein - Stimulation causes PLCβ activation and calcium mobilization	Undefined role	Jin et. al, 1998 Jacob et. al, 2013
$P2Y_2$	ATP	Gq protein - Increase cytosolic Ca^{2+}through interaction with the actin cytoskeleton	↑ Chemotaxis ↑ Orientation in chemoattractant gradients ↑ Migration ↑ Superoxide production	Corriden et al., 2010 Kukulski et. al, 2010 Önnheim et al. 2014 Gabl et. al, 2015 Ivar von Kügelgen, 2019

$P2Y_6$	UDP	Gq protein	↑ Chemotaxis	Jacob et. al, 2013 Ivar von Kügelgen 2019
$P2Y_{11}$	ATP and NAD+	Gq protein	↑ Apoptosis	Ivar von Kügelgen 2019 Vaughan et. al, 2007
P2X1	ATP	Ion channels permeable for Na^+, K^+, and Ca^{2+}	↑ Chemotaxis	Corriden et al., 2010 Coddou et al., 2011
P2X7	ATP	Ion channels permeable for Na^+, K^+, and Ca^{2+}	Undefined role	(Coddou et. al, 2011 Jacob et. al,2013

Table 2

Overview of main **connections between human neutrophil polarization** and purinergic signaling.

Neutrophil profile	**Features**	**Experimental model**	**Physiological condition**	**Purinergic signaling**	**Ref.**
N1	Neutrophil migration guidance	In vitro Human primary culture	Remove extracellular ATP by apyrase	$P2Y_2$ and A2A-mediated autocrine signaling	Li et al., 2017
N1	Neutrophil migration guidance	In vitro Human primary culture	TLR-4 stimulation by LPS or IL-8 secreted by monocytes	$P2Y_2$-mediated action	Kukulski et al., 2010

N1	Chemotaxis and bacterial clearance impairment	In vitro Human primary culture	High levels of systemic ATP (ATP 1–10 μM)	$P2Y_2$ overstimulation on neutrophils	Li et al., 2017
N1	Neutrophil migration increase at the injured site	In vitro Cell line	Extracellular ADO decrease	Inhibition of CD73 by α,β methylene ADP on the neutrophil surface	Bou Ghanem et al., 2015
N0	Neutrophilic aging	In vitro Human primary culture	Increase intracellular NAD^+ levels and Increase ATP consumption in aged neutrophils	ATP produced by neutrophils via purinergic signaling activity	Richer et al., 2018
N2	Neutrophil apoptosis inhibition	In vitro Human primary culture	Extracellular UDP (300 μM), or HNP-1 (5, 10, 20 and 40 μg/ml)	$P2Y_6$-mediated effect	Nagaoka et al., 2010
N2	Low chemotactic activity	In vitro Human primary culture from healthy donors and patients with cancer	Lack of extracellular ADO	Impairment of extracellular ATP metabolism through ADO	Patel et al., 2018
N2	Improvement of tumor progression and metastasis		Altered release of (NETs)	A high amount of ADO	Amulic et al., 2018 Sorvillo et al., 2019

Capítulo 3: COCULTURA 3D COMO FERRAMENTA DE ANÁLISE DA PROGRESSÃO TUMORAL NA PRESENÇA DE NEUTRÓFILOS (resultados preliminares)

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1. Introdução

O tumor é um conjunto de células que estão em crescimento anormal, frequentemente associado à presença de mutações nos casos malignos. O ambiente em que essas células se encontram é composto por demais células não tumorais que influenciam diretamente no desenvolvimento e permanência do câncer [27-33]. Com isso, um olhar mais focado em compreender o microambiente tumoral e formas de manipular as células não-transformadas associadas ao tumor está em ascendência para o desenvolvimento de novas terapias antitumorais [34-35].

Estudos sobre as interações do infiltrado imune presente no microambiente tumoral emerge sob o conceito de comportamento ambíguo das células imunes, principalmente macrófagos e neutrófilos associados ao tumor [28-36]. A modulação fenotípica permite a estabilização de um ambiente imunossupressor, ao mesmo tempo que promove um processo de inflamação crônica, dando subsídio suficiente para a progressão tumoral [31].

Os neutrófilos possuem um espectro de ativação ainda pouco conhecido: podem assumir um perfil pró-inflamatório, conhecido como N1, ou anti-inflamatório, N2 [40]. Essa ativação é dependente do meio em que essa célula se encontra, visto que sua modulação ocorre por fatores do ambiente, como sinais e citocinas. No microambiente tumoral, por exemplo, há evidência dessas duas subpopulações, sendo que o perfil N1 é adquirido com o bloqueio de fator de crescimento tumoral (TGF-B), liberando citocinas que atuarão contra o tumor, como fator de necrose tumoral (TNF-α) e molécula de adesão intercelular 1 (ICAM-1), reduzindo a expressão de arginase-1, diminuindo a angiogênese e recrutando linfócitos T [40-

43]. Já o fenótipo N2 é visto com a presença de TGF-β, favorecendo a progressão tumoral através da liberação de fator de crescimento endotelial vascular (VEGF-A), metalopeptidase-9 (MMP-9), inibição do recrutamento de células T e outros [40-43]. Dessa forma, é importante ressaltar a possibilidade de o tumor ter a capacidade de induzir um perfil imunossupressor, alterando as ações neutrofílicas e podendo favorecer a progressão tumoral [35, 40-43].

Estudos *in vitro* são frequentemente utilizados para examinar como ocorre a comunicação célula-célula, bem como para avaliar a influência de fatores pontuais [10,23,31]. É importante considerar os três tipos de interação intercelular, além da sinalização entre células vizinhas, observar célula-microambiente e sinalização parácrina por fatores solúveis [10]. Contudo, culturas 2D não são capazes de fornecer todas essas respostas. A estrutura de monocamada é válida para muitos outros aspectos, entretanto, é necessário utilizar ferramentas mais complexas para investigar um formato *in vitro* 3D semelhante ao clínico (Tabela 1) [7, 10, 16, 22].

Conforme mostra a tabela 1, existem evidências suficientes sobre a utilização de esferas por agregado celular para mimetizar condições *in vivo*. Pensando dessa forma, desenvolveu-se um protocolo de cocultivo de células tumorais de diferentes linhagens (U87MG – glioma humano ou HCT-116 câncer colorretal humano) com neutrófilos extraídos de indivíduos saudáveis. O estudo visa compreender a formação do infiltrado imune, a localização dos neutrófilos na esfera 3D e a influência na viabilidade celular do tumor.

Tabela 1: Comparação entre cultura 2D e 3D

Aspectos	Cultura-2D	Cultura-3D	Ref
Formação de cultura	Minutos a horas; para alta confluência pode levar dias	1 a 4 semanas dependendo da linhagem celular	[2] [4] [13] [10]
Qualidade	Alta *performance* e reprodutibilidade, pode ser mantida por longo período, fácil de interpretar	Difícil reprodutibilidade, necessita treinamento, interpretação complexa e não pode ser mantida por muito tempo	[7] [20] [22]
Morfologia	Achatada e esticada, células em monocamada	Forma esfera por crescimento agregado natural, gerando a forma tridimensional natural	[8] [9] [11]
Características	Baixa resposta a estímulos mecânicos, o perfil genético e proteico diferente das condições *in vivo*, células moderadamente diferenciadas	Propensa a estímulos mecânicos, expressão genética e proteica similar às condições *in vivo* e células bem diferenciadas	[14] [21] [17] [3][18]
Interação	Limitada a borda das células ao lado	Formação de nichos e interações mais complexas	[24] [25]
Imitação *In vivo*	Estrutura não compatível às observadas *in vivo*	As estruturas *in vivo* são naturalmente tridimensionais, consequentemente esse modelo apresenta propriedades mecânicas e biológicas semelhantes	[6] [23] [20] [19] [4] [22]
Acesso a componentes essenciais	Ilimitado	Variável, dependendo da localização da célula na esfera	[16] [15] [11]
Viabilidade	Proliferam mais rapidamente do que observado *in vivo*	Células proliferam conforme acesso aos nutrientes e interações intercelulares, podendo ser mais rápido ou devagar	[5] [20]
Custo	Baixo custo	Dependendo da técnica pode ter custo elevado	[1] [12] [20] [13] [11] [26]

2. Materiais e métodos

2.1 Cocultura 3D linhagem tumoral-neutrófilo

Esferoide tridimensional gerado através da técnica de gota suspensa [26]. Após a dissociação enzimática da cultura celular de U87MG ou HCT116, a suspensão celular foi diluída com DMEM/10% SFB até a concentração final de 25×10^3 células tumorais (grupo controle) e 25×10^3 células tumorais associadas a 5×10^3 neutrófilos

(grupo de cocultura) em 30 µL. Cada gota foi colocada dentro da tampa de uma placa de 48 poços previamente tratada com agarose a 1,5% e preenchida com 300 µL de PBS- para evitar o ressecamento [37]. As situações em que foram observadas rolagem e/ou queda de gotas foram consideradas como falha no procedimento. As placas foram mantidas imóveis em incubadoras a 37°C e 5% CO_2 por 4 dias até o estabelecimento do agregado celular. Depois disso, cada poço foi preenchido em seu volume máximo com DMEM 10% SFB com o objetivo de fazer com que a esfera caísse suavemente no poço. Foram mantidos assim por 2 dias a 37°C e 5% CO_2.

2.2 Ensaio de infiltrado neutrofílico

O ensaio de infiltrado de neutrófilos teve como objetivo imitar a invasão de neutrófilos no microambiente tumoral. O procedimento consiste na aplicação de uma “piscina” de neutrófilos no ambiente da esfera. Após a retirada do meio de cultura do agregado celular formado, adicionou-se $7,5\times10^5$/500 µL de neutrófilos para cada esferóide tumoral. As esferas foram incubadas por 24h e 72h a 37°C e 5% CO_2.

2.3 Patologia

As esferas foram fixadas com paraformaldeído 4% por 18h e deixadas mergulhadas em PBS até o processamento histológico. O protocolo adaptado do bloco de agarose foi utilizado como molde de agarose a 2% contendo micropoços para cada esfera [38]. O molde foi inserido no cassete histológico para processamento no aparelho histotécnico do OMA pv.85 (Instrumental OMA Ldta, São Paulo, Brasil). Foi realizada a desidratação do material com uma série de soluções alcoólicas com concentrações crescentes (banho de 50%, 70%, 90% de metanol e 4 banhos em 100% de metanol, 1 hora cada), seguido pelo processo de clarificação de 3 banhos de xileno (1 hora cada) e impregnação com parafina líquida (2 banhos de 2 horas cada). Os cortes das lâminas de 4 µm foram realizadas no micrótomo Leica RM2255 (Leica Microsystems, Inc., Wetzlar, Alemanha). O material obtido foi coletado com lâminas de vidro de microscopia carregada positivamente e corado com hematoxilina e eosina ou utilizado para o ensaio imuno-histoquímico.

2.3.1 Coloração de Hematoxilina e Eosina

As lâminas foram desparafinadas com 3 banhos de xilol (5 min cada) e reidratadas em escala descendente de banhos de etanol (1 min cada). A coloração foi realizada com hematoxilina monohidratada (Merck KGaA, Darmstadt, Alemanha) e Eosina amarelada (Synth, Diadema, Brasil).

2.3.2 Imunohistoquímica

As lâminas foram submetidas ao processo de recuperação de antígenos, utilizando uma solução composta de citrato de sódio 10 mM (pH 6,0) para Ki-67 e CD45. As amostras foram incubadas com as respectivas soluções de recuperação a 98°C por 30 min. Foi realizado o bloqueio da atividade endógena da peroxidase com solução de peróxido de hidrogênio a 5% (Merck KGaA, Darmstadt, Alemanha), e o bloqueio da ligação não específica com BSA a 1% (Sigma-Aldrich, St. Louis, Missouri, EUA). Os anticorpos anti-KI-67 (1: 100, Dako, cat. M7240) e anti-CD45 foram incubados por 12h a 4°C. O kit de detecção Reveal (Spring Bioscience Corporation, Pleasanton, EUA) foi utilizado de acordo com as recomendações do fabricante.

3. Resultados preliminares e Perspectivas para continuidade

Os marcadores escolhidos são importantes para visualizar a proliferação celular (Ki67) e a presença de neutrófilos (CD45). Usualmente a marcação por Ki67 é indicador de prognóstico e preditivo [39], dessa forma, fornece informações relevantes para a avaliação da progressão tumoral em contato com neutrófilos. A padronização do cocultivo avaliou o contato em 24h, 72h e 120h. Observou-se que o tempo máximo de incubação ótima é de 72h, após este período o agregado se desfaz. Foram testadas 3 linhagens tumorais: U87MG, HCT116 e A375 – glioma, câncer colorretal e melanoma, respectivamente -, somente a linhagem A375 não formou uma esfera passível de manipulação. O estudo prevê a padronização dessas condições com outras culturas celulares de diferentes tipos tumorais.

Nessas condições, espera-se que haja alteração na quantidade de células tumorais em divisão celular naquelas mergulhadas nos neutrófilos. Observou-se nas esferas de glioma, que após 3h de incubação, as células imunes formam um halo ao redor da esfera e aproximadamente 60-65% dos neutrófilos infiltram a esfera (figura 1 a).

Assim, acredita-se que os neutrófilos que permaneceram no meio de cultura não são modulados ao fenótipo pró-tumoral como os que entram e, por isso, haveria maior viabilidade celular no centro da esfera e menor na borda (figura 1 b) enquanto a esfera sem tratamento teria uma homogeneidade na proliferação celular (figura 1 c). Não foi possível realizar a coloração com o anticorpo para CD45 e confirmar a hipótese.

Para as esferas já formadas com neutrófilos, será realizado protocolo de citometria de fluxo para comparar os marcadores (CD66b, CD15 - N0; HLA-DR - N1; CD10, CD16, LOX-1 - N2) deste com os neutrófilos tratados com TGFβ ou IFNγ, os em cocultivo com as linhagens tumorais respectivas e com os de pacientes. O principal objetivo é avaliar a eficiência da modulação dos neutrófilos pelos tumores.

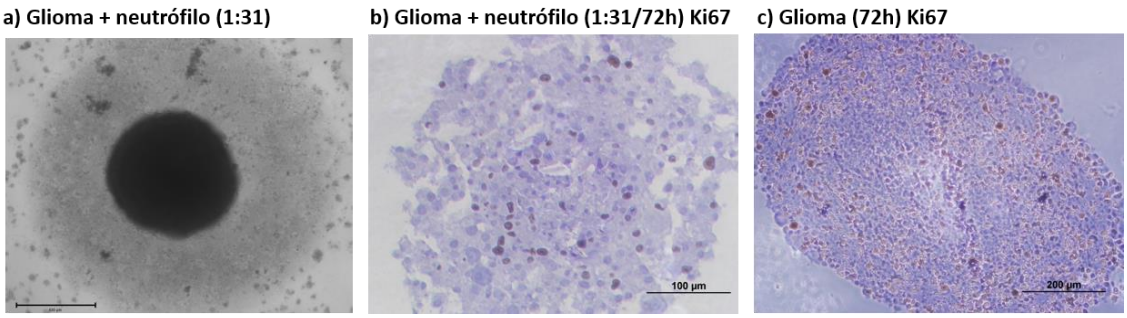

Figura 1: Resultados prévios de cocultura 3D de glioma e neutrófilo

4. Referências

[1] Aggarwal B, Danda D, Gupta S, Gehlot P. Models for prevention and treatment of cancer: problems vs promises. Biochem Pharmacol 2009; 78: 1083-94.

[2] Baker B, Chen C. Deconstructing the third dimension – how 3D culture microenvironments alter cellular cues. J Cell Sci 2012; 125: 3015-24

[3] Bingel C, Koeneke E, Ridinger J, Bittmann A, Sill M, Peterziel H, Wrobel JK, Rettig I, Milde T, Fernekorn U, Weise F, Schober A, Witt O, Oehme I. Three-dimensional tumor cell growth stimulates autophagic flux and recapitulates chemotherapy resistance. Cell Death Dis 2017; 8: e3013 [PMID

[4] Chen S, Chang Y, Nieh S, et al. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. PLoS One 2012; 7: e31864

[5] Elkayam T, Amitay-Shaprut S, Dvir-Ginzberg M, Harel T, Cohen S. Enhancing the drug metabolism activities of C3A-a human hepatocyte cell line--by tissue engineering within alginate scaffolds. Tissue Engineering. 2006;12:1357-1368

[6] Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol 2006; 7: 211-24

[7] Hickman JA, Graeser R, de Hoogt R, Vidic S, Brito C, Gutekunst M, van der Kuip H; IMI PREDECT Consortium. Three-dimensional models of cancer for pharmacology and cancer cell biology: capturing tumor complexity in vitro/ex vivo. Biotechnol J 2014; 9: 1115-1128 [PMID: 25174503 DOI: 10.1002/biot.201300492]

[8] Huang H, Ding Y, Sun XS, Nguyen TA. Peptide hydrogelation and cell encapsulation for 3D culture of MCF-7 breast cancer cells. PLoS One. 2013;8:59482

[9] Joseph JS, Malindisa ST, Ntwasa M. Two-dimensional (2D) and three-dimensional (3D) cell culturing in drug discovery. Cell Culture. 2018 Nov 28;2:1-22.

[10] Kapałczyńska M, Kolenda T, Przybyła W, Zajączkowska M, Teresiak A, Filas V, Ibbs M, Bliźniak R, Łuczewski Ł, Lamperska K. 2D and 3D cell cultures–a comparison of different types of cancer cell cultures. Archives of medical science: AMS. 2018 Jun;14(4):910.

[11] Klingelhutz AJ, Gourronc FA, Chaly A, Wadkins DA, Burand AJ, Markan KR, Idiga SO, Wu M, Potthoff MJ, Ankrum JA. Scaffold-free generation of uniform adipose spheroids for metabolism research and drug discovery. Scientific reports. 2018 Jan 11;8(1):1-2.

[12] Krishnamurthy S, Nör JE. Orosphere assay: a method for propagation of head and neck cancer stem cells. Head Neck 2013; 35: 1015-21

[13] Kuo CT, Wang JY, Lin YF, Wo AM, Chen BP, Lee H. Three-dimensional spheroid culture targeting versatile tissue bioassays using a PDMS-based hanging drop array. Scientific reports. 2017 Jun 29;7(1):1-0.

[14] Langer R, Tirrell. Desigining a material and biological and medicine. Nature. 2004;428(6982): 487-792

[15] Mehta G, Hsiao AY, Ingram M, Luker GD, Takayama S. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. J Control Release 2012; 164: 192-204 [PMID: 22613880 DOI: 10.1016/j.jconrel.2012.04.045]

[16] Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol 2007; 8: 839-845 [PMID: 17684528 DOI: 10.1038/nrm2236]

[17] Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FD. 3D cell culture systems: advantages and applications. J Cell Physiol 2015; 230: 16-26 [PMID: 24912145 DOI: 10.1002/jcp.24683]

[18] Sood D, Tang-Schomer M, Pouli D, Mizzoni C, Raia N, Tai A, Arkun K, Wu J, Black LD, Scheffler B, Georgakoudi I. 3D extracellular matrix microenvironment in bioengineered tissue models of primary

pediatric and adult brain tumors. Nature communications. 2019 Oct 4;10(1):1-4.

[19] Takai A, Fako V, Dang H, Forgues M, Yu Z, Budhu A, Wang XW. Three-dimensional organotypic culture models of human hepatocellular carcinoma. Sci Rep 2016; 6: 21174 [PMID: 26880118 DOI: 10.1038/srep21174]

[20] Weiswald L, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. Neoplasia 2015; 17: 1-15

[21] Xiang Z, Liao R, Kelly MS, Spector M. Collagen–GAG scaffolds grafted onto myocardial infarcts in a rat model: A delivery vehicle for mesenchymal stem cells. Journal of Tissue Engineering. 2006;12:2467-2478

[22] Chaicharoenaudomrung N, Kunhorm P, Noisa P. Three-dimensional cell culture systems as an in vitro platform for cancer and stem cell modeling. World Journal of Stem Cells. 2019 Dec 26;11(12):1065.

[23] Adjei IM, Blanka S. Modulation of the tumor microenvironment for cancer treatment: a biomaterials approach. Journal of functional biomaterials. 2015 Mar;6(1):81-103.

[24] Lv D, Yu SC, Ping YF, Wu H, Zhao X, Zhang H, Cui Y, Chen B, Zhang X, Dai J, Bian XW, Yao XH. A three-dimensional collagen scaffold cell culture system for screening anti-glioma therapeutics. Oncotarget 2016; 7: 56904-56914 [PMID: 27486877 DOI: 10.18632/oncotarget.10885]

[25] Kang HW, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. Nat Biotechnol 2016; 34: 312-319 [PMID: 26878319 DOI: 10.1038/nbt.3413]

[26] Gao B, Jing C, Ng K, Pingguan-Murphy B, Yang Q. Fabrication of three-dimensional islet models by the geometry-controlled hanging-drop method. Acta Mechanica Sinica. 2019 Apr 3;35(2):329-37.

[27] Heppner GH, Miller BE. Tumor heterogeneity: biological implications and therapeutic consequences. Cancer and Metastasis Reviews. 1983 Mar 1;2(1):5-23.

[28] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011 Mar 4;144(5):646-74.

[29] Chen Z and Hambardzumyan D (2018) Immune Microenvironment in Glioblastoma Subtypes. Front. Immunol. 9:1004. doi: 10.3389/fimmu.2018.01004

[30] Koi M, Carethers JM. The colorectal cancer immune microenvironment and approach to immunotherapies. Future Oncology. 2017 Aug;13(18):1633-47.

[31] Hui L, Chen Y. Tumor microenvironment: Sanctuary of the devil. Cancer letters. 2015 Nov 1;368(1):7-13.

[32] Mao, Y.; Keller, E.T.; Garfield, D.H.; Shen, K.; Wang, J. Stromal cells in tumor microenvironment and breast cancer. Cancer Metastasis Rev. 2013, 32, 303–315. 10.

[33] Otranto, M.; Sarrazy, V.; Bonté, F.; Hinz, B.; Gabbiani, G.; Desmouliere, A. The role of the myofibroblast in tumor stroma remodeling. Cell Adhes. Migr. 2012, 6, 203–219

[34] Majd N, de Groot J. Challenges and strategies for successful clinical development of immune checkpoint inhibitors in glioblastoma. Expert opinion on pharmacotherapy. 2019 Sep 2;20(13):1609-24.

[35] Németh T, Sperandio M, Mócsai A. Neutrophils as emerging therapeutic targets. Nature Reviews Drug Discovery. 2020 Jan 22:1-23.

[36] Kim J, Bae JS. Tumor-associated macrophages and neutrophils in tumor microenvironment. Mediators of inflammation. 2016;2016.

[37] Friedrich J, Seidel C, Ebner R, Kunz-Schughart L. Spheroid-based drug screen: considerations and practical approach. Nature Protocols. 2009;4(3):309-324.

[38] Ivanov D, Grabowska A. Spheroid arrays for high-throughput single-cell analysis of spatial patterns and biomarker expression in 3D. Scientific Reports. 2017;7:41160.

[39] Le Guellec S, Perallon R, Alunni J, Charitansky H, Leaha C, Gonzalez A et al.

[Neoadjuvant treatment of breast cancer: implications for the pathologist]. Annales de Pathologie. 2011;31(6):442-454.

[40] Giese MA, Hind LE, Huttenlocher A. Neutrophil plasticity in the tumor microenvironment. Blood. 2019 May 16;133(20):2159-67.

[41] Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nature reviews immunology. 2011 Aug;11(8):519-31.

[42] Liew PX, Kubes P. The neutrophil's role during health and disease. Physiological reviews. 2019 Apr 1;99(2):1223-48.

[43] Shaul ME, Fridlender ZG. Tumour-associated neutrophils in patients with cancer. Nature Reviews Clinical Oncology. 2019 Oct;16(10):601-20.

6. **CONCLUSÕES E PERSPECTIVAS**

Acredita-se que o objetivo dessa pesquisa foi cumprido ao respondermos em parte às perguntas referentes à relação entre os neutrófilos e as células tumorais de glioma humano. Nesta dissertação foram demonstradas técnicas de manipulação e cocutlivo 2D e 3D de neutrófilos humanos e da linhagem celular de GB humano (U87MG) para a observação da conversa cruzada entre essas duas células. Para o desenvolvimento do trabalho, as técnicas de isolamento e de cultivos foram padronizadas a fim de que se obtivesse o melhor resultado.

A descrição do comportamento funcional da célula imune, apresentada no capítulo 1, permitiu o estabelecimento das melhores condições de tratamento celular, revelando a importância da comunicação célula-célula utilizando um protocolo experimental que teve como objetivo mimetizar o microambiente tumoral, bem como a influência direta da célula tumoral na modulação dos neutrófilos. Deixou-se de considerar, nesta pesquisa, demais células imunes e constitutivas do microambiente tumoral devido ao aumento de variáveis as quais impediriam o controle e entendimento dos acontecimentos *in vitro*. Dentre as contribuições deste estudo, está a definição morfológica dos diferentes fenótipos avaliados, que preenche um nicho de estudos em bioinformática sobre caracterização morfológica aplicada à clínica, a qual abre portas para desenvolver softwares que facilitem a caracterização do neutrófilo e auxiliem no prognóstico dos pacientes. Além disso, mostrou-se a relevância dos estudos envolvendo microambiente tumoral e modulação do sistema imune como armas para a progressão do câncer. Por consequência, torna-se conveniente abordar as funções de migração, degranulação e formação de NETs de neutrófilos pela possível relação com o câncer.

Este estudo apresenta limitações, como o nível amostral que foi voluntário, com maior número de amostras femininas em relação às masculinas. Considerando a biologia única de cada amostra, alguns neutrófilos mostraram-se mais reativos que outros – para reduzir essa variável ao máximo, foram inseridas amostras de voluntários com ausência de sintomas gripais ou outras doenças inflamatórias agudas ou crônicas. A idade da amostra se concentra em jovens adultos e adultos, não contemplando idosos ou crianças, o que é interessante avaliar se a modulação de um número maior de neutrófilos estaria ou não relacionada à idade, uma vez que

média dos pacientes ao fazer o diagnóstico de GB é de 50-65 anos. Outro ponto importante é a escassez de abrangência demográfica, já que o perfil dos voluntários foi majoritariamente de origem europeia. Idealmente, torna-se interessante desenhar um estudo com esses objetivos de esclarecer o perfil para cada origem e faixa etária com o intuito de avaliar quaisquer alterações. Entretanto, para dar sequência com esse foco, primeiro a caracterização morfológica, bioquímica e fenotípica deve estar bem consolidada e, por isso, apesar da limitação amostral, essa pesquisa contribui de forma substancial aos estudos imunológicos relacionados ao câncer.

Como perspectivas futuras, protocolos de citometria de fluxo auxiliarão na caracterização celular de cada fenótipo, bem como a cinética de liberação de grânulos e do conteúdo intracelular na degranulação (CD63 - primário; CD66b/CD15 - secundário; CD35 - terciário) e nos NETs. A atribuição de um fenótipo específico favorece na tomada de decisões clínicas em conjunto com demais parâmetros. De todas as formas, a comunicação entre os neutrófilos e as células tumorais mostrou-se relevante no processo de ativação e modulação fenotípica, bem como o desenvolvimento e nicho propício do tumor. A imunohistoquímica das esferas de U87MG e PMN poderá fornecer dados mais sólidos sobre a viabilidade e proliferação do GB e corroborar com os dados deste estudo.

No capítulo 2, observa-se a importância do sistema purinérgico em relação à ativação de neutrófilos. As ectonoclueotidases CD39 e CD73 hidrolisam ATP e geram ADO, importantes sinalizadores que são capazes de modular proliferação, diferenciação, morte celular e inflamação. Essas enzimas estão presentes tanto em células imunes quanto em tumorais. Com isso, a modulação do neutrófilo pode estar relacionada com o sistema purinérgico: a célula apresenta diferentes respostas frente à liberação de ATP, interferindo em sua migração e ativação. Surge a hipótese, desse modo, que os fenótipos anti- (N1) e pró-tumorais (N2) tenham uma relação com os receptores purinérgicos, podendo estar ligados com a progressão tumoral.

A revisão sobre a relação do sistema purinérgico na ativação e migração dos neutrófilos mostra que há poucos estudos com células humanas e não se encontram relatórios sobre pacientes. Em suma, a quantidade de artigos final foi reduzida, pois

a maioria dos estudos publicados são *in vivo* e, apesar do sistema purinérgico já ter papel importante na migração, poucos retratam a sua ação nas demais funções e associando ao fenótipo N2.

Em GB, o sistema purinérgico é amplamente estudado e atribuiu-se a adenosina extracelular como fator solúvel importante na imunossupressão do microambiente tumoral. A resposta imune está intimamente associada ao infiltrado inflamatório mantido pelo tumor. A sinalização purinérgica tem papel importante na modulação da resposta imune através de ATP e adenosina, principalmente. O primeiro apresenta-se como fator desencadeante da ativação e migração de neutrófilos ao mesmo tempo em que a adenosina auxilia na movimentação. Contudo, os seus excessos promovem hiperativação e morte celular e imunossupressão, respectivamente. A via purinérgica pode ser uma opção interessante para alvos farmacológicos, uma vez que está ativa tanto nas células tumorais quanto nas imunes. Esse artigo serve de base para os próximos experimentos focando na participação dos receptores purinérgicos na ativação de neutrófilos e, interferência na modulação deste.

Por fim, mais estudos são necessários nessa mesma linha de pesquisa para que se compreenda o potencial do neutrófilo frente à progressão do GB, bem como a sua influência na fisiopatologia tumoral de acordo com o seu fenótipo.

7. **REFERÊNCIAS**

1. DYKE J, WEAVER I. The Emergence of Environmental Homeostasis in Complex Ecosystems. PLoS Computational Biology. 2013; cap 9(5).
2. BASANTA D et al. Homeostasis Back and Forth: An ecoevolutionary perspective of cancer. Cold Spring Harbor Perspectives in Biology. 2017.
3. MERLO LM et al. Cancer as an evolutionary and ecological process. Nature Reviews. 2006; cap 6 (12): 924-35.
4. KAREVA I. Prisoner's Dilemma in Cancer Metabolism. Plos One. 2011; Cap 6(12).
5. GREAVES M. Evolutionary determinants of cancer. Cancer Discovery. 2015; cap 5(8) 806-20.
6. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008; 454: 428-435
7. GIESE M A, et al. Neutrophil plasticity in the tumor microenvironment. Blood Journal. 2019.
8. ABBAS AK, LICHTMAN AH, PILLAI S. Imunologia celular e molecular. Elsevier Brasil; 2012. 7 edição: cap 6 (109-138); cap 14 (319-344)
9. GARG AD, et al. Pathogen response-like recruitment and activation of neutrophils by sterile immunogenic dying cells drives neutrophil-mediated residual cell killing. Cell Death Differ. 2017; 24(5): 832-843.
10. ORTEGA-GOMEZ A, PERETTI M, et al. Resolution of Inflammation: an integrated view. EMBO Molecular Medicine. 2013; cap 5: 661-674
11. SUGIMOTO MA, SOUSA LP, et al. Resolution of inflammation: what controls its onset? Front Immunology. 2016; cap 7: 160.
12. SMOLEN et al. Rheumatoid arthritis. Lancet. 2016; 388: 2023-2038.
13. HANAHAN D, WEINBERG RA. Hallmarks of cancer: the next generation. Leading Edge. 2011.
14. GUSTAFSON MP, et al. Systemic immune suppression in glioblastoma: the interplay between CD14+HLA-DRlo/neg monocytes, tumor factors, and dexamethasone. Neuro-oncology. 2010; cap 12(7): 631-44.
15. AMERATUNGA M, et al. Anti-angiogenic therapy for high-grade glioma. Cochrane Systematic Review. 2018.
16. TURVEY SE, BROIDE DH. Innate Immunity. Allergy Clinical Immunology. 2010; 125: 524-532.
17. BEUTLER B. Innate immunity: an overview. Molecular Immunology. 2004; 40: 845- 859
18. MAHLAPPU et al. Antimicrobial peptides: an emerging category of therapeutic agents. Frontiers in Cellular and Infection Microbiology. 2016; cap 6: 194.
19. RICKLIN D, et al. Complement: a key system for immune surveillance and homeostasis. Nature Immunology. 2010; 11:785-797.
20. HAJISHENGALLIS G, et al. Novel Mechanisms and functions of complement. Nature Immunology. 2017; 18: 1288-1298.
21. BRUBAKER SW et al. Innate immune pattern recognition: a cell biological perspective. Annu. Rev. Immunology. 2015; 33: 257-290
22. MÓCSAI A. Diverse novel functions of neutrophils in immunity, inflammation,

and beyond. J Exp Med. 2013; 210: 1283-1299.

23. BRINCKMANN, V et al. Neutrophil extracellular traps kill bacteria. Science. 2004; 303, 1532–1535.
24. AMULIC B, CAZALET C, HAYES G L, METZLER KD & ZYCHLINSKY A. Neutrophil function: from mechanisms to disease. Annu. Rev. Immunol. 2012; 30, 459–489.
25. LEY, K., LAUDANNA, et al. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nature Rev. Immunol. 7, 678–689 (2007).
26. MANTOVANI, A et al. Neutrophils in the activation and regulation of innate and adaptive immunity. Nature Rev. Immunol. 11, 519–531 (2011).
27. PHILLIPSON, M. & KUBES, P. The neutrophil in vascular inflammation. Nature Med. 17, 1381–1390 (2011).
28. SADIK, C. D., KIM, N. D. & LUSTER, A. D. Neutrophils cascading their way to inflammation. Trends Immunol. 32, 452–460 (2011).
29. SOEHNLEIN O. & LINDBOM, L. Phagocyte partnership during the onset and resolution of inflammation. Nature Rev. Immunol. 10, 427–439 (2010).
30. BORREGAARD N, SEHESTED M, NIELSEN BS, SENGELOV H, KJELDSEN L. Biosynthesis of granule proteins in normal human bone marrow cells. Gelatinase is a marker of terminal neutrophil differentiation. Blood. 1995; 85: 812-817.
31. MOLINEDO. Neutrophil degranulation, plasticity and cancer metastasis (cell press). 2019.
32. SOEHNLEIN O, et al Neutrophils as protagonists and targets in chronic inflammation. Nat Rev Immunol. 2017; 17: 248-261.
33. Borregaard N. Neutrophils, from marrow to microbes. Immunity. 2010; 33: 657-670.
34. MAYADAS TN, et al. The multifaceted functions of neutrophils. Annu Rev Pathol. 2014; 9: 181-218.
35. SHAUL ME, FRIDLENDER ZG. Neutrophils as active regulators of the immune system in the tumor microenvironment. J Leukoc Biol. 2017; 102 (2): 343-349
36. MORTAZ. Update on neutrophil function in severe inflammation. 2018.
37. NATHAN C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006; 6: 173-182.
38. DONSKOV F. Immunomonitoring and prognostic relevance of neutrophils in clinical trials. InSeminars in cancer biology. 2013 Jun; (Vol. 23, No. 3, pp. 200-207).
39. DUMITRU CA, LANG S, BRANDAU S. Modulation of neutrophil granulocytes in the tumor microenvironment: mechanisms and consequences for tumor progression. InSeminars in cancer biology. 2013 Jun; (Vol. 23, No. 3, pp. 141-148).
40. KOLACZKOWSKA E, KUBES P. Neutrophil recruitment and function in health and inflammation. Nature Reviews Immunology. 2013; Mar;13(3):159.
41. SCAPINI P, CASSATELLA MA. Social networking of human neutrophils within the immune system. Blood. 2014; 124: 710-719.
42. SCAPINI P, MARINI O, TECCHIO C, CASSATELLA MA. Human neutrophils in the saga of cellular heterogeneity: insights and open questions. Immunol Rev. 2016; 273. 48-60
43. KOBAYASHI SD, MALACHOWA N & DELEO FR. Influence of microbes on

neutrophil life and death. Front. Cell Infect. 2017; Microbiol. 7, 159.

44. SHAUL ME, et al. Tumor-associated neutrophils display a distinct N1 profile following TGFβ modulation: A transcriptomics analysis of pro-vs. antitumor TANs. Oncoimmunology. 2016; Nov 1;5(11).
45. NÉMETH T, et al. Neutrophils as emerging therapeutic targets. Nature. 2020.
46. CHAWLA A, et al. Neutrophil elastase enhances antigen presentation by upregulating human leukocyte antigen class I expression on tumor cells. Cancer Immunol Immunother. 2016 Jun; 65(6): 741–75.
47. COX S, HURT C, et al. The prognostic value of derived neutrophil to lymphocyte ratio in oesophageal cancer treated with definitive chemoradiotherapy. Radiotherapy and Oncology. 2017; 125(1): 154–159.
48. GALDIERO MR, et al. Cancer Inflammation and Cytokines. Cold Spring Harb Perspect Biol. 2018 Aug 1;10(8)
49. DI VIRGILIO F. Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. Nat Rev Cancer. 2018 Oct;(10):601-618.
50. KEPP O, LOOS F, LIU P. & KROEMER G. Extracellular nucleosides and nucleotides as immunomodulators. Immunol. Rev. 2017; 280, 83–92.
51. JUNGER WG., et al. Immune cell regulation by autocrine purinergic signaling. Nat Rev Immunol. 2011 Mar; 11(3):201-12.
52. Fuller SJ., et al. Genetics of the P2X7 receptor and human disease. Purinergic Signal. 2009 Jun; 5(2): 257–262.
53. Di Virgilio F., et al. Purinergic signaling in inflammation of the central nervous system. Trends Neurosci. 2009 Feb; 32(2):79-87.
54. HASKÓ G., et al. Therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov. 2008 Sep; 7(9): 759–770.
55. CHEN Y, et al. Purinergic Signaling: A Fundamental Mechanism in Neutrophil Activation. Sci Signal. 2010 Jun; 8;3(125).
56. CHEN Y, et al. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science. 2006; 314: 1792-1795.
57. WANG X, CHEN D. Purinergic regulation of neutrophil function. Frontiers in immunology. 2018 Mar; 1;9:399.
58. DOSCH M, GERBER J, JEBBAWI F, BELDI G. Mechanisms of ATP release by inflammatory cells. International journal of molecular sciences. 2018 Apr; 19(4):1222.
59. SIL P, et al. P2Y6 Receptor Antagonist MRS2578 Inhibits Neutrophil Activation and Aggregated Neutrophil Extracellular Trap Formation Induced by Gout-Associated Monosodium Urate Crystals. J Immunol. 2017 Jan 1;198(1):428-442.
60. BAO Y, LEDDEROSE C, SEIER T, GRAF A F, BRIX B, CHONG E, & JUNGER W G. Mitochondria regulate Neutrophil activation by generating ATP for Autocrine Purinergic signaling. *Journal of Biological Chemistry*. 2014; *289*(39), 26794–26803.
61. DI VIRGILIO F, et al. P2 receptors in cancer progression and metastatic spreading. Curr. Opin. Pharmacol. 2016; 29, 17–25.
62. BASANTA D, et al. The evolution of robust development and homeostasis in

artificial organisms. PLoS computational biology. 2008.

63. GERLEE P, BASANTA D, ANDERSON AR. 2011. Evolving homeostatic tissue using genetic algorithms ProgBiophysMolBiol. 2011; 106: 414–425.
64. NOWELL PC. The clonal evolution of tumor cell populations. Science. 1976 Oct 1; 194(4260):23-8.
65. GERLINGER M, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012 Mar 8;366(10):883-892.
66. GREAVES M. Evolutionary Determinants of Cancer. Cancer Discoery. 2015.
67. WEI R, et al. Cellular and extracellular components in tumor microenvironment and their application in early diagnosis of cancer. 2020.
68. WU S, et al. The Prognostic Landscape of Tumor Infiltrating Immune Cells and Immune Checkpoints in Glioblastoma. Technol Cancer Res Treat. 2019; 18.
69. Turner JS. Homeostasis and the physiological dimension of niche construction theory in ecology and evolution. Evol Ecol. 2016; 30: 303–319.
70. ZHANG S, et al. Interplay between inflammatory tumor microenvironment and cancer stem cells. Oncol Lett. 2018 Jul;16(1):679-686.
71. LIANG J, et al. Neutrophils promote the malignante glioma phenotype through A100A4. Clin Cancer Res. 2014 Jan 1;20(1):187-98
72. HAAS L, et al. Allies or enemies – the multifaceted role of the myeloid cells in the tumor miroenvironment. Front Immunol. 2019; 10: 2746.
73. CHEN Z, et al. Imune microenvironment in gliomas subtypes. Front Immunol. 2018; 9: 1004
74. MICHAELI J, et al. Tumor-associated neutrophils induce apoptosis of non activated cd8 t cells in a tnf alfalfa and NO dependent mechanism, promoting a tumor supportive environment. Oncoimmunology. 2017 Aug 16;6(11).
75. RODRIGUES SF, GRANGER DN. Blood cells and endothelial barrier function. Tissue Barriers. 2015 Apr 3;3(1-2)
76. VERANO-BRAGA T, et al. SuperQuant-assisted comparative proteome analysis of glioblastoma subpopulations allows for identification of potential novel therapeutic targets and cell markers. Oncotarget. 2018; Feb 6;9(10):9400
77. LOUIS DN, et al. The 2007 WHO classification of tumours of the central nervous system. Acta neuropathologica. 2007 Aug; 1;114(2):97-109.
78. DAI C., HOLLAND EC. Tumors of the central nervous system. Biochim. Biophys. Acta (2001) 1551:M19-M27.
79. HUSE JT, HOLLAND EC. Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma. Nature reviews cancer. 2010 May; 10(5):319.
80. BURGER PC, COHEN KJ, ROSENBLUM MK, TIHAN T. Pathology of diencephalic astrocytomas. Pediatric neurosurgery. 2000; 32(4):214-9.
81. STUPP R, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005; 352:987-996.
82. STUPP R, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year

analysis of the EORTC-NCIC trial. Lancet Oncol. 2009; 10:459–66

83. BAO S, WU Q, MCLENDON RE, HAO Y, SHI Q, HJELMELAND AB, DEWHIRST MW, BIGNER DD, RICH JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006;444:756–60
84. JUN HJ, BRONSON RT, CHAREST A. Inhibition of EGFR induces a c-MET-driven stem cell population in glioblastoma. Stem Cells. 2014;32:338–48.
85. HOLLAND EC. Glioblastoma multiforme: the terminator. Proceedings of the National Academy of Sciences. 2000 Jun 6;97(12):6242-4.
86. KAPAŁCZYŃSKA M, et al. 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. Arch Med Sci. 2018 Jun; 14(4): 910–919.
87. CHAICHAROENAUDOMRUN N, et al. Three-dimensional cell culture systems as an in vitro platform for cancer and stem cell modeling. World J Stem Cells. 2019 Dec 26; 11(12): 1065–1083.
88. ADJEI M, BLANKA S. Modulation of the tumor microenvironment for cancer treatment: a biomaterials approach. J Funct Biomater. 2015 Feb 17;6(1):81-103.
89. Gao B, et al. Fabrication of three-dimensional islets models by the geometry-controlled hanging-drop method. 2019. Acta Mechanica Sinica volume 35, pages329–337.
90. JOSEPH JS, et al. Two-Dimensional (2D) and Three-Dimensional (3D) Cell Culturing in Drug Discovery. IntechOpen. 2018.
91. Braganhol E, Kukulski F, Lévesque SA, Fausther M, Lavoie EG, Zanotto-Filho A, Bergamin LS, Pelletier J, Bahrami F, Yebdri FB, Moreira JC. Nucleotide receptors control IL-8/CXCL8 and MCP-1/CCL2 secretions as well as proliferation in human glioma cells. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2015 Jan 1;1852(1):120-30.

CEP

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Determinação de uma assinatura imune em glioblastoma
Pesquisador: Elizandra Braganhol
Área Temática:
Versão: 4
CAAE: 78664117.0.0000.5345
Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.204.937

Apresentação do Projeto:
Trata-se de emenda com a finalidade de incluir pacientes do hospital São José e do hospital Santa Rita da ISCMPA.

Objetivo da Pesquisa:
Inclusão de pacientes dos hospitais São José e Santa Rita

Avaliação dos Riscos e Benefícios:
Não há

Comentários e Considerações sobre a Pesquisa:
Não há

Considerações sobre os Termos de apresentação obrigatória:
Apresentados adequadamente

Conclusões ou Pendências e Lista de Inadequações:
Aprovado

Considerações Finais a critério do CEP:
De acordo com o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1274581_E1.pdf	18/01/2019 21:16:11		Aceito
Outros	FORMULARIODEPROJETOSDEPESQUISA.pdf	18/01/2019 20:54:48	ALINE MORAES DE ABREU	Aceito
Outros	FORMULARIODECADASTRODEPROJETOSNAUNIDADEDEPESQUISA.pdf	18/01/2019 20:54:15	ALINE MORAES DE ABREU	Aceito
Outros	DECLARACAODEUTILIZACAODEDADOSDEPRONTUARIOEUSODEPUBLICACAO.pdf	18/01/2019 20:52:47	ALINE MORAES DE ABREU	Aceito
Outros	DECLARACAODEISENCAODEONUSAINSTITUICAO.pdf	18/01/2019 20:51:21	ALINE MORAES DE ABREU	Aceito
Outros	DECLARACAODECONFIDENCIALIDADE.pdf	18/01/2019 20:50:59	ALINE MORAES DE ABREU	Aceito
Outros	DECLARACAODECOMPROMISSOPARAUTILIZACAODEDADOSDEMATERIALBIOLOGICO.pdf	18/01/2019 20:50:08	ALINE MORAES DE ABREU	Aceito
Outros	DECLARACAODACHEFIARESPONSAVEL.pdf	18/01/2019 20:48:13	ALINE MORAES DE ABREU	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_ISCMPA.pdf	11/12/2018 11:46:30	Elizandra Braganhol	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_GBMCEP_ISCM.pdf	11/12/2018 11:46:03	Elizandra Braganhol	Aceito
Outros	anuenciaHDP.pdf	10/03/2018 05:58:46	Elizandra Braganhol	Aceito
Outros	CartaCEPR2.pdf	10/03/2018 05:57:10	Elizandra Braganhol	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLEreadequadoR2.pdf	10/03/2018 05:56:35	Elizandra Braganhol	Aceito
Projeto Detalhado / Brochura Investigador	ProjGBMCEPR2.pdf	10/03/2018 05:54:16	Elizandra Braganhol	Aceito
Outros	CartaanuenciaGliomasPF.pdf	02/01/2018 02:26:09	Elizandra Braganhol	Aceito
Outros	Termo_entrega_relatorio.pdf	09/10/2017 17:08:24	Elizandra Braganhol	Aceito
Folha de Rosto	folha_rosto_assinada.pdf	16/06/2017 14:45:59	Elizandra Braganhol	Aceito

Situação do Parecer:

Endereço: Rua Sarmento Leite ,245
Bairro: Sarmento **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303-8804 **E-mail:** cep@ufcspa.edu.br

Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 18 de Março de 2019

Assinado por:
Luciane Dalcanale Moussalle
(Coordenador(a))

Endereço: Rua Sarmento Leite ,245
Bairro: Sarmento **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303-8804 **E-mail:** cep@ufcspa.edu.br

Dominique Santos Rubenich

Endereço para acessar este CV: http://lattes.cnpq.br/9855726322767371
ID Lattes: **9855726322767371**
Última atualização do currículo em 22/10/2019

Bacharel em Biomedicina pela Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), bolsista na graduação sanduíche do programa Ciências sem Fronteiras na cidade de Melbourne, Austrália, em um período de 14 meses, pela Monash University. Voluntária no laboratório de análises clínicas e doenças tropicais infecto-contagiosas no hospital de Msambweni, Ukunda - Quênia. Habilitada em genética, imunologia e análises clínicas. Atualmente mestranda do Programa de Pós-Graduação em Biociências, orientada pela prof. dra. Elizandra Braganhol, com o projeto intitulado "Investigação da participação dos neutrófilos na progressão dos glioblastomas" **(Texto informado pelo autor)**

Identificação

Nome Dominique Santos Rubenich
Nome em citações bibliográficas RUBENICH, D. S.;RUBENICH, DOMINIQUE S.
Lattes iD http://lattes.cnpq.br/9855726322767371

Endereço

Formação acadêmica/titulação

2019
Mestrado em andamento em BIOCIÊNCIAS (Conceito CAPES 4).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
Título: Investigação da participação dos neutrófilos na progressão dos glioblastomas,Orientador: Elizandra Braganhol.
Bolsista do(a): Instituicional - UFCSPA, INSTITUCIONAL, Brasil.
Palavras-chave: neutrofilos; glioblastomas; cancer; sistema imunologico; perfil bioquimico.
Grande área: Ciências Biológicas
Setores de atividade: Pesquisa e desenvolvimento científico.

2012 - 2017
Graduação em Biomedicina.
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
com **período sanduíche** em Monash University (Orientador: Dra Angela de Mattos Dutra).
Título: Análise de polimorfismos da região 3' UTR do gene HLA-G e expressão proteica em pacientes com câncer de próstata e hiperplasia prostática benigna.
Orientador: Alessandra Peres e José Artur Bogo Chies.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.

2008 - 2010
Ensino Médio (2º grau).
Centro de Ensino Médio Pastor Dohms, DOHMS, Brasil.

Formação Complementar

2019 - 2019
Abordagem de biologia celular e molecular dos diferentes tipos de cânceres. (Carga horária: 7h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

2018 - 2018
Extensão universitária em Gestão da Qualidade. (Carga horária: 40h).
Universidade de Brasília, UnB, Brasil.

2018 - 2018
Extensão universitária em Excel Intermediário. (Carga horária: 40h).
Universidade de Brasília, UnB, Brasil.

2018 - 2018 Extensão universitária em Excel Básico. (Carga horária: 40h).
Universidade de Brasília, UnB, Brasil.

2018 - 2018 Workshop Redação de Patentes, além dos guias. (Carga horária: 6h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2018 - 2018 Workshop Redação de Patentes, além dos guias. (Carga horária: 8h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2015 - 2015 Extensão universitária em Oficina de Dissecação 2015. (Carga horária: 40h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

2015 - 2015 Cellularised Scaffolds Engineering Vascular Wall. (Carga horária: 2h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

2015 - 2015 PPG Biologia Celular e Molecular aplicada à Saúde. (Carga horária: 15h).
Universidade Luterana do Brasil, ULBRA, Brasil.

2014 - 2014 Global Health: Opportunities and Challenges. (Carga horária: 90h).
Monash University, MONASH, Austrália.

2014 - 2014 Principles of Organ and Body Design. (Carga horária: 90h).
Monash University, MONASH, Austrália.

2014 - 2014 Academic Writing. (Carga horária: 90h).
Monash University, MONASH, Austrália.

2013 - 2014 Monash English Bridging for Undergraduate Degrees. (Carga horária: 96h).
Monash University, MONASH, Austrália.

2012 - 2012 Extensão universitária em Modulo de Primeiros Socorros da UFCSPA. (Carga horária: 8h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

2012 - 2012 Extensão universitária em Doutores Palhaços em Ambiente Hospitalar. (Carga horária: 30h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

2010 - 2010 XII Curso de Primeiros Socorros. (Carga horária: 24h).
Refinaria Alberto Pasqualini, REFAP, Brasil.

2010 - 2010 Papel da Proliferação Celular no Câncer. (Carga horária: 4h).
Faculdade de Odontologia - Departamento de Ciências Morfológicas - UFRGS, ODONTO - UFRGS, Brasil.

2010 - 2010 English language study. (Carga horária: 672h).
International Language Schools of Canada, ILSC, Canadá.

2009 - 2009 Programa Miniempresa Junior Achievement. (Carga horária: 53h).
Centro de Ensino Médio Pastor Dohms, DOHMS, Brasil.

Atuação Profissional

Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

Vínculo institucional

2019 - Atual Vínculo: Bolsista, Enquadramento Funcional: Mestranda, Regime: Dedicação exclusiva.

Outras informações Projeto intitulado "Investigação da participação dos neutrófilos na progressão dos glioblastomas" Estudos em pacientes com glioblastoma levantaram a hipótese de queos neutrófilos podem ter efeitos que favorecem a progressão tumoral. As células imunes exibem plasticidade funcional, alterando-se fenotipicamente e favorecendo a progressão tumoral. Há evidências da presença de fenótipos N1 e N2, porém pouco compreendidos os mecanismos efetores que alteram o comportamento funcional do leucócito. As subpopulações dos neutrófilos são polarizadas em estados de ativação diferentes, um antitumoral (N1) e um pró-tumoral (N2), em resposta a sinais e citocinas vindas no microambiente. O objetivo é diferenciar os dois fenótipos e entender quais são as suas reais diferenças, sejam elas morfológicas ou fisiológicas.

Vínculo institucional

2015 - 2016 Vínculo: Bolsista, Enquadramento Funcional: Voluntaria, Carga horária: 20

Outras informações Atuação no desenvolvimento dos projetos 'Prevalence of cytomegalovirus infection in cirrhotic patients immediately before hepatic transplantation: risk factors and prognosis', 'Evaluation of the effect of hemolysis on the galactomannan quantification test' e 'Diagnosis of latent tuberculosis pre-renal transplantation - new possibilities with serological tests'

Vínculo institucional

2012 - 2012 Vínculo: Bolsista, Enquadramento Funcional: Voluntária, Carga horária: 20

Outras informações Bolsista de iniciação científica voluntária no projeto "Efeito da administração do ômega3 sobre o estresse oxidativo e inflamação no intestino de ratos diabéticos"

Atividades

07/2012 - 12/2012 Ensino, Biomedicina, Nível: Graduação
Disciplinas ministradas
Citologia

GENEX - Instituto de Exames Genéticos, GENEX, Brasil.

Vínculo institucional

2017 - 2017 Vínculo: Estágio Obrigatório, Enquadramento Funcional: Estagiária, Carga horária: 30

Atividades

01/2017 - 05/2017 Estágios , GENEX - Instituto de Exames Genéticos, .
Estágio realizado
Citogenética.

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Vínculo institucional

2016 - 2017 Vínculo: Projeto TCC, Enquadramento Funcional: Voluntária, Carga horária: 20

Outras informações Project titled "Analysis of HLA-G expression in prostate cancer and benign prostatic hyperplasia", under the guidance of Professor Alessandra Peres and co-directed by Professor José Artur Bogo Chies.

Laboratório Endocrimeta, ENDOCRIMETA, Brasil.

Vínculo institucional

2017 - 2017 Vínculo: Estagiária, Enquadramento Funcional: Estagiária, Carga horária: 30

Atividades

07/2017 - 11/2017 Estágios , Laboratório Endocrimeta, .
Estágio realizado
Patologia Clínica (Análises Clínicas).

Msambweni County Refferal Hospital, MSAMBWENI H, Quênia.

Vínculo institucional

2017 - 2017 Vínculo: Voluntária, Enquadramento Funcional: Voluntária, Carga horária: 30

Outras informações Trabalho voluntário através da empresa Exchange do Bem para atuar como Biomédica (Bioquímica) nos laboratórios de análises clínicas e de doenças tropicais infecto-contagiosas, com foco no diagnóstico das diferentes especies de malária.

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Genética / Subárea: Genética Humana e Médica.
2. Grande área: Ciências Biológicas / Área: Bioquímica / Subárea: Biologia Molecular.
3. Grande área: Ciências Biológicas / Área: Imunologia / Subárea: Imunogenética.
4. Grande área: Ciências da Saúde / Área: Farmácia / Subárea: Patologia Clínica (Análises Clínicas).
5. Grande área: Ciências Biológicas / Área: Morfologia / Subárea: Citologia e Biologia Celular.
6. Grande área: Ciências da Saúde / Área: Medicina / Subárea: Clínica Médica/Especialidade: Cancerologia.

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Alemão Compreende Pouco, Fala Pouco, Lê Pouco, Escreve Pouco.
Espanhol Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Pouco.
Português Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.

Prêmios e títulos

2019 Destaque apresentação de pôster - IV Encontro PPG Biociências, PPG Biociências UFCSPA.
2019 Travel Award IUBMB focused Meeting - Tissue Environment in Health and Disease, Champalimaud Foundation.
2014 IELTS, Monash University.
2013 TOEFL ITP, UFRGS.
2012 Bolsa Mérito no curso de Enfermagem, PUCRS.
2007 Zentrale Deutschprufung/Grundstufe (Exame de Alemão/Nível Básico), Centro de Ensino Médio Pastor Dohms.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. MONTEIRO, ALEXANDRE A. ; **RUBENICH, DOMINIQUE S.** ; ZANDONÁ, MARILIA R. ; PASQUALOTTO, ALESSANDRO C. . Impact of pre-analytical variables in the determination of serum galactomannan. MEDICAL MYCOLOGY JCR, v. 6, p. myw123-635-641, 2016.

Citações: WEB OF SCIENCE 1 | SCOPUS 1

Apresentações de Trabalho

1. **RUBENICH, D. S.**; de Souza, P. O. ; OMIZZOLLO, N. ; BRAGANHOL, E. . Glioma-Neutrophil crosstalk modulates tumor microenvironment and neutrophil polarization. 2019. (Apresentação de Trabalho/Simpósio).
2. **RUBENICH, D. S.**; de Souza, P. O. ; BRAGANHOL, E. . THE POLARIZATION OF NEUTROPHILS ASSOCIATED WITH TUMOR ANALIZYS WHEN CULTIVATED IN VITRO WITH GLIOMA CELLS. 2019. (Apresentação de Trabalho/Outra).
3. **RUBENICH, D. S.**; de Souza, P. O. ; BRAGANHOL, E. . Analysis of neutrophil participation at the progression of glioblastomas. 2019. (Apresentação de Trabalho/Outra).

Produção técnica

Redes sociais, websites e blogs

1. BRAGANHOL, E. ; **RUBENICH, D. S.** . IV Encontro PPG Biociências. 2019; Tema: Encontro Biociências. (Site).
2. Vedovatto, S. ; **RUBENICH, D. S.** . @biocelufcspa. 2019; Tema: Divulgação Científica. (Rede social).

Demais tipos de produção técnica

Eventos

Participação em eventos, congressos, exposições e feiras

1. Curso de Bacterologia - XV Congresso Brasileiro de Biomedicina. 2016. (Congresso).
2. Curso de Microbiologia de Alimentos - XV Congresso Brasileiro de Biomedicina. 2016. (Congresso).
3. XV Congresso Brasileiro e III Congresso Internacional de Biomedicina. 2016. (Congresso).
4. I Mostra de trabalhos de ensino, pesquisa e extensão da UFCSPA. 2015. (Outra).
5. 30º Seminário de Extensão Universitária da Região Sul - SEURS.Saúde Bucal para Crianças. 2012. (Oficina).
6. Semana Acadêmica - UFCSPA. 2012. (Outra).
7. XIII Congresso Brasileiro e I Internacional de Biomedicina. 2012. (Congresso).

Outras informações relevantes

.