UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" FACULDADE DE MEDICINA

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O bloqueio da autofagia induzida por drogas aumenta a imunogenicidade das células de câncer colorretal

Tese apresentada à Faculdade de Medicina de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Ramon Kaneno

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Dedico este trabalho a minha família.

"La incertidumbre es una margarita cuyos pétalos no se terminan jamás de deshojar".

Mario Vargas Llosa

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CAPÍTULO I

Vacinas baseadas em células dendríticas como imunoterapia personalizada (Revisão)

Vacinas baseadas em células dendríticas como imunoterapia personalizada

Resumo

As células dendríticas (DCs) são uma população heterogênea de células imunes responsáveis pelo processamento e apresentação de antígenos para os linfócitos T. São divididas em várias subpopulações com características fenotípicas e funcionais distintas e podem ser encontradas no sangue periférico, pele, mucosas, e nos tecidos linfoides e não-linfoides. DCs têm um papel central no desenvolvimento da resposta imune tanto contra agentes infecciosos e câncer, quanto na regulação da resposta em doenças autoimunes e alergias. Considerando que a função das DCs está prejudicada nos pacientes com câncer, vários pesquisadores propuseram o desenvolvimento de vacina antitumorais terapêuticas baseadas nas DCs, objetivando restaurar a capacidade de resposta imune e melhorar a capacidade defensiva de linfócitos T CD4+ e CD8+ contra o câncer. Na presente revisão, apresentamos uma visão geral da biologia das DCs e de seu papel na imunidade antitumoral, os diferentes protocolos de preparação das vacinas e os ensaios clínicos realizados com diferentes tipos de tumor, evidenciando a viabilidade de uso dessas células para personalizar a terapia antitumoral.

<u>Abstract</u>

Dendritic cells (DCs) are a heterogenous population of immune cells responsible for antigens processing and presentation to T lymphocytes. They are divided in several subsets with distinct phenotypic and functional features and can be found in the blood, skin, mucosa, lymphoid and non-lymphoid tissues. DCs have a central role in the development of an immune response both against infectious and malignant diseases, and in the regulation of autoimmune and allergic responsiveness. Considering that DC function is usually impaired in cancer patients, several authors have proposed the development of DC-based antitumor therapeutic vaccines, aiming to restart the immune system and improve the CD4+ and CD8+ lymphocytes responsiveness against cancer. In the present review, we overviewed the DCs' biology and their role in the antitumor immunity, the distinct protocols for preparing DC-based vaccines, and the clinical trials involving different types of cancer, evidencing the feasibility of this approach to personalize the anticancer therapy.

I. **Introdução**

As células dendríticas (DCs) foram observadas pela primeira vez por Paul Langerhans em 1868, que as descreveu como células nervosas cutâneas, devido a sua forma de estrela com ramificações de membrana que se assemelhavam aos neurônios(1). Apenas em 1973, Ralph Steinman e Zanvil A. Cohn estabeleceram formalmente a denominação "células dendríticas"(2) em um artigo que iniciou a uma série de publicações, descrevendo as características morfológicas e funcionais que estabeleceram as bases para a nova era de estudos sobre as DCs. Assim, pode-se citar a capacidade dessas células em ativar e estimular a proliferação de linfócitos T, além de sua grande capacidade de fagocitose (2-5) . Posteriormente, a demonstração por Sallusto e Lanzavecchia (6) de que DCs poderiam ser diferenciadas *in vitro* a partir de monócitos de sangue periférico, rompeu a limitação técnica para o entendimento da biologia das DCs humanas, bem como para os estudos sobre a possibilidade de manipulação *in vivo* ou *ex vivo* dessas células para fins terapêuticos ou profiláticos.

A denominação dessas células decorre da observação de amplas ramificações de sua membrana plasmática à semelhança de dendritos ou ramos de uma árvore. Essas células apresentam um tamanho médio entre 13 a 15 µm e uma superfície externa muito ampla, devido às ramificações da membrana. Essa ampla superfície permite que as DCs interajam e endocitem concomitantemente vários antígenos solúveis ou particulados, que serão processados e posteriormente, apresentados aos linfócitos T. Essa alta capacidade fagocítica e de processamento antigênico torna as DCs as principais células apresentadoras de antígenos (APCs) (7), sendo as únicas células capazes de apresentar antígenos para os linfócitos T virgens e promover sua ativação e proliferação (7).

As DCs se originam principalmente de progenitores hematopoiéticos CD34+ da medula óssea, estimulados pelo ligante de tirosina quinase 3 relacionada a Fms (FLT3L) e GM-CSF

(8). Esses precursores CD34+ diferenciam-se em células progenitoras mielóides comuns e células progenitoras linfóides comuns. As primeiras diferenciam-se em precursores de monócitos-DC, o que dará origem aos monócitos e aos precursores comuns de DC que posteriormente diferenciaram-se em DC pré-clássicas (pré-mDC) ou em DC plasmacitóides (pDC). Estas últimas podem se originar também das células progenitoras linfóides comuns. Essas pDC e pré-mDC deixam a medula óssea e migram via circulação sanguínea para os tecidos periféricos, impulsionadas por quimiocinas. As pré-mDC diferenciam se em mDC1 ou mDC2 dependendo das citocinas do entorno (9) e, no caso de atingirem a pele, podem diferenciar-se em células de Langerhans ou DCs dérmicas intersticiais (10). Portanto, as DCs podem ser classificadas em 3 subgrupos: DCs plasmacitóides, DCs dérmicas e as DCs mielóides (11) (Figura 1).

As DC plasmacitóides são caracterizadas pela expressão de CD123+, CD303+, BDCA-4+ e CD11c-. Essas DCs estão bem equipadas para produzir IFN Tipo 1 (IFN-I) rapidamente, em resposta a infecções virais, devido à alta expressão dos receptores *Toll* similes (TLR) 7 e 9 e são usualmente associadas à resposta anti-infecciosa. Elas também são capazes de ativar as células T CD8 citotóxicas (12). Várias doenças autoimunes estão associadas a níveis elevados de IFN-I, o que implica que as pDCs desempenham um papel importante no desenvolvimento dessas doenças (13), como é o caso da psoríase e do lupus eritematoso sistêmico. Na psoríase, as lesões cutâneas são altamente infiltradas por pDCs ativadas com elevada expressão de BDCA-2 (14). Pacientes com lupus apresentam diminuição dos pDCs circulantes e acúmulo dessas células ativadas nos tecidos afetados, como a pele (15). Além disso, induzem a produção de anticorpos antinucleares e expressam altos níveis de TRL7/9, explicando assim seu alto nível de produção de IFN-I (16).
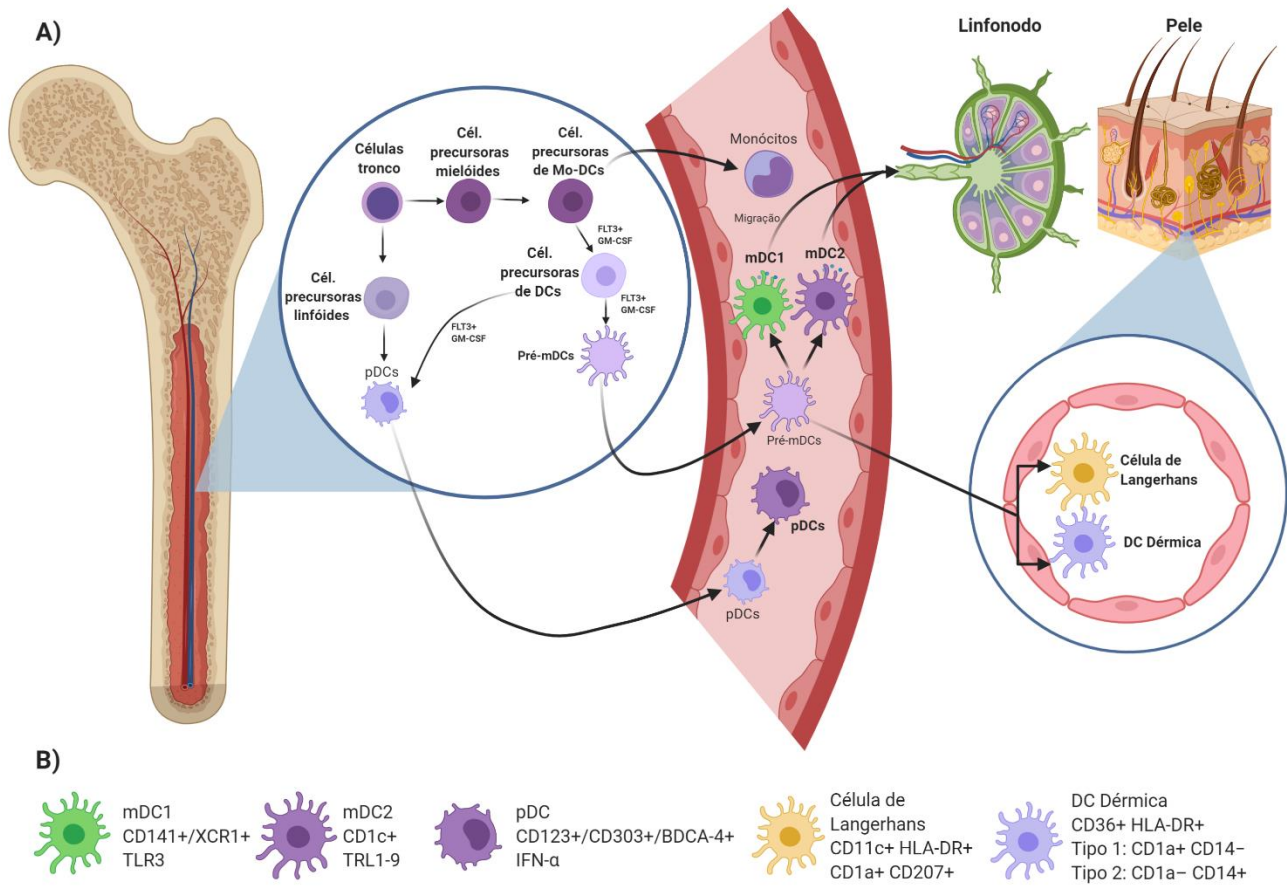
Figura 1. A) Dendropoese: Células tronco hematopoiéticas dão origem a células precursoras mielóides comuns e células precursoras linfoides comuns. As precursoras mielóides se diferenciam em células precursoras de monócitos-DCs e, que que podem se diferenciar em monócitos ou celular precursoras de Mo-DCs. Essas por sua vez, darão origem às pre-mDCs, que posteriormente serão diferenciadas nas principais subpopulações de DCs: mDC1 e mDC2. Essas células são encontradas na corrente sanguínea em sua forma imatura, e uma vez que reconhecem o antígeno, migram aos linfonodos para apresentar os antígenos aos linfócitos. Aquelas que atingem a pele, diferenciam-se em células de Langerhans ou em DCs dérmicas intersticiais. Finalmente, as DCs plasmacitóides (pDCs) podem ser originadas a partir de células precursoras mielóides e linfóides, podendo ser encontradas na corrente sanguínea, tecidos linfóides e não linfóides.

B) Principais marcadores das subpopulações DC: As DCs mielóides convencionais (mDCs) podem ser subdivididas em mDC1 e mDC2, diferenciando-se de acordo com a expressão de CD141, CD1c e TLRs. As pDCs são caracterizados pela expressão de CD123, CD303, BDCA-4 e produção de IFN-α. As DCs que habitam a pele são as células de Langerhans, que expressam níveis elevados de CD11c, CD207 e HLA-DR, e as DCs dérmicas que se subdividem de acordo com a expressão de CD1a e CD14. FLT3: Tirosina quinase 3 relacionada a FMS; GM-CSF: fator estimulador de colônias de granulócitos e monócitos. (Figura elaborada pelo autor).

As pDCs também têm um papel importante na imunidade antitumoral. Por exemplo, no estudo de Wu et al. (2017) os autores mostraram que as pDCs ativadas, obtidas a partir de modelos murinos com câncer de mama, podem eliminar células tumorais HER2/Neu+ *in vitro*, através da liberação de TRAIL e granzima B (17), conhecidos por ativar células NK e T CD8 + (18). Outro estudo mostrou que a ativação intratumoral de pDCs por meio de oligodeoxinucleotídeos CpG (reconhecido por TLR9 (19)), ativou e recrutou células NK, induzindo a regressão tumoral de melanoma *in vivo* (20). Além disso, as pDCs diferenciadas *in vitro* têm maior capacidade de induzir células NK a matar células de leucemia linfoblástica aguda devido à produção de maiores quantidades de IFN-λ2 (21), um tipo de interferon conhecido por desempenhar um papel crítico na indução de células NK antitumorais (18).

As DCs dérmicas, originadas de um progenitor mielóide, são divididas em 2 subgrupos, as células de Langerhans e as DCs dérmicas intersticiais(10). As células de Langerhans são células residentes da epiderme tipicamente caracterizadas como CD1a+ e CD207+, sendo altamente relevantes em infecções cutâneas, pois migram para os linfonodos para interagir com as células T (22). As DC dérmicas intersticiais estão localizadas na matriz extracelular da derme, são caracterizadas pela expressão na superfície de CD36 e a produção do fator de coagulação XIIIa. Estas também são divididas em duas subpopulações de acordo com sua expressão de CD1a e CD14. As células CD1a+ e CD14- estimulam e ativam células TCD8+ enquanto as CD1a- e CD14+ são especializadas em estimular a resposta imune humoral, produzindo CXCL13, e induzindo a geração de células T auxiliares foliculares (Thf) (10, 23).

As DCs mielóides ou DCs "convencionais" são subdivididas em 2 grupos de acordo com a expressão diferencial de CD1c e CD141. As mDC1 expressam CD141, mas não CD1c, enquanto as mDC2, são CD1c$^+$/CD141$^-$. Além disso as mDC2 expressam também moléculas de histocompatibilidade de classe (MHC)-II, CD11c, SIRPα, TRLs (1 a 9), e produzem várias citocinas inflamatórias (24). Estão distribuídas na corrente sanguínea e nos gânglios linfáticos

(25), geralmente associados à apresentação de antígenos para as células T CD4 (26). São produtores das interleucinas (IL)-12p70, IL-1β, IL-6 e IL-23 e são capazes de induzir perfis Th1 e Th17(27). Por último, expressam níveis elevados de dectina-1 e 2 (CLEC7A/CLEC6A) (28), receptores de lectina do tipo C e receptores de manose que reconhecem PAMPS fúngicos (29). Um exemplo disso é o estudo de Fernandes et al. onde desafiaram DCs geradas *in vitro* com antígenos fúngicos derivados de *Paracoccidioides brasiliensis* (Pb18 e Pb265). Observaram que as DCs reconheceram os antígenos fúngicos por meio do receptor de manose, inibindo a produção de TNF-α (30). Em estudo posterior, estimularam o TLR 9 (TLR que reconhece PAMPS fúngicos) presentes nas DCs *in vitro*, aumentando assim sua capacidade antifúngica contra os *P. brasiliensis*, produzindo grandes quantidades de peróxido de hidrogênio (31).

As mDC1, como todas as células apresentadoras de antígenos profissionais, capturam antígenos e os processam pela via endocítica, o que leva à apresentação de peptídeos associados às moléculas de histocompatibilidade de classe II. Os antígenos tumor-associados, gerados no interior das células tumorais, são processados pela via citosólica, sofrendo degradação no proteassoma, cujos peptídeos resultantes passam para o retículo endoplasmático com auxílio das proteínas de transporte TAP-1 e TAP-2. No interior do retículos, os componentes da maquinaria de processamento antigênico, tapasina, calnexina, calreticulina e ERp57, promovem a acomodação dos peptídeos na fenda da cadeia pesada α das moléculas de classe I nascentes, bem como a ligação com a cadeia leve β2-microglobulina, para subsequente expressão na superfície das células tumorais (32). Esses antígenos presentes na superfície das células tumorais são os alvos prioritários dos linfócitos T citotóxicos CD8^{+}. Porém, para que tais células imunes sejam geradas, é necessário que o os antígenos tumorais sejam previamente capturados e processados pelas APCs, que os apresentarão aos linfócitos. Assim, via de regra, células tumorais mortas, fragmentos dessas células ou antígenos solúveis secretados pelas

células tumorais são endocitados pelas APCs profissionais e processados no interior de fagolisossomos por ação das enzimas lisossomais, gerando peptídeos que permanecem no interior dos vacúolos. Esses vacúolos fundem-se com outros que contém moléculas nascentes de MHC classe II, em cuja fenda formada pelas cadeias α e β serão acomodados os peptídeos gerados durante o processamento antigênico (33). Assim, os antígenos processados e apresentados pelas APCs serão reconhecidos por células CD4+. Uma característica essencial das mDC1 é sua capacidade de apresentar antígenos exógenos em associação com MHC-I, fenômeno denominado apresentação cruzada (*cross-priming* ou *cross-presentation*) (11), capacidade hoje reconhecida também em macrófagos(34).

O cross-priming consiste, basicamente, na transferência de peptídeos processados pela via endocítica para compartimentos subcelulares envolvidos na associação de peptídeos citosólicos às moléculas de MHC-I. Este fenômeno foi observado pela primeira vez nos experimentos de compatibilidade de MHC de Bevan et al. (35) em que injetaram no camundongo, células esplênicas de outro camundongo com antígenos de histocompatibilidade menores diferentes. Os autores observaram que esses antígenos menores induziram a geração de linfócitos T citotóxicos (CTL) específicos e concluíram que as células do camundongo receptor poderiam ativar seu sistema imunológico, apresentando os antígenos exógenos menores presentes nas células esplênicas do animal doador. Portanto, havia a apresentação de peptídeos do MHC do doador (antígeno exógeno), associados a moléculas do MHC do receptor, evento que recebeu a referida denominação (35, 36). Anos depois, Rock et al. (37) mostraram que apenas as DCs podem processar e apresentar antígenos extracelulares associados ao MHC classe I.

Atualmente são considerados dois modelos sobre o processo de transferência dos peptídeos para os compartimentos da via citosólica de processamento, referidos como via citosólica e via vacuolar de apresentação cruzada. No modelo da via citosólica, os antígenos

são liberados do lúmen dos compartimentos fagocíticos para o citoplasma, onde são posteriormente processados pelos proteassomos(38), seguindo então o mesmo caminho do processamento de antígenos endógenos. No modelo vacuolar, os antígenos extracelulares são internalizados e degradados nos endossomos por enzimas lisossomais e os peptídeos resultantes são ligados às moléculas de MHC-I presentes nesses mesmos endossomos, por um processo similar aos que ocorre com a apresentação via MHC-II, independente da ação das moléculas TAP e envolvendo a atividade de catepsinas (39, 40).

Uma vez que o antígeno é transportado e expresso no MHC-I das DCs, esse interage diretamente com o receptor de células T (TCR) das células T que usam a molécula CD8 para estabilizar essa interação (41). Esta ligação é aumentada pelas moléculas de co-estimulação CD80 e CD86 presentes nas DCs que se ligam ao CD28 presente nas células T CD8+ (42). A estimulação do TCR e CD28 leva à ativação das vias AMPK (43) e mTOR (44), que regulam o crescimento, proliferação e metabolismo celular, o que resulta na expansão desses linfócitos (45).

Todas as DCs mielóides podem fazer apresentação cruzada, mas o mDC1 é considerado o "especialista" nesse processo (46), não apenas por sua elevada expressão de MHC-I (47) mas também pela expressão de altos níveis de CLEC9A e TLR3 (48). O TLR3 reconhece principalmente PAMPs virais (49) e, quando ativado, ocorre um aumento na produção de IFN-α/β (49) , estimulando a expressão do MHC-I (50) e do TAP1 (51). O CLEC9A, por sua vez, é um receptor endocítico que reconhece filamentos de actina de células necróticas (52) e sua principal função é transportar os antígenos exógenos capturados para os endosomos contendo o MHC-I, promovendo apresentação cruzada (48, 52).

As mDC1 podem ser encontradas nos tecidos linfóides e não linfóides no estado imaturo (iDC), fase caracterizada pela alta expressão de MHC II intracelular, baixa expressão de

moléculas co-estimulatórias e de receptores de quimiocinas (7). Além disso, iDCs têm capacidade de captura e absorção de antígenos por meio de endocitose, pinocitose e fagocitose superior à de sua correspondente madura. Essa característica permite que essas células patrulhem constantemente o ambiente interno, procurando e ativando-se ao reconhecer antígenos estranhos (7). Essa ativação ocorre após o reconhecimento de padrões moleculares associados a danos (DAMP) ou à estimulação de receptores de reconhecimento de padrões (PRR), como os receptores *Toll*-símiles (TLR). Esses sinais iniciam programas de transcrição metabólica, regulando positivamente receptores de quimiocinas como o CCR7, que permitem a migração das DCs dos tecidos periféricos para as áreas dependentes de T dos linfonodos secundários, onde ocorre a apresentação dos antígenos processados pelas DCs maduras às células T *naive* (53). Durante esse processo de maturação, as DCs diminuem sua capacidade fagocítica e de adesão e também reorganizam seu citoesqueleto, aumentando sua mobilidade. A principal mudança observada nas células maduras é o aumento na expressão de MHC-I/II e moléculas co-estimuladoras, como CD40, CD80 e CD86 (2).

Em comum, todas as subpopulações de DCs têm a capacidade de se comunicarem constantemente com outras células do sistema imunológico. Essa comunicação pode ser alcançada por meio da interação direta entre receptores e ligantes de superfície celular (41), ou de modo indireto e remotamente por meio de citocinas. As DCs podem produzir diferentes tipos de citocinas dependendo do estímulo recebido ou do ambiente onde se encontram, sendo as mais relevantes as IL-12, IL-23, IL-27, IL-6, IL -1, o fator de necrose tumoral alfa (TNFα) e o interferon alfa (IFN-α) (54).

Na resposta antitumoral destaca-se a produção de IL-12, citocina composta por duas subunidades covalentemente ligadas, as IL-12p35 e IL-12p40, constituindo a forma bioativa IL-12p70 (55). Sua principal ação é induzir a produção de IFN-γ pelas células T CD4 [+] (gerando uma resposta Th1), células NK e T CD8 (56). Essa citocina aumenta a capacidade citotóxica,

proliferação e migração de células T CD8$^+$ (56) e de células NK (57) favorecendo a fase efetora da atividade antitumoral. A IL-12 também possui atividade anti-angiogênica, através da produção de IFN-γ, que por sua vez aumenta a produção da proteína induzível por interferon - 10 (IP-10) (58), um inibidor da neovascularização que bloqueia a proliferação de células endoteliais (59, 60), evidenciando ainda mais a atividade antitumoral da IL-12.

O TNF-α é uma potente citocina pró-inflamatória, produzida principalmente por macrófagos, mas também pode ser produzida por células NK, linfócitos B e DCs (61). Entre os estímulos que induzem a expressão do TNF-α pelas DC estão as infecções virais e bacterianas, ligação com CD40L, estresse oxidativo, proteínas de choque térmico, HMGB1 e interação com células NK ativadas (62). O TNF-α tem função autócrina e afeta as DCs de várias maneiras por exemplo, melhorando a viabilidade e a migração das células de Langerhans (63) aumentando a expressão do MHC-II nas DCs (64) e promovendo sua a maturação/ativação (65). A maioria dos tipos celulares pode produzir essa citocina em resposta à infecção por vírus (66), mas são as pDCs, que expressam altos níveis de TLR7/9 e IRF- 7, as principais DCs produtoras dessa citocina (66, 67).

A IL-1 é liberada por macrófagos, monócitos e DCs em resposta ao TNF-α´(68). Apresenta três formas, a IL-1α (intracelular) e a IL-1β (extracelular), que apresentam efeitos pró-inflamatórios em decorrência da liberação de histamina pelos mastócitos, causando vasodilatação e sinais de inflamação localizada, além de possuir atividade quimiotática sobre os neutrófilos. A terceira forma é a IL-1RA, que possui atividade inibitória e atua impedindo a ligação de IL-1α e β aos seus respectivos receptores (68). A IL-1 também estimula a produção de IL-6, outra citocina produzida por DCs, macrófagos, células T, células endoteliais e fibroblastos (69), cujas principais funções são promover a produção do fator de crescimento transformador (TGF) -β, a diferenciação de células T CD4 + naive em Th17 (69, 70), estimular a diferenciação de células T foliculares auxiliares e, assim, aumentar a produção de IL-21 (71).

Em oposição às citocinas supracitadas, a IL-10 é uma citocina conhecida pelos seus múltiplos efeitos inibitórios e imunorreguladores(72). De fato, essa citocina pode ser considerada um marcador de DC supressoras pois além de sua baixa capacidade de apresentação de antígenos, as DCs produtoras dessa citocina suprimem a capacidade apresentadora das outras DCs (73). A IL-10 atua regulando negativamente a expressão de citocinas inflamatórias das células Th1, a expressão de antígenos MHC classe II e das moléculas co-estimulatórias em APCs (74). Além disso, em conjunto com TGF-β, estimula a diferenciação das células T reguladoras (Treg) via Foxp3 (75), estabelecendo-se, portanto, uma correlação inversa com a IL-12 e limitando a resposta antitumoral (76, 77).

II. <u>Vacinas antitumorais baseadas em células dendríticas</u>

Durante os estágios iniciais da carcinogênese, os tumores têm a capacidade de modificar e silenciar a resposta imune, especialmente por não expressarem antígenos relevantes para a ativação dos sistema imune (78) e pela produção de citocinas reguladoras (79-81). As vacinas baseadas em DC destinam-se principalmente a corrigir esse estado de anergia, reativando o sistema imunológico e estimulando-o a reconhecer as células tumorais.

O primeiro ensaio clínico de vacinas baseadas em DC em imunoterapia foi publicado em 1996 por Hsu et al. (82), em que quatro pacientes com linfoma folicular de células B receberam uma série de três injeções subcutâneas de DCs pulsadas com antígenos obtidos a partir de biópsias dos gânglios linfáticos em conjunto com KLH (Keyhole limpet hemocyanin), uma proteína altamente imunogênica. Os autores observaram que os quatro pacientes desenvolveram resposta imune contra KLH sendo que um deles apresentou regressão tumoral completa e outro regressão parcial (82).

Apesar do grande número de estudos realizados para o desenvolvimento das vacinas terapêuticas antitumorais de DC e, que vários centros em todo o mundo utilizem clinicamente as preparações vacinais, não há ainda um protocolo de consenso para a sensibilização dessas células. Alguns grupos defendem o uso de células progenitoras hematopoéticas CD34+ diferenciadas *in vitro* em DC pela cultura com Flt3-L (83), mas o protocolo mais amplamente utilizado e com maior volume de dados clínicos é baseado na diferenciação *in vitro* de monócitos isolados das células mononucleares de sangue periférico (PBMC) obtidos por leucoférese (84). A suspensão celular, rica em monócitos, obtida por aderência ao plástico ou por seleção de CD14+ é diferenciada em DCs imaturas por ação de citocinas recombinantes, classicamente o GM-CSF e a IL-4 (85) (Figura 2).
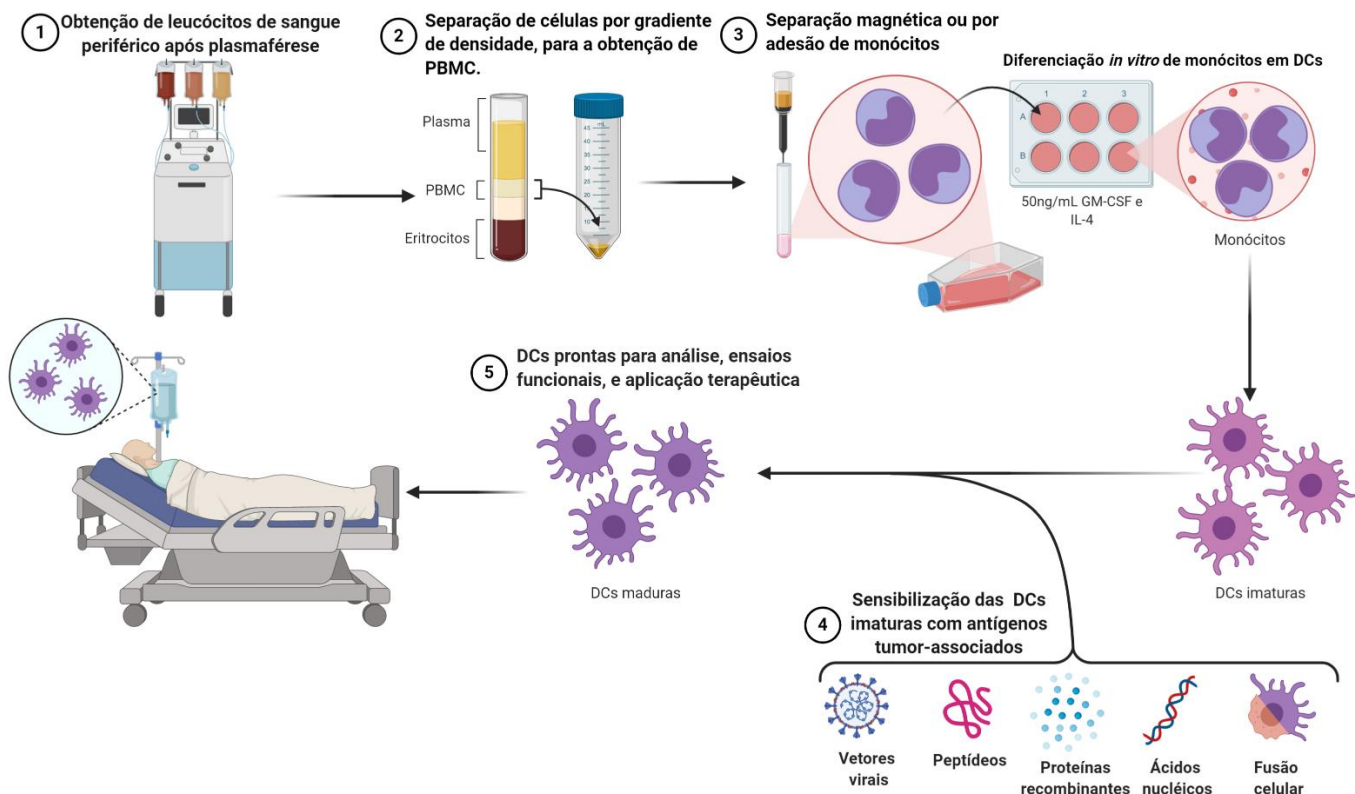
Figura 2. Protocolo básico para diferenciação *in vitro* de células dendríticas a partir de monócitos de sangue periférico. Amostras ricas em leucócitos do paciente, obtidas por plasmaférese, são submetidas a separação por centrifugação em gradiente de densidade para obtenção de células mononucleares (PBMC). Essa suspensão é então submetida a enriquecimento de monócitos, por adesão a superfície plástica de placas de cultura, ou seleção de células CD14+ por separação magnética ou *cell sorter*. Essas células são então cultivadas na presença de GM-CSF e IL-4 recombinantes por 5 dias, para obtenção das iDC. A maturação e ativação dessas células é induzida pela exposição aos antígenos tumor-associados, sendo possível otimizar esse processo com a adição de citocinas ou fatores de crescimento diversos. Após 2 dias de incubação, essas células devem estar ativadas e prontas para inoculação nos pacientes (Figura elaborada pelo autor).

Anguille et al. (86) compararam o protocolo clássico descrito acima e o seu próprio, no qual a IL-4 foi substituída por IL-15 (87), seguindo-se cultura por 5 dias e subsequente ativação da maturação pela adição de citocinas pró-inflamatórias (TNF-α, IL- 1β, IL-6, PGE2) e um coquetel de TRL 7/8 solúvel. Os autores constataram que as DCs de seu protocolo apresentaram expressão superior de CD83, CD40, e as moléculas de co-estimulação (CD80, CD86) além de secretar mais IFN-γ e IL-12p70. Em outro protocolo, foi proposta a combinação de IL-4 com IL-3, no lugar de GM-CSF, com base no fato de que monócitos possuem receptores para essa interleucina e as DCs utilizam IL-3 para proliferar (88). Essa combinação, entretanto, gerou

DCs que produziam menos IL-12p70 e mais IL-10 em comparação com aquelas que foram diferenciadas com GM-CSF ou por co-cultura com células T CD4+ alogênicas, bem como menor capacidade de induzir a produção de IFN-γ (89). Além disso, o uso de citocinas pró-inflamatórias como TNF-α e IFN-γ para complementar a ativação de iDCs é uma opção a ser considerada, pois estimulam a maturação, como evidenciado pelo aumento da expressão de marcadores de superfície como CD83, CD86, HLA-DR, CD40 e CD25 (90, 91).

Estudos prévios de nosso grupo evidenciaram que quimioterápicos selecionados também podem favorecer a maturação das DCs (92). Observamos que baixas concentrações não citotóxicas de quimioterápicos como vinblastina, paclitaxel, azacitidina, metotrexato e mitomicina C aumentam a expressão de CD83 e CD40 nas DCs geradas *in vitro* (93) e de MHC-II, bem como a produção de IL-12p70 (94). Também observamos que o uso de paclitaxel (PTX) e doxorrubicina (DOX) em baixas concentrações aumentam a expressão de proteínas da maquinaria de processamento de antígeno, como calmodulina, LMP2, LMP7, TAP1 e tapasina (95) por células de câncer de cólon humano HCT-116.

Os protocolos de sensibilização de DCs podem variar de acordo com a natureza do antígeno, sendo comumente utilizadas proteínas purificadas, proteínas recombinantes, peptídeos, e ácidos nucléicos. Proteínas purificadas e eventualmente peptídeos, são geralmente obtidos de lisados de células tumorais do próprio paciente, objetivando a obtenção da maior variedade possível de antígenos únicos do indivíduo para sensibilizar as DCs autólogas. O racional dessa abordagem é possibilitar a estimulação de uma imunidade específica ao repertório de antígenos presentes no câncer do paciente, eventualmente ainda não caracterizados (96). Além de seu custo relativamente baixo, as principais vantagens do uso de lisado tumoral autólogo são a ausência de restrição pelo HLA e o curto tempo de preparação, comparado às estratégias artificiais de produção de neoantígenos.

Em nosso grupo, usamos esse tipo de preparação para avaliar se o tratamento de células tumorais com baixas concentrações de quimioterápicos aumenta a imunogenicidade dos antígenos expressos na superfície. Para isso, DCs geradas *in vitro* foram expostas ao lisado tumoral de células de câncer colorretal HCT-116 pré-tratadas com baixas concentrações de PTX e DOX. Essas DC foram então co-cultivadas com linfócitos autólogos por duas semanas, posteriormente testadas quanto presença de células citotóxicas específicas para HCT-116. Observamos que as DCs tratadas com esse lisado induziam células T citotóxicas (CTL) com atividade citotóxica mais elevado do que as DCs tratadas com lisado de células tumorais sem tratamento (95). Usando esse mesmo protocolo, observamos, em outro estudo, que o lisado de células HCT-116 previamente tratadas com 5-fluororacil e cloroquina (bloqueador de autofagia) aumenta a expressão dos marcadores de maturação de DC (CD80, CD83, CD86 e HLA-DR), bem como em sua capacidade de estimular a proliferação de células TCD8+ e TCD4+ específicas para células de câncer colorretal, além de elevar a expressão de IFN-γ e diminuir a expressão de IL-10 (97).

Lisados tumorais também já foram utilizados em estudo clínico de fase I em pacientes pediátricos com gliomas de alto grau, para sensibilizar DCs derivadas de monócitos *in vitro*. Essas DCs foram então usadas como vacinas terapêuticas e, após a administração de nove doses, os pacientes apresentaram pico de produção de IL-4, IL-6, IL-10 TNF-alfa e INF-γ ao 28° dia, porém sem melhora clínica diretamente relacionada à vacina (98).

As proteínas recombinantes utilizadas em protocolos de sensibilização de DCs são geralmente proteínas quiméricas ou fundidas com múltiplos epítopos que têm a capacidade de estimular a resposta imune de forma mais intensa, além de não serem restritas pelo HLA, como alguns peptídeos. Em geral, essas proteínas estão altamente expressas em alguns tipos de câncer, como é o caso da proteína de fusão manana-MUC1 (MFP), presente em vários adenocarcinomas (99). Em um estudo clínico, dez pacientes com diferentes tipos de

adenocarcinomas (pulmão, ovário, rim, mama, cólon e estômago) com elevada expressão de MUC1 foram tratados com DCs autólogas sensibilizadas com a essa proteína de fusão. Após 3 doses mensais da vacina observou-se aumento da produção de IFN-γ em todos os pacientes e estabilização da doença naqueles com a doença em progressão (câncer de ovário e rim), embora níveis elevados de anticorpos anti-MUC1 só tenham sido observados em 3 pacientes (100).

Outra proteína recombinante a ser considerada é o antígeno 3 associado ao melanoma (MAGE-A3), que é um antígeno altamente expresso em melanomas e no carcinoma de células não-pequenas do pulmão, neuroblastomas, sarcomas, câncer de bexiga e nos hepatocarcinomas (101). Em estudo clínico, observou-se regressão tumoral em seis pacientes com melanoma tratados com injeções subcutâneas de um peptídeo MAGE-A3 em solução salina em três doses por três meses (102). Em estudo subsequente, esse protocolo foi testado em 39 pacientes com melanoma metastático nos estágios III e IV, observando-se que em 14 deles a doença regrediu rapidamente a partir da segunda dose (103). Esses resultados promissores estimularam os pesquisadores a criar uma proteína recombinante denominada MAGE-A3 CI, sintetizada pela combinação de MAGE-A3 combinada com o adjuvante AS15 (104). A atividade antitumoral e de estimulação imunológica de MAGE-A3 CI foi demonstrada em vários estudos, incluindo um ensaio clínico, realizado em 71 pacientes com câncer de pulmão de células pequenas, nos quais foram detectados anticorpos anti-MAGE-A3 em todos os indivíduos. Além disso, mais da metade dos pacientes apresentaram níveis elevados de células TCD4+ específicas e células T CD8+ especificas em quatro dos 71 pacientes. Em outro estudo, 25 pacientes com melanoma no estágio IIB-IV MAGE-A3+ foram tratados com MAGE-A3 CI, administrado por via intramuscular (13 pacientes) ou intradérmica (12 pacientes), esperando estimular diretamente DCs convencionais, DCs dérmicas e células de Langerhans. Constatou-se que um paciente de cada grupo apresentou resposta específica e proliferativa de linfócitos TCD8+ contra MAGE-A3. Além disso, quatro pacientes do primeiro grupo e sete do outro apresentaram aumento da

proliferação de linfócitos TCD4+. A contagem de DCs convencionais, DCs dérmicas e células de Langerhans não variou entre as vias de administração, embora a via intradérmica tenha produzido maior resposta de TCD4+. Finalmente, ambas as vias foram bem toleradas, e todos os pacientes geraram anticorpos específicos para MAGE-A3 (NCT01425749)(105).

A transfecção viral também tem sido utilizada como meio de sensibilização de DCs. Por exemplo, no estudo de Di Nicola et al. o gene da tirosinase (MVA-hTyr) foi inserido em vírus da vaccínia de Ancara (versão atenuada do poxvírus) com o objetivo de transfectar DCs com esse gene, visto que a tirosina quinase é um dos antígenos tumorais associados ao melanoma. Essa vacina foi testada em seis pacientes com melanoma maligno nos estágios 3 e 4, observando-se aumento gradual nas células TCD8+ específicas parao antígeno tumoral e aumento de IFN-γ na corrente sanguínea dos pacientes a partir do 5º dia após a injeção (106). Outro vírus usado como um vetor de transfecção é o adenovírus, mais especificamente o vírus com replicação defeituosa que codifica o antígeno de melanoma MART-1. Este é um antígeno imunogênico, normalmente expresso em células pigmentadas normais e que tem expressão exacerbada em células malignas, incluindo melanomas. Este vírus foi utilizado para sensibilizar DCs de 14 pacientes com melanoma metastático, com o objetivo de ativar as células T CD8+ e CD4+ específicas ao MART-1. Após a administração de três doses de 10^7 DCs, os autores observaram aumento nos níveis séricos de IFN-γ e aumento de TCD8+ circulantes específicos para MART-1, compatíveis com a estabilização da doença em quatro dos 14 pacientes e aumento do tempo de sobrevida média de 27 para 42 meses em outros 4 (107).

DCs podem ser também sensibilizadas com vetores artificiais, como as células de vetor adjuvante artificial (VAA) que são fibroblastos que expressam artificialmente α-galactosilceramida (α-GalCer), uma estrutura morfologicamente similar a CD1d, que estimulam células NK invariantes (NKi). Este subtipo de NK é caracterizado por expressar altamente as cadeias αβ invariantes do receptor de células T (TCR) e produzir IL-4 e IFN-γ,

estimulando uma resposta Th1 (108). Ao contrário das células T convencionais, que reconhecem peptídeos ligados a moléculas de MHC, as células NKi reconhecem glicolipídios ligados às moléculas do MHC de classe I não polimórficas, chamadas CD1d (109). As NKi, ao reconhecerem os VAA, além de destruí-los, ativam as DC que fagocitam os fragmentos celulares desses vetores (110). Em um ensaio experimental *in vivo,* o grupo observou aumento no IFN-γ e redução da massa tumoral (111) e no estudo clínico de fase I/II, 17 dos 18 pacientes com câncer de pulmão avançado tratados com o VAA tiveram aumento no IFN-γ, com aumento da sobrevida em 18 meses em comparação com o grupo controle (112).

A transfecção de ácidos nucléicos com vetores não-virais e sua subsequente expressão proteica em DCs também tem sido amplamente utilizada. A técnica consiste em isolar material genético de células tumorais, unindo-as a um veículo de transfecção não-viral que introduz essa informação nas DCs, que posteriormente poderá expressar as proteínas correspondentes para apresentação antigênica, permitindo que antígenos tumorais (exógenos), geralmente apresentados em associação com moléculas de MHC classe II, sejam agora apresentados no contexto das moléculas de classe I (após processamento citosólico) (113). Em nossos estudos, transfectamos iDC com RNA total de células de câncer de colón HCT-116, previamente tratadas com 5-fluorouracil. Observamos que essas DCs apresentaram aumento da expressão dos marcadores de maturação e co-estimulação (CD83, HLA-DR, CD80 e CD86), em comparação com DCs transfectadas com RNA de células tumorais não tratadas. Além disso, observamos melhora funcional das DCs, evidenciada pelo aumento da capacidade de induzir a proliferação de células T alogênicas, gerar células T CD8+ específicas contra HCT-116 e aumentar a produção *in vitro* de IFN-γ (114). Em estudo similar, utilizando um modelo animal, observamos que camundongos tratados com DCs sensibilizadas com RNA total das células tumorais expostas ao 5-fluorouracil, reduziu de modo significativo o crescimento das células tumorais implantados sub-cutaneamente (115). Em estudo clínico de fase II, 43 pacientes com

câncer de próstata resistente à castração, previamente tratados com docetaxel, foram tratados com vacinas autólogas de DC transfectadas com mRNA que codifica o antígeno específico da próstata (PSA) e fosfatase ácida da próstata (PAP). Os autores observaram que a sobrevida média dos pacientes e a sobrevida livre de progressão tiveram leve aumento em relação ao uso de docetaxel isoladamente, com 78% dos pacientes apresentando resposta específica para PSA e PAP (116).

Outro método alternativo para sensibilizar DCs, com resultados bastante promissores, é a criação de hibridomas compostos por DCs e células tumorais. Este método de fusão celular permite que as DCs sejam expostas a uma ampla gama de antígenos associados a tumores (ATTs) provenientes das células tumorais(117). As DCs então processam endogenamente os ATTs e os apresentam simultaneamente através do MHC classe I e II, resultando na ativação simultânea das células T CD4+ e CD8+(118). Para demonstrar isso, Pinho e Barbuto et al. usaram células de câncer de mama que expressam constitutivamente um antígeno de citomegalovírus (CMV) e as fundiu por eletrofusão com células dendríticas geradas *in vitro*. As DCs híbridas resultantes expressaram CD80, CD86, CD83, HLA-DR e MHC classe I em maior quantidade do que as DCs não fundidas. Além disso, as DCs híbridas geraram uma expansão de linfócitos T citotóxicos específicos para CMV (CTL) e uma alta produção de IFN-γ quando expostas a células tumorais CMV positivas(119). Os ensaios clínicos usando essa técnica em pacientes com câncer tiveram resultados igualmente promissores entre eles, por exemplo, é o estudo de fase I de Avigan et al. onde DCs geradas a partir de monócitos *in vitro* e células tumorais autólogas obtidas de pacientes com câncer renal e de mama metastático foram fundidos. Essas DCs híbridas foram inoculadas em 23 pacientes, descobrindo que em 5 pacientes com câncer de mama e 5 com câncer renal a doença se estabilizou, e em 2 pacientes com esses cânceres, a doença regrediu quase totalmente, reduzindo suas massas tumorais. Além disso, na maioria dos pacientes, foi encontrada uma grande quantidade de células T CD4 + e

CD8 + que expressam IFN-γ(120). Em outro estudo de fase I/II, DCs geradas *in vitro* foram fundidas com células de pacientes com gliobastoma, as DCs híbridas obtidas foram inoculadas intradermicamente na região cervical em 32 pacientes, divididos em 2 grupos, sendo o grupo "R" composto por pacientes com gliobastoma recorrente resistente à temozolomida (n = 10), e o grupo "N" de pacientes recém diagnosticados com glioblastoma (n = 22). Descobrindo que a terapia foi bem tolerada em ambos os grupos de pacientes. As medias da sobrevivência livre de progressão e a sobrevivência global para o grupo R foram de 10,3 e 18,0 meses, e do grupo N foi de 18,3 e 30,5 meses, respectivamente. Isto evidência um aumento de ambas as sobrevivências nos dois grupos, em relação ao tratamento convencional com temozolomida(121), este aumento ainda mais evidente no grupo N quando o glioblastoma é diagnosticado recentemente. Deve-se notar que o grupo "N" foi tratado imediatamente após o diagnóstico. Além disso, foi demonstrado que as DCs híbridas do grupo R apresentaram respostas imunes específicas contra antígenos associados à quimiorresistência (WT-1, gp-100 e MAGE-A3) altamente expressos em gliomas resistentes a temozolomida(122).

Assim, apesar de não haver um protocolo padrão para a preparação de DCs de grau clínico, todas as propostas vacinais apresentam um ponto comum bastante positivo que é sua baixa toxicidade. Segundo a maioria dos relatos, os efeitos colaterais mais comuns em ensaios clínicos foram eventos de grau 1 ou 2, incluindo febre, dor e inflamação no local da injeção (123), possibilitando o uso seguro em diferentes tipos de câncer.

1. **Câncer de próstata**

O câncer de próstata é o segundo câncer mais prevalente em homens, excluindo o câncer de pele não-melanoma, com uma incidência estimada de 65.840 casos em 2020 no Brasil (124). Para o diagnóstico, são utilizados o antígeno prostático específico (PSA) e a fosfatase ácida prostática (PAP), ambos quase exclusivamente expressos nas células epiteliais da próstata e

aumentados nos tecidos do câncer, portanto, ambos representam os marcadores séricos mais utilizados para o diagnóstico e monitoramento do câncer de próstata (125). A maioria dos pacientes é diagnosticada quando o tumor ainda é delimitado pelo órgão, de modo que a prostatectomia radical, a radioterapia e a quimioterapia são modalidades eficazes de tratamento para esses casos (126). Embora a maior parte dos pacientes seja tratada com sucesso com prostatectomia radical ou quimioterapia, quase 30% dos pacientes apresentam recidiva (127), motivando a busca por vacinas terapêuticas. Uma das primeiras tentativas de imunização antitumoral foi baseada na afinidade dos peptídeos PSA ao HLA-A2 (128). Ao combinar vários peptídeos PSA conhecidos e obtidos de amostras de pacientes, ligadas por um oligopeptídeo, foi demonstrada a possibilidade de indução simultânea de células T citotóxicas específicas para diferentes epítopos, embora dependendo do painel HLA do paciente para sua eficácia (129). Os primeiros resultados das vacinas autólogas DC foram esperançosos, uma vez que foi observada a diminuição nos níveis séricos de PSA sem a ocorrência de efeitos colaterais(130).

Anos depois, em 2010, o FDA aprovaria a primeira vacina terapêutica baseada em DCs, o Sipuleucel-T, após 4 anos de estudos clínicos. Esta vacina é composta por DC autólogas originados a partir de monócitos obtidos por leucoférese e sensibilizadas com uma proteína de fusão recombinante de GM-CSF e PAP. Nas duas primeiras fases dos estudos clínicos envolvendo 31 pacientes com câncer de próstata metastático, não foram relatados efeitos colaterais, e também foi encontrada uma diminuição nos níveis séricos de PSA (131). Depois, no ensaio clínico randomizado de fase III, 127 pacientes com câncer de próstata metastático foram avaliados, constatando que após 36 meses de tratamento 34% dos pacientes estavam vivos, em comparação com 11% no grupo controle, que apresentaram sobrevida média de 4,5 meses. A contagem média de linfócitos T foi oito vezes maior nos pacientes tratados que nos controles (132). Esses resultados foram tão bem recebidos que seu uso como terapia em conjunto com outros quimioterápicos de primeira linha (133, 134) (NCT01981122) e

radioterapia (135) (NCT01818986) também tiveram resultados notáveis. A maioria de estudos clínicos atuais trabalham na mesma linha, sempre usando vacinas baseadas em DCs em associação com outros medicamentos antineoplásicos, incluindo imunoterápicos como Nivolumab ou Pembrolizumabe (bloqueadores PD-1) (NCT03525652, NCT03600350, NCT04090528) (136).

2. Câncer de mama

O câncer de mama representa aproximadamente 25% de todos os cânceres no mundo (137). Estima-se para 2020 cerca de 66.280 casos de câncer de mama apenas no Brasil. Além disso, o risco de ocorrência dessa doença nas mulheres é estimado entre 10% e 12,8% (124). Os marcadores prognósticos mais comumente usados para o diagnóstico desta doença são os receptores hormonais de estrogênio (ER), progesterona (PR) e receptor 2 do fator de crescimento epidérmico humano (HER2), que geralmente são expressos na superfície das células de câncer de mama (138, 139). Nos últimos anos, a mucina 1 (MUC1) foi adicionada ao painel de marcadores, pois é superexpressa em pacientes com prognóstico ruim (140). Os principais métodos utilizados na terapia contra o câncer de mama são cirurgia, radioterapia, quimioterapia e terapia hormonal (141). A vacina DC mais amplamente utilizada contra o câncer de mama foi a Lapuleucel-T, na qual o se utiliza um princípio semelhante ao peptídeo Sipuleucel-T, recombinando-se HER2 ao adjuvante GM-CSF. Observou-se que os 18 pacientes toleraram muito bem a terapia, sem apresentar efeitos adversos e sem aumentar a contagem de linfócitos T (142). No entanto, uma das desvantagens desta vacina é que sua eficácia depende da expressão de HER2 ou MUC-1 nos tecidos cancerígenos dos pacientes (143). Portanto, em pacientes cujo tumor tem baixa expressão desses marcadores, foi proposto o uso de DC sensibilizadas *in vitro* com lisados de tumor autólogo, resultando em alta produção de CTL específicos e produção de IFN-γ *in vitro* (144), bem como proliferação de células T CD4 + e CD8 + (145). Atualmente, os ensaios clínicos usam vacinas de DC pulsadas com peptídeo

HER-2, além da quimioterapia neoadjuvante (NCT03387553, NCT02063724, NCT02061423) e novas abordagens, como o uso de neoantígenos (peptídeos sintéticos formados a partir de biópsias de pacientes) para pulsar DC autólogas (NCT04105582).

3. Câncer colorretal

As estatísticas mais recentes indicam que o câncer colorretal (CCR) é o terceiro câncer mais comum no mundo (137) e afetará mais de 40.990 pessoas até o final de 2020 no Brasil (124). Uma terapia convencional para pacientes com CCR é baseada na colectomia total ou parcial, geralmente seguida de quimioterapia neoadjuvante ou adjuvante (146). Embora 80% dos pacientes com CCR submetidos à cirurgia juntamente com quimioterapia tenham uma resposta majoritariamente favorável (147), um estudo recente mostra que 50% dos casos de CCR tratados com cirurgia e quimioterapia apresentam recidiva tumoral (148). Sob essa premissa, foi avaliado o uso da imunoterapia como tratamento complementar no combate a esta doença. Na década passada, houve relativamente poucos estudos de vacinas contra DC aplicadas ao CRC em comparação com outros tipos de câncer.

Os três maiores estudos concluídos foram estudos randomizados de fase II, realizados em pacientes com câncer colorretal que foram submetidos à ressecção cirúrgica de metástases, seguida de vacinação com DC. Em ordem cronológica o primeiro envolveu dez pacientes com CCR e metástases hepáticas e vacinação por via intradérmica e intravenosa com três doses de DCs pulsadas *in vitro* com peptídeo CEA (antígeno carcinoembrionário), antes da ressecção das metástases hepáticas. Biópsias hepáticas de sete dos 10 pacientes, revelaram número elevado de células T específicas para CEA com produção de grandes quantidades de IFN-γ. Outro achado interessante foi a presença de células T CEA-específicas em linfonodos próximos às metástases hepáticas em um paciente, mas não no sangue periférico (149). No segundo estudo, 26 pacientes com metástases hepáticas provenientes da CCR, receberam vacinas DC

aleatoriamente e previamente ativadas pelo ligante de CD40 solúvel (CD40L), com seguimento de cinco anos. O CD40L ao ligar as CD40 na superfície das DCs, estimula a produção de IL-12, ativando as células T em direção ao perfil Th1 (150). A análise *in vitro* mostrou que, embora a adição de CD40L não aumentasse a expressão de MHC I ou II em DC, aumentou a expressão de CD86. Infelizmente, esses achados não foram suficientes para melhorar a resposta imune do paciente ou a sobrevida livre de recorrência (RFS), que foi de 58% no primeiro ano e 38% em 5 anos. Trinta e três por cento dos pacientes tratados apresentaram uma resposta favorável contra o tumor, uma semana após a vacinação, com níveis aumentados de IFN-γ, e esses mesmos pacientes apresentaram maior tempo livre de recorrência (63%) em 5 anos do que aqueles com níveis de IFN-γ baixo (18%). Infelizmente esses achados não foram significativamente melhores do que os observados no grupo controle (151).

No terceiro estudo, dois tipos de tratamento foram utilizados, o primeiro usando DCs tratadas *ex vivo* com um vetor viral que codifica CEA e MUC1 denominado "PANVAC" (DC/PANVAC) e o segundo injetado diretamente PANVAC junto com GM-CSF (PANVAC-P), em 76 pacientes com CCR metastático. Os resultados mostram que a RFS foi semelhante entre os dois grupos (47% DC/PANVAC vs 55% PANVAC-P), com um aumento na sobrevida global em 2 anos no grupo vacinado com DCs que foi de 94% (DC / PANVAC) vs. 87% (PANVAC-P). Finalmente, a resposta específica das células T contra o CEA foi semelhante nos dois grupos (152). Os resultados indicam que os melhores resultados são alcançados com o uso de DC como terapia pós-operatória complementar. Visto que os tumores são diferentes em cada paciente, o uso de lisados obtidos a partir de biópsias tumorais como fonte dos antígenos tumorais únicos desses pacientes aumentaria sua eficiência (NCT02919644, NCT04147078).

4. Outros tipos de câncer

Atualmente o uso clínico de vacinas baseadas em DCs tem sido testado em todos os tipos de câncer em diferentes estágios (153, 154), entre estes, um que apresentou resultados promissores foi o melanoma.

No estudo de fase II de Boudewijns et al. (155) foram incluídos pacientes com melanoma em estágio III (n = 22) e IV (n = 32). Eles receberam 3 doses de vacinas DC transfectadas com mRNA de gp100 e tirosinase (antígenos tumorais altamente expressos em melanoma(156)) tratados com ou sem cisplatina. Os resultados foram variados, por um lado, as DC transfectadas em combinação com cisplatina funcionaram melhor, produzindo um aumento nas células TCD8+ em pacientes na fase IV em comparação com a monoterapia com DC (59% vs 40%, respectivamente), mas naqueles em fase III, não foi observada nenhuma diferença significativa entre os tratamentos. No entanto, não foram observados efeitos adversos relacionados ao uso de vacinas DC. Em outro estudo em andamento randomizado com placebo de fase IIb, foram incluidos 144 pacientes com melanoma de alto risco em estágios III e IV. Os pacientes foram tratados com DCs pré-sensibilizadas com lisado de tumor autólogo por 18 meses. Os resultados preliminares mostraram que, em comparação com o placebo, o uso de DCs sensibilizadas resultou em uma melhora estatisticamente significativa na sobrevida livre da doença (51,8% vs 27,1%) e sobrevida geral (92,9% vs 70,3%). Os pacientes no estágio IV mostraram um aumento na sobrevida livre de 49,7% em comparação com 29,4% do controle placebo, e os pacientes no estágio III mostraram um aumento de 68,6% em comparação com 9,4%, do placebo. Além disso, o tratamento foi bem tolerado pelos pacientes, apenas 34,7% dos pacientes relataram reações adversas leves relacionadas à vacinação (NCT02301611).

Resultados semelhantes foram observados no tratamento do glioblastoma, que é um dos cânceres com maior índice de complicações em cirurgia(157), portanto, o uso de vacinas de

DC é uma das alternativas a se considerar para o tratamento dessa doença. No estudo de fase II randomizado e com placebo de Wen et al., 124 pacientes com glioblastoma recém-diagnosticado foram contatados. Estes foram divididos em 2 grupos, o primeiro (n=81) tratado com DCs sensibilizadas com antígenos tumorais expressos em glioblastoma (AIM2, MAGE1, TRP-2, HER2, IL-13Rα2 e gp100) e o segundo (n=43) foi o grupo controle tratado com placebo. As doses foram 4 vezes por semana durante 12 meses. Resultando em aumento da sobrevida global mediana e sobrevida livre de progressão em 2 meses e 2,2 meses em comparação com o placebo, respectivamente. Além disso, nenhum efeito adverso foi observado em nenhum grupo(158).

Como a terapia convencional do glioblastoma consiste na ressecção do tumor (em casos possíveis) seguida de radioterapia e temozolomida, ela tem uma baixa sobrevida global média de cerca de 15 meses(159). Devido a esse problema, Inogés et al. propôs a adição de vacinas DCs à terapia convencional de glioblastoma. Em seu estudo de fase II foram usadas DCs sensibilizadas com lisado de tumor autólogo, seguido por terapia convencional contra glioblastoma multiforme em 32 pacientes recém-diagnosticados cujo volume tumoral residual após a ressecção fora baixo. A vacinação começou antes da radioterapia e continuou durante o tratamento com temozolomida. Os resultados foram interessantes, a mediana da sobrevida global foi de 23,4 meses. Em comparação com a terapia convencional, melhorou a sobrevida do paciente em quase 9 meses. Além disso, foi detectado aumento da proliferação de células TCD8+ e liberação de IFN-alfa e IL-12 após a vacinação em 11 pacientes(160).

Outro dos problemas mais comuns no tratamento do câncer é a recidiva, e um dos cânceres mais associados à esta é a leucemia mieloide aguda (LMA)(161). Portanto, em um estudo de fase II, o efeito das vacinas DC transfectadas com mRNA de WT1, um antígeno superexpresso na LMA(162), foi investigado em 30 pacientes geriátricos com um risco muito alto de recidiva. Resultando em uma resposta antileucêmica (diminuição dos blastos no sangue

periférico e estabilização da medula óssea vermelha) em 13 pacientes, e em 4 pacientes a doença estabilizou-se. A taxa de sobrevida global foi maior nos 13 pacientes que responderam em comparação com os que não responderam (53,8% vs. 25,0%). Para demonstrar uma correlação entre a resposta clínica e a estimulação imune causada pela vacinação com DC, foi avaliada a frequência de células T citotóxicas e a produção de citocinas. Encontrando um aumento na proliferação de linfócitos TCD8 + específicos para WT1 e um aumento na produção de IFN-γ e TNF-α em todos os pacientes, que foi maior nos em pacientes que responderam positivamente ao tratamento. Além disso, nenhuma toxicidade relacionada à vacina foi observada(163).

WT1 também está superexpresso na leucemia linfóide aguda (LLA)(164), portanto, seus peptídeos foram usados para sensibilizar DCs no tratamento de LLA em pacientes pediátricos. Como foi no caso clínico relatado por de Saito et al. onde foram analisadas as respostas de uma menina de 15 anos submetida a 14 vacinas de DCs alogênicas (o doador era sua irmã com HLA compatível) sensibilizada com o antígeno WT1 além do transplante de células-tronco hematopoiéticas. A partir da sétima vacinação, foram detectadas células TCD8+ específicas contra WT1 e sua proliferação foi aumentada de acordo com as vacinações subsequentes. Além disso, uma remissão de blastos no sangue periférico foi observada após a quarta dose de vacinação, que durou 44 meses. Por fim, não foram observadas reações adversas de nenhum tipo no paciente, demonstrando mais uma vez a pouca ou quase nenhuma toxicidade das vacinas baseadas em DCs(165).

III. Conclusão

Os dados da literatura nos permitem concluir que as vacinas antitumorais baseadas em DC são seguras, sendo sua produção factível em muitos centros médicos. Comparada a outros

protocolos mais invasivos que envolvem efeitos colaterais graves, o uso de DCs como vacinas terapêuticas raramente produz eventos adversos quando combinada com outras terapias. Esse recurso permite que essas vacinas terapêuticas sejam usadas como terapia complementar em pacientes com câncer de difícil remoção ou para a profilaxia de recidivas. No entanto, apesar de sua eficácia comprovada e resultados promissores, o uso clínico dessas vacinas não é definitivo. Principalmente devido ao grande número de protocolos existentes atualmente, o que dificulta sua escolha e sua padronização. Portanto, encontrar novas maneiras de aumentar a imunogenicidade das fontes de antígenos tumorais e novas terapias combinatórias com as DCs continuam a ser um desafio para tornar mais eficiente e factível a aplicação clínica dessas vacinas.

IV. Referências bibliográficas

1.   Langerhans P. Ueber die Nerven der menschlichen Haut. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin. 1868;44(2):325-37.
2.   Steinman RM, Adams JC, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. J Exp Med. 1975;141(4):804-20.
3.   Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc Natl Acad Sci U S A. 1978;75(10):5132-6.
4.   Steinman RM, Lustig DS, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. J Exp Med. 1974;139(6):1431-45.
5.   Sunshine GH, Katz DR, Feldmann M. Dendritic cells induce T cell proliferation to synthetic antigens under Ir gene control. J Exp Med. 1980;152(6):1817-22.
6.   Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 1994;179(4):1109-18.
7.   Abbas AL, Andrew. Chapter 1: Properties and Overview of Immune Responses. Cellular and Molecular Immunology 8th Edition. ELSEVIER, editor2017.
8.   Ferlazzo G, Klein J, Paliard X, Wei W-Z, Galy A. Dendritic Cells Generated From CD34+ Progenitor Cells With flt3 Ligand, c-Kit Ligand, GM-CSF, IL-4, and TNF-α Are Functional Antigen-Presenting Cells Resembling Mature Monocyte-Derived Dendritic Cells. Journal of Immunotherapy. 2000;23(1).
9.   Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018;154(1):3-20.
10.   Valladeau J, Saeland S. Cutaneous dendritic cells. Seminars in immunology. 2005;17(4):273-83.
11.   O'Keeffe M, Mok WH, Radford KJ. Human dendritic cell subsets and function in health and disease. Cellular and molecular life sciences : CMLS. 2015;72(22):4309-25.

12.   Ye Y, Gaugler B, Mohty M, Malard F. Plasmacytoid dendritic cell biology and its role in immune-mediated diseases. Clin Transl Immunology. 2020;9(5):e1139-e.

13.   Panda SK, Kolbeck R, Sanjuan MA. Plasmacytoid dendritic cells in autoimmunity. Current opinion in immunology. 2017;44:20-5.

14.   Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med. 2005;202(1):135-43.

15.   Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. The American journal of pathology. 2001;159(1):237-43.

16.   Hancharou AY, Chyzh KA, Titov LP, Soroka NF, DuBuske LM. Characterization Of Plasmacytoid Dendritic Cells From Blood Of Patients With Systemic Lupus Erythematosus. Journal of Allergy and Clinical Immunology. 2012;129(2):AB163.

17.   Wu J, Li S, Yang Y, Zhu S, Zhang M, Qiao Y, et al. TLR-activated plasmacytoid dendritic cells inhibit breast cancer cell growth in vitro and in vivo. Oncotarget. 2017;8(7):11708-18.

18.   Souza-Fonseca-Guimaraes F, Young A, Mittal D, Martinet L, Bruedigam C, Takeda K, et al. NK cells require IL-28R for optimal in vivo activity. Proc Natl Acad Sci U S A. 2015;112(18):E2376-E84.

19.   Volpi C, Fallarino F, Pallotta MT, Bianchi R, Vacca C, Belladonna ML, et al. High doses of CpG oligodeoxynucleotides stimulate a tolerogenic TLR9–TRIF pathway. Nature Communications. 2013;4(1):1852.

20.   Liu C, Lou Y, Lizée G, Qin H, Liu S, Rabinovich B, et al. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. The Journal of clinical investigation. 2008;118(3):1165-75.

21.   Díaz-Rodríguez Y, Cordeiro P, Belounis A, Herblot S, Duval M. In vitro differentiated plasmacytoid dendritic cells as a tool to induce anti-leukemia activity of natural killer cells. Cancer immunology, immunotherapy : CII. 2017;66(10):1307-20.

22.   Clayton K, Vallejo AF, Davies J, Sirvent S, Polak ME. Langerhans Cells—Programmed by the Epidermis. 2017;8(1676).

23.   Toebak MJ, Gibbs S, Bruynzeel DP, Scheper RJ, Rustemeyer T. Dendritic cells: biology of the skin. Contact dermatitis. 2009;60(1):2-20.

24.   Nizzoli G, Krietsch J, Weick A, Steinfelder S, Facciotti F, Gruarin P, et al. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood. 2013;122(6):932-42.

25.   Granot T, Senda T, Carpenter DJ, Matsuoka N, Weiner J, Gordon CL, et al. Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. Immunity. 2017;46(3):504-15.

26.   Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med. 2010;207(6):1247-60.

27.   Leal Rojas IM, Mok W-H, Pearson FE, Minoda Y, Kenna TJ, Barnard RT, et al. Human Blood CD1c(+) Dendritic Cells Promote Th1 and Th17 Effector Function in Memory CD4(+) T Cells. Front Immunol. 2017;8:971-.

28.   Lundberg K, Albrekt A-S, Nelissen I, Santegoets S, de Gruijl TD, Gibbs S, et al. Transcriptional profiling of human dendritic cell populations and models--unique profiles of in vitro dendritic cells and implications on functionality and applicability. PLoS One. 2013;8(1):e52875-e.

29.   Huysamen C, Brown GD. The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. FEMS Microbiol Lett. 2009;290(2):121-8.

30.   Fernandes RK, Bachiega TF, Rodrigues DR, Golim MdA, Dias-Melicio LA, Balderramas HdA, et al. Paracoccidioides brasiliensis interferes on dendritic cells maturation by inhibiting PGE2 production. PLoS One. 2015;10(3):e0120948-e.

31.   Vieira IR, Fernandes RK, Rodrigues DR, Gorgulho CM, Kaneno R, Soares ÂMVC. TLR9 stimulation induces increase in fungicidal activity of human dendritic cells challenged with Paracoccidioides brasiliensis. Medical Mycology. 2018;56(7):911-5.

32.   Leone P, Shin E-C, Perosa F, Vacca A, Dammacco F, Racanelli V. MHC Class I Antigen Processing and Presenting Machinery: Organization, Function, and Defects in Tumor Cells. JNCI: Journal of the National Cancer Institute. 2013;105(16):1172-87.

33.   Goldberg AC, Rizzo LV. MHC structure and function   antigen presentation. Part 1 %J Einstein (São Paulo). 2015;13:153-6.

34.   Muntjewerff EM, Meesters LD, van den Bogaart G. Antigen Cross-Presentation by Macrophages. Front Immunol. 2020;11:1276.

35.   Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med. 1976;143(5):1283-8.

36.   Carbone FR, Heath WR. Cross-Priming: Its Beginnings. The Journal of Immunology. 2010;185(3):1353.

37.   Rock K, Gamble S, Rothstein L. Presentation of exogenous antigen with class I major histocompatibility complex molecules. 1990;249(4971):918-21.

38.   Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annual review of immunology. 2013;31:443-73.

39.   Pfeifer JD, Wick MJ, Roberts RL, Findlay K, Normark SJ, Harding CV. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature. 1993;361(6410):359-62.

40.   Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity. 2004;21(2):155-65.

41.   Abbas AK, Lichtman AH, Pillai S. Chapter 6: Antigen Processing and Presentation to T Lymphocytes. Cellular and molecular immunology2017.

42.   Lanier LL, O'Fallon S, Somoza C, Phillips JH, Linsley PS, Okumura K, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. Journal of immunology (Baltimore, Md : 1950). 1995;154(1):97-105.

43.   Salmond RJ, Emery J, Okkenhaug K, Zamoyska R. MAPK, phosphatidylinositol 3-kinase, and mammalian target of rapamycin pathways converge at the level of ribosomal protein S6 phosphorylation to control metabolic signaling in CD8 T cells. Journal of immunology (Baltimore, Md : 1950). 2009;183(11):7388-97.

44.   Powell JD, Delgoffe GM. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. Immunity. 2010;33(3):301-11.

45.   Henrickson SE, Perro M, Loughhead SM, Senman B, Stutte S, Quigley M, et al. Antigen availability determines CD8+ T cell-dendritic cell interaction kinetics and memory fate decisions. Immunity. 2013;39(3):496-507.

46.   Noubade R, Majri-Morrison S, Tarbell KV. Beyond cDC1: Emerging Roles of DC Crosstalk in Cancer Immunity. Front Immunol. 2019;10:1014-.

47.   Theisen D, Murphy K. The role of cDC1s in vivo: CD8 T cell priming through cross-presentation. F1000Research. 2017;6:98.

48.   Schreibelt G, Klinkenberg LJJ, Cruz LJ, Tacken PJ, Tel J, Kreutz M, et al. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. Blood. 2012;119(10):2284-92.

49.   Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 2005;433(7028):887-92.

50.   Goodbourn S, Didcock L, Randall RE. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. 2000;81(10):2341-64.

51.   Agrawal S, Reemtsma K, Bagiella E, Oluwole SF, Braunstein NS. Role of TAP-1 and/or TAP-2 antigen presentation defects in tumorigenicity of mouse melanoma. Cellular immunology. 2004;228(2):130-7.

52.	Zhang JG, Czabotar PE, Policheni AN, Caminschi I, Wan SS, Kitsoulis S, et al. The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. Immunity. 2012;36(4):646-57.

53.	Riol-Blanco L, Sánchez-Sánchez N, Torres A, Tejedor A, Narumiya S, Corbí AL, et al. The Chemokine Receptor CCR7 Activates in Dendritic Cells Two Signaling Modules That Independently Regulate Chemotaxis and Migratory Speed. The Journal of Immunology. 2005;174(7):4070.

54.	Manfred B. Lutz NR. Cytokines Produced by Dendritic Cells. Handbook of Dendritic Cells2006. p. 355-83.

55.	Floss DM, Moll JM, Scheller J. IL-12 and IL-23-Close Relatives with Structural Homologies but Distinct Immunological Functions. Cells. 2020;9(10).

56.	Henry CJ, Ornelles DA, Mitchell LM, Brzoza-Lewis KL, Hiltbold EM. IL-12 produced by dendritic cells augments CD8+ T cell activation through the production of the chemokines CCL1 and CCL17. Journal of immunology (Baltimore, Md : 1950). 2008;181(12):8576-84.

57.	Parihar R, Dierksheide J, Hu Y, Carson WE. IL-12 enhances the natural killer cell cytokine response to Ab-coated tumor cells. The Journal of clinical investigation. 2002;110(7):983-92.

58.	Sgadari C, Angiolillo AL, Tosato G. Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10. Blood. 1996;87(9):3877-82.

59.	Campanella GSV, Colvin RA, Luster AD. CXCL10 Can Inhibit Endothelial Cell Proliferation Independently of CXCR3. PLoS One. 2010;5(9):e12700.

60.	Gao N, Liu X, Wu J, Li J, Dong C, Wu X, et al. CXCL10 suppression of hem- and lymph-angiogenesis in inflamed corneas through MMP13. Angiogenesis. 2017;20(4):505-18.

61.	Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot Gene Expr. 2010;20(2):87-103.

62.	Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. Immunity. 2003;19(1):59-70.

63.	Cumberbatch M, Griffiths CE, Tucker SC, Dearman RJ, Kimber I. Tumour necrosis factor-alpha induces Langerhans cell migration in humans. The British journal of dermatology. 1999;141(2):192-200.

64.	Simmons DP, Wearsch PA, Canaday DH, Meyerson HJ, Liu YC, Wang Y, et al. Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing. Journal of immunology (Baltimore, Md : 1950). 2012;188(7):3116-26.

65.	Maney NJ, Reynolds G, Krippner-Heidenreich A, Hilkens CMU. Dendritic cell maturation and survival are differentially regulated by TNFR1 and TNFR2. Journal of immunology (Baltimore, Md : 1950). 2014;193(10):4914-23.

66.	Fitzgerald-Bocarsly P, Feng D. The role of type I interferon production by dendritic cells in host defense. Biochimie. 2007;89(6-7):843-55.

67.	Asselin-Paturel C, Brizard G, Chemin K, Boonstra A, O'Garra A, Vicari A, et al. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. J Exp Med. 2005;201(7):1157-67.

68.	Lie PPY, Cheng CY, Mruk DD. The biology of interleukin-1: emerging concepts in the regulation of the actin cytoskeleton and cell junction dynamics. Cellular and molecular life sciences : CMLS. 2012;69(4):487-500.

69.	Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014;6(10):a016295-a.

70.	Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annual review of immunology. 2009;27:485-517.

71.	Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nature immunology. 2007;8(9):967-74.

72.	Sabat R, Grütz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. Cytokine & growth factor reviews. 2010;21(5):331-44.

73.   Wilson EB, Kidani Y, Elsaesser H, Barnard J, Raff L, Karp CL, et al. Emergence of distinct multiarmed immunoregulatory antigen-presenting cells during persistent viral infection. Cell Host Microbe. 2012;11(5):481-91.

74.   Howes A, Gabryšová L, O'Garra A. Role of IL-10 and the IL-10 Receptor in Immune Responses. Reference Module in Biomedical Sciences: Elsevier; 2014.

75.   Hsu P, Santner-Nanan B, Hu M, Skarratt K, Lee CH, Stormon M, et al. IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. Journal of immunology (Baltimore, Md : 1950). 2015;195(8):3665-74.

76.   Rivas JR, Alhakeem SS, Eckenrode JM, Zhang Y, Collard JP, Hildebrandt GC, et al. Enhancing Anti-Tumor Immunity and Responses to Immune Checkpoint Blockade By Suppressing Interleukin-10 in Chronic Lymphocytic Leukemia. Blood. 2019;134(Supplement_1):5486-.

77.   Rivas JR, Alhakeem SS, Liu Y, Eckenrode JM, Marti F, Collard JP, et al. Interleukin-10 suppression enhances T-cell antitumor immunity and responses to checkpoint blockade in chronic lymphocytic leukemia. bioRxiv. 2020:2020.07.15.204560.

78.   McGranahan N, Rosenthal R, Hiley CT, Rowan AJ, Watkins TBK, Wilson GA, et al. Allele-Specific HLA Loss and Immune Escape in Lung Cancer Evolution. Cell. 2017;171(6):1259-71.e11.

79.   Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. Genes Dev. 2018;32(19-20):1267-84.

80.   Domínguez-Soto A, Sierra-Filardi E, Puig-Kröger A, Pérez-Maceda B, Gómez-Aguado F, Corcuera MT, et al. Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. Journal of immunology (Baltimore, Md : 1950). 2011;186(4):2192-200.

81.   Jarnicki AG, Lysaght J, Todryk S, Mills KH. Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. Journal of immunology (Baltimore, Md : 1950). 2006;177(2):896-904.

82.   Hsu FJ, Benike C, Fagnoni F, Liles TM, Czerwinski D, Taidi B, et al. Vaccination of patients with B–cell lymphoma using autologous antigen–pulsed dendritic cells. Nature Medicine. 1996;2(1):52-8.

83.   Fay JW, Palucka AK, Paczesny S, Dhodapkar M, Johnston DA, Burkeholder S, et al. Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed CD34(+) progenitor-derived dendritic cells. Cancer immunology, immunotherapy : CII. 2006;55(10):1209-18.

84.   Sabado RL, Balan S, Bhardwaj N. Dendritic cell-based immunotherapy. Cell Research. 2017;27(1):74-95.

85.   CECHIM G, CHIES JAB. In vitro generation of human monocyte-derived dendritic cells methodological aspects in a comprehensive review %J Anais da Academia Brasileira de Ciências. 2019;91.

86.   Anguille S, Smits EL, Cools N, Goossens H, Berneman ZN, Van Tendeloo VF. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. Journal of translational medicine. 2009;7:109.

87.   Anguille S, Smits ELJM, Cools N, Goossens H, Berneman ZN, Van Tendeloo VFI. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. Journal of translational medicine. 2009;7:109-.

88.   Lutz MB. IL-3 in dendritic cell development and function: a comparison with GM-CSF and IL-4. Immunobiology. 2004;209(1-2):79-87.

89.   Ebner S, Hofer S, Nguyen VA, Fürhapter C, Herold M, Fritsch P, et al. A novel role for IL-3: human monocytes cultured in the presence of IL-3 and IL-4 differentiate into dendritic cells that produce less IL-12 and shift Th cell responses toward a Th2 cytokine pattern. Journal of immunology (Baltimore, Md : 1950). 2002;168(12):6199-207.

90.   Chabot V, Martin L, Meley D, Sensebé L, Baron C, Lebranchu Y, et al. Unexpected impairment of TNF-α-induced maturation of human dendritic cells in vitro by IL-4. Journal of translational medicine. 2016;14:93-.

91.   Decker WK, Li S, Xing D, Robinson SN, Yang H, Steiner D, et al. Deficient T(H)-1 responses from TNF-alpha-matured and alpha-CD40-matured dendritic cells. Journal of immunotherapy (Hagerstown, Md : 1997). 2008;31(2):157-65.

92.   Nars MS, Kaneno R. Immunomodulatory effects of low dose chemotherapy and perspectives of its combination with immunotherapy. Int J Cancer. 2013;132(11):2471-8.

93.   Kaneno R, Shurin GV, Tourkova IL, Shurin MR. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. Journal of translational medicine. 2009;7:58.

94.   Shurin GV, Tourkova IL, Kaneno R, Shurin MR. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. Journal of immunology (Baltimore, Md : 1950). 2009;183(1):137-44.

95.   Kaneno R, Shurin GV, Kaneno FM, Naiditch H, Luo J, Shurin MR. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. Cellular Oncology. 2011;34(2):97-106.

96.   Lesimple T, Neidhard E-M, Vignard V, Lefeuvre C, Adamski H, Labarrière N, et al. Immunologic and Clinical Effects of Injecting Mature Peptide-Loaded Dendritic Cells by Intralymphatic and Intranodal Routes in Metastatic Melanoma Patients. Clinical Cancer Research. 2006;12(24):7380.

97.   Zamame Ramirez JA, Romagnoli GG, Falasco BF, Gorgulho CM, Sanzochi Fogolin C, Dos Santos DC, et al. Blocking drug-induced autophagy with chloroquine in HCT-116 colon cancer cells enhances DC maturation and T cell responses induced by tumor cell lysate. Int Immunopharmacol. 2020;84:106495-.

98.   Lasky JL, 3rd, Panosyan EH, Plant A, Davidson T, Yong WH, Prins RM, et al. Autologous tumor lysate-pulsed dendritic cell immunotherapy for pediatric patients with newly diagnosed or recurrent high-grade gliomas. Anticancer research. 2013;33(5):2047-56.

99.   Lau SK, Weiss LM, Chu PG. Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. Am J Clin Pathol. 2004;122(1):61-9.

100.   Loveland BE, Zhao A, White S, Gan H, Hamilton K, Xing P-X, et al. Mannan-MUC1–Pulsed Dendritic Cell Immunotherapy: A Phase I Trial in Patients with Adenocarcinoma. Clinical Cancer Research. 2006;12(3):869.

101.   Roeder C, Schuler-Thurner B, Berchtold S, Vieth G, Driesch P, Schuler G, et al. MAGE-A3 is a frequent tumor antigen of metastasized melanoma. Archives of dermatological research. 2005;296(7):314-9.

102.   Marchand M, Weynants P, Rankin E, Arienti F, Belli F, Parmiani G, et al. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. Int J Cancer. 1995;63(6):883-5.

103.   Marchand M, van Baren N, Weynants P, Brichard V, Dréno B, Tessier MH, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer. 1999;80(2):219-30.

104.   Kruit WH, Suciu S, Dreno B, Mortier L, Robert C, Chiarion-Sileni V, et al. Selection of immunostimulant AS15 for active immunization with MAGE-A3 protein: results of a randomized phase II study of the European Organisation for Research and Treatment of Cancer Melanoma Group in Metastatic Melanoma. J Clin Oncol. 2013;31(19):2413-20.

105.   Slingluff CL, Jr., Petroni GR, Olson WC, Smolkin ME, Chianese-Bullock KA, Mauldin IS, et al. A randomized pilot trial testing the safety and immunologic effects of a MAGE-A3 protein plus AS15 immunostimulant administered into muscle or into dermal/subcutaneous sites. Cancer immunology, immunotherapy : CII. 2016;65(1):25-36.

106.   Di Nicola M, Carlo-Stella C, Mortarini R, Baldassari P, Guidetti A, Gallino GF, et al. Boosting T Cell-Mediated Immunity to Tyrosinase by Vaccinia Virus-Transduced, CD34<sup>+</sup>-Derived Dendritic Cell Vaccination. Clinical Cancer Research. 2004;10(16):5381.

107.   Butterfield LH, Comin-Anduix B, Vujanovic L, Lee Y, Dissette VB, Yang J-Q, et al. Adenovirus MART-1-engineered autologous dendritic cell vaccine for metastatic melanoma. Journal of immunotherapy (Hagerstown, Md : 1997). 2008;31(3):294-309.

108.   Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nature Reviews Immunology. 2013;13(2):101-17.

109.   Krovi SH, Gapin L. Invariant Natural Killer T Cell Subsets—More Than Just Developmental Intermediates. 2018;9(1393).

110.   Fujii S-I, Shimizu K. Immunotherapy with artificial adjuvant vector cells: Harnessing both arms of the immune response. Oncoimmunology. 2013;2(4):e23432-e.

111.   Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nature immunology. 2002;3(9):867-74.

112.   Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. Journal of immunology (Baltimore, Md : 1950). 2009;182(4):2492-501.

113.   Mitchell DA, Nair SK. RNA-transfected dendritic cells in cancer immunotherapy. The Journal of clinical investigation. 2000;106(9):1065-9.

114.   De Almeida CV, Zamame JA, Romagnoli GG, Rodrigues CP, Magalhães MB, Amedei A, et al. Treatment of colon cancer cells with 5-fluorouracil can improve the effectiveness of RNA-transfected antitumor dendritic cell vaccine. Oncol Rep. 2017;38(1):561-8.

115.   de Camargo MR, Gorgulho CM, Rodrigues CP, Penitenti M, Frederico JCL, Rodrigues MAM, et al. Low Concentration of 5-Fluorouracil Increases the Effectiveness of Tumor RNA to Activate Murine Dendritic Cells. Cancer biotherapy & radiopharmaceuticals. 2017;32(8):302-8.

116.   Kongsted P, Borch TH, Ellebaek E, Iversen TZ, Andersen R, Met Ö, et al. Dendritic cell vaccination in combination with docetaxel for patients with metastatic castration-resistant prostate cancer: A randomized phase II study. Cytotherapy. 2017;19(4):500-13.

117.   Takakura K, Kajihara M, Ito Z, Ohkusa T, Gong J, Koido S. Dendritic-tumor fusion cells in cancer immunotherapy. Discov Med. 2015;19(104):169-74.

118.   Koido S. Dendritic-Tumor Fusion Cell-Based Cancer Vaccines. Int J Mol Sci. 2016;17(6):828.

119.   Pinho MP, Sundarasetty BS, Bergami-Santos PC, Steponavicius-Cruz K, Ferreira AK, Stripecke R, et al. Dendritic-tumor cell hybrids induce tumor-specific immune responses more effectively than the simple mixture of dendritic and tumor cells. Cytotherapy. 2016;18(4):570-80.

120.   Avigan D, Vasir B, Gong J, Borges V, Wu Z, Uhl L, et al. Fusion Cell Vaccination of Patients with Metastatic Breast and Renal Cancer Induces Immunological and Clinical Responses. Clinical Cancer Research. 2004;10(14):4699.

121.   Rønning PA, Helseth E, Meling TR, Johannesen TB. A population-based study on the effect of temozolomide in the treatment of glioblastoma multiforme. Neuro Oncol. 2012;14(9):1178-84.

122.   Akasaki Y, Kikuchi T, Homma S, Koido S, Ohkusa T, Tasaki T, et al. Phase I/II trial of combination of temozolomide chemotherapy and immunotherapy with fusions of dendritic and glioma cells in patients with glioblastoma. Cancer Immunology, Immunotherapy. 2016;65(12):1499-509.

123.   Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. The Lancet Oncology. 2014;15(7):e257-e67.

124.   INCA. Estimativa 2020: incidência de câncer no Brasil. Instituto Nacional de Câncer José Alencar Gomes da Silva/ Ministério da Saúde.; 2019.

125.   Xu H, Wang F, Li H, Ji J, Cao Z, Lyu J, et al. Prostatic Acid Phosphatase (PAP) Predicts Prostate Cancer Progress in a Population-Based Study: The Renewal of PAP? Disease Markers. 2019;2019:7090545.

126.   Litwin MS, Tan HJ. The Diagnosis and Treatment of Prostate Cancer: A Review. Jama. 2017;317(24):2532-42.

127.   Ward JF, Blute ML, Slezak J, Bergstralh EJ, Zincke H. The long-term clinical impact of biochemical recurrence of prostate cancer 5 or more years after radical prostatectomy. The Journal of urology. 2003;170(5):1872-6.

128.   Olson BM, Frye TP, Johnson LE, Fong L, Knutson KL, Disis ML, et al. HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase. Cancer immunology, immunotherapy : CII. 2010;59(6):943-53.

129.   Correale P, Walmsley K, Zaremba S, Zhu M, Schlom J, Tsang KY. Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide. Journal of immunology (Baltimore, Md : 1950). 1998;161(6):3186-94.

130.   Xue BH, Zhang Y, Sosman JA, Peace DJ. Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen. The Prostate. 1997;30(2):73-8.

131.   Small EJ, Fratesi P, Reese DM, Strang G, Laus R, Peshwa MV, et al. Immunotherapy of Hormone-Refractory Prostate Cancer With Antigen-Loaded Dendritic Cells. Journal of Clinical Oncology. 2000;18(23):3894-903.

132.   Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, et al. Placebo-Controlled Phase III Trial of Immunologic Therapy with Sipuleucel-T (APC8015) in Patients with Metastatic, Asymptomatic Hormone Refractory Prostate Cancer. Journal of Clinical Oncology. 2006;24(19):3089-94.

133.   Rini BI, Weinberg V, Fong L, Conry S, Hershberg RM, Small EJ. Combination immunotherapy with prostatic acid phosphatase pulsed antigen-presenting cells (provenge) plus bevacizumab in patients with serologic progression of prostate cancer after definitive local therapy. Cancer. 2006;107(1):67-74.

134.   Small EJ, Lance RS, Gardner TA, Karsh LI, Fong L, McCoy C, et al. A Randomized Phase II Trial of Sipuleucel-T with Concurrent versus Sequential Abiraterone Acetate plus Prednisone in Metastatic Castration-Resistant Prostate Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(17):3862-9.

135.   Twardowski P, Wong JYC, Pal SK, Maughan BL, Frankel PH, Franklin K, et al. Randomized phase II trial of sipuleucel-T immunotherapy preceded by sensitizing radiation therapy and sipuleucel-T alone in patients with metastatic castrate resistant prostate cancer. Cancer treatment and research communications. 2019;19:100116.

136.   Westdorp H, Creemers JHA, van Oort IM, Schreibelt G, Gorris MAJ, Mehra N, et al. Blood-derived dendritic cell vaccinations induce immune responses that correlate with clinical outcome in patients with chemo-naive castration-resistant prostate cancer. Journal for ImmunoTherapy of Cancer. 2019;7(1):302.

137.   Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: A Cancer Journal for Clinicians. 2020;70(1):7-30.

138.   Parise CA, Caggiano V. Breast Cancer Survival Defined by the ER/PR/HER2 Subtypes and a Surrogate Classification according to Tumor Grade and Immunohistochemical Biomarkers. Journal of Cancer Epidemiology. 2014;2014:469251.

139.   Iqbal B, Buch A. Hormone receptor (ER, PR, HER2/neu) status and proliferation index marker (Ki-67) in breast cancers: Their onco-pathological correlation, shortcomings and future trends. 2016;9(6):674-9.

140.   Jing X, Liang H, Hao C, Yang X, Cui X. Overexpression of MUC1 predicts poor prognosis in patients with breast cancer. Oncol Rep. 2019;41(2):801-10.

141.   Moo T-A, Sanford R, Dang C, Morrow M. Overview of Breast Cancer Therapy. PET Clin. 2018;13(3):339-54.

142.   Peethambaram PP, Melisko ME, Rinn KJ, Alberts SR, Provost NM, Jones LA, et al. A phase I trial of immunotherapy with lapuleucel-T (APC8024) in patients with refractory metastatic tumors that express HER-2/neu. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(18):5937-44.

143.   Miglietta F, Dieci MV, Griguolo G, Guarneri V, Conte PF. Chemotherapy for advanced HER2-negative breast cancer: Can one algorithm fit all? Cancer treatment reviews. 2017;60:100-8.

144.   Delirezh N, Moazzeni SM, Shokri F, Shokrgozar MA, Atri M, Kokhaei P. Autologous dendritic cells loaded with apoptotic tumor cells induce T cell-mediated immune responses against breast cancer in vitro. Cellular immunology. 2009;257(1-2):23-31.

145.   Gao Y, Chen X, Gao W, Yang Y, Ma H, Ren X. A new purification method for enhancing the immunogenicity of heat shock protein 70-peptide complexes. Oncol Rep. 2012;28(6):1977-83.

146.   Brenner H, Kloor M, Pox CP. Colorectal cancer. The Lancet. 2014;383(9927):1490-502.

147.   Tebbutt NC, Cattell E, Midgley R, Cunningham D, Kerr D. Systemic treatment of colorectal cancer. European journal of cancer (Oxford, England : 1990). 2002;38(7):1000-15.

148.   Sevá-Pereira G, Cypreste RN, Oliveira Filho JJ, Moraes SPd, Tarabay PB. Recurrence pattern of rectal cancer after surgical treatment. Analysis of 122 patients in a tertiary care center %J Journal of Coloproctology (Rio de Janeiro). 2018;38:18-23.

149.   Lesterhuis WJ, de Vries IJ, Schuurhuis DH, Boullart AC, Jacobs JF, de Boer AJ, et al. Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests. Annals of oncology : official journal of the European Society for Medical Oncology. 2006;17(6):974-80.

150.   Ma DY, Clark EA. The role of CD40 and CD154/CD40L in dendritic cells. Seminars in immunology. 2009;21(5):265-72.

151.   Barth RJ, Jr., Fisher DA, Wallace PK, Channon JY, Noelle RJ, Gui J, et al. A randomized trial of ex vivo CD40L activation of a dendritic cell vaccine in colorectal cancer patients: tumor-specific immune responses are associated with improved survival. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(22):5548-56.

152.   Morse MA, Niedzwiecki D, Marshall JL, Garrett C, Chang DZ, Aklilu M, et al. A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. Ann Surg. 2013;258(6):879-86.

153.   Garg AD, Vara Perez M, Schaaf M, Agostinis P, Zitvogel L, Kroemer G, et al. Trial watch: Dendritic cell-based anticancer immunotherapy. Oncoimmunology. 2017;6(7):e1328341-e.

154.   Versteven M, Van den Bergh JMJ, Marcq E, Smits ELJ, Van Tendeloo VFI, Hobo W, et al. Dendritic Cells and Programmed Death-1 Blockade: A Joint Venture to Combat Cancer. Front Immunol. 2018;9:394.

155.   Boudewijns S, Bloemendal M, de Haas N, Westdorp H, Bol KF, Schreibelt G, et al. Autologous monocyte-derived DC vaccination combined with cisplatin in stage III and IV melanoma patients: a prospective, randomized phase 2 trial. Cancer immunology, immunotherapy : CII. 2020;69(3):477-88.

156.   de Vries TJ, Smeets M, de Graaf R, Hou-Jensen K, Bröcker EB, Renard N, et al. Expression of gp100, MART-1, tyrosinase, and S100 in paraffin-embedded primary melanomas and locoregional, lymph node, and visceral metastases: implications for diagnosis and immunotherapy. A study conducted by the EORTC Melanoma Cooperative Group. The Journal of pathology. 2001;193(1):13-20.

157.   Jackson C, Westphal M, Quiñones-Hinojosa A. Complications of glioma surgery. Handbook of clinical neurology. 2016;134:201-18.

158.   Wen PY, Reardon DA, Armstrong TS, Phuphanich S, Aiken RD, Landolfi JC, et al. A Randomized Double-Blind Placebo-Controlled Phase II Trial of Dendritic Cell Vaccine ICT-107 in Newly Diagnosed Patients with Glioblastoma. Clinical Cancer Research. 2019;25(19):5799.

159.   Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England journal of medicine. 2005;352(10):987-96.

160.   Inogés S, Tejada S, de Cerio AL-D, Gállego Pérez-Larraya J, Espinós J, Idoate MA, et al. A phase II trial of autologous dendritic cell vaccination and radiochemotherapy following fluorescence-guided surgery in newly diagnosed glioblastoma patients. Journal of translational medicine. 2017;15(1):104-.

161.   Oliva EN, Franek J, Patel D, Zaidi O, Nehme SA, Almeida AM. The Real-World Incidence of Relapse in Acute Myeloid Leukemia (AML): A Systematic Literature Review (SLR). Blood. 2018;132(Supplement 1):5188-.

162.   Rampal R, Figueroa ME. Wilms tumor 1 mutations in the pathogenesis of acute myeloid leukemia. Haematologica. 2016;101(6):672-9.

163.   Anguille S, Van de Velde AL, Smits EL, Van Tendeloo VF, Juliusson G, Cools N, et al. Dendritic cell vaccination as postremission treatment to prevent or delay relapse in acute myeloid leukemia. Blood. 2017;130(15):1713-21.

164.   Uckun FM, Qazi S. Wilms' tumor gene (<i>WT1</i>) is strongly expressed in high-risk subsets of pediatric acute lymphoblastic leukemia. Cancer Drug Resistance. 2018;1(4):250-65.

165.   Saito S, Yanagisawa R, Yoshikawa K, Higuchi Y, Koya T, Yoshizawa K, et al. Safety and tolerability of allogeneic dendritic cell vaccination with induction of Wilms tumor 1-specific T cells in a pediatric donor and pediatric patient with relapsed leukemia: a case report and review of the literature. Cytotherapy. 2015;17(3):330-5.

CAPÍTULO II

O bloqueio da autofagia induzida por drogas aumenta a imunogenicidade das células de câncer colorretal (trabalho original)

Lista de abreviaturas

5-FU	**5-Fluorouracil**
CCR	**Câncer colorretal**
CD69	*Cluster of differentiation 69*
CQ	**Cloroquina**
HCQ	**Hidroxicloroquina**
CTL	**Células T citotóxicas**
DC	**Célula dendrítica**
GM-CSF	*Granulocyte and macrophage-Colony Stimulating Factor*
IFN-γ	**Interferon-gamma**
IL-10	**Interleucina 10**
IL-4	**Interleucina 4**
MLR	**Reação mista leucocitária**
NTC	**Concentração não toxica**
PD-1	*Programmed cell death protein 1*
WT	**Wild type**

"O bloqueio da autofagia induzida por drogas aumenta a imunogenicidade das células de câncer colorretal"

Resumo

O câncer colorretal (CCR) é o terceiro tipo mais frequente de tumores no Brasil e no mundo e, embora haja esquemas quimioterápicos bem definidos, observam-se muitos casos de recidivas e metástases. Um dos mecanismos de escape tumoral é a autofagia, que pode contribuir para o desenvolvimento de quimiorresistência. Portanto, a associação de drogas inibidoras de autofagia, com os quimioterápicos convencionais, pode contribuir para aumentar a eficiência dos agentes antineoplásicos. Observamos previamente que a exposição de células tumorais a baixas concentrações de agentes antineoplásicos selecionados, além de estimular a autofagia, aumenta sua imunogenicidade e sua capacidade de estimular a maturação das células dendríticas (DC). Assim, no presente estudo testamos a hipótese de que o bloqueio farmacológico da autofagia com cloroquina (CQ), em combinação com baixas concentrações de 5-fluorouracil (5-FU), favorece a imunogenicidade das células tumorais. Nossos resultados mostram que as DCs sensibilizadas com o lisado de células tumorais previamente expostas a combinação de 5-FU e CQ aumentam seus marcadores de maturação e ativação (CD80, CD83, CD86, HLA-DR). Essas DCs maturas também aumentaram a capacidade de induzir resposta alogênica das células T CD4+ e CD8+, que manifestaram uma resposta proliferativa superior de àquelas induzidas por DCs sensibilizadas com lisados sem tratamento prévio. Ademais, as células T geradas nessas co-culturas apresentam aumento do marcador de ativação CD69, enquanto a expressão de PD-1 é reduzida. Observamos também níveis mais altos de IFN-γ e reduzida produção de IL-10, sendo consistente com uma ativação preferencial das células Th1. A co-cultura de DCs e linfócitos autólogos melhorou a geração de linfócitos T citotóxicos (CTL), avaliada pela expressão de perforina e granzima B. Além disso, a análise molecular das células tumorais expostas a combinação de 5-FU e CQ aumentou a expressão de genes relacionados à família CEACAM (CEACAM1, CEACAM5, CEACAM6 e CEACAM7) autofagia (BECN1, ATGs, MAPLC3B, ULK1, SQSTM1) e supressores de tumor (PCBP1). Ademais, houve diminuição da expressão de genes relacionados à metástase e progressão do

tumor (BNIP3, BNIP3L, FOSL2, HES1, LAMB, LOXL2, NDRG1, P4HA1, PIK3R2). Concluímos, portanto, que a combinação de uma baixa concentração de 5-FU e CQ aumenta a capacidade imunogênica das células tumorais, favorecendo a sensibilização e maturação de DCs e aumentando sua capacidade de estimular a proliferação de linfócitos T.

Abstract

Autophagy is an important mechanism for tumor escape, allowing tumor cells to recover from the damage induced by chemotherapy, radiation therapy, immunotherapy and contributing to the development of resistance. The pharmacological inhibition of autophagy contributes to increase the efficacy of antineoplastic agents. The exposure of the tumor cells to low concentrations of select autophagy-inducing antineoplastic agents increases their immunogenicity and enhances their ability to stimulate dendritic cell (DC) maturation. We tested whether the application of an autophagy-inhibiting agent, chloroquine (CQ), in combination with low concentrations of 5-fluorouracil (5-FU) increases the ability of tumor cells to induce DC maturation. DCs sensitized with the lysate of HCT-116 cells previously exposed to such a combination enhanced the DC maturation/activation ability. These matured DCs also increased the allogeneic responsiveness of both CD4+ and CD8+ T cells, which showed a greater proliferative response than those from DCs sensitized with control lysates. The T cells expanded in such cocultures were CD69+ and PD-1- and produced higher levels of IFN-γ and lower levels of IL-10, consistent with the preferential activation of Th1 cells. Cocultures of autologous DCs and lymphocytes improved the generation of cytotoxic T lymphocytes, as assessed by the expression of CD107a, perforin, and granzyme B. The drug combination increased the expression of genes related to the CEACAM family (BECN1, ATGs, MAPLC3B, ULK1, SQSTM1) and tumor suppressors (PCBP1). Furthermore, the decreased expression of genes related to metastasis and tumor progression (BNIP3, BNIP3L, FOSL2, HES1, LAMB3, LOXL2, NDRG1, P4HA1, PIK3R2) was noted. The combination of 5-FU and CQ increases the ability of tumor cells to drive DC maturation and enhances the ability of DCs to stimulate T cell responses.

I.-Introdução

De acordo com as estimativas mais recentes, no Brasil, o câncer colorretal (CCR) afetará mais de 40.990 pessoas ao final de 2020, sendo o terceiro câncer mais frequente no país (1). Esta doença pode ter origem hereditária e esporádica. A forma "herdada" que surge com síndromes transmitidas pelos pais, entre elas a principal associada é a síndrome de Lynch ou também conhecida como câncer colorretal hereditário, não associado à pólipos. É uma síndrome com padrão de herança autossômica dominante, caracterizada por instabilidade microssatélites como resultado da falha do sistema de reparo de bases pouco compatíveis, devido à inativação de genes da família MSH e MLH. Com a falha desse sistema, responsável por monitorar e corrigir os erros introduzidos nas sequências microssatélites pela DNA polimerase, aumenta o risco de desenvolver vários tipos de câncer, principalmente o câncer colorretal (2). Dessa maneira, células normais do cólon, com instabilidade genômica, desenvolvem adenomas tubulares que podem progredir para adenocarcinomas (3). E a forma "esporádica", que geralmente não tem histórico familiar e é a mais comum (incidência de 60%). Principalmente atribuídos à presença de variações genéticas estocásticas, juntamente com a exposição de agentes carcinogênicos, entre eles, podemos citar a fumaça do cigarro, consumo excessivo de carne vermelha, gorduras e álcool, que estimulam o aparecimento dessa doença (4). O dano a longo prazo, causado por esses agentes, gera um acúmulo progressivo de alterações genéticas e epigenéticas que ativam oncogenes e inativam genes supressores de tumores. Isso estimula a transformação maligna das células normais de cólon através de ciclos de expansão clonal que selecionam as células com comportamento mais agressivo e maligno. Dessa maneira, as células normais do cólon são transformadas em pólipos serrilhados e sua progressão para o câncer colorretal leva entre 5 a 10 anos. As vias mais comumente alteradas em ambas formas são MAPK quinase, Wnt/β-catenina e PIK3 (3, 5).

A terapia convencional para pacientes com CCR é baseada em colectomia total ou parcial, geralmente seguida de quimioterapia neoadjuvante ou adjuvante (tratamento pré- ou pós-operatório, respectivamente)(6), com drogas como 5-Fluorouracil (5-FU), capecitabina (Xeloda), irinotecan (Camptosar), oxaliplatina (Eloxatin), trifluridina e tipiracil (Lonsurf)(7). O sucesso da quimioterapia é frequentemente complicado por metástases e doenças recorrentes associadas ao desenvolvimento de resistência à quimioterapia (8, 9). Um dos mecanismos responsáveis pela quimioresistência é a autofagia, um processo seletivo de degradação celular, no qual proteínas e organelas citosólicas são sequestradas em autofagosomos de membrana dupla, que se fundem com o lisossomo para reciclar macromoléculas (10-12). Este é um processo que, em níveis basais, estão presentes em todas as células do corpo(10). Este processo é necessário para a manutenção da homeostase celular e geração de aminoácidos para períodos prolongados de privação de nutrientes, sendo essa condição o gatilho mais típico de autofagia(13). Outros tipos de estresse, como estresse genômico, estresse do reticulo endoplasmático, infecções intracelulares e exposição a drogas, também podem desencadear a autofagia(12-15). As células tumorais, devido ao seu metabolismo acelerado, tem uma capacidade autofágica aumentada, utilizando-a como mecanismo de escape das condições adversas causadas pelo uso de agentes antineoplásicos ou mesmo dos mecanismos imunológicos de defesa.

O papel da autofagia na carcinogênese é polêmico, havendo tanto evidências da inibição da transformação tumoral, quanto da promoção e adaptação das células tumorais(16). Assim, nos estágios iniciais do câncer, a autofagia parece ter papel protetor, regulando genes e moléculas importantes no desenvolvimento do câncer. Por exemplo, a alta produção de espécies reativas de oxigeno (ROS) provoca deaminação de bases de DNA, acelerando a mutagênese, aumentando a ativação de oncogenes e estimulando a carcinogênese. As mitocôndrias são consideradas a principal fonte de ROS intracelular, e a produção de ROS

aumenta à medida que essas organelas envelhecem ou são expostas a dano externo. Nesse contexto, a autofagia ajuda a evitar danos pela degradação seletiva de mitocôndrias defeituosas (mitofagia), suprimindo a carcinogênese (17, 18). Além disso, há evidências de que o silenciamento de genes relacionados aos estágios iniciais da autofagia estimula a progressão do tumor, por exemplo: BECN1(19).

Em oposição ao exposto existe evidências de que a autofagia é promotora da carcinogênese, baseadas principalmente no fato de que diferentes genes associados a esse processo estão também altamente expressos em tumores, como é o caso do oncogene Ras, uma proteína globular monomérica (GDP-Ras) encontrada na membrana de células humanas. Uma vez ativada (GTP-Ras), estimula diversos efetores que acionam uma matriz de redes de sinalização celular, incluindo a via de autofagia da AMPK(20). Mutações ativadoras de Ras são encontradas em 10-30% de todos os cânceres humanos (21, 22). Outro gene relacionado à autofagia é o ULK1 (um dos três principais genes necessários para a formação de fagóforo). Níveis aumentados de ULK1 significam níveis mais altos de autofagia e consequente facilitação do crescimento de células tumorais. Altos níveis de ULK1 estão associados à quimioresistência (23, 24), sendo plausível pensar que as células tumorais utilizariam a autofagia como mecanismo de correção dos danos provocados pela exposição a quimioterápicos, conseguindo, desse modo, desenvolver resistência aos agentes antitumorais.

Em estudos prévios, observamos que agentes antineoplásicos selecionados em baixas concentrações modulam as vias de sinalização e produzem citocinas como IL-12, IL-10, IL-4 e TNF-α (25), sem induzir apoptose. O tratamento de células cancerígenas de linhagem colorretal HCT-116 com uma concentração não tóxica de paclitaxel (droga comumente usada contra o CCR) altera a expressão de vários genes, especialmente os responsáveis pela síntese de proteínas de choque térmico, componentes do mecanismo de processamento de antígenos (componentes APM) e antígenos associados a tumores(26). O aumento da imunogenicidade

das células tumorais induzidas pela exposição a drogas depende do início da morte celular imunogênica, com aumento da expressão de sinais de perigo (DAMP), como calreticulina, proteínas de choque térmico, ATP e high mobility group box 1 (HMGB -1) (27-30). Consistentemente, também observamos que a exposição das HCT-116 a uma concentração não tóxica de paclitaxel ou doxorrubicina causa alterações transcricionais de vários genes associados à expressão de antígenos tumorais(31). Além disso, células dendríticas (DCs) sensibilizadas com o lisado de células tumorais previamente tratadas com baixas concentrações de paclitaxel induziram células T citotóxicas (CTLs) com maior potencial lítico do que as DCs sensibilizadas com células tumorais não tratadas(25). Mais recentemente, alguns grupos propuseram a associação de agentes antineoplásicos com bloqueadores de autofagia como uma abordagem terapêutica para o tratamento de pacientes com câncer(32-34). Um desses quimioterápicos é o 5-fluororacil (5-FU), comumente utilizado no tratamento contra o CCR (7). Descoberto por Charles Heidelberger em 1957(35), atua inibindo a timidilato-sintase, e desse modo, bloqueia a reação de metilação de ácido desoxiuridílico impedindo a conversão em ácido timidílico, promovendo assim o desequilíbrio metabólico que resulta na morte da célula tumoral alvo por apoptose(35). Além disso, devido a seu estímulo apoptótico é conhecido também por estimular a autofagia tanto em modelos *in vitro* como *in vivo* (36, 37).

A exposição das células tumorais aos fármacos antimaláricos cloroquina, hidroxicloroquina ou mefloquina (quinolonas) reduz a autofagia e aumenta a suscetibilidade das células à quimioterapia (38). As quinolonas e seus derivados são uma família de antibióticos de amplo espectro, isolados a partir de fontes naturais e podem atuar como agentes antimicrobianos naturais(39). Quando utilizados no tratamento da malária, devido a sua natureza alcalina, funcionam como uma base fraca que se difunde passivamente nos lisossomos, onde são protonadas e impedidas de deixar essa vesícula. Aumenta o pH do lisossomo, interrompendo suas funções e impedindo o processo autofágico (40, 41). A

associação de CQ com drogas antineoplásicas melhora a resposta em pacientes com câncer de mama (42), rim(43) e pulmão(44), com um efeito sinérgico na ativação do mTOR (alvo de rapamicina em mamíferos), inibindo a autofagia e aumentando a morte das células tumorais (45).

Não há estudos disponíveis sobre a indução de autofagia em células tumorais ao usar doses baixas de quimioterápicos. Pouco se sabe sobre o efeito do fenômeno na imunogenicidade das células tumorais. Não existem relatos na literatura sobre os efeitos da associação de CQ com outros fármacos sobre as funções de células imunocompetentes, como linfócitos ou células dendríticas.

As células dendríticas (DC) são células apresentadoras de antígenos profissionais, que realizam constante reconhecimento do microambiente em que se encontram, apresentando uma grande capacidade de endocitar ou fagocitar antígenos, processando-os em peptídeos (46). No nosso corpo, encontram-se dois grandes subconjuntos de DCs: as DCs plasmocitóides (pDCs) e as DCs mielóides (mDCs). As primeiras se caracterizam por expressar CD4+, HLA-DR+, CD123+, *Toll-like* receptor (TLR) 7, TLR9 e são CD11c+ e, CD14-(47). Durante infecções produzem altas quantidades de IFN-I (IFN-α e IFN-β), favorecendo a resistência antiviral(48). As DCs mielóides (também conhecidos como DCs convencionais ou DCs clássicas) estão subdivididas em mDC-1, que é um grande estimulador de células T e o mDC-2 menos comum que ajuda a combater infecções(48). As mDCs secretam a interleucina (IL)-12 e exibem os marcadores CD1c+, CD11c+, HLA-DR+, CD80+, CD83+ e CD86+ e os receptores TLR 2 e TLR 4 (46, 48).

Devido ao papel central das DCs na apresentação e indução de imunidade antitumoral (49) sustenta-se a proposta de uso dessas células como vacinas terapêuticas antitumorais (50). As DCs podem ser sensibilizadas com diferentes fontes de antígenos, desde lisados de células tumorais obtidos por sonicação(51, 52), ou ciclos de congelamento e descongelamento(53, 54),

até a geração de híbridos celulares por fusão de DC e células tumorais(55, 56). Seja qual for o método de preparação, o principal objetivo destas vacinas é gerar uma resposta de células T CD4+ e TCD8+ específica no paciente com câncer, para conseguir combater a progressão do tumor.

Os lisados de células tumorais são fontes de uma ampla variedade de antígenos que podem-se associar às moléculas de MHC de classe I/II para induzir uma resposta imunológica mais ampla (57). É importante destacar que durante a realização dos protocolos de vacina, seja o método de obtenção do lisado, como tratamentos prévios (fármacos ou quimioterápicos), ou o uso de adjuvantes (KLH), pode afetar a imunogenicidade do lisado e a eficiência das DCs na terapia antitumoral (51, 52, 58). Esta eficácia varia entre cada paciente e, existem relatos com resultados muito promissores, como no caso do uso de vacinas de DCs no tratamento de melanoma (59), câncer renal (60), câncer de mama (61) ou câncer de ovário (62). Lamentavelmente, em estudos do CCR, as vacinas baseadas em DCs tiveram um impacto relativamente pequeno (63), principalmente devido ao fato de que a maioria dos pacientes apresentam doença avançada. Além disso, a imunoterapia não demonstrou ser uma modalidade eficaz nesse cenário de doença devido ao tempo necessário para gerar uma resposta imunológica (64-66). O sistema imunológico desses pacientes poderia estar esgotado, devido aos agentes antineoplásicos recebidos antes da inscrição nos estudos de vacina. E por último, existem relatórios de mudanças produzidas pelas vacinas aumentando os níveis de TNF-α, IFN-γ e IL-2 (citocinas inibitórias), que refletem a polarização em relação às respostas Th1 que são direcionadas contra as células de CCR (67).

Portanto, no presente estudo, hipotetizamos que a aplicação de CQ com baixas concentrações de 5-fluorouracil (5-FU) poderia bloquear a autofagia em células colorretais HCT-116, preservando assim os antígenos tumorais, aumentando a imunogenicidade dessas

células. Além disso, o lisado originado dessas células poderia promover a maturação das DCs, aumentando sua capacidade de induzir proliferação de linfócitos T citotóxicos.

II.-Delineamento Experimental

A. Cultura e exposição de células HCT-116 a CQ e 5-FU
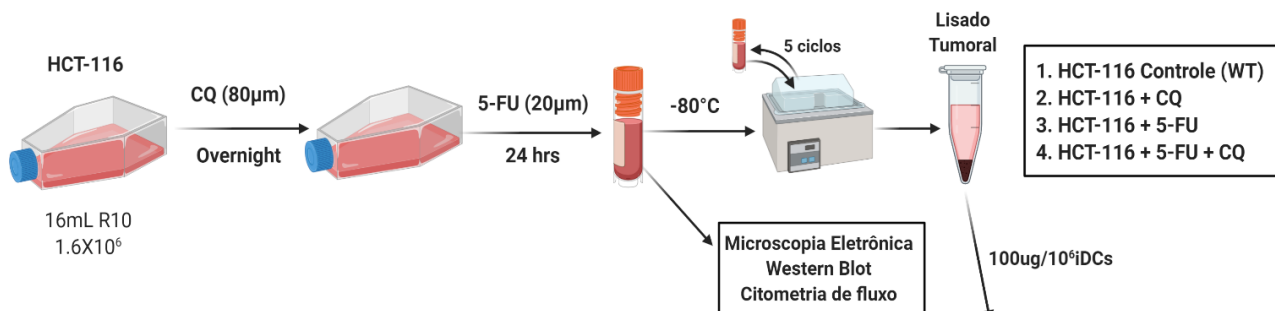
B. Diferenciação e sensibilização de células dendríticas (DC)
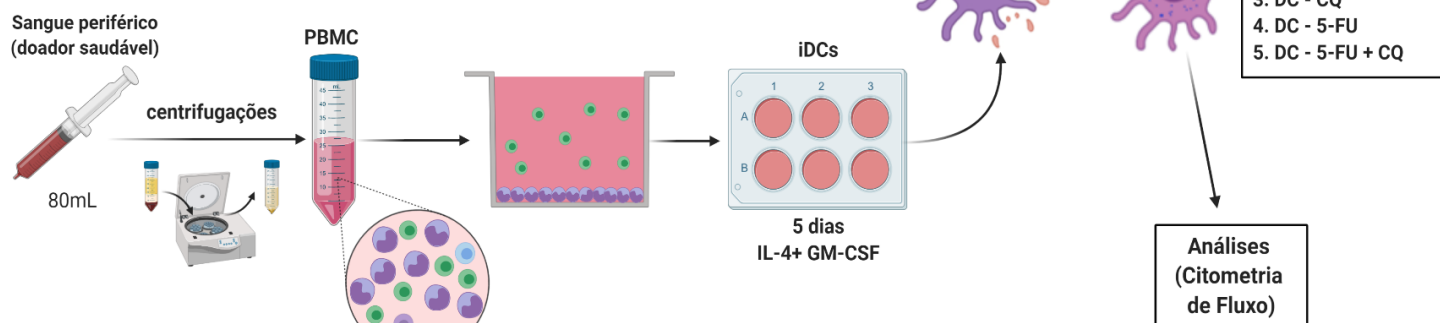
C. Reação mista de leucócitos (MLR)
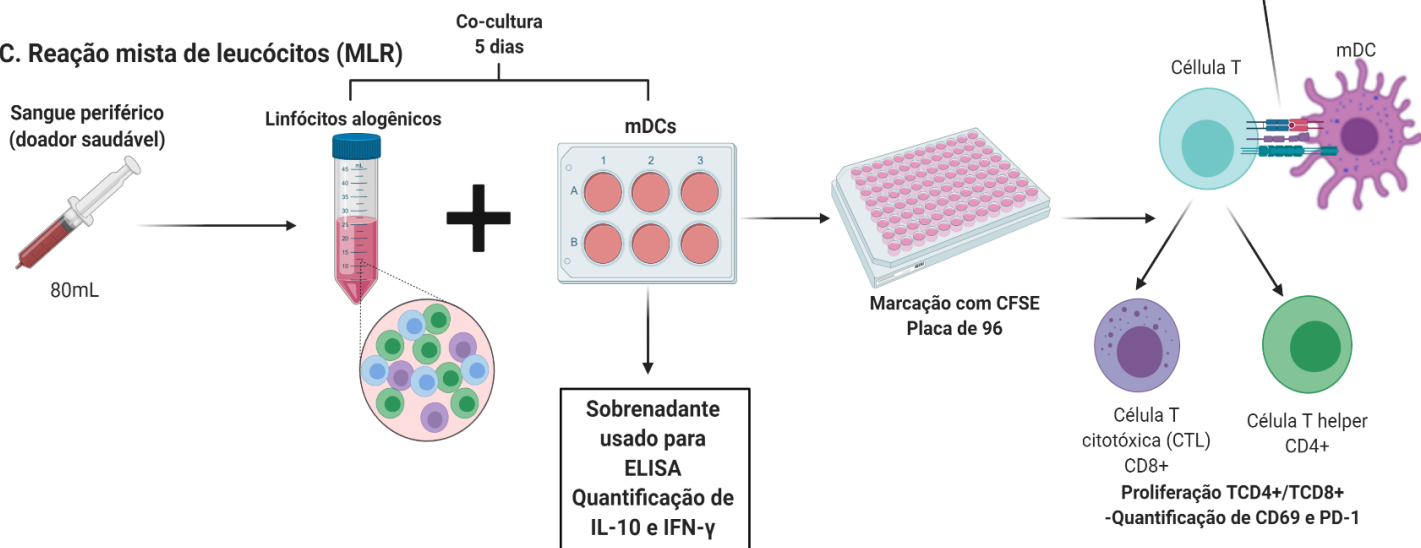
D. Geração de linfócitos T citotóxicos e ensaio de citotoxicidade antitumoral (CTL)

Co-cultura
14 dias
IL-2 + IL-7

Co-cultura por
5 horas
Marcado com
Anti-CD107a-APC

Sangue periférico
(doador saudável)

Linfócitos autólogos

mDCs

80mL

Tratamento com
Cytofix/CytoPerm kit

Análises
(Citometria
de Fluxo)

Marcação com
anti-Perforin-Alexa 488 e
anti-Granzime-PE
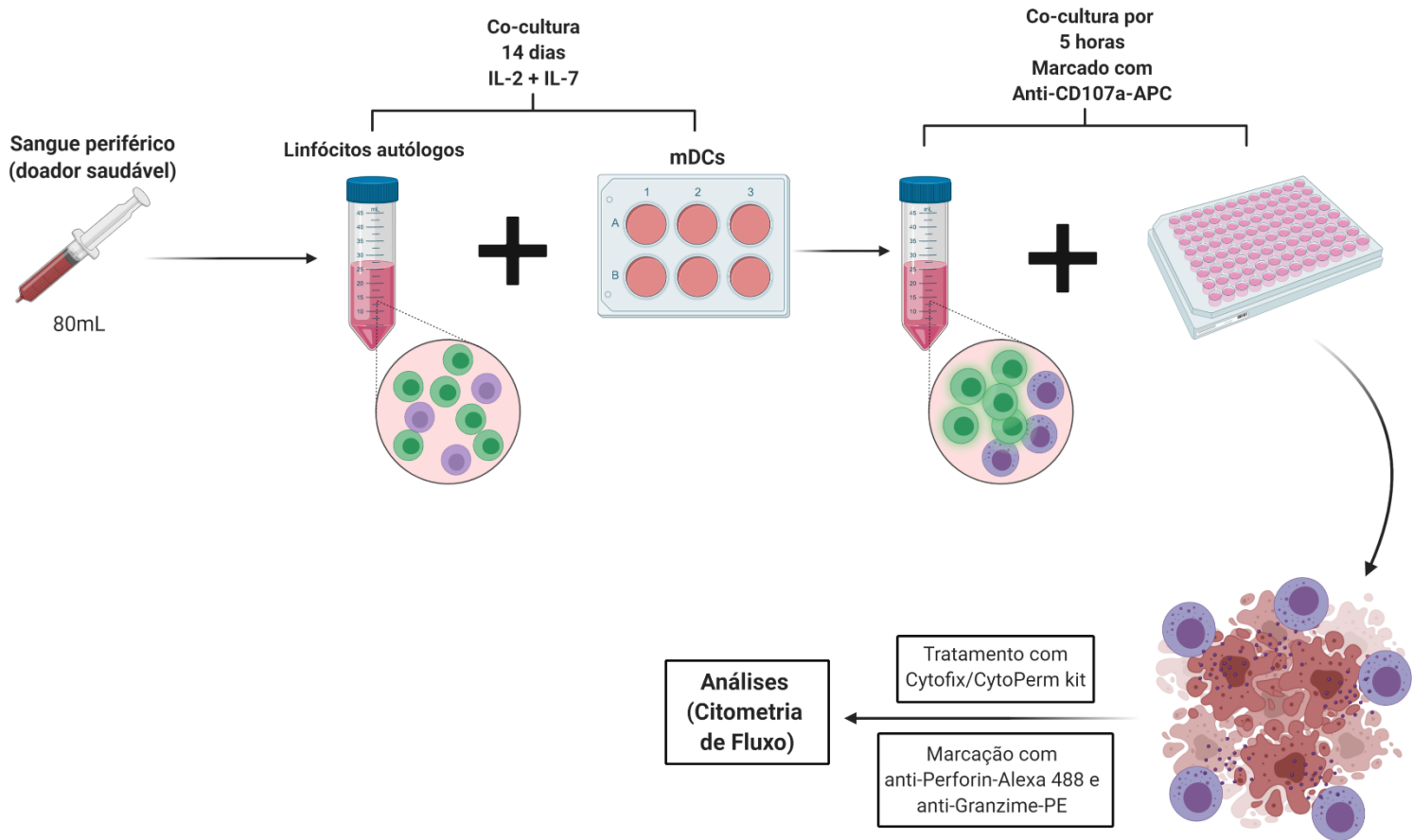
III.- **Materiais e Métodos**

1.- **Cultura de células**

Células tumorais da linhagem HCT-116 (carcinoma colorretal humano) foram autenticadas pelo DNA STR usando o GenePrint 10 commercial system (Promega, Madison, WI, EUA) pelo grupo de Pesquisa em Carcinogênese Viral e Biologia do Câncer do Instituto de Biotecnologia (IBTEC) da Universidade Estadual de São Paulo (UNESP). Confirmado a ausência de micoplasma e cultivadas em meio RPMI-1640 (*Gibco*) suplementado com 10% de soro fetal bovino (SFB) (*Gibco)*, 1% de piruvato de sódio, 1% de aminoácidos não essenciais e 25uM HEPES (Sigma), 1% de antibióticos e antimicóticos (*Life Technologies*) (meio completo) a 37°C e sob tensão de 5% de CO_2, até completarem a monocamada. As células foram então soltas da superfície das garrafas de cultura utilizando-se solução de tripsina-EDTA (*Gibco*) e lavadas com meio de cultura completo para uso nos ensaios propostos.

2.- **Determinação das concentrações de trabalho de cloroquina e 5-fluororacil**

As células tumorais foram ajustadas a concentrações de 10^4 e cultivadas por 24 horas em placas de 96 poços de fundo chato. Como já foi demonstrado por Park, a cloroquina (CQ) inicia seu efeito bloqueador perder da sobrevivência celular em concentrações acima de 80 μM(68). Sendo assim, nós fizemos os testes com concentrações: 80, 100, 150 ou 200 μM de cloroquina e, depois de 18 h, os sobrenadantes de todos os poços foram substituídos por 100μl da solução de brometo de difeniltetrazólio (MTT) a 1mg/mL em meio de cultura completo. Após duas horas, o sobrenadante foi novamente removido e adicionado 100μl de dimetilsulfoxido (DMSO) em cada poço para dissolução do MTT incorporado pelas células. A avaliação de citotoxicidade dos tratamentos foi realizada a partir da capacidade de redução do MTT em cristais de formazan pelas células viáveis, cuja densidade ótica foi medida por espectrofotometria a 540nm. Foi estabelecida a concentração de 80μM para a CQ, e para determinar a concentração efetiva mínima do 5-fluororacil (5-FU) (*EuroFarma, Brasil*), as

HCT-116 foram tratadas com as concentrações de 0.06, 0.12, 0.25, 0.5,1, 2.5, 5, 10, 20, 50 ou 100 µM por 24 horas. Em seguida, foi repetido o procedimento do teste de MTT, estabelecendo-se 20µM como a concentração efetiva mínima, isto é a concentração capaz de inibir a proliferação das células tumorais *in vitro*.
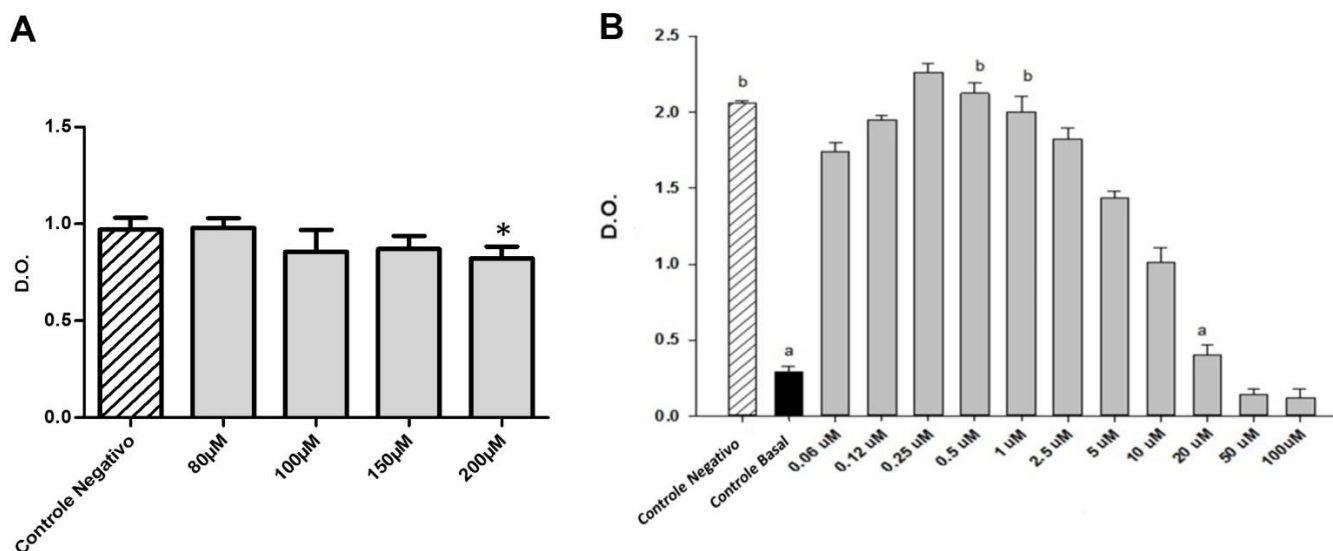
Figura 1 - Determinação das concentrações de trabalho de CQ e 5-FU. (A) As células HCT-116 foram submetidas ao tratamento com CQ durante 18 horas, com as concentrações variando de 80µM a 200µM. Após esse período, as células viáveis foram quantificadas por coloração com MTT. (B). As células HCT-116 foram tratadas com 5-FU durante 24 horas, com as concentrações variando de 0,06µM a 100µM. Após esse período, as células viáveis foram quantificadas por coloração com MTT. Dados representativos de três experimentos independentes e expressos em D.O (a<b, p<0,05). Dados representativos de um ensaio realizado em quintuplicata. *p<0,05 em relação ao controle negativo. Os experimentos realizados para determinação das concentrações de trabalho foram previamente realizadas em monografia associada ao projeto para a obtenção do título de Bacharel em Ciências Biomédicas de Bianca Francisco Falasco(69).

3.- Tratamento das células tumorais com a combinação de drogas

Estabelecidas as condições de trabalho, preparou-se uma garrafa de cultura (75 cm2) de HCT-116 até atingir 60% de confluência para cada tipo de tratamento (Tabela 1). CQ foi adicionada à garrafa 6 h após o cultivo, seguindo-se incubação por 18 h adicionais. Depois da incubação, o meio de cultura foi removido e substituído por meio completo fresco.

Esperou-se 6 horas para estabilização da cultura e subsequente adição de 5-FU, seguindo-se exposição por 24 h. As células foram criopreservadas a -80°C para posterior

obtenção de lisados de células tumorais. As células do grupo controle WT não foram tratadas e os grupos CQ e 5-FU foram tratados apenas com as concentrações estabelecidas dessas drogas isoladamente. Assim, foram constituídos os grupos descritos na Tabela 1.

Tabela 1 – Grupos de tratamento das HCT-116

DENOMINAÇÃO	TRATAMENTO
WT	HCT-116 Controle (Wild Type)
CQ	HCT-116 + Cloroquina (80uM)
5-FU	HCT-116 + 5-FU (20μM)
5-FU+CQ	HCT-116 + 5-FU (20μM) + CQ (80uM)

4.- Análise de morte celular por citometria de fluxo

Células HCT-116 ajustadas a 10^5 cel/ml, foram cultivadas em placas de 24 poços, seguindo-se tratamento com CQ, 5-FU ou a combinação das drogas conforme ao descrito no item 3. Como controle positivo de morte, uma alíquota das células a 10^5 cel/mL foi mantida em banho maria a 59°C por 20 min. As células foram destacadas por tripsinização e centrifugadas a 1200 rpm por 10 min e o "pellet" foi novamente lavado com PBS frio e centrifugado a 10.000 rpm por 30 s. O sedimento celular foi ressuspenso em 100 mL de solução tampão de anexina e incubado com anexina V à temperatura ambiente e protegido da luz. Após a incubação, foram adicionados 200 μl do tampão de anexina. Finalmente, as células foram coradas com 7-amino-actinomicina D (7AAD). A anexina V é uma proteína que na presença de íons cálcio, se liga à fosfatidilserina (PS) externalizada nos estágios iniciais de apoptose ou necrose, enquanto o 7AAD é um intercalante de DNA que penetra nas células que perderam a integridade da membrana plasmática e, por tanto, marca células em apoptose tardia e/ou necrose. As HCT-116 coradas foram analisadas por citometria de fluxo FACSCantoTM II (BD

Biosciences) com software FACSDiva (BD Biosciences). Os resultados foram analisados no software FlowJo, versão vX.10.7 (Tree Stars Inc.).

5.- Preparação de lisado tumoral e quantificação de proteínas

As alíquotas criopreservadas foram descongeladas em banho maria a 37°C por 3 min, e logo recongeladas em nitrogênio líquido por 3 min (ciclo de congelamento/descongelamento), processo repetido 5 vezes. Depois, as amostras foram centrifugadas a 14000rpm por 15 min; e o sobrenadante recolhido, ao qual se adicionou inibidor de protease (*Halt™ Protease Inhibitor Cocktail Thermofisher*) 70 µl IP/mL, seguindo-se armazenamento a -80°C.

A quantificação de proteínas foi feita com uso do o Kit *"BCA Protein assay" Thermofisher*©, e as amostras foram distribuídas em alíquotas de 100µg de proteína e armazenadas a -80°C.

6.- Western blot

Todos os experimentos envolvendo a técnica de western blot foram realizados sob supervisão do prof. Dr. Rodrigo Portes Ureshino, do Departamento de Farmacologia da EPM da Universidade Federal de São Paulo – UNFESP.

As culturas dos quatro grupos de tratamento (WT, CQ, 5-FU e 5-FU + CQ) foram submetidas à lise celular empregando-se tampão de lise RIPA – glicina 1M, com os inibidores: fluoreto de sódio (NaF), na proporção 1:50 e ortovanadato de sódio (NaOt), molibdato de sódio (NaMo), inibidor de protease (IP) e fluoreto de fenilmetilsulfonil (PMSF), na proporção 1:100. As células tratadas mantidas em banho de gelo foram lavadas com PBS gelado e em seguida foi adicionado 100µL do tampão RIPA com os inibidores. O lisado foi retirado da placa com o auxílio de *cell scrapers* e aliquotado em microtubos de 1,5mL. Os microtubos foram mantidos no gelo por 30 min, a cada 10 min no vórtex, e em seguida centrifugados a 13.200rpm a 4ºC

por 10 min. Retirou-se o sobrenadante e a determinação da concentração de proteínas foi feita pelo método de Bradford, com leitura em espectrofotômetro no comprimento de onda 595nM. Após a determinação da concentração proteica, 40µg de proteínas de cada um dos grupos foram diluídas em tampão de amostra para corrida de gel, de poliacrilamida. As amostras foram submetidas à corrida eletroforetica a 95°c por 10 min. As proteínas separadas no gel foram transferidas para a membrana de difluoreto de polivinilideno (PVDF). A ligação inespecífica de proteínas foi bloqueada através da incubação das membranas em leite desnatado 5% em tampão PBS Tween por 1h à temperatura ambiente. As membranas foram subsequentemente incubadas, "overnight", com os anticorpos primários antígeno-específicos: LC3 (#2775s Cell signal) e o controle tubulina (#T8203 Sigma). Após lavagem sob agitação por 30 min, as membranas foram incubadas em anticorpo secundário específico anti-rabbit (#A0545 Sigma) para o LC3 e anti-mouse para a tubulina (#A9044 Sigma) diluído em PBS-Tween por 1h. As membranas foram lavadas por 30 min e reveladas com componentes imunorreativos ECL (enhanced chemiluminescent) e lidas em fotodocumentador UVITEC Cambridge.

7.- Análise Ultraestrutural das HCT-116 por Microscopia Eletrônica de Transmissão

A preparação das HCT-116 e análise dos resultados relacionados à Microscopia Eletrônica de Transmissão foram realizados sob supervisão da profa. Dra. Daniela Carvalho dos Santos do Centro de Microscopia Eletrônica do Instituto de Biociências de Botucatu– Universidade Estadual de São Paulo (UNESP).

Após tripsinizadas, cerca de 10^6 células permaneceram em cultura em placa de 6 poços "overnight" e receberam os tratamentos com 5-FU e CQ conforme o item 3. Após a incubação, as monocamadas de células foram tratadas com tripsina e as células lavadas por 10 min. a 1200 rpm a 20ºC. As amostras foram fixadas em 1 mL de solução de Karnovsky, pós-fixadas em tetróxido de ósmio a 1% em tampão fosfato 0,1M, pH 7,3, incubadas em solução aquosa de acetato de uranila 0,5%; desidratadas em série crescente de soluções de acetona e incluídas em

araldite. As secções ultrafinas (50nm) foram contrastadas com solução saturada de acetato de uranila e citrato de chumbo e observadas ao microscópio eletrônico de transmissão Tecnai Spirit da FEI Company, a 80kV.

8.- Geração *in vitro* de células dendríticas derivadas de monócitos humanos

Amostras de 80ml de sangue foram obtidas por venóclise de 6 doadores voluntários saudáveis após esclarecimentos sobre o estudo e assinatura do termo de consentimento livre e esclarecido (TCLE), aprovado pelo comitê de ética em pesquisa da Faculdade de Medicina de Botucatu (Parecer N°2.258.145).

As células foram submetidas à centrifugação em gradiente de Ficoll-Hypaque^{TM} PLUS (*GE Heathcare*) por 30' a 900xg. O anel de células mononucleares foi recolhido, lavado e ressuspenso em PBS 5% de soro fetal bovino (SFB), para posterior centrifugação em gradiente de Percoll^{TM} (*GE Heathcare*) por 20' a 800xg para enriquecimento da população de monócitos. As células obtidas foram então cultivadas em placas de 6 poços em meio AIM-V, livre de SFB, e deixadas em aderência por 90 min. As células não aderentes (consideradas como linfócitos totais) foram removidas e criopreservadas para uso em ensaios posteriores. As aderentes (em sua maioria monócitos) foram cultivadas na presença de 50ng/mL de IL-4 e GM-CSF recombinantes humanos (*Peprotech- USA*), para diferenciação em DCs imaturas (iDCs). No 5º dia, as iDCs foram contadas e cultivadas com o lisado de células HCT-116 submetidas aos diferentes tratamentos, conforme a tabela 2, na proporção de 100ug de lisado:10^6 iDCs. No 7º dia as DC foram coletadas para realização dos ensaios propostos.

Tabela 2– Esquema de sensibilização das iDC com lisados de células tumorais sob diferentes tratamentos.

DENOMINAÇÃO	TRATAMENTO DAS CÉLULAS TUMORAIS
DC CONTROLE	Célula dendrítica sem tratamento
DC WT	Wild Type (Controle)
DC CQ	Cloroquina (80uM)
DC 5-FU	5-FU (20μM)
DC 5-FU+CQ	5-FU (20μM)+ CQ (80uM)

9.- Fenotipagem de DCs

Cerca de 10^5 DCs foram colocadas em um tubo eppendorf, centrifugadas por 20 seg a 10000 rpm. Anticorpos fluoromarcados específicos para os marcadores de interesse foram adicionados ao "pellet" em concentração pré-estabelecida, homogeneizados e os tubos incubados por 20 min a 4° C, ao abrigo de luz. Depois da incubação, os tubos foram lavados duas vezes com 200μL de solução diluente (PBS, contendo 0,5% de BSA e 0,02% de azida sódica). Após as lavagens, as células foram ressuspendidas em 300uL de tampão para citometria e adquiridas no citômetro FACS CantoTMII (BD Biosciences) com software FACSDiva. Os resultados foram analisados no software FlowJo, versão vX.10.7 (Tree Stars Inc.).

As moléculas analisadas em DCs foram: CD11c (envolvida na adesão do monócito ao endotélio e a proteínas da matriz extracelular), utilizada como marcador da população de DCs, CD86 e CD80 (moléculas de co-estimulação); HLA-DR (molécula do Complexo Principal de Histocompatibilidade de classe II) e CD83 (expressão aumentada em DCs maduras). A viabilidade celular foi avaliada com uso de 7AAD. Os fluorocromos utilizados em cada

anticorpo estão detalhados na Tabela 3 e todos os anticorpos e respectivos controles isotípicos foram adquiridos da BD (Becton e Dickinson).

Tabela 3– Painel de fenotipagem das DCs após 48h de cultivo com lisado de células tumorais submetidas a diferentes tratamentos. Todos os anticorpos foram adquiridos da *BD Biosciences.*

ANTICORPOS	FLUOROCROMOS
CD11C	APC (Aloficocianina)
HLA-DR	PE (Ficoeritrina)
CD86	FITC (Isotiocianato de fluoresceína)
CD80	APCH7 (Aloficocianina 7)
CD83	PECy7 (Ficoeritrina-cianina 7)
7AAD	PerCP (Peridinina clorofila)

10.- Reação mista de leucócitos (MLR)

A atividade funcional das DCs foi inicialmente avaliada pela capacidade de estimular a proliferação de linfócitos T alogênicos normais. A reação mista de leucócitos foi realizada através do co-cultivo de DCs com linfócitos alogênicos de 6 doadores saudáveis. Os linfócitos foram obtidos por centrifugação de sangue periférico em gradiente de Ficoll-Hypaque seguido por centrifugação em gradiente de Percoll, conforme descrito no item III-5. O "pellet" resultante, composto principalmente de linfócitos, foi lavado duas vezes com meio completo e marcados com succinil éster de carboxifluoresceína (CSFE *Lyfe Technologist*). Essas células foram ajustadas a 10^6 cel/ml e co-cultivadas com DCs alogênicos a 10^5cel/ml (preparação de 10:1) em placas de 96 wells de fundo em "U" por 5 dias a 37°C e sob tensão constante de 5% de CO_2.

Ao final desse tempo, as células foram recolhidas para análise de proliferação por citometria de fluxo baseada na diluição do CFSE nas células. Os linfócitos em expansão

foram analisados quanto à subpopulação (CD4/CD8) e expressão de marcadores de ativação/inibição (CD69 e PD-1, respectivamente) cujos fluorocromos estão detalhados na Tabela 4.

Tabela 4– Painel de avaliação da proliferação nos linfócitos T alogênicos co-cultivados com DC previamente tratados com lisados. Todos os anticorpos utilizados foram adquiridos da *BD Biosciences.*

ANTICORPOS	FLUOROCROMOS
CD3	APC (Aloficocianina)
CD4	PE (Ficoeritrina)
CD8	PerCP (Peridinina clorofila)
CD69	APCH7 (Aloficocianina 7)
PD-1	PE (Ficoeritrina)

11.- Quantificação de citocinas produzidas *in vitro*

Os sobrenadantes das reações mistas de leucócitos foram recolhidos e criopreservados para posterior quantificação de IFN-γ e de IL-10, através da técnica de ELISA, seguindo as recomendações de fabricante (Biolegend®). Brevemente, placas de 96 poços de fundo plano (Sigma-Aldrich), foram sensibilizadas com o anticorpo de captura de IFN-γ ou IL-10, por incubação overnight a 4ºC. Após esse tempo, as placas foram lavadas com tampão de lavagem (BioLegend ELISA Wash Buffer 1X) e incubadas por duas horas com o diluente de ensaio para o bloqueio dos sítios de ligação inespecífica. Após quatro lavagens com tampão de lavagem foram adicionadas 100μl das amostras de sobrenadante e as soluções padrão em diferentes concentrações (500 a 16pg/mL), seguindo-se incubação por duas horas à temperatura ambiente. Os poços foram novamente lavados com o tampão de lavagem por quatro vezes e em seguida,

foi adicionado o anticorpo de detecção anti-IFN-γ ou anti-IL-10, biotinilado, seguindo-se incubação por uma hora à temperatura ambiente. Após quatro lavagens para a remoção dos anticorpos não ligados, adicionou-se aos poços 100μL da solução de estreptoavidina-peroxidasse diluído em diluente de ensaio, durante 30 min. Depois de cinco lavagens com tampão de lavagem, foi adicionada a solução de substrato tetrametillbenzidina (TMB), que desenvolve cor azul ao ser degradada pela enzima peroxidasse. Após 30 min, a reação foi interrompida com uma solução de H_2SO_4 2N. A leitura foi realizada em espectrofotômetro a 450nm (Elisa reader com software Bio-Tek Gen 5). A concentração das citocinas das amostras foi calculada com base na curva padrão de cada reação e os dados expressos em pg/mL.

12.- Geração de linfócitos T citotóxicos e ensaio de citotoxicidade antitumoral (CTL)

A capacidade de gerar células T citotóxicas a partir de DCs sensibilizadas foi avaliada pelo ensaio de micro linfocitotoxicidade antitumoral. Para a geração de células T citotóxicas autólogas específicas, as DCs pré-sensibilizadas foram co-cultivadas com suspensão autóloga rica em linfócitos T em uma proporção DC: linfócitos de 1:10 (10^4: 10^5) em meio de cultura completo suplementado com IL-7 (5 ng / ml), IL-2 (25 UI / ml) e IL-15 (5ng/ml). A cultura foi pulsada com um mix de citocina (IL-7, IL-2 e IL-15) a cada dois dias durante 14 dias. No dia 7, dois poços aleatórios, nos quais foram observados aumento da proliferação de linfócitos, foram aspirados e as celulas contadas. Quando necessário, as células foram distribuídas em 2 ou mais poços, para evitar sofrimento celular por superpopulação. No dia 14, os linfócitos foram colhidos e avaliados quanto à atividade citotóxica contra células-alvo HCT-116.

O ensaio de citotoxicidade foi realizado colocando os linfócitos gerados *in vitro* na monocamada alvo de HCT-116 (10^4 em 100ul) em diferentes proporções (15: 1, 7,5: 1 e 3,25: 1 / efetor: alvo). O anti-CD107a-APC foi adicionado a esta co-cultura e mantido por 5h a 37 ° C, sob 5% de CO_2. Em seguida, as células foram colhidas e lavadas com PBS + BSA a 0,1% e tratadas com o kit Cytofix/CytoPerm (BD Pharmingen). Finalmente, as células foram

marcadas com anticorpos anti-perforina-Ale 488 e anti-granzima-PE e analisadas por citometria de fluxo.

13.- Transcriptoma de células tumorais tratadas com 5-FU e CQ

Para investigar como o tratamento de células tumorais com a combinação de CQ e 5-FU altera a expressão gênica, realizamos a análise de transcrição dessas células. O RNA total foi extraído das células sob diferentes tratamentos com o QIAGEN © "RNeasy Plus Micro Kit". A quantificação foi realizada pelo "RNA HS Assay Kit" (INVITROGEN®). A quantificação do RNA foi realizada pelo sistema QBIT® e, finalmente, a qualidade do RNA foi analisada usando um chip ALIGENT® RNA 6000 no sistema Bioanalyzer 2100 ". Apenas as amostras com número de integridade do RNA (RIN) maior que 8,0 (qualidade ótima) foram processadas.

Todas as indicações e etapas do " Sure Select Strand-Specific RNA Library Preparation Kit" foram seguidas e as bibliotecas dsDNA de cada grupo de tratamento com os *primers* e *índex* foram analisadas na plataforma Illumina Miseq. Após a obtenção dos dados, as leituras foram montadas com base nas sequências de transcrição de cada gene de interesse usando o programa CLC Genomics Workbench. O efeito dos tratamentos na expressão gênica foi avaliado comparando-se os *fold-change* das alterações genéticas das amostras com o controle sem tratamento.

14.- Análise estatística

Os dados obtidos foram inicialmente analisados quanto à homogeneidade da variância, utilizando-se o teste de Bartlett. Os conjuntos de dados que passaram neste teste foram submetidos ao teste de análise de variância (ANOVA), seguida pelo teste de comparações múltiplas de Tukey. Os conjuntos de dados que não passaram pelo teste de Barlett foram submetidos à análise de dados não paramétrico de Kruskall-Wallis. As diferenças foram consideradas significativas quando a probabilidade de erro foi menor ou igual a 5% ($p \leq 0,05$).

IV.- <u>Resultados</u>

1.- A combinação de cloroquina e 5-FU bloqueia a autofagia

A capacidade de bloqueio da autofagia induzida nas células tumorais pela exposição do 5-FU e CQ foi avaliada pela detecção do acúmulo de LC3-II e SQSTM1. Observamos expressão aumentada de LC3-II devido ao tratamento com CQ, enquanto sua combinação com 5-FU potencializou sua expressão. É interessante notar que o tratamento apenas com 5-FU induziu um ligeiro aumento da expressão de LC3-II, indicando a indução de autofagia, como confirmado pela menor intensidade do SQSTM1 nesse grupo (Figura 2).
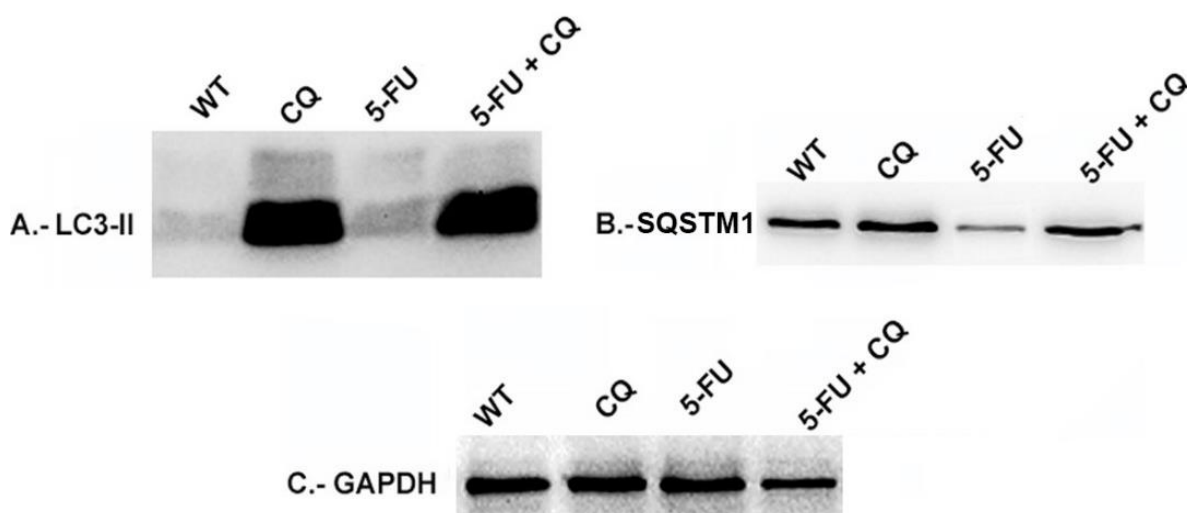
Figura 2 - O tratamento com CQ ou combinado com 5-FU induziu aumento de LC3-II nas células HCT-116. As células foram tratadas com 80μM CQ durante a noite e, em seguida, tratadas com a concentração efetiva mínima de 5-FU (20μM) por 24h. As células foram lisadas e as proteínas totais analisadas por western blot para LC3-II (A) e p62 (B). GAPDH foi utilizado como controle (C), para validação do ensaio (n=3).

2.- Alterações ultraestruturais induzidas em células HCT-116 por 5-FU e CQ

Para reforçar os resultados de indução e inibição de autofagia analisamos as células sob diferentes tratamentos por microscopia eletrônica de transmissão. As células tratadas com 5-FU apresentam várias vesículas de membrana dupla (autofagossomos) e vesículas de membrana única com material degradado (autolissosomo) no citoplasma (Figura 3C), enquanto

as células de controle mostram um número muito pequeno de autofagossomos com material floculado e degradado, além de mitocôndrias e algumas cisternas de retículo endoplasmático rugoso (Figura 3A). Quando o 5-FU foi associado à CQ, os autofagossomos parecem aumentar em quantidade dentro do citoplasma das HCT116 e essas vesículas exibem diferentes conteúdos fagocitados. Algumas vesículas possuem material heterogêneo denso no interior e outras mostram estruturas de mielina e material degradado mais claro, indicando que alguns autofagossomos não estão envolvidos no processo de digestão (autofagia bloqueada). Isso mostrou claramente um acúmulo de autofagossomos com organelas não digeridas, demonstrando uma falha ou inibição da formação de autolissosomo (Figura 3D), coincidindo com o que foi mostrado em estudos anteriores (70-73).
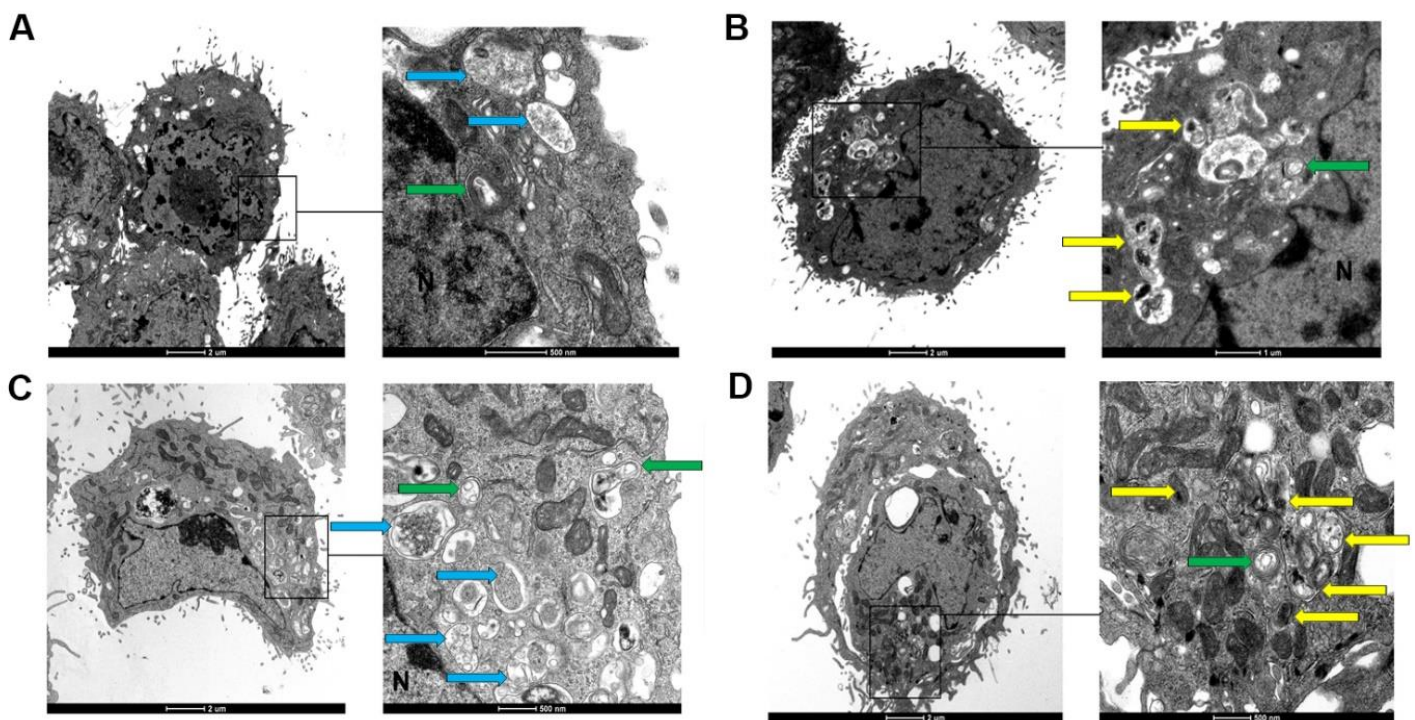
Figura 3 - Os tratamentos induziram alterações ultraestruturais nas células HCT-116. As células tumorais HCT-116 foram tratadas ou não com 80μM de CQ overnight. Após este período foram tratadas ou não com 20μm de 5-Fu. Decorridas 24 horas as células foram tripsinizadas, fixadas com Karnovsky e preparadas para aquisição de imagens em microscopia eletrônica de transmissão. N = núcleo; seta verde: autofagossomo; seta amarela: autofagossomo com organelas não digeridas; seta ciana: autolissosomo (vesícula com material degradado). Grupos: Controle (A), CQ (B), 5-FU (C), 5-FU + CQ (D) (n=3).

3.- A inibição de autofagia potencializa a ação citotóxica de 5-FU

O mecanismo de ação das fluoropirimidinas como o 5-FU ocorre a partir da indução de apoptose nas células tumorais. A inibição da autofagia pela CQ é capaz de potencializar a ação citotóxica do 5-FU, visto que, o acúmulo de autofagossomos no citoplasma, gerado pela obstrução do processo, causa estresse oxidativo na célula levando-a a morte.

A contagem do número de células HCT-116 viáveis após exposição as drogas, utilizando o teste de exclusão do azul de trypan, mostrou que tratamento apenas com CQ teve um efeito discreto na diminuição do crescimento celular enquanto o 5-FU reduziu o número de células para aproximadamente 50% comparada com cultura controle. A combinação de 5-FU e CQ inibiu fortemente o crescimento celular (Tabela 6).

Tabela 6– **Contagem celular das HCT-116 pelo azul de trypan**

CONTAGEM CELULAR (X 10^7)

TRATAMENTO	Ensaio 1	Ensaio 2	Ensaio 3	Ensaio 4	Ensaio 5	Médias
CTRL	6.7	6.5	6.3	6.4	6.4	6.46
CQ	5.7	5.1	5.6	5.2	5.3	5.38
5-FU	3.0	3.4	3.5	3.1	3.3	3.26
5-FU+CQ	1.2	1.5	1.3	1.2	1.4	1.32

CTRL: Células não tratadas cultivadas em meio de cultura regular. Cada coluna refere-se a um ensaio independente (cultivado em dias diferentes).

A análise de células apoptóticas em fase inicial (anexina V+), apoptóticas tardias/necróticas (anexina V+ e 7AAD+), por citometria de fluxo mostrou que o tipo de morte predominantemente induzida após tratamento adotado foi a apoptose em fase inicial (anexina V+).

O 5-FU sozinho (Figura 4D) aumentou quatro vezes a marcação da anexina V em comparação com o controle (Figura 4B), enquanto sua combinação com CQ aumentou a apoptose precoce em dez vezes em comparação com o mesmo controle (Figura 4E), e cerca de duas vezes em comparação com 5-FU sozinho.
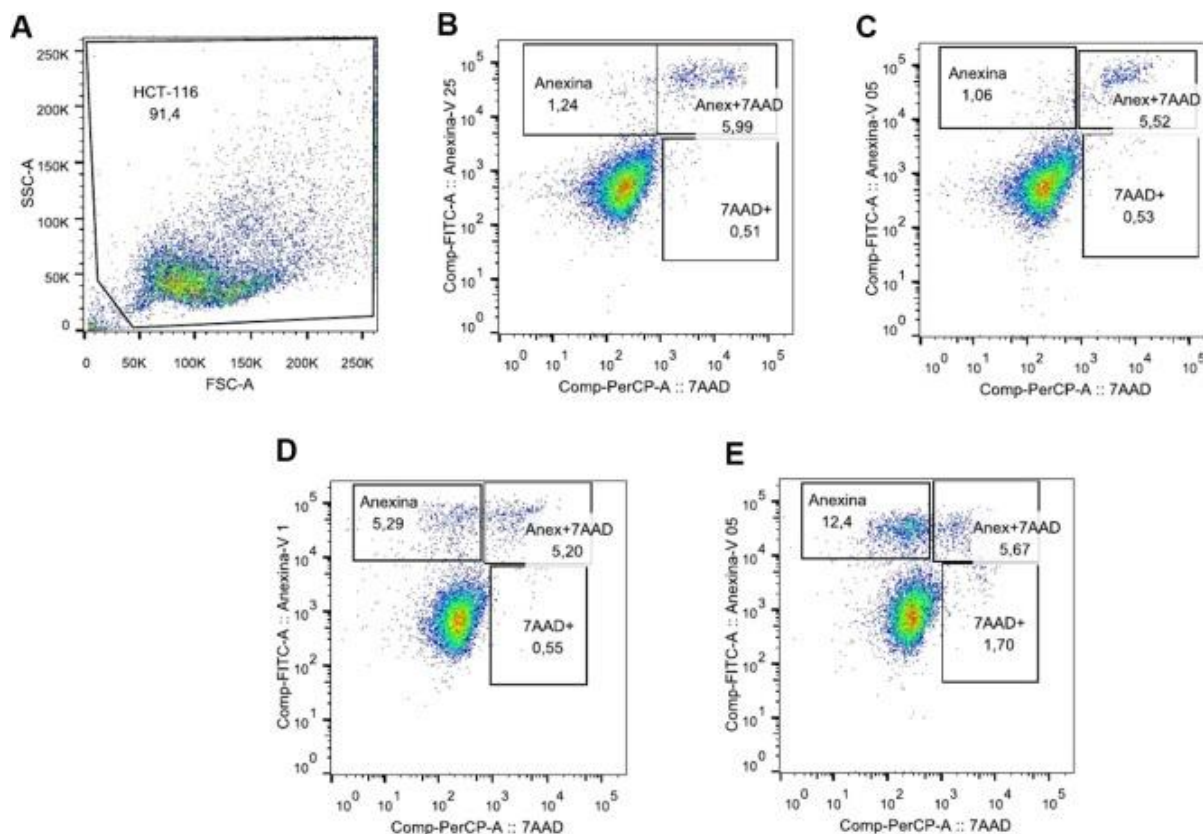
Figura 4 - A combinação de cloroquina e 5-FU induziu apoptose em células HCT-116. As células tumorais HCT-116 foram tratadas ou não com CQ a 80 µM por 18 horas. Posteriormente, foram tratados ou não com 5-FU a 20 µM. Após 24 h, as células foram tripsinizadas e coradas com anexina V e 7AAD para avaliar apoptose e necrose. (A) Pseudocolor mostrando tamanho de célula (FSC) e granularidade (SSC), com *gate* de análise. (B) Controle Negativo. (C) Tratamento com CQ. (D) Tratamento com 5-FU. (E) Tratamento com 5-FU e CQ (n=5).

4.- Lisado de células tumorais tratadas com CQ e 5-FU são mais imunogênicas

A análise fenotípica das DCs sensibilizadas *in vitro* com os diferentes lisados tumorais foi realizada seguindo a estratégia de *gating* ilustrada na Figura 5. Primeiramente, a análise foi feita com base no tamanho e morfologia característicos de DCs (Figura 5A); o segundo gate

corresponde as ocorrências de *singlets* e exclusão de células agregadas (*doublets*) (Figura 5B); as células mortas foram excluídas da análise por marcação com 7AAD (Figura 5C) e as moléculas de interesse foram analisadas dentro da população 7AAD-/CD11c+ (Figura 5D).

Os resultados representados na Figura 4 E-H, ilustram o efeito do lisado tumoral no fenótipo das DC, destacando a tendência de elevação sobre a percentagem de expressão de todos os marcadores em comparação com o controle. Nossos resultados mostram que a associação de CQ com baixas concentrações do 5-FU tornam as células tumorais mais imunogênicas, potencializando sua capacidade de induzir a maturação de DC. Desse modo, observamos que a porcentagem de células que expressam o marcador CD83 aumentou de 74 ± 3,4% no WT a mais de 90% nos grupos em que associamos 5-FU com CQ. Os marcadores celulares associados à maturação de DCs, CD80, CD86 e HLA-DR aumentaram de 53,5 ± 2,7%; 74,5 ± 1,2% e 65,9 ± 3,2% do controle WT, para 63,7 ± 5,1%; 92,7 ± 1,5%, respectivamente, e para 68,7 ± 2,5%, 93,5 ± 1,12% e 86,3 ± 2,4% nas células tratadas com 5-FU+CQ, respectivamente. Embora o tratamento de HCT-116 apenas com CQ ou 5-FU aumenta o efeito imunogênico nas DCs, esse efeito não é tão intenso quanto a combinação de drogas.
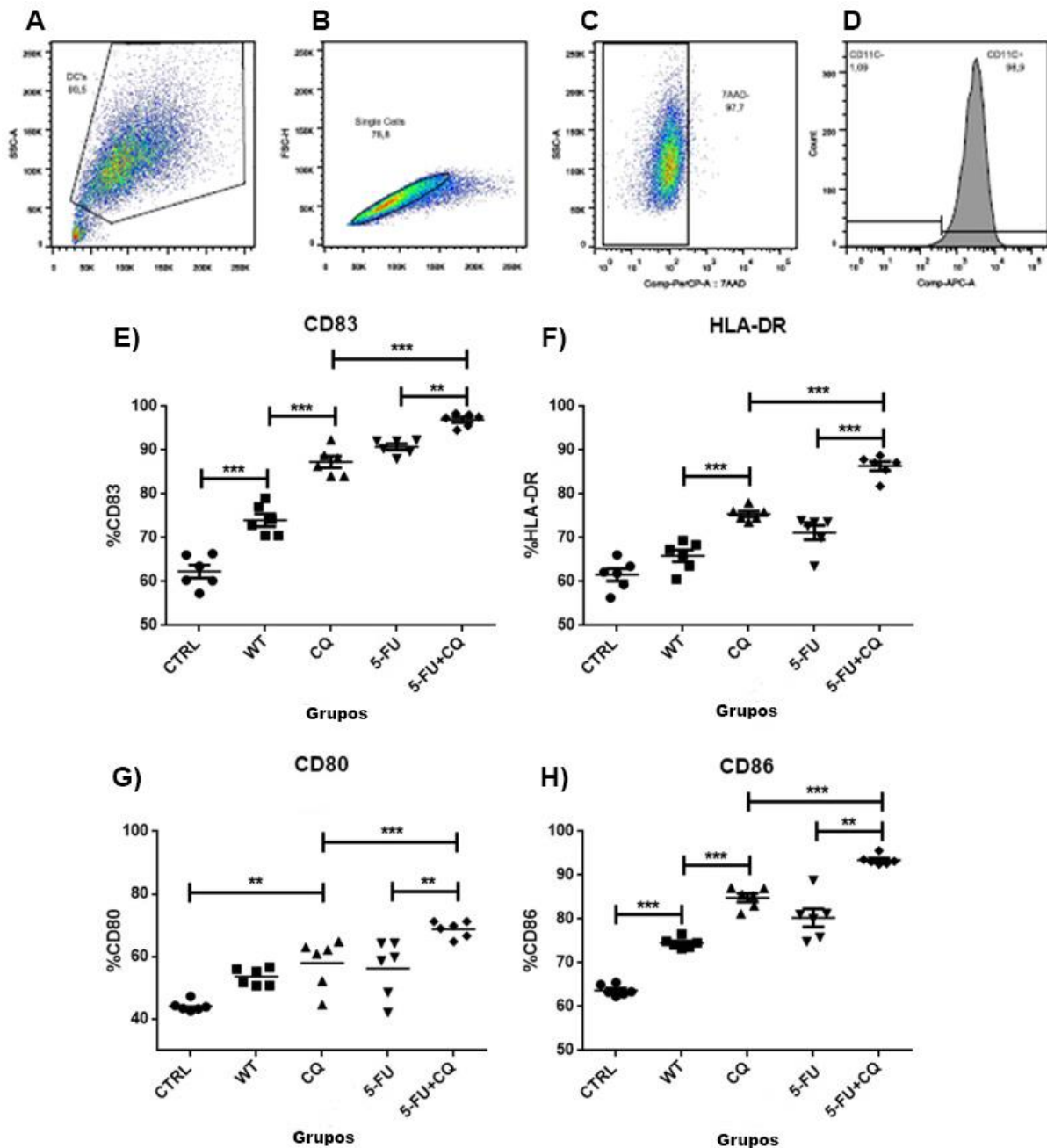
Figura 5 - Estratégia de *gating* de análise de DCs, baseado em tamanho e granulosidade da população de interesse. (A)*Gate de singlets.* (B)Representando eventos que passaram individualmente pelo laser, *gate* feito nos eventos 7AAD⁻ para exclusão de células mortas. (C)G*ate* feito dentro da população CD11c⁺. (D)Expressão das moléculas de interesse em DCs de 6 doadores saudáveis, após sensibilização com lisado tumoral de células tumorais HCT 116 previamente tratadas com 5-FU e CQ. (E-H) Os gráficos de dispersão ilustram as porcentagens de células dendríticas (CD11c+) que co-expressam os marcadores CD83, HLA-DR, CD80 e CD86. Análises estatísticas feitas por Kruskall-Wallis, indicando o efeito dos tratamentos sobre a imunogenicidade das células tumorais (n=6).

5. DCs sensibilizadas com lisados de células tumorais pré-tratadas com a combinação de CQ e 5-FU induz resposta alogênica mais intensa

Em vista do efeito dos lisados de células tumorais, sobre a maturação e ativação das DCs de indivíduos saudáveis, nosso passo seguinte foi avaliar se as alterações fenotípicas eram acompanhadas da mudança no perfil funcional das DCs.

Assim, o ensaio de MLR foi efetuado para avaliar o efeito do lisado tumoral sobre a capacidade das DC de induzirem resposta alogênica, um ensaio clássico para investigar o potencial de indução de resposta imune celular. Esse estudo foi realizado apenas com os tratamentos combinados de CQ e 5-FU que foi o grupo que apresentou as alterações fenotípicas mais intensas em comparação com os controles DC WT e DC CQ, devido à escassez de DCs. As figuras 6B e 6C ilustram as médias de atividade proliferativa dos linfócitos T CD4+ e CD8+ respectivamente.

Embora a exposição das HCT-116 a CQ, isoladamente, também tenha aumentado o efeito imunogênico sobre as DC em comparação com WT, esse efeito não foi tão intenso quanto a combinações das drogas. Nossos resultados mostram efeito especialmente marcante na proliferação dos linfócitos T CD4+, quando as DCs foram sensibilizadas com lisados das células pré-tratadas com 5-FU+CQ.

6. Linfócitos que respondem ao estímulo alogênico têm perfil de células ativadas

Para entender em pouco melhor o perfil de linfócitos gerados em resposta às DCs, decidimos avaliar a expressão de 2 marcadores na superfície dessas células, o CD69, um marcador de linfócitos T ativados e o PD-1, uma molécula que ao interagir com seu ligante PD-L1 presentes em APCs e nas células tumorais, induz supressão linfocitária.

Nossos resultados, mostrados na Figura 6D-G, indicam que os linfócitos gerados pela exposição ao DC 5-FU+CQ expressam uma alta frequência de células CD69+, tanto entre os linfócitos T CD4+ (30,7 ± 2,7) quanto no T CD8+ (41,6 ± 3,2%, 42,5 ± 2,9%). Em contraste,

as células co-cultivadas com DC 5-FU+CQ, apresentam menor número de linfócitos PD-1+, principalmente nas células CD4+ (5-FU+CQ (16,5 ± 1,3)).

7. DCs sensibilizadas com lisado de células tumorais expostas a combinação de drogas, favorecem a resposta do tipo Th1

A Figura 6H mostra um incremento da secreção de IFN-γ; comparando o grupos que foram tratado com o lisado sem tratar (WT=171,1±11,4) e os grupos que foram tratados com nossa combinação; CQ=195,7±16,5; 5-FU+CQ=389,3±19,8. Na figura 6I, contrariamente mostra que os linfócitos que foram co-cultivados com as DCs sensibilizadas com lisado tumoral produziram uma quantidade menor de IL-10 quando foram tratadas com a combinação de 5-FU e CQ (WT= 46,8±6,3; CQ=28,7±7,2; 5-FU+CQ=8,2±3,7).
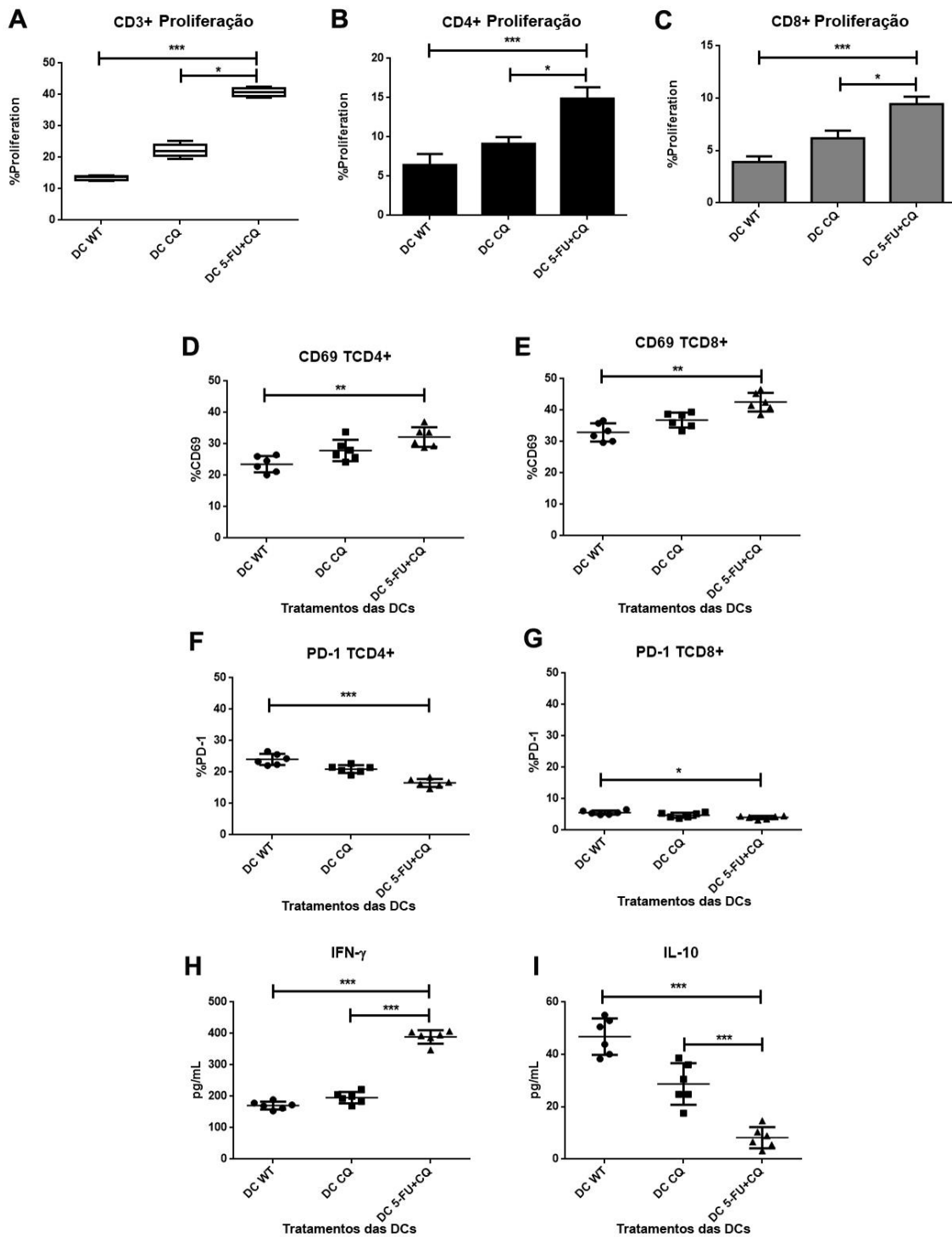
Figura 6 - Melhora da função apresentadora de antígenos das DC induzida por lisados de HCT-116 pré-tratados com 5-FU+CQ. As DCs foram expostas a lisados HCT-116 e co-cultivadas com linfócitos alogênicos por 5 dias. Os linfócitos totais proliferados (células CD3+) foram quantificados por citometria de fluxo (A), assim como as células CD4 + e CD8 + (B; C) (N = 9; * p ≤ 0,05; ** p ≤ 0,01). Os linfócitos T CD4+ e CD8+ foram depois analisados separadamente seus níveis de expressão de CD69 (marcador de ativação) e PD-1 (molécula reguladora) (D-G; * p ≤ 0,05; ** p ≤ 0,02). Os sobrenadantes de MLR foram coletados e analisados quanto à secreção de IFN-γ (H) e IL-10 (I) por ELISA (*** p ≤ 0,01) (n=6).

8. DCs tratadas com lisado aumentam a geração *in vitro* de CTL

O cultivo de linfócitos com DCs autólogas pre-sensibilizadas com diferentes lisados tumorais levou à geração de CTL tumor específicos cuja atividade citotóxica foi evidenciada pela expressão de moléculas de perforina e granzima B. Observamos que os linfócitos cultivados com DCs sensibilizados com lisado de células expostas a 5-FU e CQ induziram a geração de linfócitos com níveis mais altos de perforina e granzima B, como representando na Figura 7 que mostra a intensidade de fluorescência (MFI) para esses marcadores. Não foram observadas diferenças para a marcação com anti-CD107a.
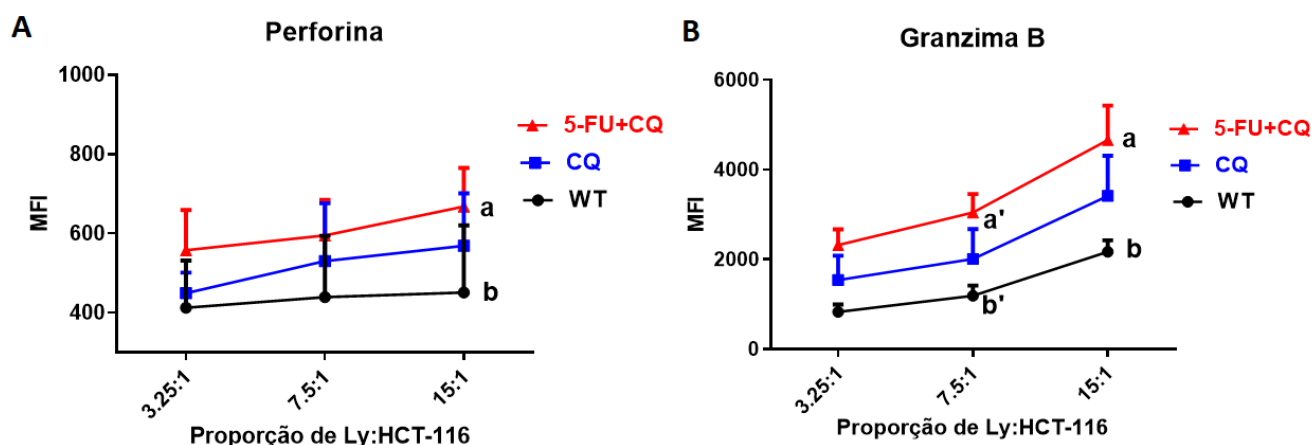
Figura 7 - A geração *in vitro* de células T citotóxicas (CTL) é melhorada por DCs sensibilizadas com lisados de células expostas a combinação de 5-FU e CQ. Intensidade média de fluorescência (MFI) de células CD8 + em proliferação (A) de quatro doadores saudáveis com proporções individuais de efetor: alvo (3,25: 1, 7,5: 1 e 15: 1). Esses linfócitos mostram maior expressão de marcadores de citotoxicidade perforina (MFI da proporção 15:1, 5-FU+CQ (a)>WT (b) ($p<0.05$)) e granzima B (MFI da proporção 7.5:1, 5-FU+CQ (a')>WT (b') ($p<0.05$); MFI da proporção 15:1, 5-FU+CQ (a)>WT (b) ($p<0.05$)) que o grupo controle WT (n=6).

9. Alterações transcricionais associadas ao bloqueio da autofagia

Para entender melhor o aumento da maturação de DC associado ao bloqueio da autofagia, avaliamos as células HCT-116 tratadas com 5-FU, CQ ou sua combinação. Consideramos a alteração do fold-change para comparar a expressão genica e procuramos diferenças significativas entre os grupos (Tabela 7). Observamos que as células tratadas com CQ apresentaram um aumento modesto na expressão dos genes de autofagia ATGs, SQSTM1,

MAP1LC3B e ULK1, e uma diminuição considerável nos genes relacionados à progressão tumoral (BNIP3, BNIP3L, FOSL2, HES1, LAMB3, LOXL2, NDRG1, P4HA1 e PIK3R2), bem como antígenos tumorais da família CEA (CEACAM 1, 5, 6 e 7). O tratamento com 5-FU induziu um aumento nos genes de autofagia. Em contraste com o grupo CQ, não observamos uma diminuição tão intensa nos genes relacionados à progressão tumoral, enquanto a expressão dos genes CEA foi aumentada. As células tratadas com a combinação de 5-FU e CQ tiveram aumento na expressão dos genes da autofagia, bem como os genes da família CEA. Corroborando nossa hipótese a expressão dos genes associados à progressão tumoral foi diminuída pela combinação de drogas.

Tabela 7– Expressão do *fold-change* de genes selecionados por células tumorais, comparando células sem tratar (WT) com aquelas tratadas com CQ, 5-FU ou CQ com 5FU.

Função do gene	Gene	WT vs CQ	WT vs 5-FU	WT vs 5-FU + CQ
Autofagia	**ATG12**	1.7	3.2	**7.1**
	ATG5	1.9	4.5	**6.4**
	MAP1LC3B	1.8	2.3	**5.7**
	ULK1	1.2	2.4	**5.2**
	SQSTM1	2.1	1.9	**4.8**
	BECN1	0.8	**5.8**	2.5
	BNIP3	**-4.36**	-1.36	**-4.11**
	BNIP3L	**-4.06**	-1.2	**-3.85**
Adesão celular	**CEACAM5**	-0.9	1.5	**8.4**
	CEACAM6	-1.3	2.4	**6.5**
	CEACAM7	-2.1	1.7	**5.2**
	CEACAM1	-1.2	2.1	**5.7**
	HES1	**-3.16**	-0.5	**-7.1**
	FOSL2	**-4.72**	-0.72	**-3.6**
	NDRG1	**-10.43**	-1.43	**-6.11**
Atividade catalítica	**PIK3R2**	-1.2	-3.97	**-4.2**
	PGK1	-2.59	-0.2	-2.59
	LOXL2	-4.8	-0.9	-2.85
	PGK1	-2.59	-0.2	-2.59
	ALDOC	**-8.14**	-1.14	**-7.52**
	ANGPTL4	**-6.25**	-1.25	**-5.93**
Estrutura molecular	**LAMB3**	**-4.26**	-0.76	**-3.59**
Proteinas de choque térmico e HMGBs	**HMGB1**	1.02	1.9	1.12
	HMGB2	0.98	1.87	1.05
	HMGB3	0.2	0.5	0.347
	DNAJB1 (HSP40)	1.17	1.5	0.85
	HSP1A (HSP70)	2.67	2.82	**3.38**
	HSP90AB1 (HSP90)	1.45	2.97	1.52
Oxidoredutase	**P4HA1**	**-6.53**	-1.1	**-6.89**
Ligação de RNA	**PCBP1**	1.2	2.86	**8.26**

V.- <u>Discussão</u>

O desenvolvimento de resistência a agentes antitumorais é um dos desafios da terapia antitumoral e a capacidade de adaptação das células tumorais exige que muitos esquemas terapêuticos façam a associação de fármacos, com o objetivo de eliminar o maior número possível de células transformadas. Neste trabalho, investigamos os efeitos da associação do 5-FU, droga de primeira linha na terapia contra o câncer de cólon, com a CQ um agente antimalárico capaz de inibir a autofagia. A concentração de 20µM foi escolhida por ser a menor concentração capaz de inibir o crescimento tumoral, aproximando-se, portanto, de uma dose metrônomica utilizada na clínica(74). Embora a ação da CQ, em combinação com agentes antineoplásicos, já tinha sido investigada por outros autores, não há relatos dessa associação com baixas concentrações de drogas antineoplásicas. Outro aspecto inédito em nossa abordagem, está na investigação do efeito do tratamento sobre a imunogenicidade das células tumorais e não de seu efeito citotóxico direito.

Nos protocolos atuais de avaliação autofágica, o aumento de LC3-II e a diminuição do SQSTM1 são usados como os principais marcadores de inibição da autofagia em etapas tardias. O LC3-II é uma proteína presente na parede dos autofagossomos que normalmente é degradada junto com o autolissosomo ao final da autofagia. Quando a autofagia é bloqueada pela CQ, os lisossomos não conseguem se unir com o autofagossomos e por tanto há acumulo de LC3-II (75). O SQSTM1 se liga a proteínas ubiquitinadas, especialmente LC3-II, formando parte da parede do autofagossomo. Se a autofagia é estimulada, o SQSTM1 será degradado ao final da autofagia. Assim, a inibição da autofagia promove o acúmulo de SQSTM1, bem como de LC3-II (76). Baixas concentrações de 5-FU aumentam a autofagia nas células HCT-116, enquanto o CQ foi capaz de inibir o processo, como evidenciado pelo acúmulo de LC3-II. A análise da expressão de SQSTM1 é consistente com estudos anteriores da autofagia no HCT-116 (77).

A indução de autofagia por 5-FU também foi confirmada por análise do transcriptoma que mostra um aumento na expressão dos genes associados à autofagia ATGs, SQSTM1, MAP1LC3B, BECN1 e ULK1. Um aumento maior da expressão foi observado no grupo da combinação de 5-FU e CQ. O aumento da expressão de LC3B e BECN1 sob condições de estresse é consistente com os dados da literatura (78, 79).

A análise ultraestrutural por microscopia eletrônica revelou que as células de controle sem tratamento têm uma formação desprezível de autofagossomos e autolisosomo, enquanto as células tratadas com CQ possuem um elevado número de autofagossomos no citoplasma. Nas HCT-116 tratadas apenas com 5-FU observamos um aumento evidente no número de autolissosomo com material degradado, indicando indução de autofagia e a conclusão do processo, consistente com relatos anteriores (32, 71-73, 80).

Após confirmarmos a capacidade da CQ em bloquear a autofagia e do 5-FU em estimulá-la, avaliamos se este bloqueio é capaz de potencializar a ação citotóxica do quimioterápico, induzindo morte celular. Em consonância com os resultados de indução e inibição de autofagia e, morfologia das células tratadas, os resultados da marcação das células positivas para anexina V e 7AAD mostraram que a necrose (anexina V+ e 7AAD+) não é um tipo de morte celular predominante neste cenário, mas sim a apoptose (anexina V+), caracterizada pela externalização da fosfatidilserina na membrana plasmática (81). Foi possível também, confirmar o efeito não tóxico da 5-FU a 20µM e da CQ a 80µM quando administradas isoladamente. Contudo, a combinação com CQ foi capaz de potencializar a ação do 5-FU na indução da morte celular apoptótica.

Analisando os efeitos da sinergia entre o 5-FU e a CQ na imunogenicidade de células tumorais, observamos que a exposição de DC ao lisado de células tumorais previamente tratadas com a combinação aumentou a expressão marcadores de co-estimulação, maturação e apresentação antigênica dessas células. Antes de serem estimuladas as DCs se encontram num

estágio imaturo (iDC), possuem uma enorme capacidade fagocítica e endocítica, porém uma baixa capacidade de estimular linfócitos T naive, por não terem os níveis necessários de sinais co-estimuladores(48). O processo de maturação das DCs culmina com a redução de sua capacidade endocítica e migração em direção as áreas de linfócitos T naive em órgão linfóides secundários(82). Essa migração resulta de uma resposta quimiotática controlada pela expressão de receptores para quimiocinas, como CCR7, com aumento de moléculas envolvidas diretamente na ativação linfocitária e a produção de citocinas inflamatórias (83, 84). A maturação das DCs é caracterizada fenotipicamente pelo aumento da expressão de MHC de classe II, marcadores de maturação (CD83) e das moléculas co-estimuladoras (CD40, CD80, CD86), essências para a correta apresentação de antígenos pelos linfócitos T (85). Além de ser um marcador de maturação, o CD83 modula a resposta imune melhorando as respostas imunológicas, aumentando a liberação intracelular de cálcio nos linfócitos T naive e de memória (86). A expressão de CD83 também foi incrementada pela nossa combinação de drogas.

Em estudos anteriores do nosso grupo, descobrimos que o RNA total extraído das HCT-116 tratadas com 5-FU transfectado as DCs aumentou a expressão de marcadores de maturação e moléculas coestimuladoras e melhorou sua capacidade de estimulação alogênica(87) Ademais, a exposição das HCT-116 a doses baixas de antineoplásicos como paclitaxel ou doxorrubicina aumenta a expressão de genes de proteínas de choque térmico (HSPs), calmodulina, e aumenta a expressão de genes envolvidos no mecanismo de processamento de antígenos pela via citosólica, como TAP1, tapasina e calnexina; aumentando a imunogenicidade das células tumorais (26). Essa visão foi reforçada pela observação de que nosso lisado pré-tratado com a combinação de 5-FU e CQ incrementou a expressão de proteínas de choque térmico, entre elas ao HSP70 e HSP90, que aumentam a capacidade fagocítica e promovem a apresentação cruzada de antígenos (88). Os HSPs, junto com o HMGB1,

desempenham um papel importante na maturação fenotípica das DCs, aumentando a expressão de HLA-DR, CD80 e CD86 e a maturação funcional, além de melhorar a secreção de IL-6, IL-12 e IFN-γ (88, 89).

A reação mista de linfócitos (MLR) nos permitiu demonstrar que as alterações fenotípicas observadas nas DC sensibilizadas são acompanhadas por uma melhora na capacidade de estimular linfócitos T alogênicos. Especificamente, as DCs sensibilizadas com os lisados de tumor pré-tratados com 5-FU junto com CQ promoveram uma proliferação aumentada de linfócitos na MLR. Esse efeito proliferativo foi mais intenso nos linfócitos T CD4+ também é coerente com sua capacidade de interagir com as moléculas de HLA-DR expressas nas DCs. Em adição, o aumento observado nos linfócitos T CD8+, sugere a geração de linfócitos T citotóxicos que são específicos para as células tumorais (visto que sua proliferação foi estimulada por DC sensibilizadas com antígenos tumorais). Fato confirmado pelo cultivo de DC e linfócitos autólogos para geração de CTL (90). A perforina e a granzima B são consideradas marcadores funcionais em células com capacidade citotóxica (91), pois a polimerização da perforina perfura a membrana da célula alvo, enquanto a granzima B ativa as caspases, induzindo apoptose (92). No nosso estudo, encontramos um aumento significativo na intensidade média de fluorescência (MFI) da população T CD8+ proliferadas pelas DC sensibilizadas com lisado tumoral pré-tratado com 5-FU+CQ. A expressão de perforina e granzima B foi maior nesta população de T CD8+.

Também observamos que os linfócitos gerados em resposta às DCs sensibilizadas apresentaram perfil de células ativadas, expressando níveis mais altos do CD69+, marcador associado à estimulação eficaz dos linfócitos T pelas DCs (93). Nossos resultados também indicaram que não há ativação seletiva das células T CD4+ ou CD8+, visto que ambas as subpopulações mostraram aumento na porcentagem de CD69+. Consequentemente, esses mesmos tratamentos resultaram na redução da expressão de PD-1+ entre os linfócitos T CD4+.

PD-1 é um membro da família CD28 e sua função principal é limitar o agrupamento de TCR e moléculas co-estimulatórias na sinapse imunológica enviando sinais inibitórios ao interagir com os receptores PD-L1 e PD-L2, presentes tanto em células apresentadoras de antígenos, como as DCs, quanto em células tumorais (94). A expressão de PD-1 é aumentada nas células T de pacientes com câncer de pâncreas, câncer de pulmão, várias leucemias e linfomas (95-97). Portanto, a expressão de PD-1 nas células T e a expressão de PD-L1 nas células tumorais constituem um mecanismo de escape do tumor. Nós observamos a redução da expressão de PD-1 em linfócitos em proliferação pela co-cultura com DC pré-sensibilizadas com lisado tumoral. Esse aspecto é particularmente interessante, pois os sinais associados ao PD-1 e PD-L1 estão entre os principais bloqueios do ponto de verificação da resposta antitumoral (*checkpoint blockage*). Assim, além do anticorpo anti-CTLA-4 (ipilimumab), os anticorpos anti-PD-1 (nivolumab) (98) estão entre os principais agentes imunoterápicos antitumorais da atualidade (99).

Os linfócitos co-cultivados com DCs sensibilizadas com lisados de células tumorais expostas ao 5-FU e CQ produziram maiores quantidades de IFN-γ que os controles, enquanto diminuíam a produção de IL-10. Os linfócitos T CD4+ e CD8+ naives podem se diferenciar em células T efetoras específicas, com funções diferentes, em parte diferenciadas de acordo as citocinas que produzem(100). Assim, os linfócitos T CD4+ podem se diferenciar em linfócitos T helper 1 (Th1) que secretam IFN-γ e TNF-α para estimular a geração de linfócitos T CD8+, estando envolvidas no desenvolvimento da resposta imune antitumoral. O IFN-γ também recruta monócitos, induzindo a diferenciação de linfócitos T CD4+ em linfócitos Th1 (100, 101). A IL-10 é uma citocina inibidora por excelência das DCs que pode ser secretada por Tregs e Th2 e sua principal função é inibir a progressão da resposta Th1 reduzindo a expressão do MHC-II e das moléculas co-estimuladoras CD80 e CD86(102). Assim, as DCs

sensibilizadas com o lisado da combinação, promovem a ativação preferencial dos linfócitos de perfil Th1, com pouca estimulação de Th2 ou Treg.
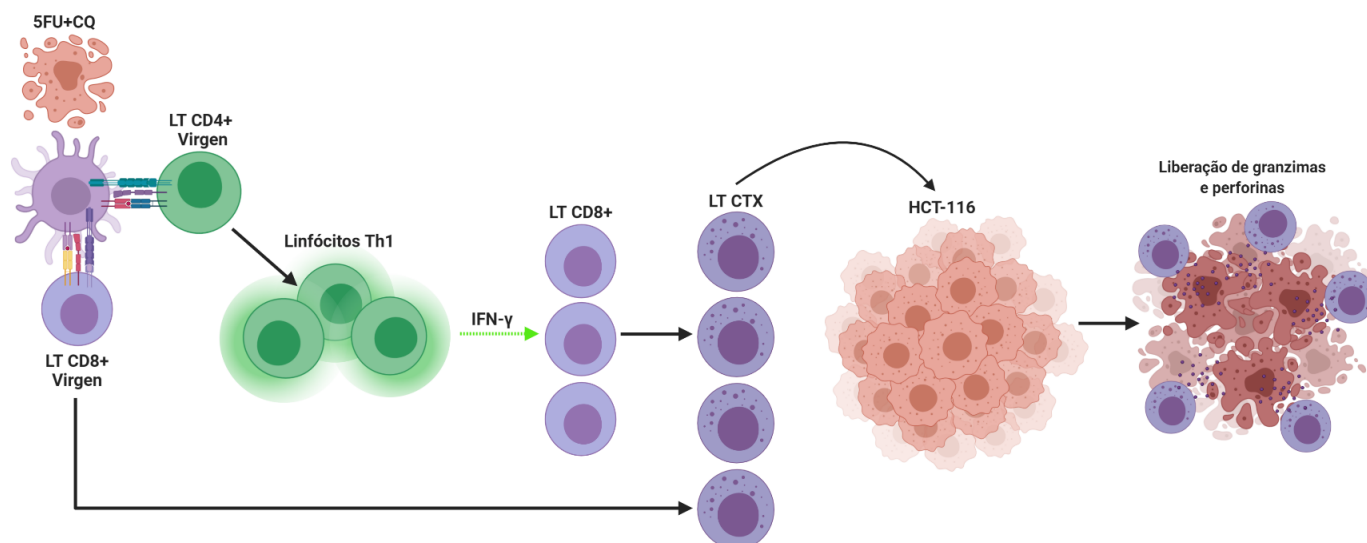
Figura 8 - Efeito imunoestimulador do lisado tumoral. As células dendríticas sensibilizadas com o lisado de células pré-tratadas com a combinação de 5-FU e CQ aumentaram seus marcadores de co-estimulação, maturação e apresentação antigênica, produzindo assim uma alta proliferação de linfócitos T CD4+ e CD8+ (via *cross-priming*). Esses linfócitos T CD4+ gerados eram predominantemente de perfil Th1, produtores de IFN-γ, estimulando a diferenciação dos linfócitos TCD8+ em linfócitos T citotóxicos (CTL). Quando desafiados com as HCT-116, os CTLs gerados *in vitro*, liberaram granzimas e perforinas, marcadores funcionais de CTL, destruindo As células tumorais alvo.

A análise de outras citocinas, como IL-12, IL-2, TNF, IL-4, IL-6, IL-17 e IL-23, seria útil para fornecer uma visão mais clara sobre como a combinação de 5-FU e CQ afetam a modulação das outras populações de linfócitos T. Por fim, a evidência de que a combinação de 5-FU e CQ pode modular a biologia das HCT-116, bem como o lisado celular obtido a partir destas, é reforçada pelo aumento da expressão de genes de membros da família CEA associados a tumores (CEACAM 1, 5,6 e 7), coincidindo com resultados prévios observados em outro linhagem de células de câncer colorretal humano (HT-29) (103), e a expressão diminuída de genes associados à progressão tumoral como: BNIP3, BNIP3L, FOSL2, HES1, LAMB3, LOXL2, NDRG1, P4HA1 e PIK3R2.

Em conclusão, os resultados aqui apresentados demonstram que o uso de 5-FU em combinação com a CQ provoca alterações genéticas e fenotípicas nas células tumorais aumentando sua imunogenicidade, tornando-as mais eficientes em sensibilizar as DCs. Estas, por sua vez mostram maior habilidade em induzir resposta específica contra as células tumorais. Por fim, em continuidade à linha de investigação sobre a combinação de quimioterápicos antitumorais com a imunoterapia baseada na estimulação de DC, nossos resultados indicam o potencial para uso clínico deste protocolo de sensibilização *in vitro* de DCs, bem como o uso de bloqueadores autofágicos combinados com quimioterápicos convencionais para o tratamento de pacientes com câncer colorretal, estimulando-nos a continuar investigando novas abordagens para combater essa doença.

VI. **Referências bibliográficas**

1.   INCA. Estimativa 2020: incidência de câncer no Brasil. Instituto Nacional de Câncer José Alencar Gomes da Silva/ Ministério da Saúde.; 2019.
2.   Castro-Mujica MdC, Barletta-Carrillo C. Síndrome de Lynch: aspectos genéticos, clínicos y diagnósticos %J Revista de Gastroenterología del Perú. 2018;38:265-79.
3.   Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, et al. Colorectal cancer. Nat Rev Dis Primers. 2015;1:15065-.
4.   Society AC. Colorectal Cancer Risk Factors 2020 [Available from: https://www.cancer.org/cancer/colon-rectal-cancer/causes-risks-prevention/risk-factors.html.
5.   Colussi D, Brandi G, Bazzoli F, Ricciardiello L. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. Int J Mol Sci. 2013;14(8):16365-85.
6.   Andre T, de Gramont A, Study Group of Clinical Research in Radiotherapies Oncology OMRG. An overview of adjuvant systemic chemotherapy for colon cancer. Clin Colorectal Cancer. 2004;4 Suppl 1:S22-8.
7.   Libutti SK SL, Willett CG, Levine RA. Chapter 57: Cancer of the colon. Wilkins LW, editor. Philadelphia2015.
8.   Fares J, Fares MY, Khachfe HH, Salhab HA, Fares Y. Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduction and Targeted Therapy. 2020;5(1):28.
9.   Piawah S, Venook AP. Targeted therapy for colorectal cancer metastases: A review of current methods of molecularly targeted therapy and the use of tumor biomarkers in the treatment of metastatic colorectal cancer. Cancer. 2019;125(23):4139-47.
10.   Khandia R, Dadar M, Munjal A, Dhama K, Karthik K, Tiwari R, et al. A Comprehensive Review of Autophagy and Its Various Roles in Infectious, Non-Infectious, and Lifestyle Diseases: Current Knowledge and Prospects for Disease Prevention, Novel Drug Design, and Therapy. Cells. 2019;8(7):674.
11.   Kardideh B, Samimi Z, Norooznezhad F, Kiani S, Mansouri K. Autophagy, cancer and angiogenesis: where is the link? Cell & Bioscience. 2019;9(1):65.

12.   Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1):1-222.

13.   Gross AS, Graef M. Mechanisms of Autophagy in Metabolic Stress Response. Journal of Molecular Biology. 2020;432(1):28-52.

14.   McDonnell SJ, Spiller DG, White MRH, Prior IA, Paraoan L. ER stress-linked autophagy stabilizes apoptosis effector PERP and triggers its co-localization with SERCA2b at ER-plasma membrane junctions. Cell Death Discov. 2019;5:132-.

15.   Mao J, Lin E, He L, Yu J, Tan P, Zhou Y. Autophagy and Viral Infection. Adv Exp Med Biol. 2019;1209:55-78.

16.   Yun CW, Lee SH. The Roles of Autophagy in Cancer. Int J Mol Sci. 2018;19(11):3466.

17.   Yun HR, Jo YH, Kim J, Shin Y, Kim SS, Choi TG. Roles of Autophagy in Oxidative Stress. Int J Mol Sci. 2020;21(9).

18.   Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Developmental cell. 2009;17(1):87-97.

19.   Qin W, Li C, Zheng W, Guo Q, Zhang Y, Kang M, et al. Inhibition of autophagy promotes metastasis and glycolysis by inducing ROS in gastric cancer cells. Oncotarget. 2015;6(37):39839-54.

20.   Schmukler E, Kloog Y, Pinkas-Kramarski R. Ras and autophagy in cancer development and therapy. Oncotarget. 2014;5(3):577-86.

21.   Prior IA, Hood FE, Hartley JL. The frequency of Ras mutations in cancer. Cancer Research. 2020:canres.3682.2019.

22.   Chen S-H, Tsai H-L, Jiang J-K, Sung Y-C, Huang C-W, Yeh Y-M, et al. Emergence of RAS mutations in patients with metastatic colorectal cancer receiving cetuximab-based treatment: a study protocol. BMC Cancer. 2019;19(1):640.

23.   Yun M, Bai HY, Zhang JX, Rong J, Weng HW, Zheng ZS, et al. ULK1: a promising biomarker in predicting poor prognosis and therapeutic response in human nasopharygeal carcinoma. PLoS One. 2015;10(2):e0117375.

24.   Lu J, Zhu L, Zheng LP, Cui Q, Zhu HH, Zhao H, et al. Overexpression of ULK1 Represents a Potential Diagnostic Marker for Clear Cell Renal Carcinoma and the Antitumor Effects of SBI-0206965. EBioMedicine. 2018;34:85-93.

25.   Shurin GV TI, Kaneno R, et al. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. The Journal of Immunology. 2009(183):137-44.

26.   Kaneno R, Shurin GV, Kaneno FM, Naiditch H, Luo J, Shurin MR. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. Cell Oncol (Dordr). 2011;34(2):97-106.

27.   Zitvogel L AL, Ghiringhelli F, et al. Immunological aspects of cancer chemotherapy. Nature Reviews Immunology 2008(8):59-73.

28.   Zitvogel L CN, Pequignot MO, et al. Immune response against dying tumor cells. Advances in Immunology. 2004(84):131-79.

29.   Zitvogel L KG. Anticancer immunochemotherapy using adjuvants with direct cytotoxic effects. Journal of Clinical Investigation. 2009(119):2127-30.

30.   Pitt JM, Kroemer G, Zitvogel L. Immunogenic and Non-immunogenic Cell Death in the Tumor Microenvironment. In: Kalinski P, editor. Tumor Immune Microenvironment in Cancer Progression and Cancer Therapy. Cham: Springer International Publishing; 2017. p. 65-79.

31.   Kaneno R SG, Tourkova IL, et al. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. Journal of translational medicine. 2009(7):58-66.

32.   Sasaki K, Tsuno NH, Sunami E, Tsurita G, Kawai K, Okaji Y, et al. Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells. BMC cancer. 2010;10:370-.

33.   Dolgin E. Anticancer autophagy inhibitors attract 'resurgent' interest. Nat Rev Drug Discov. 2019;18(6):408-10.

34.   Cudjoe EK, Lauren Kyte S, Saleh T, Landry JW, Gewirtz DA. Chapter 12 - Autophagy Inhibition and Chemosensitization in Cancer Therapy. In: Johnson DE, editor. Targeting Cell Survival Pathways to Enhance Response to Chemotherapy. 3: Academic Press; 2019. p. 259-73.

35.   Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nature reviews Cancer. 2003;3(5):330-8.

36.   Tang J-C, Feng Y-L, Liang X, Cai X-J. Autophagy in 5-Fluorouracil Therapy in Gastrointestinal Cancer: Trends and Challenges. Chin Med J (Engl). 2016;129(4):456-63.

37.   Sasaki K, Tsuno NH, Sunami E, Tsurita G, Kawai K, Okaji Y, et al. Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells. BMC Cancer. 2010;10:370.

38.   Verbaanderd C, Maes H, Schaaf MB, Sukhatme VP, Pantziarka P, Sukhatme V, et al. Repurposing Drugs in Oncology (ReDO)-chloroquine and hydroxychloroquine as anti-cancer agents. Ecancermedicalscience. 2017;11:781-.

39.   Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M. Quinolones: from antibiotics to autoinducers. FEMS Microbiol Rev. 2011;35(2):247-74.

40.   Slater AFG. Chloroquine: Mechanism of drug action and resistance in plasmodium falciparum. Pharmacology & Therapeutics. 1993;57(2):203-35.

41.   Homewood CA, Warhurst DC, Peters W, Baggaley VC. Lysosomes, pH and the Anti-malarial Action of Chloroquine. Nature. 1972;235(5332):50-2.

42.   Groh N BI, Beckmann M, Schrauder M, Loehberg C. Evaluation for synergistic suppression of breast cancer proliferation by Chloroquine in combination with the rapamycin derivative, Rad001. Geburtshilfe und Frauenheilkunde. 2009;5(69):214-8.

43.   Grimaldi A SD, Zappavigna S, Lombardi A, Misso G, Boccellino M, et al. Antagonistic effects of chloroquine on autophagy occurrence potentiate the anticancer effects of everolimus on renal cancer cells. Cancer Biology & Therapy. 2015;4(16):567–79.

44.   Chou H-L, Lin Y-H, Liu W, Wu C-Y, Li R-N, Huang H-W, et al. Combination Therapy of Chloroquine and C_2-Ceramide Enhances Cytotoxicity in Lung Cancer H460 and H1299 Cells. Cancers (Basel). 2019;11(3):370.

45.   Iljoong O, Hyunchul C, Yonghoon L, Minseok C, Deokbae P, Youngki L. Blockage of Autophagy Rescues the Dual PI3K/mTOR Inhibitor BEZ235-induced Growth Inhibition of Colorectal Cancer Cells. Development & Reproduction. 2016;20(1):1-10.

46.   Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.

47.   McKenna K, Beignon AS, Bhardwaj N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. Journal of virology. 2005;79(1):17-27.

48.   Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annual review of immunology. 2000;18:767-811.

49.   Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. Nature Reviews Immunology. 2020;20(1):7-24.

50.   González FE, Gleisner A, Falcón-Beas F, Osorio F, López MN, Salazar-Onfray F. Tumor cell lysates as immunogenic sources for cancer vaccine design. Human vaccines & immunotherapeutics. 2014;10(11):3261-9.

51.   Herzog GI, Solgi G, Wiegmann DS, Nienhaus C, Schrezenmeier H, Yildiz T, et al. Quality of tumor lysates used for pulsing dendritic cells is influenced by the method used to harvest adherent tumor cells. BMC Res Notes. 2011;4:153-.

52.   Cerezo D, Peña MJ, Mijares M, Martínez G, Blanca I, De Sanctis JB. Peptide vaccines for cancer therapy. Recent patents on inflammation & allergy drug discovery. 2015;9(1):38-45.

53.   Win SJ, McMillan DGG, Errington-Mais F, Ward VK, Young SL, Baird MA, et al. Enhancing the immunogenicity of tumour lysate-loaded dendritic cell vaccines by conjugation to virus-like particles. Br J Cancer. 2012;106(1):92-8.

54.   Herzog GI, Solgi G, Wiegmann DS, Nienhaus C, Schrezenmeier H, Yildiz T, et al. Quality of tumor lysates used for pulsing dendritic cells is influenced by the method used to harvest adherent tumor cells. BMC Res Notes. 2011;4(1):153.

55.   Parkhurst MR, DePan C, Riley JP, Rosenberg SA, Shu S. Hybrids of dendritic cells and tumor cells generated by electrofusion simultaneously present immunodominant epitopes from multiple human tumor-associated antigens in the context of MHC class I and class II molecules. Journal of immunology (Baltimore, Md : 1950). 2003;170(10):5317-25.

56.   Barbuto JA, Ensina LF, Neves AR, Bergami-Santos P, Leite KR, Marques R, et al. Dendritic cell-tumor cell hybrid vaccination for metastatic cancer. Cancer immunology, immunotherapy : CII. 2004;53(12):1111-8.

57.   Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med. 1995;182(2):389-400.

58.   González FE, Gleisner A, Falcón-Beas F, Osorio F, López MN, Salazar-Onfray F. Tumor cell lysates as immunogenic sources for cancer vaccine design. Human vaccines & immunotherapeutics. 2014;10(11):3261-9.

59.   Alvarez-Dominguez C, Calderón-Gonzalez R, Terán-Navarro H, Salcines-Cuevas D, Garcia-Castaño A, Freire J, et al. Dendritic cell therapy in melanoma. Ann Transl Med. 2017;5(19):386-.

60.   Dall'Oglio M, Srougi M, Barbuto JAM. Complete response of metastatic renal cancer with dendritic cell vaccine %J International braz j urol. 2003;29:517-9.

61.   Ni J, Song J, Wang B, Hua H, Zhu H, Guo X, et al. Dendritic cell vaccine for the effective immunotherapy of breast cancer. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2020;126:110046.

62.   Sarivalasis A, Boudousquié C, Balint K, Stevenson BJ, Gannon PO, Iancu EM, et al. A Phase I/II trial comparing autologous dendritic cell vaccine pulsed either with personalized peptides (PEP-DC) or with tumor lysate (OC-DC) in patients with advanced high-grade ovarian serous carcinoma. Journal of translational medicine. 2019;17(1):391-.

63.   Rahma OaM, Zaw Win and Estfan, Bassam. Dendritic Cell Cancer Vaccines for Treatment of Colon Cancer. Current Colorectal Cancer Reports. 2014;10.

64.   Sakakibara M, Kanto T, Hayakawa M, Kuroda S, Miyatake H, Itose I, et al. Comprehensive immunological analyses of colorectal cancer patients in the phase I/II study of quickly matured dendritic cell vaccine pulsed with carcinoembryonic antigen peptide. Cancer immunology, immunotherapy : CII. 2011;60(11):1565-75.

65.   Lesterhuis WJ, de Vries IJ, Aarntzen EA, de Boer A, Scharenborg NM, van de Rakt M, et al. A pilot study on the immunogenicity of dendritic cell vaccination during adjuvant oxaliplatin/capecitabine chemotherapy in colon cancer patients. Br J Cancer. 2010;103(9):1415-21.

66.   Burgdorf SK. Dendritic cell vaccination of patients with metastatic colorectal cancer. Danish medical bulletin. 2010;57(9):B4171.

67.   Burgdorf SK, Claesson MH, Nielsen HJ, Rosenberg J. Changes in cytokine and biomarker blood levels in patients with colorectal cancer during dendritic cell-based vaccination. Acta oncologica (Stockholm, Sweden). 2009;48(8):1157-64.

68.   Deokbae P, Youngki L. Biphasic Activity of Chloroquine in Human Colorectal Cancer Cells. Development & Reproduciton. 2014;18(4):225-31.

69.   Bianca Francisco Falasco GGRC, Ramon Kaneno. Aumento da suscetibilidade de células tumorais a baixas concentrações de 5-fluorouracil pelo bloqueio da autofagia com cloroquina. In: UNESP, editor. 2015.

70.   Eskelinen EL, Prescott AR, Cooper J, Brachmann SM, Wang L, Tang X, et al. Inhibition of autophagy in mitotic animal cells. Traffic. 2002;3(12):878-93.

71.   Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell. 2010;140(3):313-26.

72.   Jia L, Dourmashkin RR, Allen PD, Gray AB, Newland AC, Kelsey SM. Inhibition of autophagy abrogates tumour necrosis factor α induced apoptosis in human T-lymphoblastic leukaemic cells. British Journal of Haematology. 1997;98(3):673-85.

73.   Moon E-K, Kim S-H, Hong Y, Chung D-I, Goo Y-K, Kong H-H. Autophagy inhibitors as a potential antiamoebic treatment for Acanthamoeba keratitis. Antimicrob Agents Chemother. 2015;59(7):4020-5.

74.   Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer. 2003;3:330.

75.   Mizushima N, Yoshimori T. How to Interpret LC3 Immunoblotting. Autophagy. 2007;3(6):542-5.

76.   Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T. Chapter 12 Monitoring Autophagic Degradation of p62/SQSTM1. Methods in Enzymology. Volume 452: Academic Press; 2009. p. 181-97.

77.   Kucharewicz K, Dudkowska M, Zawadzka A, Ogrodnik M, Szczepankiewicz AA, Czarnocki Z, et al. Simultaneous induction and blockade of autophagy by a single agent. Cell Death & Disease. 2018;9(3):353.

78.   Scherz-Shouval R, Weidberg H, Gonen C, Wilder S, Elazar Z, Oren M. p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation. Proc Natl Acad Sci U S A. 2010;107(43):18511-6.

79.   Yang J-w, Zhang Q-h, Liu T. Autophagy facilitates anticancer effect of 5-fluorouracil in HCT-116 cells. Journal of Cancer Research and Therapeutics. 2018;14(12):1141-7.

80.   Choi J-H, Yoon JS, Won Y-W, Park B-B, Lee YY. Chloroquine enhances the chemotherapeutic activity of 5-fluorouracil in a colon cancer cell line via cell cycle alteration. APMIS. 2012;120(7):597-604.

81.   Lee SH, Meng XW, Flatten KS, Loegering DA, Kaufmann SH. Phosphatidylserine exposure during apoptosis reflects bidirectional trafficking between plasma membrane and cytoplasm. Cell Death & Differentiation. 2013;20(1):64-76.

82.   de Winde CM, Munday C, Acton SE. Molecular mechanisms of dendritic cell migration in immunity and cancer. Medical Microbiology and Immunology. 2020.

83.   Yanagihara S, Komura E, Nagafune J, Watarai H, Yamaguchi Y. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. Journal of immunology (Baltimore, Md : 1950). 1998;161(6):3096-102.

84.   Burgoyne P, Hayes AJ, Cooper RS, Le Brocq ML, Hansell CAH, Campbell JDM, et al. Sorting for CCR7-positivity enriches for a dendritic cell population with enhanced antigen-presenting capacity and anti-tumour potency. bioRxiv. 2019:755488.

85.   Adema GJ HF, Verstraten R, et al. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. Nature. 1997(387):713-7.

86.   Pinho MP, Migliori IK, Flatow EA, Barbuto JA. Dendritic cell membrane CD83 enhances immune responses by boosting intracellular calcium release in T lymphocytes. Journal of leukocyte biology. 2014;95(5):755-62.

87.   De Almeida CV, Zamame JA, Romagnoli GG, Rodrigues CP, Magalhaes MB, Amedei A, et al. Treatment of colon cancer cells with 5-fluorouracil can improve the effectiveness of RNA-transfected antitumor dendritic cell vaccine. Oncol Rep. 2017;38(1):561-8.

88.   Zhu H, Fang X, Zhang D, Wu W, Shao M, Wang L, et al. Membrane-bound heat shock proteins facilitate the uptake of dying cells and cross-presentation of cellular antigen. Apoptosis : an international journal on programmed cell death. 2016;21(1):96-109.

89.   Aarntzen EHJG VI, Lesterhuis WJ, Schuurhuis D, Jacobs JFM, Bol K, et al. Targeting CD4+ T-Helper Cells Improves the Induction of Antitumor Responses in Dendritic Cell–Based Vaccination. Cancer Research. 2013;1(73):19–29.

90.   Burleson GR, Burleson FG, Dietert RR. The cytotoxic T lymphocyte assay for evaluating cell-mediated immune function. Methods in molecular biology. 2010;598:195-205.

91.   Bryceson YT, Fauriat C, Nunes JM, Wood SM, Björkström NK, Long EO, et al. Functional Analysis of Human NK cells by Flow Cytometry. Methods in molecular biology (Clifton, NJ). 2010;612:335-52.

92.   Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nature Reviews Immunology. 2002;2:735.

93.   Cibrián D, Sánchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. 2017;47(6):946-53.

94.   Patsoukis N, Sari D, Boussiotis VA. PD-1 inhibits T cell proliferation by upregulating p27 and p15 and suppressing Cdc25A. Cell Cycle. 2012;11(23):4305-9.

95.	Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. Am J Cancer Res. 2020;10(3):727-42.

96.	Yao H, Wang H, Li C, Fang JY, Xu J. Cancer Cell-Intrinsic PD-1 and Implications in Combinatorial Immunotherapy. Front Immunol. 2018;9:1774.

97.	Waki K, Yamada T, Yoshiyama K, Terazaki Y, Sakamoto S, Matsueda S, et al. PD-1 expression on peripheral blood T-cell subsets correlates with prognosis in non-small cell lung cancer. Cancer Sci. 2014;105(10):1229-35.

98.	Sundar R CB-C, Brahmer JR, Soo RA. Nivolumab in NSCLC: latest evidence and clinical potential. Therapeutic Advances in Medical Oncology. 2015;7(2):85-95.

99.	Rotte A. Combination of CTLA-4 and PD-1 blockers for treatment of cancer. Journal of Experimental & Clinical Cancer Research. 2019;38(1):255.

100.	Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nature immunology. 2003;4(9):835-42.

101.	Ostroumov D, Fekete-Drimusz N, Saborowski M, Kühnel F, Woller N. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. Cellular and Molecular Life Sciences. 2018;75(4):689-713.

102.	Margarida Saraiva AOG, et al. The regulation of IL-10 production by immune cells. Nature Reviews Immunology. 2010;10(1):170-81.

103.	Aquino A, Prete SP, Guadagni F, Greiner JW, Giuliani A, Orlando L, et al. Effect of 5-fluorouracil on carcinoembryonic antigen expression and shedding at clonal level in colon cancer cells. Anticancer research. 2000;20(5b):3475-84.

VII. <u>Anexos</u>

1.

Blocking drug-induced autophagy with chloroquine in HCT-116 colon cancer cells enhances DC maturation and T cell responses induced by tumor cell lysate

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ABSTRACT

Autophagy is an important mechanism for tumor escape, allowing tumor cells to recover from the damage induced by chemotherapy, radiation therapy, and immunotherapy and contributing to the development of resistance. The pharmacological inhibition of autophagy contributes to increase the efficacy of antineoplastic agents. Exposing tumor cells to low concentrations of select autophagy-inducing antineoplastic agents increases their immunogenicity and enhances their ability to stimulate dendritic cell (DC) maturation. We tested whether the application of an autophagy-inhibiting agent, chloroquine (CQ), in combination with low concentrations of 5-fluorouracil (5-FU) increases the ability of tumor cells to induce DC maturation. DCs sensitized with the lysate of HCT-116 cells previously exposed to such a combination enhanced the DC maturation/activation ability. These matured DCs also increased the allogeneic responsiveness of both CD4+ and CD8+ T cells, which showed a greater proliferative response than those from DCs sensitized with control lysates. The T cells expanded in such cocultures were CD69+ and PD-1- and produced higher levels of IFN-γ and lower levels of IL-10, consistent with the preferential activation of Th1 cells. Cocultures of autologous DCs and lymphocytes improved the generation of cytotoxic T lymphocytes, as assessed by the expression of CD107a, perforin, and granzyme B. The drug combination increased the expression of genes related to the CEACAM family (BECN1, ATGs, MAPLC3B, ULK1, SQSTM1) and tumor suppressors (PCBP1). Furthermore, the decreased expression of genes related to metastasis and tumor progression (BNIP3, BNIP3L, FOSL2, HES1, LAMB3, LOXL2, NDRG1, P4HA1, PIK3R2) was noted. The combination of 5-FU and CQ increases the ability of tumor cells to drive DC maturation and enhances the ability of DCs to stimulate T cell responses.

1. Introduction

Colorectal cancer (CRC) is the third most frequent cancer observed worldwide [1], with 40,990 new cases expected for 2020 in Brazil [2]. Conventional therapy for patients with CRC is based on total or partial colectomy, usually followed by neoadjuvant or adjuvant chemotherapy [3] with a fluoropyrimidine, oxaliplatin, irinotecan and/or taxanes [4]. The success of chemotherapy is often complicated by metastases and relapsing disease associated with the development of drug resistance [5]. One of the mechanisms responsible for drug resistance is autophagy, a selective cellular degradation process in which cytosolic proteins and organelles are sequestered into double-membrane autophagosomes that fuse with lysosome for the recycling of macromolecules [6,7]. This process is also triggered to generate amino acids, nucleotides, and fatty acids under conditions of nutrient deprivation [8]. Other types of stress can also trigger autophagy, such as genomic stress,

endoplasmic reticulum stress, intracellular infections, and exposure to drugs [9].

We have previously observed that certain chemotherapeutic agents at ultra-low concentrations can modulate signaling pathways and induce cytokines, such as IL-12, IL-10, IL-4, and TNF-α [10], without inducing apoptosis. Treatment of HCT-116 colorectal cancer cells with a nontoxic concentration of paclitaxel alters the expression of several genes, especially those responsible for the synthesis of heat shock proteins, components of the antigen processing machinery (APM) and tumor-associated antigens [11]. The increased immunogenicity of tumor cells induced by drug exposure is dependent on the onset of so-called immunogenic cell death, with increased expression of danger signals (e.g., danger-associated molecular patterns; DAMPs) such as calreticulin, heat shock proteins, ATP, and high mobility group box 1 (HMGB-1) [12–16]. Consistent with this notion, we also observed that exposing HCT-116 cells to a nontoxic concentration of paclitaxel or doxorubicin causes transcriptional alterations in several genes associated with the expression of tumor antigens [17]. In addition, dendritic cells (DCs) sensitized with the lysate of HCT-116 cells that were previously treated with low concentrations of paclitaxel induced cytotoxic T lymphocytes (CTLs) with higher lytic potential than did the DCs sensitized with untreated tumor cells [17].

More recently, combining antineoplastic agents with autophagy blockers as a therapeutic approach for treating cancer patients has been proposed. Exposing human colorectal cancer cells to 5-fluorouracil (5-FU) enhances autophagy in a considerable portion of these cells [18]. Exposure of these cells to the antimalarial drugs chloroquine (CQ), hydroxychloroquine, or mefloquine (quinolones) reduces autophagy and increases the susceptibility of cells to chemotherapy [19]. Quinolones function as weak bases that passively diffuse into the lysosomes where they are protonated and prevented from leaving this vesicle. Their presence increases the lysosome pH, interrupting its functions and preventing the end of autophagy [20,21]. As anti-inflammatory drugs, quinolones have been used to treat some autoimmune diseases [22]. Currently, there are a number of clinical trials and experimental studies focusing on the feasibility of using CQ and hydroxychloroquine against SARS-CoV-2 (COVID-19) [23,24] . In addition, the combination of CQ and antineoplastic drugs enhances the clinical response in patients with breast [25] and kidney cancer [26], showing a synergistic effect on the activation of mTOR (mammalian target of rapamycin), inhibiting autophagy, and thus increasing tumor cell death. No studies on the induction of autophagy in tumor cells treated with ultra-low doses of chemotherapeutic agents are available, and little is known about the effect of this phenomenon on the immunogenicity of tumor cells. Furthermore, there are no reports on the effects of using CQ combined with other drugs on the functions of immunocompetent cells such as lymphocytes or DCs.

In the present study, we tested the hypothesis that the application of CQ in combination with low concentrations of 5-FU blocks autophagy in tumor cells and promotes DC maturation, increasing their ability to enhance T lymphocyte cytotoxic granule expression.

We found that exposing HCT-116 cells to this combination induced transcriptional changes, and DCs treated with HCT-116 lysate showed an improved ability to stimulate the proliferation of allogeneic T cells and to enhance the generation of cytotoxic CD8+ T cells. These results lead us to conclude that antigenic and transcriptional changes induced in tumor cells by the combination of CQ and low concentrations of 5-FU can be used as a basis for developing better DC-based antitumor reactive T cells.

2. Materials and methods

2.1. Culture and treatments of colorectal cancer HCT-116 cells

The human colon cancer cell line HCT-116 was authenticated by DNA STR profiling using the GenePrint 10 commercial system (Promega, Madison, WI, USA) at the Viral Carcinogenesis and Cancer Biology Research Group, Institute of Biotechnology (IBTEC), Sao Paulo State University (UNESP). These cells were authenticated as mycoplasma-free and were cultured in $150 \, cm^2$ culture bottles in RMPI-1640 medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, and 1% antibiotics/antimycotics solution (Life Technologies). The cells were cultured at 37°C under a 5% CO_2 atmosphere. After 24 h of culturing, the cells were treated with 80 μM CQ for 18 h, and then the minimum effective concentration of 5-fluorouracil (20 μM) (Eurofarma) was added and the cells were cultured for a further 24 h. The cells were then detached with trypsin and washed before subsequent analysis or were cryopreserved for the further preparation of cell lysates. The CQ and 5-FU concentrations and the incubation times were previously determined by MTT-based cytotoxicity assay. For this, $2x10^3$ cells were plated in 96-well flat-bottomed culture plates and incubated with variable concentrations of CQ (80 to 200 μM) and 5-FU (0.06 to 100 μM). Increased optical density due to formazan reduction was read by a spectrophotometer to evaluate the toxic effect of the drug concentrations.

2.2. Analysis of cell viability and death

The concentration of HCT-116 cells was adjusted to 10^5 cells/mL and cultured overnight in a 24-well plate. The cells were treated with CQ overnight, followed by exposure to 5-FU for 24 h. As a positive control of cell death, cells were heated at 59 °C for 20 min. The cells were detached by trypsinization and centrifuged at 1200 rpm for 10 min. The pellet was suspended in 100 μl of cold PBS and centrifuged at 10,000 rpm for 30 sec. The cell pellet was resuspended in 100 μl of annexin buffer solution and incubated with annexin V at room temperature and protected from light. After incubation, 200 μl of the annexin buffer was added. Finally, the cells were stained with 7-aminoactinomycin D (7AAD) to assess viability and were analyzed by flow cytometry.

2.3. HCT-116 lysate

Cryopreserved aliquots were thawed in a 37 °C water bath for 3 min and then quickly refrozen in liquid nitrogen for 3 min (freeze/thaw cycle). This process was repeated five times. The samples were then centrifuged at 14,000 rpm for 15 min, and the supernatant was treated with 70 μl/mL of protease inhibitor (Halt ™ Protease Inhibitor Cocktail ThermoFisher), followed by storage at −80 °C. The total protein was quantified using a ThermoFisher BCA Protein Assay Kit, and samples were aliquoted (100 μg protein) and stored at −80 °C.

2.4. Western blot

Briefly, 40 μg of cell lysate proteins derived from each of the four treatment groups (WT, CQ, 5-FU, and 5-FU + CQ) were diluted in gel electrophoresis sample buffer and heated at 95 °C for 10 min. The proteins were separated on a polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. Nonspecific binding of proteins was blocked by incubating the membranes in 5% low-fat milk in PBS-Tween buffer for 1 h at room temperature. The membranes were subsequently incubated overnight with antigen-specific primary antibodies for autophagy markers: LC3-II (#2775 s Cell Signaling Technologies) and SQSTM1 (#pm045 MBL Intern. Corp.); secondary anti-rabbit (#A0545 Sigma) and anti-mouse (#A9044 Sigma) antibodies were applied, respectively. GAPDH (#g8795 Sigma) was used as a housekeeping gene. The membranes were washed with PBS-Tween buffer for 30 min and developed with enhanced chemoluminescence immunoreactive reagent and read on a UVITEC Cambridge photodocumentation system.

2.5. Ultrastructural analysis by transmission electron microscopy

Approximately 10^6 cells were cultured overnight in a 6-well culture plate and were treated with CQ and 5-FU according to the above-mentioned protocol . After incubation, cell monolayers were trypsinized, and the collected cells were centrifuged for 10 min at 1,200 rpm at 20 °C. The samples were fixed in 1 mL of Karnovsky's solution, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.3, incubated in 0.5% uranyl acetate in aqueous solution; dehydrated in a gradient series of acetone solutions, and embedded in Araldite glue. The ultrafine sections (50 nm) were contrasted with saturated uranyl acetate and lead citrate solutions and observed using a Tecnai Spirit (FEI Company) electron microscope at 80 kV.

2.6. Human monocyte-derived dendritic cells

DCs were differentiated *in vitro* from the peripheral blood adherent mononuclear cells (PBMCs) of six healthy donors. The PBMCs were obtained by centrifugation through a Ficoll-isopaque gradient, suspended in AIM-V culture medium (Invitrogen), and seeded in 6-well culture plates ($1x10^6$/per well). After incubating for 1 h at 37°C, non-adherent cells were removed and adherent monocytes were cultured in complete culture medium containing 80 ng/ml recombinant human GM-CSF and IL-4 (PeproTech) for 5 days. They were then treated with HCT-116 lysate (100 μg per 10^6 DCs) and kept in culture for an additional 48 h. Immature DCs were submitted to seven different culture conditions: DCs (untreated immature DCs); WT (DCs exposed to the lysate of untreated HCT-116 cells); CQ (DCs exposed to the lysate of HCT-116 cells that were pretreated with 80 μM of CQ), 5-FU (DCs exposed to the lysate of HCT-116 cells that were pretreated with 20 μM of 5-FU), and 5-FU + CQ (DCs exposed to the lysate of HCT-116 cells that were pretreated with 20 μM of 5-FU and 80 μM of CQ). All procedures involving both normal and transformed human cells were approved by the Ethics Committee of the Botucatu School of Medicine – UNESP (CEP # 2.258.145).

2.7. DC phenotyping

The lysate-exposed and control DCs were incubated with fluorescent monoclonal antibodies for 30 min and washed with PBS containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. The DCs were incubated with labeled antibodies (HLA-DR-PE, CD11c-APC, CD83-PE-Cy7, CD80-APC-H7, and CD86-FITC (BD Biosciences) for 20 min at 4 °C and then washed with PBS-BSA. The cells were suspended in 100 μl of PBS-BSA, and the samples were read in a FACSCanto II cytometer (Becton-Dickinson) and analyzed using FlowJo, version 7.2.4.

2.8. Mixed lymphocyte reaction assay (MLR)

The functional activity of DCs was first evaluated through their ability to stimulate the proliferation of normal allogeneic T lymphocytes. DCs from six different donors were cocultured with allogeneic T lymphocytes (previously marked with carboxyfluorescein succinyl ester (CFSE) in flat-bottomed 96-well plates in a 1:10 (10^4:10^5) DCs: lymphocyte ratio. Cells were harvested five days later, and the lymphocyte proliferation was analyzed by flow cytometry based on the dilution of CFSE in the replicant cells. We also analyzed the expression of PD-1 and CD69 on CD3+ cells using anti-PD-1-PE and CD69-APC-H7 (BD Pharmingen).

2.9. IFN-γ and IL-10 detection

Supernatants of the MLR assay were collected and preserved at −80°C. These samples were analyzed for the *in vitro* synthesis of IFN-γ and IL-10 using an ELISA kit according to the manufacturer's instructions (R&D Systems).

2.10. Generation of cytolytic T lymphocytes and antitumor cytotoxicity assay

To generate specific antitumor T cells, DCs were cocultured with an autologous T lymphocyte-rich suspension in a 1:10 DC:lymphocyte ratio (10^4:10^5) in complete culture medium supplemented with IL-7 (5 ng/ml) and IL-2 (40 IU/ml). The culture was pulsed with IL-2 every two days for 14 days. On day 14, the lymphocytes were harvested and evaluated for their cytotoxic activity against HCT-116 target cells. A lymphocytotoxicity assay was performed by adding the *in vitro*-generated lymphocytes to HCT-116 monolayer cells in different effector: target ratios (15:1, 7.5:1 and 3.25:1). Anti-CD107a-APC was added to these cocultures and incubated for 5 h at 37°C in an atmosphere containing 5% CO_2. The cells were then harvested and washed with 0.1% BSA in PBS and treated with the Cytofix/CytoPerm kit (BD Pharmingen). Finally, the cells were labeled with anti-perforin-Alexa 488 and anti-granzyme-PE antibodies and analyzed by flow cytometry.

2.11. Tumor cell transcription

To investigate how treatment with the combination of CQ and 5-FU changes tumor cell gene expression, we performed a transcription analysis of these cells. Total RNA was extracted using a QIAGEN RNeasy Plus Micro Kit. RNA was quantified using an RNA HS Assay Kit (Invitrogen) and a QBIT® system. The quality of RNA was analyzed using an Agilent RNA 6000 chip in a Bioanalyzer 2100 system. Only samples with an RNA integrity number higher than 8.0 (optimal quality) were processed.

All indications and steps of the Sure Select Strand-Specific RNA Library Preparation Kit were followed, and the dsDNA libraries of each treatment group with the adapters and index were analyzed on the Illumina Miseq platform. After obtaining the data, the reads were assembled based on the sequences of each transcript of interest using the CLC Genomics Workbench program. The effect of the treatments on gene expression was evaluated by comparing the fold change in RNA expression of the samples with that of the untreated control.

2.12. Statistical analysis

Homogeneity of variance was accessed by the Bartlett test, and the data were analyzed by analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. Differences were considered significant when the error probability was less than or equal to 5% ($p \leq 0.05$).

3. Results

3.1. Combination of chloroquine and 5-FU blocks autophagy

We first verified whether the working concentrations of CQ and 5-FU were able to block autophagy. Proteins extracted from untreated tumor cells (WT) or cells treated with either chloroquine (80 μM) or 5-fluorouracil (20 μM) or those treated with the combination of drugs (5-FU + CQ) were analyzed by western blot to evaluate the accumulation of LC3-II and SQSTM1 (both autophagic markers). We observed slightly increased LC3-II expression when treating with CQ alone, while the combination of CQ and 5-FU resulted in higher LC3-II expression. Interestingly, treatment with 5-FU alone induced a slight increase of LC3-II expression, indicating the induction of autophagy, as confirmed by the lower intensity of SQSTM1 in this group (Fig. 1A).

3.2. Ultrastructural changes induced by 5-FU and CQ in HCT-116 cells

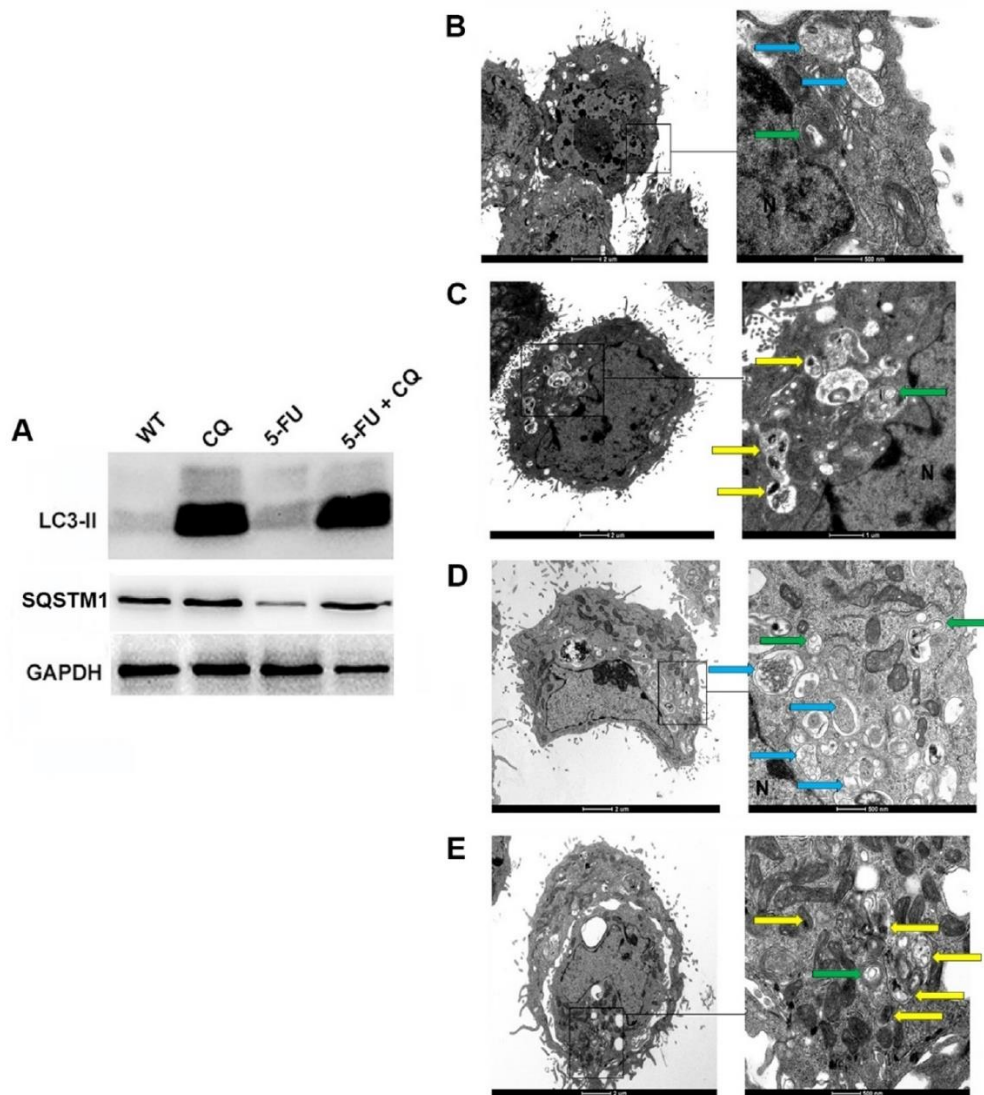To reinforce the cytometric results of the induction and inhibition of

Fig. 1. Autophagy Inhibition prior to Chemotherapy Treatment of HCT-116 cells. Cells were treated with 80 μM CQ overnight and then treated with the minimum effective concentration of 5-FU (20 μM) for 24 h. A portion of these cells was lysed, and the total proteins were analyzed by western blot for LC3-II and SQSTM1 (A). GAPDH was used as a housekeeping gene (C). The remaining cells were analyzed by transmission electron microscopy (B-E). N = nucleus; green arrow: autophagosome; yellow arrow: autophagosome with undigested organelles; blue arrow: autophagolysosomes (vesicles with degraded material). Groups: Control (B), CQ (C), 5-FU (D), 5-FU + CQ (E).

autophagy and the induction of apoptosis, we used transmission electron microscopy to analyze the cells under different treatments. Cells treated with 5-FU showed double-membrane vesicles (autophagosomes) and single-membrane vesicles containing degraded material (autophagolysosomes) in the cytoplasm (Fig. 1D). By contrast, the control cells showed a very small number of autophagosomes containing flocculated and degraded material in addition to containing mitochondria and some rough endoplasmic reticulum cisterns (Fig. 1B). In the 5-FU + CQ combined treatment, the size of autophagosomes appeared to increase within the cytoplasm, and these vesicles exhibited different filler contents. Some vesicles had heterogeneous electron-dense material inside them, and others showed myelin figures and electron-lucent degraded material, indicating that some autophagosomes were not engaged in the digestion process and that the process of digestion was blocked. There was a clear accumulation of autophagosomes containing undigested organelles, demonstrating the failure or inhibition of autophagolysosome formation (Fig. 1E). This finding is in agreement with previous studies [27–30].

3.3. Enhanced cytotoxic action of 5-FU by inhibiting autophagy with chloroquine

Table 1 shows the number of HCT-116 cells after incubation with

Table 1
Number of HCT-116 cells following treatment with CQ, 5-FU, or the combination of both drugs. CTRL refers to untreated cells cultured in regular culture medium. Each column refers to an independent assay (cultured on different days).

HC-T116 cell counting (x 10^7)

Treatment	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5
CTRL	6.7	6.5	6.3	6.4	6.4
CQ	5.7	5.1	5.6	5.2	5.3
5-FU	3.0	3.4	3.5	3.1	3.3
5-FU + CQ	1.2	1.5	1.3	1.2	1.4

Cells were cultured in 75 cm^2 culture flasks until achieving 60% confluence and then either CQ 20 mM (24 h), 5-FU 20 mM (24 h), or the combination of both drugs (24 h + 24 h) was added. Cells were detached by trypsinization and counted. The cell viability was evaluated by the trypan blue exclusion test, and no differences of viability were observed among the cell suspensions (all greater than 94%). CTRL refers to untreated cells cultured in regular culture medium. Each column refers to an independent assay (cultured on different days).

CQ, 5-FU or the combination of both drugs (five independent assays). While CQ alone had a discrete effect on cell growth, 5-FU reduced the number of cells to approximately 50% of the control culture. The
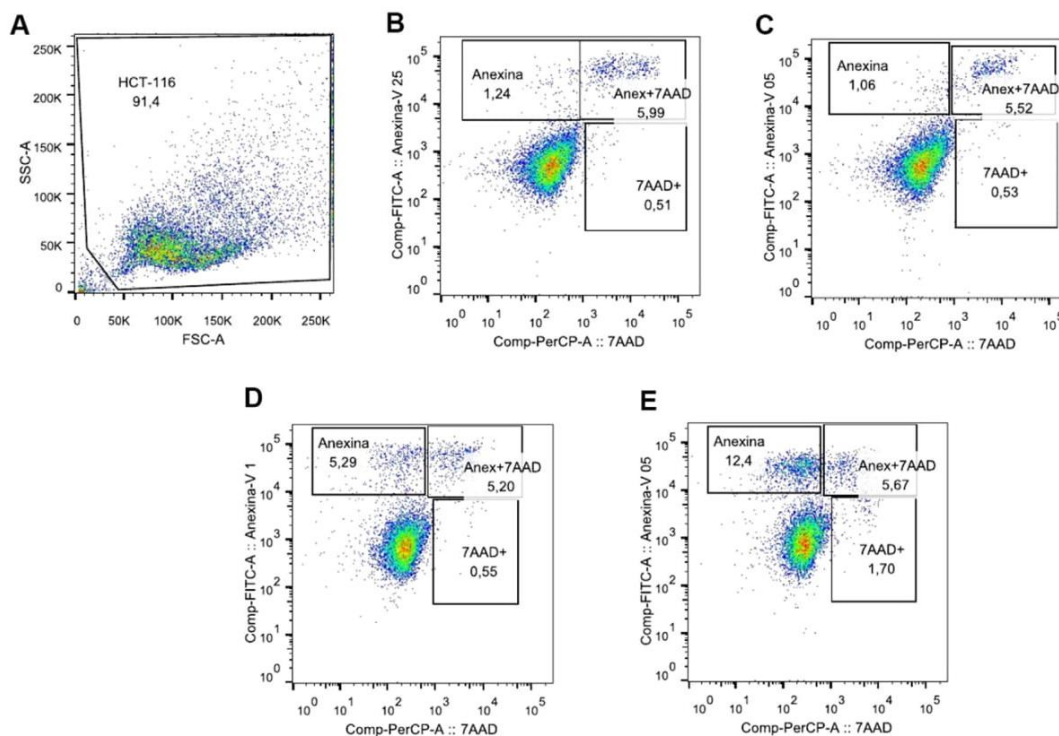
Fig. 2. The combination of chloroquine and 5-FU induced apoptosis in HCT-116 cells. HCT-116 tumor cells were treated or not with 80 μM CQ overnight. Subsequently, they were treated or not with the minimum nontoxic or effective dose of 5-FU. After 24 h, the cells were trypsinized and stained with annexin V and 7AAD to assess apoptosis and necrosis (A). Pseudocolor showing cell size (FSC) and granularity (SSC), with analysis gate (B). Negative Control (C). Treatment with QC (D). Treatment with 5-FU (E). Treatment with QC + 5-FU (F).

combination of 5-FU and CQ strongly inhibited cell growth in the cultures.

Fig. 2 shows that early apoptosis (annexin V+) was the predominant type of cell death induced by the treatments. 5-FU alone (Fig. 2D) increased by four times the annexin V labeling compared to the control (Fig. 2B), while its combination with CQ increased early apoptosis in ten times compared with the same control (Fig. 2E), and around twice compared with 5-FU alone.

3.4. Treatment of tumor cells with CQ plus 5-FU enhances their ability to induce DC maturation

Fig. 3 illustrates the effect of the HCT-116 lysates on the DC phenotype, highlighting the overall increase in the percentage of cells expressing maturation or activation markers CD83, CD80, CD86, and HLA-DR. The percentage of cells expressing the CD83 marker increased from $74 \pm 3.4\%$ in WT to more than 90% in the groups exposed to 5-FU + CQ. Cell markers associated with DC maturation (CD80, CD86, and HLA-DR) increased from $53.5 \pm 2.7\%$; $74.5 \pm 1.2\%$, and $65.9 \pm 3.2\%$ in the WT control to $63.7 \pm 5.1\%$; $92.7 \pm 1.5\%$, respectively, and to $68.7 \pm 2.5\%$, $93.5 \pm 1.12\%$ and $86.3 \pm 2.4\%$ in cells treated with 5-FU + CQ, respectively. Although treating HCT-116 cells with CQ or 5-FU alone increase the immunogenic effect on DCs, this effect was not as intense as seen in the drug combination.

3.5. DCs sensitized with lysates from HCT-116 cells pretreated with CQ and 5-FU showed an enhanced allogeneic response

Given the effect of HCT-116 lysates on the maturation and activation of DCs from healthy individuals, we analyzed whether phenotypic changes were accompanied by changes in DC function. The MLR assay was used to evaluate the effect of HCT-116 lysates on the ability of DCs

to induce an allogeneic response. This study was performed only with the combination of CQ and 5-FU, which presented the most relevant phenotypic changes. Fig. 4A shows the proliferative response of total CD3 lymphocytes, while Fig. 4B and C show the proliferation of CD4+ and CD8+ cells, respectively. Although the exposure of HCT-116 cells to CQ alone increased the DC maturation compared to WT, this effect was not as intense as the drug combination. Our results showed a marked effect on the proliferation of CD4+ T lymphocytes when DCs were sensitized with lysates from HCT-116 cells that were pretreated with 20 μM 5-FU + CQ.

3.6. Lymphocytes responding to allogeneic stimulation showed an activated cell profile

CD69, a marker of activated T lymphocytes, and PD-1, the checkpoint ligand of PD-L1 expressed by tumor cells, were assessed on DC-stimulated T cells. Fig. 4D-G indicates that lymphocytes generated through exposure to DC 5-FU + CQ had a high frequency of CD69+ expression, both among CD4+ T lymphocytes ($30.7 \pm 2.7\%$) and CD8+ lymphocytes ($41.6 \pm 3.2\%$ and $42.5 \pm 2.9\%$, respectively). In contrast, the expression of the regulatory marker PD-1 was lower in these lymphocytes, especially in CD4+ cells (5-FU + CQ: $16.5 \pm 1.3\%$).

3.7. HCT-116 treated with 5-FU + CQ showed an increased ability to induce a Th1 response

As shown in Fig. 4H, DCs exposed to the lysates of HCT-116 cells that were treated with 5-FU + CQ had an increased ability to induce the production of IFN-γ via allogeneic lymphocytes. The levels of IFN-γ increased from 171.1 ± 11.4 pg/ml (WT) to 195.7 ± 16.5 pg/ml (CQ) and 389.3 ± 19.8 pg/ml (5-FU + CQ). Conversely, Fig. 4I shows
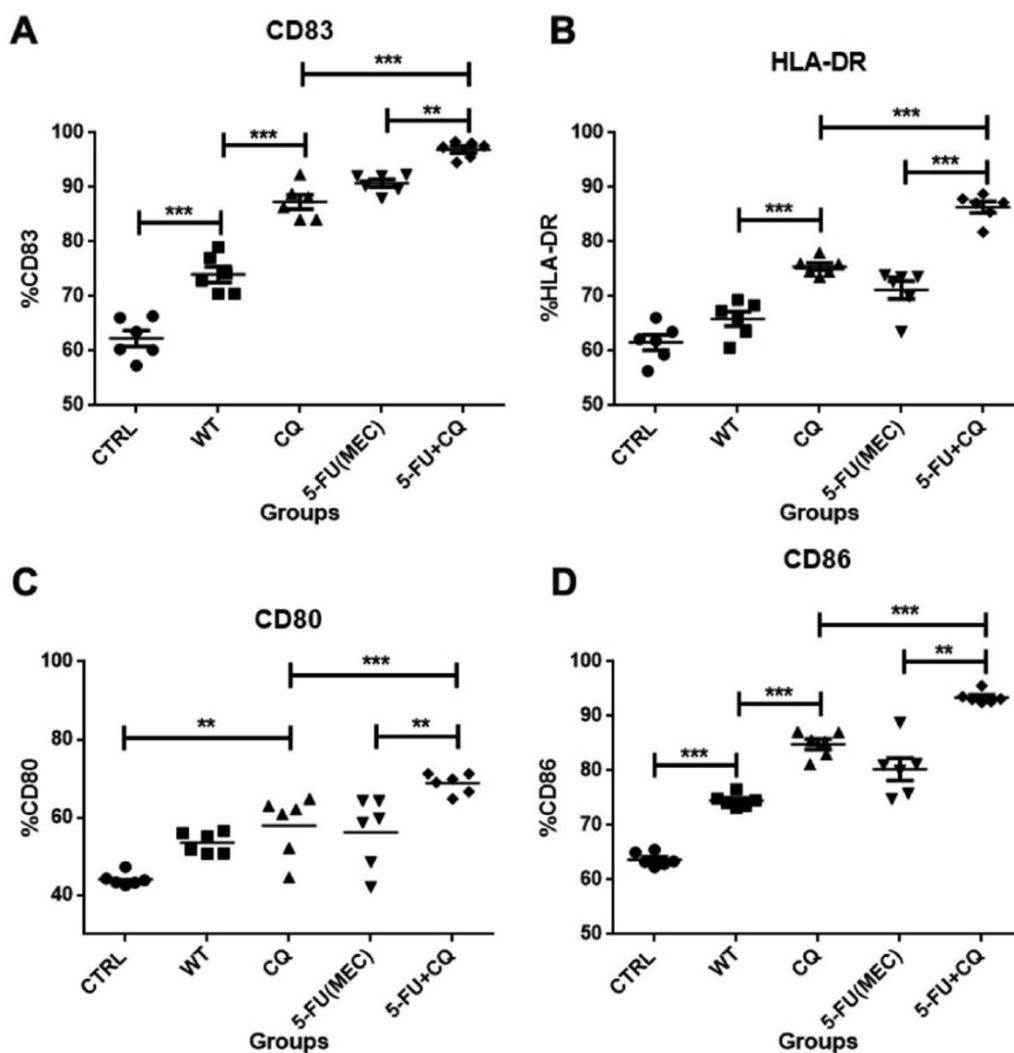
Fig. 3. Lysates of HCT-116 exposed to 5-FU + CQ increase DC maturation and activation. DCs from six healthy donors were exposed to the lysates of HCT-116 cells previously treated with the minimal effective concentrations of 5-FU or its combination with CQ (5-FU + CQ). WT refers to DCs pulsed with the lysate of untreated HCT-116 cells, and CTRL refers to unstimulated DCs. Scatter plots illustrate the percentages of DCs (CD11c+) that co-express the markers CD83 (A), HLA-DR (B), CD80 (C), and CD86 (D). The mean and standard deviation of the six individual donors was analyzed by ANOVA. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.005.

that DC exposure to the lysates decreased the ability of DC-stimulated T cells to secrete IL-10, reducing the levels from 46.8 ± 6.3 pg/ml (WT) to 28.7 ± 7.2 pg/ml (CQ) and 8.2 ± 3.7 pg/ml (5-FU + CQ).

3.8. Dcs treated with lysate enhanced the in vitro generation of CTLs

Our analysis of cytotoxic T lymphocytes was restricted to the expression of perforin and granzyme B molecules. We tested the efficiency of HCT-116 lysate-treated DCs to generate autologous tumor-reactive T cells. We found that lymphocytes cultured with DCs exposed to lysates of HCT-116 cells treated with 5-FU + CQ induced the generation of lymphocytes with higher levels of perforin and granzyme B than in those cultured with control DCs (Fig. 5). No differences were observed upon labeling with anti-CD107a (data not shown).

3.9. Transcriptional changes associated with autophagy blockade

To better understand the increase of DC maturation associated with blocking autophagy, we evaluated HCT-116 cells treated with 5-FU, CQ and their combination. The gene fold change was used to identify significant differences in gene expression among the groups (Table 2). CQ-

treated cells showed a modest increase in the expression of the autophagy genes ATGs, SQSTM1, MAP1LC3B, and ULK1 and a considerable decrease in genes related to tumor progression (BNIP3, BNIP3L, FOSL2, HES1, LAMB3, LOXL2, NDRG1, P4HA1, and PIK3R2), as well as a decrease in nominal tumor antigens (members of the CEA family). Treatment with 5-FU induced an increase in autophagy genes. In contrast with the CQ group, we did not observe such an intense decrease in the genes related to tumor progression, while the expression of CEA genes was increased. Cells treated with 5-FU + CQ showed an increased expression of autophagy genes, as well as an increase in genes of the CEA family. Unlike the 5-FU group, the expression of genes associated with tumor progression was decreased.

4. Discussion

The development of resistance to antitumor agents is one of the primary challenges of modern cancer therapy. Here, we investigated the effects of treating tumor cells with a combination of 5-FU and CQ. The concentration of 20 μM 5-FU was chosen because it was the lowest concentration capable of inhibiting tumor growth and may, therefore, represent a suitable *in vitro* model of the metronomic dose used in the
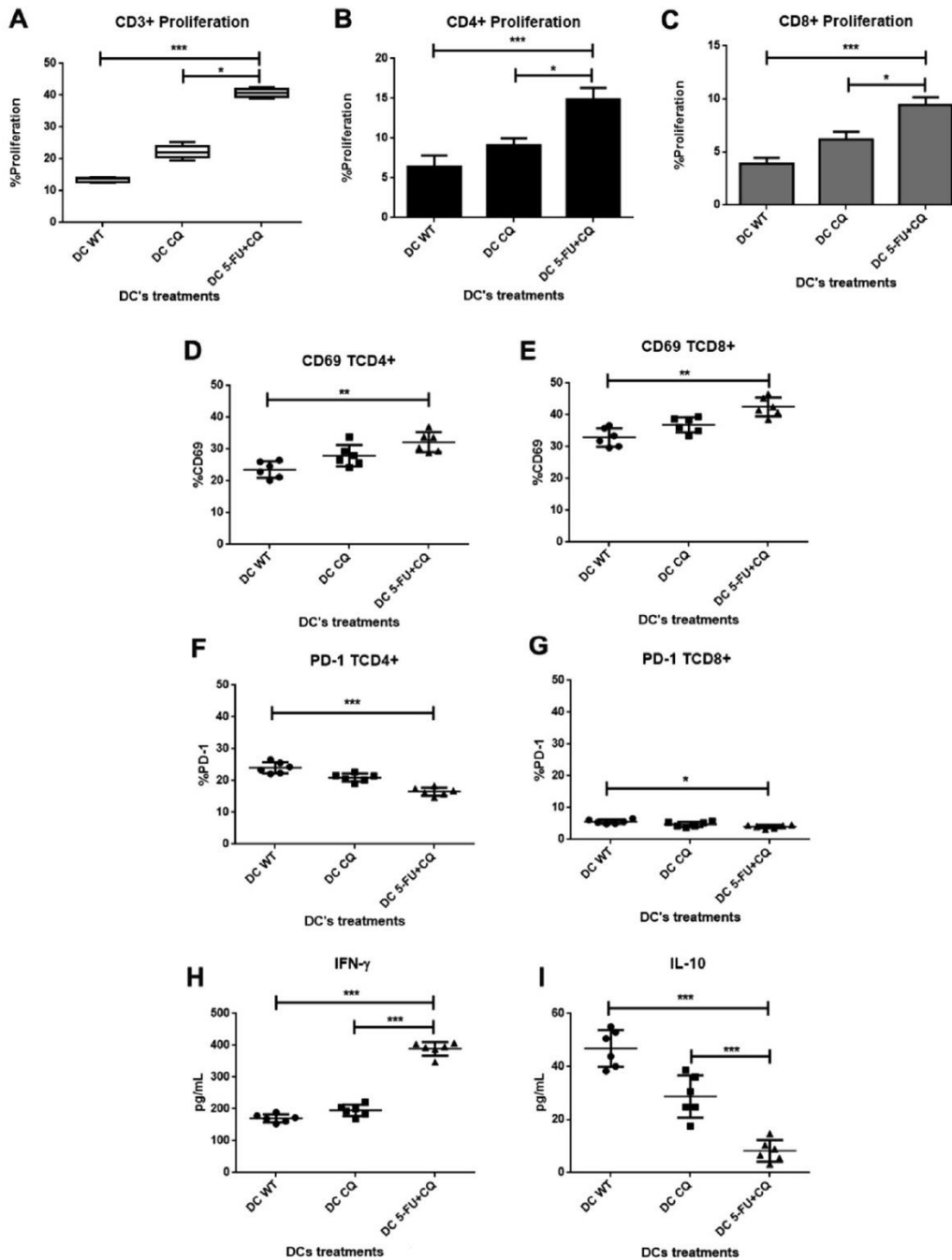
Fig. 4. Improved DC antigen-presenting function induced by lysates of HCT-116 treated with 5-FU + CQ. DCs were exposed to HCT-116 lysates and cocultured with allogeneic lymphocytes for 5 days. Growing total lymphocytes (CD3 + cells) were quantified by flow cytometry (A), as were CD4 + and CD8 + cells (B; C) (N = 9; * $p \leq 0.05$; ** $p \leq 0.01$). The CD4 + and CD8 + T lymphocytes were further analyzed separately for the expression of CD69 (activation marker) and PD-1 (regulatory molecule) (D-G; * $p \leq 0.05$; ** $p \leq 0.02$). MLR supernatants were collected and analyzed for the secretion of IFN-γ (H) and IL-10 (I) by ELISA (*** $p \leq 0.01$).

clinic [31]. Although CQ has previously been applied in combination with several antineoplastic agents, a study using low concentrations of the drug has not yet been reported.

Increased LC3-II and SQSTM1 levels are currently used as suitable markers of effective autophagy inhibition. When autophagy is blocked by CQ, LC3-II accumulates [32]. SQSTM1 binds to ubiquitinated proteins, especially LC3-II, forming part of the autophagosome wall. Thus, if autophagy is enhanced, SQSTM1 is degraded. Inhibiting autophagy promotes the accumulation of SQSTM1, as well as LC3-II [33]. Low concentrations of 5-FU increased autophagy in HCT-116 cells, while CQ was able to inhibit the process, as evidenced by the accumulation of

LC3-II. The analysis of SQSTM1 expression is consistent with constitutive autophagy in HCT-116 cells [34].

Autophagy induction by 5-FU was also confirmed by transcriptome analysis that showed an increase in expression of the autophagy-associated genes ATGs, SQSTM1, MAP1LC3B, BECN1, and ULK1. Their expression was enhanced when applying the combination of 5-FU + CQ. The increased expression of LC3B and BECN1 under stress conditions is consistent with previous reports [35,36].

Ultrastructural analysis revealed that the untreated control cells had negligible expression of autophagosomes and autophagolysosomes, while those treated with CQ showed increased numbers of

A

B

Fig. 5. *In vitro* generation of cytotoxic T lymphocytes (CTLs) is improved by DCs exposed to lysates of HCT-116 previously treated with 5-FU + CQ. Mean fluoresc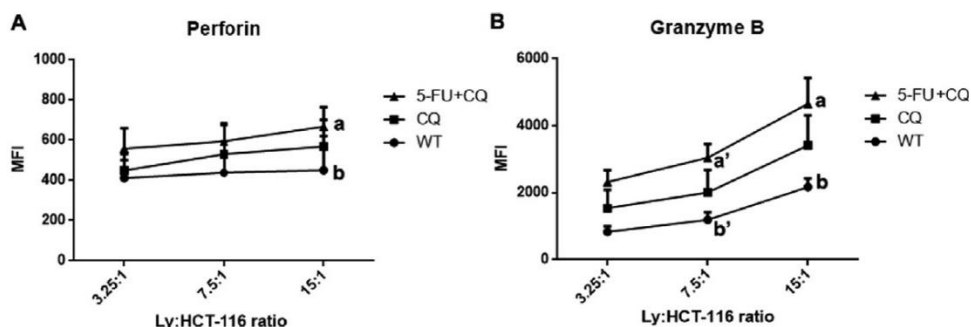ence intensity (MFI) of proliferating CD8+ cells (A) of four healthy donors at individual effector:target ratios (3.25:1, 7.5:1, and 15:1). These lymphocytes showed higher expression levels of the cytotoxicity markers perforin (MFI 15:1 ratio, 5-FU + CQ > WT (p < 0.05)) and granzyme B (MFI 7.5:1 ratio, 5-FU + CQ > WT (p < 0.05); MFI 15:1 ratio, 5-FU + CQ > WT (p < 0.05)) compared to the WT control group.

Table 2
Fold change expression of selected genes by tumor cells, comparing untreated cells (WT) with those treated with CQ, 5FU or CQ + 5FU.

Gene functions	Gene name	WT vs CQ	WT vs 5-FU	WT vs 5-FU + CQ
Autophagy-	ATG12	1.7	3.2	7.1
	ATG5	1.9	4.5	6.4
	MAP1LC3B	1.8	2.3	5.7
	ULK1	1.2	2.4	5.2
	SQSTM1	2.1	1.9	4.8
	BECN1	0.8	5.8	2.5
	BNIP3	−4.36	−1.36	−4.11
	BNIP3L	−4.06	−1.2	−3.85
Cell adhesion	CEACAM5	−0.9	1.5	8.4
	CEACAM6	−1.3	2.4	6.5
	CEACAM7	−2.1	1.7	5.2
	CEACAM1	−1.2	2.1	5.7
	HES1	−3.16	−0.5	−7.1
	FOSL2	−4.72	−0.72	−3.6
	NDRG1	−10.43	−1.43	−6.11
Catalytic activity	PIK3R2	−1.2	−3.97	−4.2
	PGK1	−2.59	−0.2	−2.59
	LOXL2	−4.8	−0.9	−2.85
	PGK1	−2.59	−0.2	−2.59
	ALDOC	−8.14	−1.14	−7.52
	ANGPTL4	−6.25	−1.25	−5.93
Structural molecule activity	LAMB3	−4.26	−0.76	−3.59
Heat shock proteins and HMGBs	HMGB1	1.02	1.9	1.12
	HMGB2	0.98	1.87	1.05
	HMGB3	0.2	0.5	0.347
	DNAJB1 (HSP40)	1.17	1.5	0.85
	HSP1A (HSP70)	2.67	2.82	3.38
	HSP90AB1 (HSP90)	1.45	2.97	1.52
Oxidoreductase	P4HA1	−6.53	−1.1	−6.89
RNA binding	PCBP1	1.2	2.86	8.26

autophagosomes in the cytoplasm. Cells treated with 5-FU showed an evident increase in the number of autophagolysosomes containing degraded material, indicating the induction of autophagy and the completion of the process, consistent with prior reports [28–30,37,38]. The combined treatment of CQ and 20 μM 5-FU induced intense accumulation of cytoplasmic vesicles in several stages, especially autophagosomes containing undigested material. We also found cells with signs of autophagic and apoptotic death, such as cytoplasmic vacuolization, chromatin rarefaction (reduction of heterochromatic groups associated with the nuclear envelope), altered mitochondrial morphology (dilation of the cristae), and dilation of the nuclear envelope, which also agrees with previous reports [39,40]. This phenomenon was confirmed by the observation that CQ + 5-FU treatment strongly reduced cell proliferation and induced early apoptosis in tumor cells, although CQ or 5-FU alone had no apparent effect on cell viability. 5-Fluorouracil induces apoptosis in cancer cells [41], and the inhibition of autophagy by CQ

can potentiate the cytotoxicity of 5-FU. This occurs because the accumulation of vesicles in the cytoplasm due to incomplete autophagy can cause oxidative stress and lead to cell death. Based on this information, we aimed to confirm whether the toxicity of low 5-FU concentrations would be enhanced by CQ. In this study, the apoptosis and cell death results showed that 5-FU + CQ increased the levels of early apoptosis even though the drug concentrations had only a negligible effect if applied alone.

Analyzing the effects of CQ + 5-FU treatment on the immunogenicity of tumor cells revealed that DC exposure to the lysate of HCT-116 cells treated with drug enhanced the maturation of antigen-presenting cells. DC maturation is required for increased antigen-presenting ability and is accompanied by phenotypic changes, such as increased expression of MHC class II and co-stimulatory molecule expression. CD40, CD80, and CD86 expression are essential for T lymphocyte stimulation [42]. In addition to being a maturation marker, CD83 modulates the immune response by stimulating both naïve and memory T cells [43]. CD83 expression was also enhanced by the drug combination. Although apoptotic cell death is considered non-immunogenic or weakly immunogenic, it is possible that apoptosis induced by the blockage of autophagy results in the expression of danger signals (e.g., DAMPs) that are usually not expressed during physiological apoptosis. In a parallel study, we observed that exposing HCT-116 cells to low concentrations of paclitaxel induces the expression of HSP-70 and 90 (unpublished data). In the present study, we observed a small increase in the expression of HSP-70 but irrelevant changes in the HSP-90 and HMGB genes. These findings indicated that the enhanced ability to sensitize DCs is mainly due to the increased expression of the tumor-associated genes of the CEA family (CEACAM 1, 5, 6, and 7).

In another study, we found that exposing HCT-116 cells to low doses of paclitaxel or doxorubicin increased the expression of several genes associated with enhanced tumor immunogenicity [11]. Total RNA extracted from HCT-116 colorectal cancer cells treated with 5-FU was more immunogenic when transfected into DCs [44]. Exposing tumor cells to other drugs such as paclitaxel and doxorubicin increases the expression of the antigen processing machinery via the cytosolic route, such as TAP1, tapasin, and calnexin [11]. This view is reinforced by the observation that chemical stress triggers the cell surface expression of calreticulin (CALR) [12], another molecule promoting the antigenic processing of tumor cells. Interestingly, CALR moves from the endoplasmic reticulum (where it functions as an APM component) to the cytoplasmic membrane, where it serves as a potent activating signal. CALR, together with HMGB1, plays an important role in the phenotypic maturation of DCs, increasing HLA-DR, CD80, and CD86 expression and functional maturation, as well as enhancing the secretion of IL-12 and INF-γ [45].

This enhancing effect on tumor cell immunogenicity was reinforced by our demonstration that the cell lysates had a functional effect on DCs. Indeed, the mixed lymphocyte reaction (MLR) allowed us to demonstrate that the phenotypic changes observed in DCs are accompanied by an improvement in their ability to stimulate allogeneic lymphocytes. Specifically, DCs sensitized with the lysates of HCT-116

cells treated with 5-FU + CQ promoted the enhanced proliferation of lymphocytes in the MLR.

The more intense proliferative effect on CD4+ T lymphocytes is also in agreement with their ability to interact with the HLA-DR molecules expressed by DCs. Furthermore, the increased proliferation of CD8+ T lymphocytes suggests the generation of cytotoxic T lymphocytes that are specific for tumor cells (since their proliferation was stimulated by DCs sensitized with HCT-116 lysates). Aiming to confirm that the combination of 5-FU and CQ can stimulate the proliferation of CD8+ lymphocytes, we performed a CTL assay, which, unlike the MLR, tests the ability of DCs to stimulate the proliferation of autologous CD8+ lymphocytes that target tumor cells [46]. Our main difficulty when evaluating the direct cytotoxicity of CTLs against target cells is that HCT-116 cells are adherent, but most available assays for this purpose work well for cells in suspension and are not reliable for adherent cell types. There are some chemiluminescence-based assays for this, but we were unable to acquire the appropriate kit. Therefore, we decided to evaluate the expression of perforin and granzyme B by CTLs challenged with tumor target cells, following the methodology proposed by Betts et al [47]. These proteins are considered to be functional markers in cells with cytotoxic capacity [48]; perforin polymerization perforates the target cell membrane, while granzyme B induces apoptosis [49]. As expected, we found increased expression (i.e., increased mean fluorescence intensity; MFI) of both perforin and granzyme B in the CD8+ population induced by DCs sensitized with 5-FU + CQ lysate.

We also observed that lymphocytes generated in response to sensitized DCs expressed higher levels of the 'very early antigen' CD69, which is associated with the effective stimulation of lymphocytes by antigen-presenting cells [50]. Consistently, lymphocytes stimulated with DCs exposed to 5-FU + CQ lysates induced earlier and more potent T cell activation. The findings also indicated that there was no selective activation of CD4+ or CD8+ T cells, since the percentage of CD69+ cells was increased in both subpopulations. Accordingly, these same treatments resulted in a reduced generation of PD-1+ cells among CD4+ T lymphocytes. PD-1 is a member of the CD28 family, and its primary function is to limit clustering of the TCR and costimulatory molecules within the immunologic synapse. PD-1 expression is increased in the T cells of patients with pancreatic cancer, lung cancer, and various leukemias and lymphomas [51]. Therefore, PD-1 expression in T cells and PD-L1 expression in tumor cells constitute a tumor escape mechanism. We observed reduced PD-1 expression on the expanding lymphocytes cocultured with DCs. This aspect is particularly interesting because the signals associated with PD-1 and PD-L1 are among the main checkpoint blockades of the antitumor response. Hence, in addition to the anti-CTLA-4 antibody, the anti-PD-1 antibodies [52] are mainstays of modern antitumor immunotherapy.

The lymphocytes cultured with DCs sensitized with drug-exposed tumor cells produced higher amounts of IFN-γ than controls while showing decreased production of IL-10. Naive CD4+ and CD8+ lymphocytes can differentiate into specific effector T cells with different functions that are attributed, in part, to the pattern of cytokines they produce [53]. CD4+ T cells can differentiate into Th1 cells that release IFN-γ and TNF-α to stimulate the generation of CD8+ cells; in this way, Th1 cells are involved in the development of the antitumor immune response. Interferon γ also recruits monocytes, inducing the differentiation of CD4+ T lymphocytes into Th1 lymphocytes [54,55]. IL-10 is a DC-inhibiting cytokine that can be secreted by both Tregs and Th2, inhibiting antigen presentation, and decreasing the expression of MHC II and the co-stimulatory molecules CD80 and CD86 [56]. Thus, DCs under the combined treatment promoted preferential activation of Th1 lymphocytes, with little Th2 or Treg stimulation. An analysis of other cytokines, such as IL-12, IL-2, TNF, IL-4, IL-6, IL17, and IL-23, would be useful to provide a clearer view regarding how the different treatments affect the modulation of T cell subsets. The quantification of these cytokines should be considered in future studies.

Finally, the evidence that the combination of 5-FU and CQ can modulate tumor cell biology, as can the corresponding HCT-116 lysate, is reinforced by the increased expression of genes of the tumor-associated CEA family (CEACAM 5, 6, and 7), as previously reported [57], and the decreased expression of genes associated with tumor progression (BNIP3, BNIP3L, FOSL2, HES1, LAMB3, LOXL2, NDRG1, P4HA1, and PIK3R2).

5. Conclusion

Taken together, our results indicate that blocking autophagy with chloroquine increases the ability of tumor cells to mature DCs that can induce an antitumor response. In addition, these results confirm the clinical application of this protocol for improving the *in vitro* stimulation of DCs, as well as the use of combined anti-autophagic agents and conventional chemotherapeutic agents for the treatment of patients with colorectal cancer.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Jofer Andree Zamame Ramirez: Methodology, Investigation, Formal analysis, Writing - original draft. **Graziela Gorete Romagnoli:** Conceptualization, Investigation, Validation. **Bianca Francisco Falasco:** Investigation. **Carolina Mendonça Gorgulho:** Investigation, Writing - review & editing. **Carla Sanzochi Fogolin:** Investigation. **Daniela Carvalho dos Santos:** Formal analysis, Writing - review & editing. **João Pessoa Araújo Junior:** Methodology, Formal analysis. **Michael Thomas Lotze:** Writing - review & editing. **Rodrigo Portes Ureshino:** Methodology. **Ramon Kaneno:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106495.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2020, CA: A Cancer J. Clinicians 70(1) (2020) 7–30.
[2] INCA, Estimativa 2020: incidência de câncer no Brasil, in: M.d. Saúde (Ed.) Estimativa, Instituto Nacional de Câncer José Alencar Gomes da Silva/ Ministério da Saúde., 2019, p. 120.
[3] T. Andre, An overview of adjuvant systemic chemotherapy for colon cancer, Clin. Colorectal Cancer 4 (1) (2004) 22–28.

[4] Derek J. Jonker, Chris J. O'Callaghan, Christos S. Karapetis, John R. Zalcberg, Dongsheng Tu, Heather-Jane Au, Scott R. Berry, Marianne Krahn, Timothy Price, R. John Simes, Niall C. Tebbutt, Guy van Hazel, Rafal Wierzbicki, Christiane Langer, Malcolm J. Moore, Cetuximab for the Treatment of Colorectal Cancer, N. Engl. J. Med. 357 (20) (2007) 2040–2048, https://doi.org/10.1056/NEJMoa071834.

[5] X. Guan, Cancer metastases: challenges and opportunities, Acta Pharmaceutica Sinica B 5 (5) (2015) 402–418.

[6] A.M. Cuervo, Autophagy, sickness and in health, Trends Cell Biol. 14 (2004) 70–77.

[7] M. Mehrpour, I. Beau, et al., Overview of macroautophagy regulation in mammalian cells, Cell Res. 20 (2010) 748–762.

[8] A. Ciechanover, Proteolysis: from the lysosome to ubiquitin and the proteasome, Nat. Rev. Mol. Cell Biol. 6 (2005) 79–87.

[9] E.L. Eskelinen, Autophagy, A lysosomal degradation pathway with a central role in health and disease, Biochimica et Biophysica Acta 1793 (2009) 664–673.

[10] G.V. Shurin, R. Kaneno, et al., Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism, J. Immunol. 183 (2009) 137–144.

[11] R. Kaneno, F.M. Kaneno, et al., Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells, Cellular Oncol. (Dordrecht) 34 (2011) 97–106.

[12] L. Zitvogel, F. Ghiringhelli, et al., Immunological aspects of cancer chemotherapy, Nat. Rev. Immunol. 8 (2008) 59–73.

[13] L. Zitvogel, M.O. Pequignot, et al., Immune response against dying tumor cells, Adv. Immunol. 84 (2004) 131–179.

[14] L. Zitvogel, G. Kroemer, Anticancer immunochemotherapy using adjuvants with direct cytotoxic effects, J. Clin. Invest. 119 (8) (2009) 2127–2130.

[15] J.M. Pitt, G. Kroemer, L. Zitvogel, Immunogenic and Non-immunogenic Cell Death in the Tumor Microenvironment, in: P. Kalinski (Ed.), Tumor Immune Microenvironment in Cancer Progression and Cancer Therapy, Springer International Publishing, Cham, 2017, pp. 65–79.

[16] I. Vanmeerbeek, J. Sprooten, D. De Ruysscher, S. Tejpar, P. Vandenberghe, J. Fucikova, R. Spisek, L. Zitvogel, G. Kroemer, L. Galluzzi, A.D. Garg, Trial watch: chemotherapy-induced immunogenic cell death in immuno-oncology, Oncoimmunology 9 (1) (2020) 1703449-1703449.

[17] R. Kaneno, G.V. Shurin, I.L. Tourkova, M.R. Shurin, Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations, J. Transl. Med. 7 (2009) 58–66.

[18] K. Sasaki, N.H. Tsuno, E. Sunami, et al., Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells, BMC Cancer 10 (2010) 370–375.

[19] R.K. Amaravadi, D. Yu, J.J. Lum, et al., Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma, J. Clin. Invest. 117 (2007) 326–336.

[20] F.G. Andrew, Chloroquine, Mechanism of drug action and resistance in plasmodium falciparum, Pharmacol. Therapeut. 57 (2) (1993) 203–235.

[21] N. Sharma, T. Simmy, E.B. Golden, et al., Inhibition of autophagy and induction of breast cancer cell death by mefloquine, an antimalarial agent, Cancer Lett. 326 (2012) 143–154.

[22] C. Ponticelli, G. Moroni, Hydroxychloroquine in systemic lupus erythematosus (SLE), Expert Opin. Drug Saf. 16 (3) (2017) 411–419.

[23] J. Liu, R. Cao, M. Xu, X. Wang, H. Zhang, H. Hu, Y. Li, Z. Hu, W. Zhong, M. Wang, Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro, Cell Discov. 6 (1) (2020) 16.

[24] Chinese Clinical Trial Register, 2020. http://www.chictr.org.cn.

[25] N. GrohBrandt, I. Beckmann, M.W. Schrauder, M. Loehberg, Evaluation for synergistic suppression of breast cancer proliferation by Chloroquine in combination with the rapamycin derivative, Rad001, Geburtshilfe Und Frauenheilkunde 5 (69) (2009) 214–218.

[26] A. Grimaldi, D. Santini, S. Zappavigna, et al., Antagonistic effects of chloroquine on autophagy occurrence potentiate the anticancer effects of everolimus on renal cancer cells, Cancer Biol. Therapy 4 (16) (2015) 567–579.

[27] E.L. Eskelinen, A.R. Prescott, J. Cooper, S.M. Brachmann, L. Wang, X. Tang, J.M. Backer, J.M. Lucocq, Inhibition of autophagy in mitotic animal cells, Traffic 3 (12) (2002) 878–893.

[28] N. Mizushima, T. Yoshimori, B. Levine, Methods in mammalian autophagy research, Cell 140 (3) (2010) 313–326.

[29] L. Jia, R.R. Dourmashkin, P.D. Allen, A.B. Gray, A.C. Newland, S.M. Kelsey, Inhibition of autophagy abrogates tumour necrosis factor α induced apoptosis in human T-lymphoblastic leukaemic cells, Br. J. Haematol. 98 (3) (1997) 673–685.

[30] E.-K. Moon, S.-H. Kim, Y. Hong, D.-I. Chung, Y.-K. Goo, H.-H. Kong, Autophagy inhibitors as a potential antiamoebic treatment for Acanthamoeba keratitis, Antimicrob Agents Chemother 59 (7) (2015) 4020–4025.

[31] D. Longley, D. Harkin, P. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 5 (3) (2003) 330–338.

[32] N. Mizushima, T. Yoshimori, How to Interpret LC3 Immunoblotting, Autophagy 3 (6) (2007) 542–545.

[33] G. Bjørkøy, T. Lamark, S. Pankiv, A. Øvervatn, A. Brech, T. Johansen, Chapter 12 Monitoring Autophagic Degradation of p62/SQSTM1, Methods in Enzymology, Academic Press, 2009, pp. 181–197.

[34] K. Kucharewicz, M. Dudkowska, A. Zawadzka, M. Ogrodnik, A.A. Szczepankiewicz, Z. Czarnocki, E. Sikora, Simultaneous induction and blockade of autophagy by a single agent, Cell Death Dis. 9 (3) (2018) 353.

[35] R. Scherz-Shouval, H. Weidberg, C. Gonen, S. Wilder, Z. Elazar, M. Oren, p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation, PNAS 107 (43) (2010) 18511–18516.

[36] J.-W. Yang, Q.-H. Zhang, T. Liu, Autophagy facilitates anticancer effect of 5-fluorouracil in HCT-116 cells, J. Cancer Res. Ther. 14 (12) (2018) 1141–1147.

[37] J.-H. Choi, J.S. Yoon, Y.-W. Won, B.-B. Park, Y.Y. Lee, Chloroquine enhances the chemotherapeutic activity of 5-fluorouracil in a colon cancer cell line via cell cycle alteration, APMIS 120 (7) (2012) 597–604.

[38] K. Sasaki, N.H. Tsuno, E. Sunami, G. Tsurita, K. Kawai, Y. Okaji, T. Nishikawa, Y. Shuno, K. Hongo, M. Hiyoshi, M. Kaneko, J. Kitayama, K. Takahashi, H. Nagawa, Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells, BMC Cancer 10 (2010) 370–370.

[39] R.K. Amaravadi, D. Yu, J.J. Lum, T. Bui, M.A. Christophorou, G.I. Evan, A. Thomas-Tikhonenko, C.B. Thompson, Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma, J. Clin. Investig. 117 (2) (2007) 326–336.

[40] P. Boya, R.-A. González-Polo, N. Casares, J.-L. Perfettini, P. Dessen, N. Larochette, D. Métivier, D. Meley, S. Souquere, T. Yoshimori, G. Pierron, P. Codogno, G. Kroemer, Inhibition of macroautophagy triggers apoptosis, Mol. Cell. Biol. 25 (3) (2005) 1025–1040.

[41] N.M. Mhaidat, M. Bouklihacene, R.F. Thorne, 5-Fluorouracil-induced apoptosis in colorectal cancer cells is caspase-9-dependent and mediated by activation of protein kinase C-δ, Oncol Lett. 8 (2) (2014) 699–704.

[42] G. Adema, F. Hartgers, R. Verstraten, et al., A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells, Nature 387 (1997) 713–717.

[43] X. Wang, J. Ji, H. Zhang, et al., Stimulation of dendritic cells by DAMPs in ALA-PDT treated SCC tumor cells, Oncotarget 42 (6) (2015) 688–702.

[44] C.V. De Almeida, J.A. Zamame, G.G. Romagnoli, C.P. Rodrigues, M.B. Magalhaes, A. Amedei, R. Kaneno, Treatment of colon cancer cells with 5-fluorouracil can improve the effectiveness of RNA-transfected antitumor dendritic cell vaccine, Oncol. Rep. 38 (1) (2017) 561–568.

[45] E.H.J.G. Aarntzen, I. Aarntzen, W.J. Lesterhuis, D. Schuurhuis, J.F.M. Jacobs, K. Bol, et al., Targeting CD4+ T-helper cells improves the induction of antitumor responses in dendritic cell–based vaccination, Cancer Res. 1 (73) (2013) 19–29.

[46] G.R. Burleson, F.G. Burleson, R.R. Dietert, The cytotoxic T lymphocyte assay for evaluating cell-mediated immune function, Methods Mol. Biol. 598 (2010) 195–205.

[47] M.R. Betts, J.M. Brenchley, D.A. Price, S.C. De Rosa, D.C. Douek, M. Roederer, R.A. Koup, Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation, J. Immunol. Methods 281 (1) (2003) 65–78.

[48] Y.T. Bryceson, C. Fauriat, J.M. Nunes, S.M. Wood, N.K. Björkström, E.O. Long, H.-G. Ljunggren, Functional Analysis of Human NK cells by Flow Cytometry, Methods Mol. Biol. (Clifton, N.J.) 612 (2010) 335–352.

[49] J.A. Trapani, M.J. Smyth, Functional significance of the perforin/granzyme cell death pathway, Nat. Rev. Immunol. 2 (2002) 735.

[50] N. Patsoukis, D. Sari, V.A. Boussiotis, PD-1 inhibits T cell proliferation by upregulating p27 and p15 and suppressing Cdc25A, Cell Cycle 23 (11) (2012) 4305–4309.

[51] L.R. Cockerham, V. Jain, E. Sinclair, et al., Programmed death-1 expression on CD4+ and CD8+ T cells in treated and untreated HIV disease, AIDS 28 (12) (2014) 1749–1758.

[52] R. Sundar, B.C. Cho, J.R. Brahmer, R.A. Soo, Nivolumab in NSCLC: latest evidence and clinical potential, Therapeut. Adv. Med. Oncol. 7 (2) (2015) 85–95.

[53] P. Guermonprez, J. Valladeau, L. Zitvogel, et al., Antigen presentation and T cell stimulation by dendritic cells, Ann. Rev. Immunol. 20 (2002) 621–667.

[54] L. Guéry, S. Hugues, Th17 Cell Plasticity and Functions in Cancer Immunity, Biomed Res. Int. 20 (2015) 31–46.

[55] K.L. Knutson, M.L. Disis, Tumor antigen-specific T helper cells in cancer immunity and immunotherapy, Cancer Immunol., Immunother. 8 (54) (2005) 721–728.

[56] A.O.G. Margarida Saraiva, et al., The regulation of IL-10 production by immune cells, Nat. Rev. Immunol. 10 (1) (2010) 170–181.

[57] A. Aquino, S.P. Prete, F. Guadagni, J.W. Greiner, A. Giuliani, L. Orlando, G. Masci, S. De Santis, E. Bonmassar, G. Graziani, Effect of 5-fluorouracil on carcinoembryonic antigen expression and shedding at clonal level in colon cancer cells, Anticancer Res. 20 (5B) (2000) 3475–3484.

2.

Review article

Inhibiting autophagy to prevent drug resistance and improve anti-tumor therapy

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A R T I C L E   I N F O

A B S T R A C T

Cytotoxic drugs remain the first-line option for cancer therapy but the development of drug-resistance by tumor cells represents a primary obstacle for successful chemotherapy. Autophagy is a physiological mechanism of cell survival efficiently used by tumor cells to avoid cell death and to induce drug-resistance. It is a macromolecular process, in which cells degrade and recycle intracellular substrates and damaged organelles to alleviate cell stress caused by nutritional deprivation, hypoxia, irradiation, and cytotoxic agents, as well. There is evidence that autophagy prevents cancer during the early steps of carcinogenesis, but once transformed, these cells show enhanced autophagy capacity and use it to survive, grow, and facilitate metastasis. Current basic studies and clinical trials show the feasibility of using pharmacological or molecular blockage of autophagy to improve the anticancer therapy efficiency. In this review, we overviewed the pathways and molecular aspects of autophagy, its role in carcinogenesis, and the evidence for its role in cancer adaptation and drug-resistance. Finally, we reviewed the clinical findings on how the autophagy interference helps to improve conventional anticancer therapy.

1. Introduction

Cancer is the second-leading cause of death worldwide, just behind coronary diseases, killing more than 56.9 million people in 2019 [1]. Breast, prostate, lung, and colon cancers are the most prevalent malignancies [1], and chemotherapy is the primary treatment option for inoperable tumors. Platinum compounds, taxanes, fluorouracil, doxorubicin, and methotrexate, among others, are used to treat various cancer types [2], usually at the maximum tolerable dose, aiming to kill massive numbers of tumor cells. However, because chemotherapeutic drugs also kill healthy cells, an interval is necessary between consecutive drug applications to alleviate the side effects and allow patients to recover. During this interval, the serum levels of chemotherapeutic agents fall, allowing the growth of drug-resistant tumor cells and resulting in future disease relapse [3]. One mechanism used by tumor cells to develop resistance against chemotherapeutic agents is autophagy, a highly conserved physiological process that allows tumor cells to avoid damage and death.

The term autophagy was coined by de Duve in 1963 [4] and was defined as "self-eating" at the subcellular level by Klionsky [5]. Autophagy refers to a conserved cellular degradation process, during which portions of the cytosol and damaged organelles are sequestered into double-membrane vesicles, called autophagosomes. These vesicles fuse with lysosomes, which subsequently degrade and eventually recycle the existing macromolecules [6–8]. Under nutrient-rich conditions, cells engage in consistent, low-level autophagy (known as basal autophagy) to control the quality of the intracellular environment, providing tissues with cytoplasmic recycling mechanisms that allow the removal of damaged or unnecessary organelles [9]. In contrast, cells under various stress conditions, such as nutrient deprivation, hypoxia, intracellular infections [7,10], or exposure to cytotoxic drugs [6], rapidly increase autophagy to maintain the amino acid pool within the cytoplasm. Cellular processes, including protein synthesis *de novo*, energy production, and gluconeogenesis [11], require these recycled materials to maintain cellular ATP production.

Three different forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy [8]. Macroautophagy (here just referred to as autophagy) is the most well-

characterized form and is defined as the sequestration of bulk cytoplasm and organelles into double-membrane vesicles, called phagophores [8].

Phagophores originate from the endoplasmic reticulum and expand into autophagosomes, which subsequently fuse with lysosomes to form autophagolysosomes. Inside autophagolysosomes, damaged organelles are degraded by lysosomal hydrolases, and the resulting ATP and peptides are eventually recycled to maintain cell viability [8]. In contrast, microautophagy involves the direct uptake of cytoplasmic substrates into the lysosome, through the invagination of the lysosomal membrane [12], whereas chaperone-induced autophagy involves the shuttling of soluble proteins into the lysosome *via* lysosomal chaperone proteins, such as *Hsc70* [8,13].

1.1. Molecular aspects of autophagy

Autophagy involves the orchestrated activation of highly conserved genes and is controlled by two pathways that share the same target and are involved in regulating cell growth and metabolism: the mTOR (mammalian target of rapamycin) and the AMPK (AMP-activated protein kinase) signaling pathways. Under normal nutrient availability, the mTOR pathway acts through the mTOR complex I (*mTORCI*) to phosphorylate autophagy-related (ATG)13, causing the disaggregation of the Unc-51-like autophagy-activating kinase 1 (ULK1) complex [formed by ULK1, ATG13, and FAK family kinase-interacting protein of 200 kDa

(FIP200)]. Because ULK1 is required to form autophagosomes, its disaggregation can prevent autophagy. Under cell starvation conditions, mTOR phosphorylation is inhibited, allowing the ULK1 complex to remain intact and able to initiate autophagosome formation [5,8]. Under cellular stress conditions, liver kinase B1 (LKB1) activates the AMPK pathway, which then acts on two fronts: 1) stimulating autophagy through the dephosphorylation and inhibition of mTORC1; and 2) phosphorylating and activating the ULK1 complex, initiating the autophagic process [14].

Thus, one of the first genes activated by autophagy stimulating signals is *BECN1* that encodes beclin-1. This protein produces phosphatidylinositol 3-phosphate [PI(3)P], which is essential for phagophore formation and the initiation of the autophagosome. Two models have been described to explain the biogenesis of phagophores. The first model, called the maturation model, states that phagophores are derived from pre-existing endoplasmic reticulum (ER) membranes, and this theory is supported by electron microscopy evidence indicating that the phagophore membrane thickness is similar to that of the ER membrane [5]. The second model, called the assembly model, is commonly observed in yeast cells and states that phagophores are assembled *de novo*, from lipids in the cytoplasm, to form a pre-autophagosomal structure (PAS) [15] that eventually forms phagophore and autophagosome membranes. Green fluorescent protein (GFP)-labeled anti-LC3 antibodies have been used to show that PAS plays a crucial role
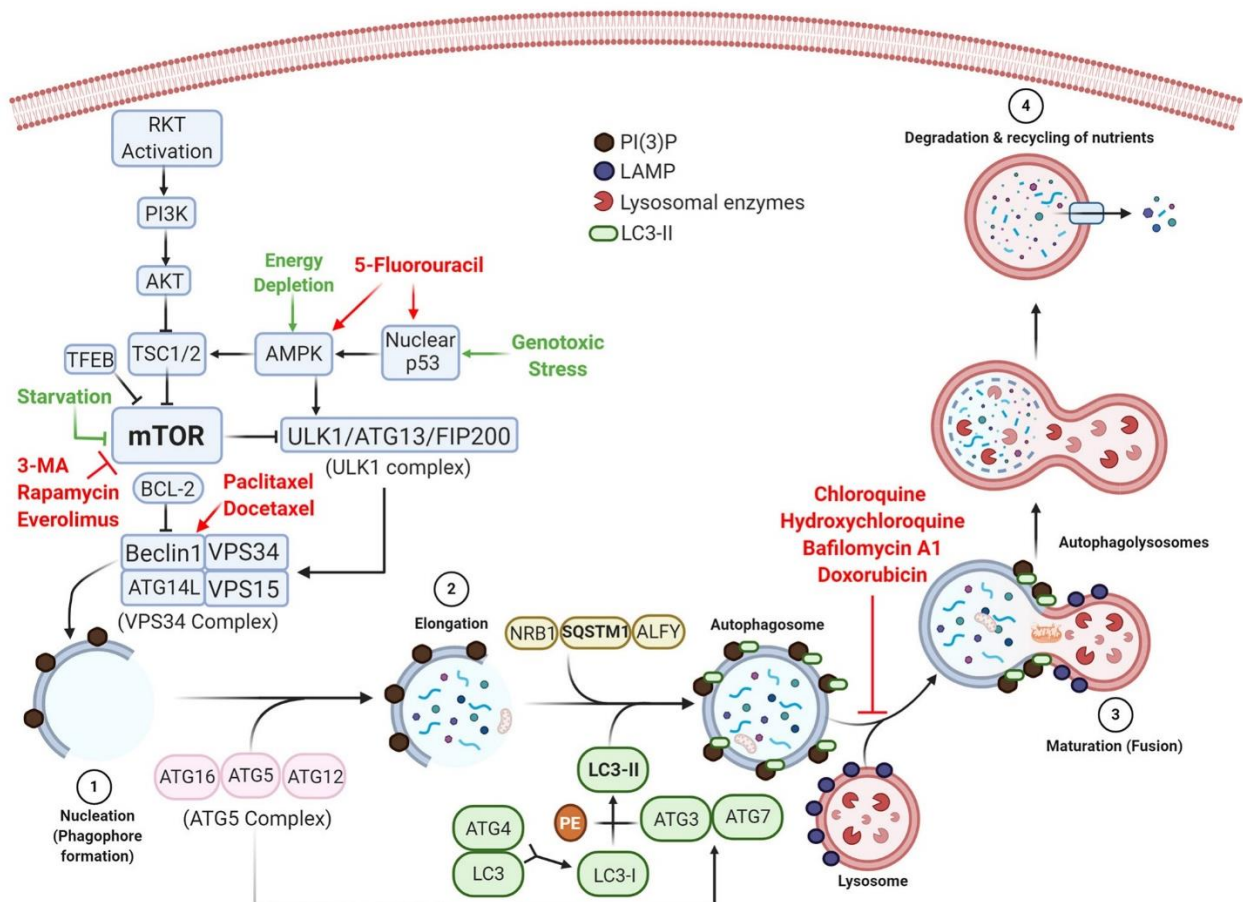
Fig. 1. The molecular control of various steps in the autophagy pathway and the roles played by drugs that activate or inhibit autophagy-associated pathways. Autophagy is triggered by several types of cellular stress, inducing the synthesis of beclin-1 and forming the VPS34 complex, which initiates phagophore formation (1). Autophagosomes, containing damaged organelles and cytosolic proteins (2), fuse with lysosomes (3) to initiate degradation and nutrient recycling (4). Anti-tumor chemotherapeutic drugs, such as 5-fluorouracil, trigger autophagy by stimulating AMPK and nuclear p53, whereas taxanes act on beclin-1 to induce the nucleation of phagophores. Autophagy can be pharmacologically inhibited during the early stage by blocking mTOR (*e.g.*, 3-methyladenine, rapamycin, and everolimus) or preventing the final step of autophagolysosome formation (*e.g.*, quinones, bafilomycin A1, and doxorubicin).

immediately prior to and during the formation of autophagosomes in yeast. Regardless of which proposed model is used, autophagy can be summarized by four steps: nucleation, elongation, maturation, and degradation, as illustrated in Fig. 1.

1.1.1. Nucleation

Nucleation refers to the initial formation of autophagosomes and requires the VPS34 complex [*BECN1*, vacuolar protein sorting (*VPS34*), *ATG14L*, and *VPS15*]. This complex is formed and regulated by the ultraviolet (UV) irradiation resistance-associated gene (*UVRAG*) and generates PI(3)P through the phosphorylation of phosphatidylinositol (PI) at position 3 of the inositol ring. The accumulation of PI(3)P generates a platform for the recruitment of effector proteins. PI(3)P-binding proteins (ATG2-ATG18) then form an isolation membrane that sequesters autophagy substrates [16,17] (Fig. 1 – step 1).

1.1.2. Elongation

It refers to the closure of the isolation membrane to form autophagosomes. This process requires the conjugation of ATG proteins, resulting in the sequestration of cytoplasmic constituents. PI(3)P recruits the ATG5 complex, consisting of ATG12, ATG5, and ATG16, which conjugates LC3-I to phosphatidylethanolamine (PE), generating LC3-II, which increases the stability of the elongating autophagosome by recruiting microtubule-associated proteins, in a process similar to using bricks to build a wall (Fig. 1 – step 2).

1.1.3. Maturation (fusion)

The molecular mechanisms that underlie autophagosome maturation remain largely unknown; however, according to some theories, this process is mediated by tectonin beta-propeller repeat-containing 1 (TECPR1), located in lysosomes, which binds to the ATG5 complex and PI(3)P to promote autophagosome-lysosome fusion and stability (Fig. 1 – step 3). Alternatively, autophagosomes can fuse with endosomal vesicles, such as endosomes and multivesicular bodies, to form amphisomes, which ultimately fuse with lysosomes.

1.1.4. Degradation

Following the fusion of autophagosomes with lysosomes, the sequestered materials are hydrolyzed, and cargoes and the inner autophagosome membrane are degraded by lysosomal enzymes. Breakdown products are released into the cytosol for further recycling (Fig. 1 – step 4).

2. The dual role played by autophagy in cancer

Autophagy and autophagic defects have been implicated in a variety of diseases, including neurodegeneration [18], myopathy [19], Crohn's disease [20], and cancer [21]. However, the role played by autophagy in carcinogenesis remains controversial, and evidence exists to suggest that autophagy can both suppress tumor transformation [22,23] and promote or facilitate tumor cell survival and adaptation [22,24,25]. Overall, autophagy appears to play a protective role during the early stages of tumor development, regulating oncogenic genes and molecules in normal cells, whereas established cancer cells appear to benefit from autophagy.

2.1. Autophagy as a tumor prevention mechanism

One of the most important links between autophagy and tumor suppression is the regulation of reactive oxygen species (ROS). Increased ROS production induces nitration and deamination reactions with DNA bases, accelerating mutagenesis, increasing the activation of oncogenes, and stimulating carcinogenesis [26]. Mitochondria are considered the primary source of intracellular ROS, and the production of ROS increases as these organelles age or become damaged. Autophagy can prevent the accumulation of damaged mitochondria through the

selective degradation of defective mitochondria (known as mitophagy) [27]. Thus, the selective removal of damaged mitochondria can prevent excessive ROS production and limit tumor-promoting effects. Accordingly, autophagy inhibition leads to the accumulation of defective mitochondria, with consequent cell transformation [28].

Evidence for this protective role of autophagy can be found not only at the mitochondrial level but also at the cellular level. An analysis of the biological changes induced by autophagy inhibition in the gastric cancer cell lines SGC-7901 and MGC-803 revealed that *BECN1* silencing (using short hairpin mRNA inhibitors, shBECN1) promoted the epithelial-mesenchymal transition, as demonstrated by the expression of N-cadherin, E-cadherin, vimentin, epithelial cell adhesion molecule (EpCAM), and Snail [29]. Furthermore, immunohistochemical analyses showed that autophagy inhibition increased the expression levels of hypoxia-inducible factor (HIF)-1α and E-cadherin by activating the ROS/nuclear factor (NF)-κB/HIF-1α pathway in both cell lines, due to the intracellular ROS accumulation caused by autophagy blockage [30]. In a murine model (SGC-7901), a much higher number of metastatic nodules was observed for shBECN1-treated cells compared with non-silenced cells. In accordance with these findings, the induction of *BECN1* knockout in adult mice results in decreased autophagy events and the increased incidence of lymphomas and carcinomas, indicating that autophagy can prevent the development of cancers [30].

Mutations in UVRAG, a protein that recruits BECN1 to increase autophagy, interferes with its tumor-suppressing functions and enhances the transformation of colorectal cancer cells, as observed in many human colon cancer cell lines (HCT15, HCT116, KM12, LIM2405, LS180, and RKO) [24]. This evidence suggests a prominent protective role for BECN1. However, the sequencing analysis of almost 10,000 human tumor samples, obtained from 24 different types of tumors in The Cancer Genome Atlas (TCGA) database, showed that the most common deletions identified in breast and ovarian cancers were long deletions in the *BECN1* genes, together with *BRCA1* on chromosome 17q21, and short deletions of *BRCA1* only but not *BECN1* [31]. These findings agree with the study that reported that the *BRCA1* mutation represents a primary mutation in breast and ovarian cancers [32]. This phenomenon may be due to the proximity of *BECN1* and *BRCA1* in the 17q21 region, suggesting that the *BRCA1* deletion represents the primary driver mutation and, therefore, BECN1 represents a passenger mutation [31,33].

Autophagy is also the process used to degrade protein aggregates, and defective autophagy can cause the accumulation of both protein aggregates and the autophagy substrate p62/sequestosome 1 (SQSTM1). p62 contains three regions: Phox and Bem1 (PB1), ubiquitin-associated domain (UBA), and LC3-interacting region (LIR). The PB1 domain enables p62 oligomerization, UBA is required for p62 to bind polyubiquitinated proteins, and LIR is necessary for p62 to associate with LC3 and phosphatidylethanolamine (PE) to form LC3-II [34]. p62 also activates NF-kB, initiating cell growth, inflammation, cell survival, and the promotion of antioxidant defense mechanisms [35]. In addition, the interaction between p62 and regulatory-associated protein of mTORC1 (RAPTOR) promotes the nutrient-sensing and cell growth activities of mTORC1 [23]. These interactions suggest that p62 plays various roles in cancer cell differentiation, inflammation, metabolism, and growth. Interestingly, reduced autophagy results in the accumulation of p62 in breast and prostatic tumors, suggesting an association between reduced autophagy and carcinogenesis [36,37]. This accumulation was also observed in a variant of Hep-2 cells (a human laryngeal carcinoma line), which were resistant against three commonly used chemotherapeutic drugs: cisplatin, 5-fluorouracil (5-FU), and docetaxel (DTX). The resistant cell variant was found to present high levels of autophagy, and these cells accumulated large amounts of ROS and p62 compared with drug treatment-susceptible Hep-2 cells [38].

2.2. Tumor-promoting role of autophagy

In contrast with the above evidence, which suggested that autophagy

protects cells from malignant transformation, several studies have implicated autophagy in the escape from anti-tumor responses. Tumor cells generally display high proliferation rates, translating into increased bioenergetic and biosynthetic requirements compared with non-transformed cells. These requirements can be satisfied by increasing autophagy levels as a mechanism for obtaining both ATP and metabolic intermediates [25]. Therefore, tumor cells may trigger autophagy as a strategy for overcoming adverse conditions more intensely than normal cells to maintain viability.

During a ten-year prospective study, LC3-II expression was investigated in 202 clinical samples from patients with colon cancer metastasis. The authors found an 87.19% positive rate for this gene among the collected tissue samples [39]. High expression levels of LC3-II were also found in 40 of 54 tissue samples obtained from patients with hypopharyngeal squamous cell carcinoma, characterized by metastasis and poor overall survival [40].

The role played by *p53* in autophagy is ambiguous and remains unclear. Gene *p53*, sometimes referred to as the "guardian of the cell genome", inhibits the cell cycle, preventing the progression of mutated cells. Therefore, defects, mutations, or the suppression of *p53* can result in genomic instability and facilitate the survival of tumorigenic cells, as observed in many *p53*-transformed cells [41]. In addition, autophagic cells degrade a portion of the available cytoplasmic p53 protein, facilitating carcinogenesis [42].

In contrast, increased autophagic flux was observed in $p53^+$ HCT-116 colon cancer cells under starvation conditions but not in $p53^{-/-}$ variants of the same cell line [43], suggesting that *p53* may be one of the genes involved in triggering autophagy. These conflicting data may be due to the subcellular location of p53. Nuclear p53 works as a nuclear factor to promote autophagy since it activates AMPK (autophagy inducer), which inhibits mTOR (the primary autophagy regulator [44]). On the other hand, cytoplasmic p53 operates in the mitochondria to repress autophagy and promote cell death [42]; therefore, the lack of p53, due to deletion, mutation, or the inhibition of gene expression, would allow tumor cells to activate autophagy and survive under adverse conditions, which is supported by the observation that the inhibition of cytoplasmic p53 degradation prevents autophagy in a variety of cancer cell lines, including HCT116 (colon) and MCF7 (breast) [45].

The activated oncogene, Ras, has also been associated with high levels of basal autophagy in tumor cells that depend on this mechanism for survival [46,47]. Ras-activating mutations have been identified in 33% of all human cancers [48] and are linked to the development of some of the most lethal cancers, including lung, colon, and pancreatic cancer [46]. Ras activation is initiated by cell surface receptors, which induce RasGEFs (guanine-nucleotide exchange factors), exchanging GDP for GTP, and activating Ras. Once activated, Ras stimulates diverse downstream effectors that trigger an array of cell signaling networks, including the AMPK autophagy pathway [49]. Because Ras acts as a positive signal in the mTOR pathway, this gene was expected to negatively regulate autophagy. However, the multifaceted role played by Ras during autophagy regulation is exemplified by the *in vitro* finding that cell lines with Ras-activating mutations exhibit high levels of basal autophagy and marked autophagy-dependent survival under nutrient deprivation conditions [50]. Ras silencing promotes the accumulation of dysfunctional mitochondria, along with low oxygen consumption and decreased cell growth [27]. Overall, current evidence has indicated that autophagy serves as a mechanism to ensure mitochondrial metabolism in Ras$^+$ cancers by supplying mitochondrial intermediates produced by the degradation of macromolecules, under both basal and starvation conditions [47].

Also contributing to tumor growth, increased levels of ULK1 were observed in two independent cohorts of patients with nasopharyngeal carcinoma, indicating a correlation between the high expression level of this protein and resistance to therapy, whereas lower ULK1 levels were observed in patients those patients who had a good therapeutic response [51]. These findings suggested that high ULK1 expression may be closely associated with an aggressive nasopharyngeal carcinoma profile. Because ULK1 (one of the three primary genes required for phagophore formation) is regulated by the mTOR complex, increased levels of ULK1 result in increased levels of autophagy and the consequent facilitation of tumor cell growth [25] (Fig. 1).

2.3. Autophagy and metastasis

Metastasis describes a multifactorial process that involves various genetic, epigenetic, and microenvironmental factors, both at the primary tumor site and in metastasis target organs. Metastatization requires cells of the primary tumor to acquire the ability to migrate and settle in adjacent or distant organs. It is a systemic process that includes changes in the target organs that co-evolve with the primary tumor, preparing them for receiving metastatic cells and sustaining their growth. These sites, called "pre-metastatic niches" show changes in the extracellular matrix and the establishment of an immunosuppressed microenvironment with the recruitment of numerous stromal cell types, population with regulatory immune cells such as myeloid-derived suppressor cells (MDSC) and Tregs, and the release of growth factors and regulatory cytokines [52,53].

A primary way for migration and communication that is used by metastatic tumor cells is the production of exosomes, which are small, membranous vesicles derived from the endosomal system, especially from late endosomes and multivesicular bodies, and are subsequently secreted through fusion with the plasma membrane [54]. Exosomes contain soluble factors, such as cytokines, integrins, and growth factors, but are also capable of releasing mRNA and microRNA [54,55].

Because autophagy acts as a component of the endolysosomal membrane system, the autophagy machinery is likely related to exosome production [56]. Therefore, blockade of autophagy in tumor cells may be associated with the upregulated release of exosomes that condition pre-metastatic niches. In a recent study, we observed that the treatment of HCT-116 colon cancer cells with a low concentration of 5-FU resulted in tumor cells that release twice the concentration of exosomes produced by untreated cells. These tumor exosomes play a regulatory role in autologous dendritic cells (DC), reducing their ability to induce cytotoxic T cells and increasing the frequency of PD-1+ lymphocytes (Romagnoli GG - personal communication).

Another important factor related to metastasis is the occurrence of cancer stem cells (CSCs), which are also responsible for tumor growth, relapse, and the development of drug resistance [57]. CSCs have been identified in several solid tumor types [58], as immortal tumor-initiating cells with self-renewing and pluripotent capacity [59]. Several chemoresistance mechanisms have been identified in CSCs [60], including autophagy, as recently reviewed by Smith and Macleod [61]. For instance, mutated beclin-1 is crucial for the maintenance of CSCs and tumor development in athymic mice, highlighting the role played by autophagic pathways in CSC maintenance and, consequently, tumor survival and growth [62]. Gastric CSCs show high levels of the autophagic marker LC3-II and the increased expression of Notch-1. The treatment of these cells with 5-FU, combined with chloroquine (CQ) and a Notch-inhibitor, significantly decreased cell viability, indicating that autophagy regulates 5-FU sensitivity in gastric CSCs *via* the Notch signaling pathway [63].

In a similar study, the assessment of autophagic levels in ovarian CSCs obtained from patients with epithelial ovarian cancer showed increased levels of LC3-II in high-level CD44$^+$ and CD117$^+$ cells (which are markers of CSCs). The treatment of these cells with CQ and the silencing of *ATG5* reduced both cell viability and the ability to form spheroidal structures *in vitro*. Furthermore, the combination of CQ and carboplatin displays synergistic effects on CSCs, *in vitro*, reducing the diameter and quantity of spheroidal tumor cells and decreasing the volume of the tumor mass and the percentage of CD44$^+$/CD117$^+$ CSCs, as measured by flow cytometry in *in vivo* models. These results demonstrated that autophagy plays a crucial role in CSC maintenance

and that autophagy blockade, combined with antineoplastic agents, may represent an improved strategy for overcoming chemoresistance [64].

3. Tumor cells use autophagy to acquire resistance to chemotherapeutic agents

In addition to its role in carcinogenesis progression, autophagy promotes chemoresistance in tumor cells. Several commonly used chemotherapeutic agents, including 5-FU, DTX, and paclitaxel (PTX), can induce autophagy in tumor cells. 5-FU acts directly on *AMPK* and *p53* [65], which are autophagy-promoting genes, resulting in increased autophagy events in tumor cells [66]. DTX and PTX bind to tubulin to simultaneously promote tubulin assembly and inhibit disassembly. The stabilization of microtubules leads to the inhibition of mitosis, resulting in cell death [67] and the inhibition of tumor growth. Therefore, DTX and PTX were expected to block autophagy; however, DTX directly increases the expression of Bcl-2 and BECN1 [68], and PTX increases the expression of BECN1 [69] and decreases p62 [70], inducing autophagy, in both cases. We also have studied the effects of pharmacological autophagy blockades, using a $BAX^{-/-}$ variant of HCT-116 cells, which display lower levels of apoptosis than wild-type HCT-116 cells. Because BAX controls the synthesis of the BCL2-associated X-protein, which regulates beclin-1 activation, this knockout variant displayed levels of autophagy than its wild-type counterpart. We observed that the blockage of 5-FU-induced autophagy with hydroxychloroquine (HCQ) increased the susceptibility of $BAX^{-/-}$ cells to 5-FU, suggesting that autophagy depends on BAX expression to enhance chemotherapy survival (Gorgulho CM – personal communication).

When autophagy is stimulated, tumor cells can use it as a resistance mechanism to escape drug treatment. Castration-resistant prostate cancer cells are particularly resistant to conventional treatments [71]. Their susceptibility to DTX was evaluated when co-treated with either inhibitors or activators of signal transducer and activator of transcription 3 (STAT3), a negative regulator of autophagy. These cancer cells were cultured for 24 h with DTX, and autophagy was evaluated by measuring the levels of p62 and LC3-I/II and by electron microscopy. The authors observed that STAT3 activators reduced autophagy and cell viability, increasing mitochondrial damage and apoptosis, whereas STAT3 inhibitors showed the opposite effects, leading to the conclusion that the activation of autophagy promoted DTX-resistance [72].

In a preliminary study, we observed that both CQ and HCQ increases the expression of pSTAT3 in HCT-116 cells exposed to 5-FU (analysis by flow cytometry showed an increase from 62% to 87% and 97%, under HCQ and CQ, respectively). These results fit with a decreased cell viability and the arrest of the cell cycle in the G0/G1 phase (Zamame JA, Sanzochi FC – personal communication).

The treatment of non-small-cell lung cancer lines A549 and Calu-3 with low concentrations of PTX (1, 3, and 10 μmol), combined with the autophagy inhibitor 3-methyladenine (3-MA), prevented PTX from increasing BECN1 expression and markedly decreased cell viability in comparison with cells treated with PTX alone. Therefore, although PTX stimulated autophagy and promoted the development of resistant variants, the use of autophagy inhibitors prevented the development of chemoresistance [73].

To evaluate the effects of autophagy blockade in breast cancer, BT-549, and MDA-MB-468 triple-negative cell lines were cultured with 5-FU, DTX, or doxorubicin, to select chemoresistant variants. The analysis of cell viability and the expression of autophagy-related genes showed that resistant variants (BT-459DOX[20] and MDA-MB-4685-FU[200]) expressed increased levels of BECN1, ATG5, and Bcl2-associated athanogene 3 (BAG3). Autophagy inhibition, through bafilomycin A1 treatment or siRNA targeting *ATG5*, decreased cell viability, and autophagy-associated gene expression [74] (Fig. 1 – step 2).

Chemoresistance may also be associated with lysosomal effects, which store enzymes capable of degrading cellular components affected by chemotherapy. One of the primary factors associated with the

development of chemoresistance is the transcription factor EB (TFEB), which stimulates lysosomal biogenesis and positively regulates autophagy by inhibiting the mTOR pathway. The role played by lysosomes in chemoresistance was investigated *in vitro*, using LoVo and HeLa cells that displayed increased TFEB expression levels after exposure to low concentrations of doxorubicin. TFEB overexpression was stimulated by transfection, with significantly decreased levels of apoptosis and cell death observed for both cell lines. TFEB silencing by RNA interference dramatically increased the level of cellular apoptosis, demonstrating a correlation between chemoresistance and autophagy [75].

Evidence for the role of autophagy in the development of chemoresistance in clinical studies was also reviewed by Mele et al. [76], with especial attention to targeted therapy. Clinical trials developed from 2015 to 2020 are summarized in Table 1. For instance, the analysis of miR-489 and lysosome-associated transmembrane protein 4B (LAPTM4B) expression in tissue samples obtained from 14 patients with breast cancer showed that those with high miR-489 levels achieved higher overall survival rates than those with low miR-489 levels. The authors also found an inverse correlation between the expression levels of miR-489 and LAPTM4B [77]. miR-489-mediated interference directly acts on ULK1 and LAPTM4B, decreasing their expression levels and reducing autophagy events. These two proteins have been associated with phagophore formation and the lysosome and autophagosome fusion, respectively, and are upregulated in various types of cancers [78,79]. In this same study, using an experimental model, athymic nude mice bearing the breast cancer cell line MDA-MB-231 were treated with doxorubicin and miR-489, delivered by nanoparticles. Increased sensitivity to doxorubicin and an improved capacity to control tumor growth was observed in these animals compared with control animals [77].

Intending to use autophagy genes as biomarkers for cancer prognosis, Koustas et al. analyzed the relationships between the levels of various autophagy markers and overall survival in patients with colorectal cancer. The immunohistochemical analysis of 68 clinical samples from patients with colorectal cancer who underwent chemotherapeutic treatment for twelve months showed that patients with low beclin-1 expression levels had a better therapeutic outcome, based on both average survival (P = 0.001) and progression-free survival (P = 0.069), compared with patients with high beclin-1 levels [80]. This result suggests that autophagy proteins can be useful indicators of chemoresistance prognosis and tumor development.

All this information support that autophagy facilitates chemoresistance but there are some conflicting data. For instance, Yao et al. cultured two lines of colon cancer cells, one of them susceptible to 5-FU and the other a resistant variant able to grow in culture medium containing the drug. Authors evaluated the amounts of acidic vesicular organelles (AVOs) and the expression levels of ATG5, BECN1, and LC3-I/II following challenge with 5-FU. They observed that the resistant variant presented 100% viability when treated with 140 μM 5-FU. The amount of AVOs and the expression levels of ATG5, BECN1, and LC3-I/II in the resistant cells were lower than those observed in the sensitive cells. The authors suggested that 5-FU susceptible cells use autophagy to develop chemoresistance and, subsequently, no longer require autophagy to maintain their viability, as demonstrated by the low levels of autophagy observed in the resistant variant [81].

4. Therapeutic interference in autophagy

Based on these studies, the inhibition of autophagy may sensitize cancer cells and increase the cytotoxic capacity of various antineoplastic agents. Quinolones, such as CQ and HCQ, are the best-known autophagy blockers and the only approved by the FDA for clinical use. These drugs are primarily used as antimalarial medications and their ability to block the union of the autophagosome with the lysosome, allow them to interfere with the final step of autophagy (Fig. 1 – step 3). Our group observed that CQ reverts autophagy induced by low concentrations of 5-FU in HCT-116 human colon cancer cells, making them

Table 1

Clinical trials developed from 2015 to 2020 using autophagy inhibitors in combination with conventional chemotherapy/radiotherapy.

Disease (study phase)	Treatment	Rationale	Results	Ref.
Breast cancer (II)	CQ	CQ accumulates inside the lysosome causing lysosomal membrane permeabilization, inhibiting autophagy, and leading to apoptosis.	15% adverse reactions. No significant differences between treatments.	[108]
Early-stage solid tumors (prostate, lung, thyroid, and squamous cell carcinoma) (I)	HCQ	HCQ work in the same way as CQ being better tolerated by patients.	Safe and well-tolerated treatment. Increase of autophagy and cancer biomarkers.	[109]
Pancreatic cancer (I/II)	HCQ + gemcitabine	Blockage of the late phase of autophagy improves the antitumor effect of gemcitabine, an inhibitor of DNA replication	Safe and well-tolerated treatment. 2.1% overall survival response.	[110]
Pancreatic cancer (I)	CQ + gemcitabine	Blockage of the late phase of autophagy improves the antitumor effect of gemcitabine, an inhibitor of DNA replication	Well-tolerated treatment. 33% overall response rate 55% tumor control rate 43% increase in overall survival	[84]
Pancreatic cancer (II)	HCQ + gemcitabine + Abraxane	Blockage of the late phase of autophagy enhances the cytotoxic effects of gemcitabine that inhibits DNA replication, and Abraxane, which blocks mitosis	Well-tolerated treatment. HCQ reduces hypercoagulability in pancreatic cancer. No improvement in overall survival.	[111,112]
Pancreatic cancer (I/II)	HCQ + gemcitabine + Nab-paclitaxel	Blockage of the late phase of autophagy enhances the effect of gemcitabine (that inhibits DNA replication), and paclitaxel (that binds to tubulin, preventing cell mitosis)	Adverse reactions related to high-dose treatment. 38.2% overall survival response.	[113]
Pancreatic cancer (II)	Gemcitabine + Nab-paclitaxel + HCQ + Avelumab	Blockage of the late phase of autophagy enhances the effect of gemcitabine (that inhibits DNA replication), paclitaxel (that binds to tubulin, preventing cell mitosis), and Avelumab (anti-PD-L1)	Toxic damage related to high-dose treatment. No improvement in overall survival.	[114]
Prostate cancer (II)	Docetaxel + pantoprazole	Docetaxel inhibits microtubular depolymerization to prevent cell mitosis, and pantoprazole inhibits autophagy by deacidifying endosomes, preventing their fusion with autophagosomes.	Well-tolerated treatment. No significant improvement in overall survival.	[115]
Renal cell carcinoma (I/II)	Everolimus + HCQ	Everolimus directly inhibits mTOR, stimulating autophagy. Its combination with HCQ would induce metabolic instability in tumor cells leading to apoptosis	Safe and well-tolerated treatment. 67% Disease control 6% Partial response	[116]
Lymphangioleiomyo-matosis (I)	HCQ + Sirolimus	Everolimus directly inhibits mTOR, stimulating autophagy. Its combination with HCQ would induce metabolic instability in tumor cells leading to apoptosis	Safe and well-tolerated treatment. Increase of autophagy and cancer biomarkers. No significant improvement in overall survival	[117,118]
Glioblastoma multiforme (I/II)	HCQ + Temozolomide + radiotherapy	HCQ would increase the antineoplastic effect of Temozolomide (that methylates DNA, and induces cell death) and radiation therapy (that uses charged photons to damage the DNA)	Toxic damage related to high-dose treatment. No improvement in overall survival.	[119]
Non-small cell lung cancer (I)	HCQ + Erlotinib	HCQ enhances the sensitivity of cells to this antibody that blocks the highly mutated EGFR in lung cancer	Safe and well-tolerated treatment. 5% overall survival response.	[120]
Non-small cell cancer (I/II)	HCQ + Bevacizumab + carboplatin + Paclitaxel	Autophagy blockage would enhance the pharmacological synergy among bevacizumab (anti-VEGF), carboplatin (that binds to DNA), and paclitaxel (binds to tubulin, inhibiting cell division	Well-tolerated treatment. 20% Disease control. 33% overall response.	[121]
Varied advanced solid tumors (colon, non-small cell lung cancer, melanoma, breast, and others) (I)	MK-2206 + HCQ	autophagy blockage would enhance the effect of MK-2206, an inhibitor of the AKT pathway	94% of adverse reactions. 15% overall survival response.	[114]

more immunogenic than untreated cells, as demonstrated by both the increased gene expression of tumor-associated antigens (CEACAM 1, 5, 6, and 7) and improved the ability of tumor lysates to sensitize monocyte-derived DCs [82]. We have also observed that a combination of HCQ and 5-FU induces the expression of CEA, HLA-ABC, and CD54 on the surface of these tumor cells (Gorgulho CM, – personal communication).

In a phase I study, CQ was used in combination with gemcitabine to treat nine patients with metastatic pancreatic cancer, resulting in three patients with a partial response and two patients who continued to present stable disease, resulting in a 33% overall response rate and a 55% tumor control rate. These rates were significantly higher than the 9.4% achieved using gemcitabine alone in patients with metastatic pancreatic cancer [83]. In addition, the median overall survival rate was 7.6 months for the combination treatment, compared with 3.3 months with gemcitabine alone [83]. These data indicated that the autophagic blockade stimulated and enhanced the anti-neoplastic activity of gemcitabine [84].

HCQ induces fewer side effects than CQ [85], but its combination with anti-neoplastic agents has resulted in varied outcomes. For

instance, HCQ combined with RAD001 (an mTOR inhibitor) enhanced the effectiveness of chemotherapy against the renal carcinoma cell lines ACHN, Caki-1, and 769-P [86]. HCQ also enhanced the anticancer effects of the anti-angiogenic monoclonal antibody of VEGFR2 in the gastric cancer cell line BGC823 [87] and potentiated the cytotoxic effects of bevacizumab (another monoclonal anti-VEGF antibody) against LN18 and LN229 glioblastoma cells [88].

In a phase II trial, patients with untreated phase IV colorectal cancer were treated with a combination of HCQ, the FOLFOX regimen (5-FU, oxaliplatin, and leucovorin), and bevacizumab. The authors observed an overall response rate of 68%, and a complete response was observed in 3 of 28 patients [89]. Hydroxychloroquine was also tested in patients with pancreatic adenocarcinoma metastasis in a phase II study, and the patients treated with this drug showed an improvement in overall survival by 69 days [90]. In another clinical trial, 112 patients with advanced pancreatic adenocarcinoma and no previous treatment history were treated with gemcitabine and PTX coated with glucose nanocaps (nab-PTX), combined with HCQ, in a twice-daily dose. This combination was well-tolerated by the patients, and HCQ increased the response rate to gemcitabine and nab-PTX compared with those who received

chemotherapy alone [2]. A meta-analysis of all 293 known clinical trials involving CQ or HCQ showed that their combination with chemotherapy agents improved the overall response rate, progression-free survival, and overall survival [91].

In contrast with these findings on cytoprotective autophagy, some studies have reported that autophagy can fight some types of cancer. Recently, Wen et al. [92] reviewed the evidence that increased autophagy flux induces the death of breast cancer *in vitro* and their sensitivity to drugs [93,94]. Cells can die both due to autophagy-dependent cell death – ADCD - formerly referred to as autophagic cell death [95], which directly involves the autophagy machinery, and autophagic-related cell death, which depends on other pathways, such as apoptosis and necrosis [96,97]. Rapamycin also induces autophagy through the selective inhibition of *mTORC1* and was found to inhibit the proliferation of murine sarcoma cells (s180) [98], neuroblastoma cells (KN-SH and SH-SY5Y) [99], and lung cancer cells (A549) [100].

Although most data concerning this subject was obtained through *in vitro* studies (reviewed by Wen et al., 2019) or in experimental models there are some clinical trials indicating that low concentrations of rapamycin increase the immune response and enhance the immunotherapeutic agents activity, such as the monoclonal anti-PD-L1 antibody used in patients with oral cavity cancers [101]. The therapeutic potential of this approach was evaluated in head and neck cancer patients (8 with malignant lesions in the oral cavity and 8 with malignant lesions in the oropharynx). After 21 days of treatment with rapamycin, these patients showed reduced *mTOR* signaling and lower tumor growth, despite the relatively short treatment duration. In addition, none of the patients presented any form of immunosuppression [102]. A similar effect was observed in another study in which low doses of rapamycin resulted in favorable immunomodulatory activity in bladder cancer patients undergoing surgery, decreasing the numbers of $CD4^+/CD8^+$ $PD1^+$ T cells, therefore preventing the immune dysfunction induced by surgery [103].

Everolimus, which inhibits *mTOR* to induce autophagy, also regulates the *FoxP3* transcription factor, modulating regulatory T cell (Tregs) expression [104]. In a phase 1 study, everolimus was combined with cyclophosphamide to deplete Tregs in patients with metastatic renal carcinoma, and the results showed that this combination not only depleted Tregs but also reduced MDSC expression [105]. In addition, this combination maintained sustained levels of $CD8^+$ T cells, reversing a decrease in peripheral blood DC subsets (cDC1, cDC2, and pDC) induced by cancer [106]. Therefore, these anti-tumor effects may not be directly linked to autophagy because *mTOR* has a wide range of functions [107].

5. Concluding remarks and perspectives

Even though the controversy over autophagy role in tumor suppression or promotion remains unresolved, both basic studies and clinical trials led us to conclude that pharmacological or molecular blockage of autophagy improves the effectiveness of cytotoxic antitumor drugs, as well as monoclonal antibodies for tumor-associated antigens. Our own results suggest that pharmacological blockage of drug-induced autophagy increases the immunogenicity of tumor cells. This view opens the perspectives for expanding the use of autophagy blockers to synergize the effects of available immunotherapeutic agents, such as checkpoint blockade antibodies and other growth receptor inhibitors. An improved understanding of the autophagy role in cancer is also required for the development of new alternatives for blocking autophagy in cancer patients.

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References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2020, CA Cancer J. Clin. 70 (2020) 7–30.

[2] T.B. Karasic, M.H. O'Hara, A. Loaiza-Bonilla, K.A. Reiss, U.R. Teitelbaum, E. Borazanci, et al., Effect of gemcitabine and nab-paclitaxel with or without hydroxychloroquine on patients with advanced pancreatic cancer: a phase 2 randomized clinical trial, JAMA Oncology 5 (2019) 993–998.

[3] M.S. Nars, R. Kaneno, Immunomodulatory effects of low dose chemotherapy and perspectives of its combination with immunotherapy 132, 2013, pp. 2471–2478.

[4] C. de Duve, Lysosomes revisited, Eur. J. Biochem. 137 (1983) 391–397.

[5] D.J. Klionsky, J.M. Cregg, W.A. Dunn Jr., S.D. Emr, Y. Sakai, I.V. Sandoval, et al., A unified nomenclature for yeast autophagy-related genes, Dev. Cell 5 (2003) 539–545.

[6] A. Ertmer, V. Huber, S. Gilch, T. Yoshimori, V. Erfle, J. Duyster, et al., The anticancer drug imatinib induces cellular autophagy, Leukemia 21 (2007) 936–942.

[7] N. Jounai, F. Takeshita, K. Kobiyama, A. Sawano, A. Miyawaki, K.-Q. Xin, et al., The Atg5–Atg12 conjugate associates with innate antiviral immune responses, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14050–14055.

[8] N. Mizushima, Y. Ohsumi, T. Yoshimori, Autophagosome formation in mammalian cells, Cell Struct. Funct. 27 (2002) 421–429.

[9] J. Seino, L. Wang, Y. Harada, C. Huang, K. Ishii, N. Mizushima, et al., Basal autophagy is required for the efficient catabolism of sialyloligosaccharides, J. Biol. Chem. 288 (2013) 26898–26907.

[10] Y. Wang, L.M. Weiss, A. Orlofsky, Host cell autophagy is induced by toxoplasma gondii and contributes to parasite growth, J. Biol. Chem. 284 (2009) 1694–1701.

[11] O.B. Kotoulas, S.A. Kalamidas, D.J. Kondomerkos, Glycogen autophagy, Microsc. Res. Tech. 64 (2004) 10–20.

[12] A. Uttenweiler, H. Schwarz, A. Mayer, Microautophagic vacuole invagination requires calmodulin in a Ca2+-independent function 280, 2005, pp. 33289–33297.

[13] R. Sahu, S. Kaushik, C.C. Clement, E.S. Cannizzo, B. Scharf, A. Follenzi, et al., Microautophagy of cytosolic proteins by late endosomes, Dev. Cell 20 (2011) 131–139.

[14] M.M. Mihaylova, R.J. Shaw, The AMPK signalling pathway coordinates cell growth, autophagy and metabolism, Nat. Cell Biol. 13 (2011) 1016–1023.

[15] K.S. Takeshi Noda, Yoshinori Ohsumi, Yeast autophagosomes: de novo formation of a membrane structure, Trends Cell Biol. 12 (2002) 231–235.

[16] C. Burman, N.T. Ktistakis, Regulation of autophagy by phosphatidylinositol 3-phosphate, FEBS Lett. 584 (2010) 1302–1312.

[17] A. Kihara, T. Noda, N. Ishihara, Y. Ohsumi, Two distinct Vps34 phosphatidylinositol 3–kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae, J. Cell Biol. 152 (2001) 519–530.

[18] R.A. Nixon, The role of autophagy in neurodegenerative disease, Nat. Med. 19 (2013) 983–997.

[19] M.C.V. Malicdan, I. Nishino, Autophagy in lysosomal myopathies, Brain Pathol. 22 (2012) 82–88.

[20] J.D. Rioux, R.J. Xavier, K.D. Taylor, M.S. Silverberg, P. Goyette, A. Huett, et al., Genome-wide association study identifies five novel susceptibility loci for Crohn's disease and implicates a role for autophagy in disease pathogenesis, Nat. Genet. 39 (2007) 596–604.

[21] X.H. Liang, S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh, et al., Induction of autophagy and inhibition of tumorigenesis by beclin 1, Nature 402 (1999) 672–676.

[22] K. Degenhardt, R. Mathew, B. Beaudoin, K. Bray, D. Anderson, G. Chen, et al., Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis, Cancer Cell 10 (2006) 51–64.

[23] A. Duran, R. Amanchy, J.F. Linares, J. Joshi, S. Abu-Baker, A. Porollo, et al., p62 is a key regulator of nutrient sensing in the mTORC1 pathway, Mol. Cell 44 (2011) 134–146.

[24] L. Galluzzi, F. Pietrocola, J.M. Bravo-San Pedro, R.K. Amaravadi, E.H. Baehrecke, F. Cecconi, et al., Autophagy in malignant transformation and cancer progression, EMBO J. 34 (2015) 856.

[25] R. Mathew, C. Karp, B. Beaudoin, N. Vuong, G. Chen, H.-Y. Chen, et al., Autophagy suppresses tumorigenesis through elimination of p62, Cell 137 (2009) 1062–1075.

[26] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, Z. Elazar, Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4, EMBO J. 26 (2007) 1749–1760.

[27] L.C. Gomes, G. Di Benedetto, L. Scorrano, During autophagy mitochondria elongate, are spared from degradation and sustain cell viability, Nat. Cell Biol. 13 (2011) 589–598.

[28] K. Okamoto, N. Kondo-Okamoto, Y. Ohsumi, Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy, Dev. Cell 17 (2009) 87–97.

[29] W. Qin, C. Li, W. Zheng, Q. Guo, Y. Zhang, M. Kang, et al., Inhibition of autophagy promotes metastasis and glycolysis by inducing ROS in gastric cancer cells, Oncotarget 6 (2015) 39839–39854.

[30] W. Qin, C. Li, W. Zheng, Q. Guo, Y. Zhang, M. Kang, et al., Inhibition of autophagy promotes metastasis and glycolysis by inducing ROS in gastric cancer cells, Oncotarget 6 (2015) 39839–39854.

[31] S.V. Laddha, S. Ganesan, C.S. Chan, E. White, Mutational landscape of the essential autophagy gene BECN1 in human cancers, Mol. Cancer Res. 12 (2014) 485–490.

[32] D.P. Silver, D.M. Livingston, Mechanisms of BRCA1 tumor suppression, Cancer Discov 2 (2012) 679–684.

[33] H. Tang, S. Sebti, R. Titone, Y. Zhou, C. Isidoro, T.S. Ross, et al., Decreased BECN1 mRNA expression in human breast cancer is associated with estrogen receptor-negative subtypes and poor prognosis, EBioMedicine 2 (2015) 255–263.

[34] S. Pankiv, T.H. Clausen, T. Lamark, A. Brech, J.-A. Bruun, H. Outzen, et al., p62/ SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, J. Biol. Chem. 282 (2007) 24131–24145.

[35] H. Wei, C. Wang, C.M. Croce, J.-L. Guan, p62/SQSTM1 synergizes with autophagy for tumor growth in vivo, Genes Dev. 28 (2014) 1204–1216.

[36] G. Bjørkøy, T. Lamark, S. Pankiv, A. Øvervatn, A. Brech, T. Johansen, Chapter 12 Monitoring Autophagic Degradation of p62/SQSTM1, Academic Press, Methods in Enzymology, 2009, pp. 181–197.

[37] Y. Inami, S. Waguri, A. Sakamoto, T. Kouno, K. Nakada, O. Hino, et al., Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells, J. Cell Biol. 193 (2011) 275–284.

[38] R.A. Battista, M. Resnati, C. Facchi, E. Ruggieri, F. Cremasco, F. Paradiso, et al., Autophagy mediates epithelial cancer chemoresistance by reducing p62/SQSTM1 accumulation, PLoS One 13 (2018), e0201621.

[39] S. Wu, C. Sun, D. Tian, Y. Li, X. Gao, S. He, et al., Expression and clinical significances of Beclin1, LC3 and mTOR in colorectal cancer, Int. J. Clin. Exp. Pathol. 8 (2015) 3882–3891.

[40] J. Wang, X.-L. Pan, L.-J. Ding, D.-Y. Liu, L. Da-Peng, T. Jin, Aberrant expression of beclin-1 and LC3 correlates with poor prognosis of human hypopharyngeal squamous cell carcinoma, PLoS One 8 (2013) e69038.

[41] C.M. Eischen, Genome stability requires p53, Cold Spring Harb Perspect Med. 6 (2016) a026096.

[42] E. Tasdemir, M.C. Maiuri, L. Galluzzi, I. Vitale, M. Djavaheri-Mergny, M. D'Amelio, et al., Regulation of autophagy by cytoplasmic p53, Nat. Cell Biol. 10 (2008) 676–687.

[43] R. Scherz-Shouval, H. Weidberg, C. Gonen, S. Wilder, Z. Elazar, M. Oren, p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 18511–18516.

[44] A.V. Budanov, M. Karin, p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling, Cell 134 (2008) 451–460.

[45] E. Tasdemir, M.C. Maiuri, L. Galluzzi, I. Vitale, M. Djavaheri-Mergny, M. D'Amelio, et al., Regulation of autophagy by cytoplasmic p53, Nat. Cell Biol. 10 (2008) 676–687.

[46] J. Liu, H. Xia, M. Kim, L. Xu, Y. Li, L. Zhang, et al., Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13, Cell 147 (2011) 223–234.

[47] Y. Pylayeva-Gupta, E. Grabocka, D. Bar-Sagi, RAS oncogenes: weaving a tumorigenic web, Nat. Rev. Cancer 11 (2011) 761–774.

[48] J. Downward, Targeting RAS signalling pathways in cancer therapy, Nat. Rev. Cancer 3 (2003) 11–22.

[49] J. Ge, Z. Chen, J. Huang, J. Chen, W. Yuan, Z. Deng, et al., Upregulation of autophagy-related gene-5 (ATG-5) is associated with chemoresistance in human gastric cancer, PLoS One 9 (2014), e110293.

[50] J.Y. Guo, H.-Y. Chen, R. Mathew, J. Fan, A.M. Strohecker, G. Karsli-Uzunbas, et al., Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis, Genes Dev. 25 (2011) 460–470.

[51] M. Yun, H.-Y. Bai, J.-X. Zhang, J. Rong, H.-W. Weng, Z.-S. Zheng, et al., ULK1: a promising biomarker in predicting poor prognosis and therapeutic response in human nasopharyngeal carcinoma, PLoS One 10 (2015), e0117375.

[52] A.C. Obenauf, J. Massagué, Surviving at a distance: organ-specific metastasis, Trends Cancer 1 (2015) 76–91.

[53] B. Psaila, D. Lyden, The metastatic niche: adapting the foreign soil, Nat. Rev. Cancer 9 (2009) 285–293.

[54] E.R. Abels, X.O. Breakefield, Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake, Cell. Mol. Neurobiol. 36 (2016) 301–312.

[55] H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J.J. Lee, J.O. Lötvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (2007) 654–659.

[56] J. Xu, R. Camfield, S.M. Gorski, The interplay between exosomes and autophagy – partners in crime, J. Cell Sci. 131 (2018) jcs215210.

[57] R. Tripathi, D. Ash, C. Shaha, Beclin-1–p53 interaction is crucial for cell fate determination in embryonal carcinoma cells, J. Cell. Mol. Med. 18 (2014) 2275–2286.

[58] J.-L. Guan, A.K. Simon, M. Prescott, J.A. Menendez, F. Liu, F. Wang, et al., Autophagy in stem cells, Autophagy 9 (2013) 830–849.

[59] S. Ma, T.K. Lee, B.J. Zheng, K.W. Chan, X.Y. Guan, CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway, Oncogene 27 (2007) 1749–1758.

[60] C.G. Sanchez, P. Penfornis, A.Z. Oskowitz, A.G. Boonjindasup, D.Z. Cai, S. S. Dhule, et al., Activation of autophagy in mesenchymal stem cells provides tumor stromal support, Carcinogenesis 32 (2011) 964–972.

[61] A.G. Smith, K.F. Macleod, Autophagy, cancer stem cells and drug resistance, J. Pathol. 247 (2019) 708–718.

[62] C. Gong, C. Bauvy, G. Tonelli, W. Yue, C. Deloménie, V. Nicolas, et al., Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/ progenitor cells, Oncogene 32 (2013) 2261–2272.

[63] L.Q. Li, D. Pan, S.W. Zhang, D. YX, X.L. Zheng, H. Chen, Autophagy regulates chemoresistance of gastric cancer stem cells via the Notch signaling pathway, Eur. Rev. Med. Pharmacol. Sci. 22 (2018) 3402–3407.

[64] A. Pagotto, G. Pilotto, E.L. Mazzoldi, M.O. Nicoletto, S. Frezzini, A. Pastò, et al., Autophagy inhibition reduces chemoresistance and tumorigenic potential of human ovarian cancer stem cells, Cell Death Dis. 8 (2017) e2943–e.

[65] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-Fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 3 (2003) 330.

[66] T. Hu, L. Wang, L. Zhang, L. Lu, J. Shen, R.L.Y. Chan, et al., Sensitivity of apoptosis-resistant colon cancer cells to tanshinones is mediated by autophagic cell death and p53-independent cytotoxicity, Phytomedicine 22 (2015) 536–544.

[67] A.-M.C. Yvon, P. Wadsworth, M.A. Jordan, Taxol suppresses dynamics of individual microtubules in living human tumor cells, Mol. Biol. Cell 10 (1999) 947–959.

[68] Q. Wang, W.-Y. He, Y.-Z. Zeng, A. Hossain, X. Gou, Inhibiting autophagy overcomes docetaxel resistance in castration-resistant prostate cancer cells, Int. Urol. Nephrol. 50 (2018) 675–686.

[69] G. Xi, X. Hu, B. Wu, H. Jiang, C.Y.F. Young, Y. Pang, et al., Autophagy inhibition promotes paclitaxel-induced apoptosis in cancer cells, Cancer Lett. 307 (2011) 141–148.

[70] Y.-F. Yu, P.-C. Hu, Y. Wang, X.-L. Xu, G.M. Rushworth, Z. Zhang, et al., Paclitaxel induces autophagy in gastric cancer BGC823 cells, Ultrastruct. Pathol. 41 (2017) 284–290.

[71] D.L. Suzman, E.S. Antonarakis, Castration-resistant prostate cancer: latest evidence and therapeutic implications, Therapeutic Advances in Medical Oncology 6 (2014) 167–179.

[72] F. Hu, Y. Zhao, Y. Yu, Fang J-m, R. Cui, Liu Z-q, et al., Docetaxel-mediated autophagy promotes chemoresistance in castration-resistant prostate cancer cells by inhibiting STAT3, Cancer Lett. 416 (2018) 24–30.

[73] K. Chen, W. Shi, Autophagy regulates resistance of non-small cell lung cancer cells to paclitaxel, Tumor Biol. 37 (2016) 10539–10544.

[74] C.K. Das, B. Linder, F. Bonn, F. Rothweiler, I. Dikic, M. Michaelis, et al., BAG3 overexpression and cytoprotective autophagy mediate apoptosis resistance in chemoresistant breast cancer cells, Neoplasia (New York, NY) 20 (2018) 263–279.

[75] L.-M. Fang, B. Li, J.-J. Guan, H.-D. Xu, G.-H. Shen, Q.-G. Gao, et al., Transcription factor EB is involved in autophagy-mediated chemoresistance to doxorubicin in human cancer cells, Acta Pharmacol. Sin. 38 (2017) 1305–1316.

[76] L. Mele, V. del Vecchio, D. Liccardo, C. Prisco, M. Schwerdtfeger, N. Robinson, et al., The role of autophagy in resistance to targeted therapies, Cancer Treat. Rev. 88 (2020), 102043.

[77] M. Soni, Y. Patel, E. Markoutsa, C. Jie, S. Liu, P. Xu, et al., Autophagy, cell viability, and chemoresistance are regulated by miR-489 in breast cancer, Mol. Cancer Res. 16 (2018) 1348.

[78] J. Lu, L. Zhu, L.-P. Zheng, Q. Cui, H.-H. Zhu, H. Zhao, et al., Overexpression of ULK1 represents a potential diagnostic marker for clear cell renal carcinoma and the antitumor effects of SBI-0206965, EBioMedicine 34 (2018) 85–93.

[79] F. Wang, H. Wu, S. Zhang, J. Lu, Y. Lu, P. Zhan, et al., LAPTM4B facilitates tumor growth and induces autophagy in hepatocellular carcinoma, Cancer Manag. Res. 11 (2019) 2485–2497.

[80] E. Koustas, P. Sarantis, S. Theoharis, A.A. Saetta, I. Chatziandreou, G. Kyriakopoulou, et al., Autophagy-related proteins as a prognostic factor of patients with colorectal cancer, Am. J. Clin. Oncol. 42 (2019) 767–776.

[81] C.W. Yao, K.A. Kang, M.J. Piao, Y.S. Ryu, P.M.D.J. Fernando, M.C. Oh, et al., Reduced autophagy in 5-fluorouracil resistant colon cancer cells, Biomol. Ther. 25 (2017) 315–320.

[82] J.A. Zamame Ramirez, G.G. Romagnoli, B.F. Falasco, C.M. Gorgulho, C. Sanzochi Fogolin, D.C. Dos Santos, et al., Blocking drug-induced autophagy with chloroquine in HCT-116 colon cancer cells enhances DC maturation and T cell responses induced by tumor cell lysate, Int. Immunopharmacol. 84 (2020), 106495.

[83] T. Conroy, F. Desseigne, M. Ychou, O. Bouché, R. Guimbaud, Y. Bécouarn, et al., FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer 364, 2011, pp. 1817–1825.

[84] P. Samaras, M. Tusup, T.D.L. Nguyen-Kim, B. Seifert, H. Bachmann, R. von Moos, et al., Phase I study of a chloroquine-gemcitabine combination in patients with metastatic or unresectable pancreatic cancer, Cancer Chemother. Pharmacol. 80 (2017) 1005–1012.

[85] RtdS Cabral, E.M. Klumb, M.I.N.N. Couto, S. Carneiro, Evaluation of toxic retinopathy caused by antimalarial medications with spectral domain optical coherence tomography, J Arquivos Brasileiros de Oftalmologia 82 (2019) 12–17.

[86] H.-O. Lee, A. Mustafa, G.R. Hudes, W.D. Kruger, Hydroxychloroquine destabilizes phospho-S6 in human renal carcinoma cells, PLoS One 10 (2015) e0131464.

[87] W. Wang, L. Liu, Y. Zhou, Q. Ye, X. Yang, J. Jiang, et al., Hydroxychloroquine enhances the antitumor effects of BC001 in gastric cancer, Int. J. Oncol. 55 (2019) 405–414.

[88] L.-Q. Liu, S.-B. Wang, Y.-F. Shao, J.-N. Shi, W. Wang, W.-Y. Chen, et al., Hydroxychloroquine potentiates the anti-cancer effect of bevacizumab on glioblastoma via the inhibition of autophagy, Biomed. Pharmacother. 118 (2019) 109339.

[89] M.H. O'Hara, T.B. Karasic, I. Vasilevskaya, M. Redlinger, A. Loaiza-Bonilla, U. R. Teitelbaum, et al., Phase II trial of the autophagy inhibitor hydroxychloroquine with FOLFOX and bevacizumab in front line treatment of metastatic colorectal cancer, J. Clin. Oncol. 35 (2017) 3545.

[90] B.M. Wolpin, D.A. Rubinson, X. Wang, J.A. Chan, J.M. Cleary, P.C. Enzinger, et al., Phase II and pharmacodynamic study of autophagy inhibition using hydroxychloroquine in patients with metastatic pancreatic adenocarcinoma, Oncologist 19 (2014) 637–638.

[91] R. Xu, Z. Ji, C. Xu, J. Zhu, The clinical value of using chloroquine or hydroxychloroquine as autophagy inhibitors in the treatment of cancers: a systematic review and meta-analysis, Medicine 97 (2018).

[92] N. Wen, Q. Lv, Z.-G. Du, MicroRNAs involved in drug resistance of breast cancer by regulating autophagy, J Zhejiang Univ Sci B 21 (2020) 690–702.

[93] L. Gonzalez-Malerva, J. Park, L. Zou, Y. Hu, Z. Moradpour, J. Pearlberg, et al., High-throughput ectopic expression screen for tamoxifen resistance identifies an atypical kinase that blocks autophagy, Proc. Natl. Acad. Sci. 108 (2011) 2058.

[94] M.Y. Wu, J. Fu, J. Xu, B.W. O'Malley, R.C. Wu, Steroid receptor coactivator 3 regulates autophagy in breast cancer cells through macrophage migration inhibitory factor, Cell Res. 22 (2012) 1003–1021.

[95] L. Galluzzi, I. Vitale, S.A. Aaronson, J.M. Abrams, D. Adam, P. Agostinis, et al., Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018, Cell Death Differ. 25 (2018) 486–541.

[96] X. Yan, R. Zhou, Z. Ma, Autophagy—cell survival and death, in: Z.-H. Qin (Ed.), Autophagy: Biology and Diseases: Basic Science, Springer Singapore, Singapore, 2019, pp. 667–696.

[97] Z. Zhong, A. Umemura, E. Sanchez-Lopez, S. Liang, S. Shalapour, J. Wong, et al., NF-κB restricts inflammasome activation via elimination of damaged mitochondria, Cell 164 (2016) 896–910.

[98] H. Shi, L. Zhang, C. Zhang, Y. Hao, X. Zhao, Rapamycin may inhibit murine S180 sarcoma growth by regulating the pathways associated with autophagy and cancer stem cells 15, 2019, pp. 398–403.

[99] X. Lin, L. Han, J. Weng, K. Wang, T. Chen, Rapamycin inhibits proliferation and induces autophagy in human neuroblastoma cells, Biosci. Rep. 38 (2018) (BSR20181822).

[100] R.Y. Jiang, H.L. Pei, W.D. Gu, J. Huang, Z.G. Wang, Autophagic inhibitor attenuates rapamycin-induced inhibition of proliferation in cultured A549 lung cancer cells, Eur. Rev. Med. Pharmacol. Sci. 18 (2014) 806–810.

[101] E.C. Moore, H.A. Cash, A.M. Caruso, R. Uppaluri, J.W. Hodge, C. Van Waes, et al., Enhanced tumor control with combination mTOR and PD-L1 inhibition in syngeneic oral cavity cancers, Cancer Immunol Res 4 (2016) 611–620.

[102] T.A. Day, K. Shirai, P.E. O'Brien, M.G. Matheus, K. Godwin, A.J. Sood, et al., Inhibition of mTOR signaling and clinical activity of rapamycin in head and neck cancer in a window of opportunity trial, Clinical cancer research: an official journal of the American Association for Cancer Research 25 (2019) 1156–1164.

[103] R.S. Svatek, N. Ji, E. de Leon, N.Z. Mukherjee, A. Kabra, V. Hurez, et al., Rapamycin prevents surgery-induced immune dysfunction in patients with bladder cancer, Cancer immunology research 7 (2019) 466–475.

[104] R. Gedaly, F. De Stefano, L. Turcios, M. Hill, G. Hidalgo, M.I. Mitov, et al., mTOR inhibitor everolimus in regulatory T cell expansion for clinical application in transplantation, Transplantation 103 (2019) 705–715.

[105] M. Ahlmann, G. Hempel, The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy, Cancer Chemother. Pharmacol. 78 (2016) 661–671.

[106] C.M. Huijts, S.M. Lougheed, Z. Bodalal, C.M. van Herpen, P. Hamberg, M. Tascilar, et al., The effect of everolimus and low-dose cyclophosphamide on immune cell subsets in patients with metastatic renal cell carcinoma: results from a phase I clinical trial, Cancer immunology, immunotherapy: CII 68 (2019) 503–515.

[107] A.W. Thomson, H.R. Turnquist, G. Raimondi, Immunoregulatory functions of mTOR inhibition, Nat. Rev. Immunol. 9 (2009) 324–337.

[108] A. Arnaout, S.J. Robertson, G.R. Pond, H. Lee, A. Jeong, L. Ianni, et al., A randomized, double-blind, window of opportunity trial evaluating the effects of chloroquine in breast cancer patients, Breast Cancer Res. Treat. 178 (2019) 327–335.

[109] P. Wang, R. Burikhanov, R. Jayswal, H.L. Weiss, S.M. Arnold, J.L. Villano, et al., Neoadjuvant administration of hydroxychloroquine in a phase 1 clinical trial induced plasma Par-4 levels and apoptosis in diverse tumors, Genes Cancer 9 (2018) 190–197.

[110] B.A. Boone, N. Bahary, A.H. Zureikat, A.J. Moser, D.P. Normolle, W.-C. Wu, et al., Safety and biologic response of pre-operative autophagy inhibition in combination with gemcitabine in patients with pancreatic adenocarcinoma, Ann. Surg. Oncol. 22 (2015) 4402–4410.

[111] B.A. Boone, P. Murthy, J. Miller-Ocuin, W.R. Doerfler, J.T. Ellis, X. Liang, et al., Chloroquine reduces hypercoagulability in pancreatic cancer through inhibition of neutrophil extracellular traps, BMC Cancer 18 (2018) (678-).

[112] J.L. Miller-Ocuin, X. Liang, B.A. Boone, W.R. Doerfler, A.D. Singhi, D. Tang, et al., DNA released from neutrophil extracellular traps (NETs) activates pancreatic stellate cells and enhances pancreatic tumor growth, Oncoimmunology 8 (2019), e1605822.

[113] T.B. Karasic, M.H. O'Hara, A. Loaiza-Bonilla, K.A. Reiss, U.R. Teitelbaum, E. Borazanci, et al., Effect of gemcitabine and nab-paclitaxel with or without hydroxychloroquine on patients with advanced pancreatic cancer: a phase 2 randomized clinical trial, JAMA oncology 5 (2019) 993–998.

[114] J.M. Mehnert, A.D. Kaveney, J. Malhotra, K. Spencer, D. Portal, S. Goodin, et al., A phase I trial of MK-2206 and hydroxychloroquine in patients with advanced solid tumors, Cancer Chemother. Pharmacol. 84 (2019) 899–907.

[115] A.R. Hansen, I.F. Tannock, A. Templeton, E. Chen, A. Evans, J. Knox, et al., Pantoprazole affecting docetaxel resistance pathways via autophagy (PANDORA): phase II trial of high dose pantoprazole (autophagy inhibitor) with docetaxel in metastatic castration-resistant prostate cancer (mCRPC), Oncologist 24 (2019) 1188–1194.

[116] N.B. Haas, L.J. Appleman, M. Stein, M. Redlinger, M. Wilks, X. Xu, et al., Autophagy inhibition to augment mTOR inhibition: a phase I/II trial of everolimus and hydroxychloroquine in patients with previously treated renal cell carcinoma, Clin. Cancer Res. 25 (2019) 2080.

[117] S. El-Chemaly, A. Taveira-Dasilva, H.J. Goldberg, E. Peters, M. Haughey, D. Bienfang, et al., Sirolimus and autophagy inhibition in lymphangioleiomyomatosis: results of a phase I clinical trial, Chest 151 (2017) 1302–1310.

[118] A.M. Lamattina, A. Taveira-Dasilva, H.J. Goldberg, S. Bagwe, Y. Cui, I.O. Rosas, et al., Circulating biomarkers from the phase 1 trial of sirolimus and autophagy inhibition for patients with lymphangioleiomyomatosis, Chest 154 (2018) 1070–1082.

[119] M.R. Rosenfeld, X. Ye, J.G. Supko, S. Desideri, S.A. Grossman, S. Brem, et al., A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme, Autophagy 10 (2014) 1359–1368.

[120] S.B. Goldberg, J.G. Supko, J.W. Neal, A. Muzikansky, S. Digumarthy, P. Fidias, et al., A phase I study of erlotinib and hydroxychloroquine in advanced non-small-cell lung cancer, Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer 7 (2012) 1602–1608.

[121] J. Malhotra, S. Jabbour, M. Orlick, G. Riedlinger, Y. Guo, E. White, et al., Phase Ib/II study of hydroxychloroquine in combination with chemotherapy in patients with metastatic non-small cell lung cancer (NSCLC), Cancer treatment and research communications 21 (2019), 100158.

3.

Dendritic cells-based vaccine as personalized immunotherapy (Review)

Dendritic cells-based vaccine as personalized immunotherapy

Abstract

Dendritic cells (DCs) are a heterogenous population of immune cells responsible for antigens processing and presentation to T lymphocytes. They are divided in several subsets with distinct phenotypic and functional features and can be found in the blood, skin, mucosa, lymphoid and non-lymphoid tissues. DCs have a central role in the development of an immune response both against infectious and malignant diseases, and in the regulation of autoimmune and allergic responsiveness. Considering that DC function is usually impaired in cancer patients, several authors have proposed the development of DC-based antitumor therapeutic vaccines, aiming to restart the immune system and improve the CD4+ and CD8+ lymphocytes responsiveness against cancer. Here, we overviewed the DCs' biology and their role in the antitumor immunity, the distinct protocols for antigen loading of DC-based vaccines, such as the use of tumor cell lysate, nucleic acid transfection, and DC-tumor cell fusion, focusing the clinical trials involving different types of cancer. Overall, there is evidence that DC-based immunotherapy is a feasible approach to personalize the anticancer fight.

I.   Introduction

Dendritic cells (DCs), formerly called Langerhans cells, were first observed by Paul Langerhans in 1868, who described them as cutaneous nerve cells, due to their star shape with membrane branches that resembled neurons [69]. In 1973, Ralph Steinman and Zanvil A. Cohn formally established the name "dendritic cells"[137] in an article that started a series of publications, describing the morphological and functional features that laid the foundation for a new era of studies on DCs, evidencing their ability to activate and stimulate the proliferation of T lymphocytes, in addition to their great capacity for phagocytosis [137-139,141]. Subsequently, the experiments conducted by Sallusto and Lanzavecchia [121] demonstrate that DCs could be differentiated *in vitro* from peripheral blood monocytes, overcoming the technical limitation for understanding the biology of human DCs, as well as for studies on the possibility of manipulation *in vivo* or *ex vivo* of these cells for therapeutic or prophylactic purposes.

The name of these cells comes from the observation of broad branches of its plasma membrane, similar to dendrites or branches of a tree. These cells have an average size between 13 to 15 µm and a very large external surface, due to the ramifications of the membrane. This large surface allows DCs to simultaneously interact and endocyte various soluble or particulate antigens, which will be processed and later presented to T lymphocytes. This high phagocytic and antigenic processing capacity makes DCs the main antigen presenting cell (APCs) [2], being the only cells capable of presenting antigens for naive T lymphocytes and promoting their activation and proliferation [2].

DCs originate mainly from CD34+ bone narrow hematopoietic progenitor cells, stimulated by the Fms-related tyrosine kinase 3 ligand (FLT3L) and GM-CSF [35]. These CD34+ precursors differentiate into common myeloid progenitor cells and common

lymphoid progenitor cells. Vommon myeloid progenitor cells differentiate into monocyte-DC precursors (Mo-DCs), which will turn in to monocytes and common DC precursors that later differentiate into pre-classical DCs (pre-mDC) or plasmacytoid DCs (pDC). These last can also originate from common lymphoid progenitor cells. Both pDC and pre-mDC leave the bone marrow and migrate through the bloodstream to peripheral tissues, driven by chemokines, where pre-mDC differenciate into mDC1 or mDC2, depending on the surrounding cytokines [22] or into Langerhans cells or interstitial dermal DCs, if they reach the skin [149]. Therefore, DCs can be classified into 3 subgroups: plasmacytoid DCs, dermal DCs and myeloid DCs [99] (Figure 1).
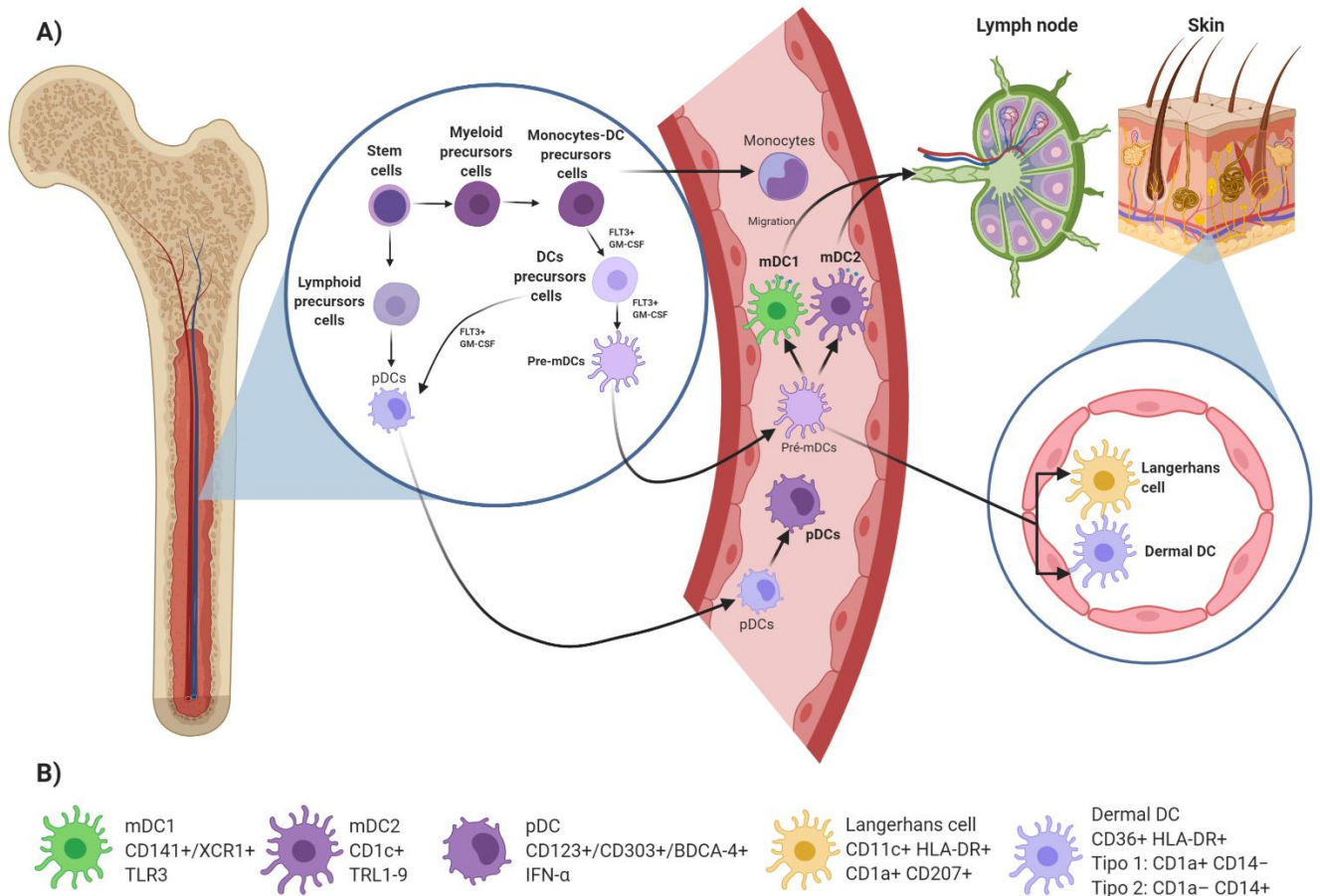
Figure 1. A) Dendropoeisis: Hematopoietic stem cells can differentiate into common myeloid precursor cells and common lymphoid precursor cells. Myeloid precursors differentiate into monocyte-DC (Mo-DCs) precursor cells that further differentiate into monocytes or DCs precursors cells. These cells will give rise to pre-mDCs, which will later differentiate into the main subpopulations of DCs: mDC1 and mDC2. These cells are found in the bloodstream in their immature state, and once they recognize the antigen, they migrate to the lymph nodes to present the antigens to lymphocytes. Those immature DC reaching the skin, differ in Langerhans cells or interstitial dermal DCs. Finally, plasmacytoid DCs (pDCs) can originate from myeloid and lymphoid precursor cells, and can be found in the bloodstream, and in lymphoid and non-lymphoid tissues.

B) Main markers of DC subpopulations: Conventional myeloid DCs (mDCs) can be subdivided into mDC1 and mDC2, differing according to the expression of CD141, CD1c and TLRs. pDCs are characterized by the expression of CD123, CD303, BDCA-4 and IFN-α production. DCs that populate the skin are the Langerhans cells, which express high levels of CD11c, CD207 and HLA-DR, and the dermal DCs that are subdivided according to the expression of CD1a and CD14. FLT3: FMS-related tyrosine kinase 3; GM-CSF: granulocyte and monocyte colony stimulating factor.

Plasmacytoid DCs are characterized by the expression of CD123+, CD303+, BDCA-4+ and CD11c-. These DCs are able to quickly produce high amount of IFN Type

1 (IFN-I) in response to viral infections, due to the high expression of *Toll*-like receptors (TLR) 7 and 9 and are usually associated with the anti-infectious response. They are also capable of activating cytotoxic CD8 T cells [160]. Several autoimmune diseases are associated with high levels of IFN-I, which implies that pDCs play an important role in the development of these diseases, such as psoriasis and systemic lupus erythematosus [102]. In psoriasis, skin lesions are highly infiltrated by activated pDCs with high expression of BDCA-2 [96]. Patients with lupus present a decrease in circulating pDCs and that accumulate as activated cells in the affected tissues, such as the skin [33]. In addition, they induce the production of antinuclear antibodies and express high levels of TRL7/9, thus explaining their high level of IFN-I production [48].

pDCs also play an important role in anti-tumor immunity as showed by Wu et al. [136] who observed that activated pDCs, obtained from mice with breast cancer, can eliminate HER2/Neu+ tumor cells *in vitro*, through the release of TRAIL and granzyme B [157], by activating NK and T CD8+ cells. Another study showed that intratumoral activation of pDCs by means of CpG oligodeoxynucleotides (recognized by TLR9 [152]), activate and recruit NK cells, inducing melanoma tumor regression *in vivo* [79]. In addition, *in vitro* differentiated pDCs have a greater ability to induce NK cells to kill acute lymphoblastic leukemia cells due to the production of higher amounts of IFN-λ2 [30], a type of interferon known to play a critical role in the induction of NK antitumor activity [136].

Dermal DCs, originating from a myeloid precursor, are divided into 2 subgroups, Langerhans cells and interstitial dermal DCs [149]. Langerhans cells are resident cells of the epidermis, typically characterized as CD1a+ and CD207+, being highly relevant in skin infections from which sites they migrate to the lymph nodes to interact with T cells [21]. The interstitial dermal DCs, located in the extracellular matrix of the dermis, are

characterized by expression on the surface of CD36 and the production of coagulation factor XIIIa. These cells are also divided into two subpopulations according to their expression of CD1a and CD14. CD1a+/CD14− cells stimulate and activate CD8+ T cells while CD1a-/CD14+ cells are specialized in stimulating the humoral immune response, producing CXCL13 and inducing the generation of follicular helper T cells (Thf)[146,149].

Myeloid DCs or "conventional" DCs are subdivided into 2 groups according to the differential expression of CD1c and CD141. mDC1 express CD141, but not CD1c, while mDC2 express CD1c+/CD141-. In addition, mDC2 also express major histocompatibility complex (MHC) class II antigens (MHC-II), CD11c, SIRPα, TRLs (1 to 9), and produce several inflammatory cytokines [97]. They are distributed in the bloodstream and in   lymph nodes [47], generally associated with the presentation of antigens for CD4 T cells [61]. They produce interleukins (IL)-12p70, IL-1β, IL-6 and IL-23 and are capable of inducing Th1 and Th17 lymphocytes [73]. Finally, they express high levels of dectin-1/2 (CLEC7A / CLEC6A)[81], type C lectin receptors and mannose receptors that recognize fungal PAMPs[54]. For instance, DCs challenged *in vitro* with *Paracoccidioides brasiliensis* fungal antigens recognized them through mannose receptors, inhibiting the production of TNF-α [36]. In a subsequent study, they stimulated DCs via TLR 9 (that recognizes fungal PAMPS) increasing their antifungal capacity by producing large amounts of hydrogen peroxide [151].

The mDC1, like all professional antigen presenting cells, capture antigens and process them by the endocytic pathway, which leads to the presentation of peptides associated with MHC-II. Tumor-associated antigens, generated inside tumor cells, are processed by the cytosolic pathway, undergoing degradation in the proteasome, whose peptides pass to the endoplasmic reticulum with the aid of transport proteins TAP-1 and

TAP-2. Within the reticulum, the components of the antigen processing machinery, tapasin, calnexin, calreticulin and ERp57, promote the accommodation of peptides in the α heavy chain gap of nascent MHC-I, as well as the link with the β2-microglobulin light chain, for subsequent expression on the surface of tumor cells [74]. These antigens present on the surface of tumor cells are the priority targets for CD8+ cytotoxic T lymphocytes. However, for such immune cells being generated, it is necessary that the tumor antigens are previously captured and processed by the APCs, which will present them to the lymphocytes. Thus, dead tumor cells, their fragments or soluble antigens secreted by tumor cells are endocitated by professional APCs and processed inside phagolysosomes where are degraded by lysosomal enzymes into peptides that remain inside the vacuoles. These vacuoles fuse with others that contain nascent MHC class II molecules, in which gap formed by the α and β chains, those peptides are accommodated [44], to be further recognized by CD4 + lymphocytes. An essential characteristic of mDC1 is its ability to present exogenous antigens in association with MHC-I, a phenomenon called cross-priming or cross-presentation, which is now also recognized in macrophages [94].

Cross-priming basically consists of transferring peptides processed by the endocytic pathway to subcellular compartments involved in the association of cytosolic peptides with MHC-I molecules. This phenomenon was observed for the first time in the MHC compatibility experiments by Bevan et al. [11] in which they injected mice with splenic cells from another mouse with minor antigens with different histocompatibility. The authors observed that these minor antigens induced the generation of specific cytotoxic T lymphocytes (CTL) and concluded that cells of the receptor mouse could activate its immune system, presenting the minor exogenous antigens present in the splenic cells of the donor animal. Therefore, there was the presentation of peptides from the donor's MHC (exogenous antigen), associated with molecules of the receptor's MHC,

an event that received the name of *cross-presentation* [11,18]. Years later, Rock et al. [115] showed that only DCs can process and present extracellular antigens associated with MHC class I and just recently this ability was also observed in macrophages [94].

Currently there are two models considered on the process of transferring the peptides to the compartments of the cytosolic processing pathway, referred to as the cytosolic pathway and the cross-presenting vacuolar pathway. In the cytosolic pathway model, antigens are released from the lumen of the phagocytic compartments to the cytoplasm, where they are later processed by proteasomes [12], then following the same path as the processing of endogenous antigens. In the vacuolar model, extracellular antigens are internalized and degraded in the endosomes by lysosomal enzymes and the resulting peptides are linked to the MHC-I molecules present in those same endosomes, in a process similar to what occurs with the presentation via MHC-II, regardless the action of TAP molecules and involving the activity of cathepsins [107,128].

Once the antigen is transported and expressed in the MHC-I of DCs, it interacts directly with the T cell receptor (TCR) of the T cells that use the CD8 molecule to stabilize this interaction [1]. This binding is increased by the co-stimulation molecules CD80 and CD86 on DCs that bind to the CD28 of CD8+ T cells [70]. The stimulation of TCR and CD28 leads to the activation of the MAPK [122] and mTOR [109] pathways, which regulate cell growth, proliferation and metabolism, which results in the expansion of these lymphocytes [49].

All myeloid DCs can cross-present, but mDC1 is considered the "expert" in this process [98], not only because of its high expression of MHC-I [145] but also because of the expression of high levels of CLEC9A and TLR3 [123]. TLR3 mainly recognizes viral PAMPs [124] and, when activated, there is an increase in the production of IFN-α/β [124],

stimulating the expression of MHC-I [46] and TAP1 [3]. CLEC9A, in turn, is an endocytic receptor that recognizes actin filaments from necrotic cells [162] and its main function is to transport the captured exogenous antigens to the endosomes containing MHC-I, promoting cross presentation [123,162].

Also, mDC1 can be found in lymphoid and non-lymphoid tissues in the immature state (iDC), a phase characterized by high expression of intracellular MHC II, low expression of co-stimulatory molecules and chemokine receptors[2]. In addition, iDCs have the ability to capture and absorb antigens through endocytosis, pinocytosis and phagocytosis superior to that of their mature counterpart. This characteristic allows these cells to constantly patrol the internal environment, looking for and being activated when they recognize foreign antigens [2]. This activation occurs after the recognition of damage-associated molecular patterns (DAMP) or the stimulation of pattern recognition receptors (PRR), such as TLR. These signals initiate metabolic transcription programs, positively regulating chemokine receptors such as CCR7, which allow the migration of DCs from peripheral tissues to the T-dependent areas of secondary lymph nodes, where mature DCs present the processed antigens to naive T cells [112]. During this maturation process, DCs decrease their phagocytic, adhesion capacity and reorganize their cytoskeleton, increasing their mobility. The main change observed in mature DCs is the increase in the expression of MHC-I/II and co-stimulatory molecules, such as CD40, CD80 and CD86[137].

In common, all subpopulations of DCs have the ability to constantly communicate with other cells of the immune system. This communication can be achieved through direct interaction between receptors and cell surface ligands [1] or indirectly and remotely through cytokines. DCs can produce different types of cytokines depending on the stimulus received or the environment in which they are found, the most relevant being

IL-12, IL-23, IL-27, IL-6, IL -1, the tumor necrosis factor alpha (TNFα) and interferon alpha (IFN-α) [85].

In the antitumor response the production of IL-12 is prominent. This cytokine is composed of two covalently linked subunits, IL-12p35 and IL-12p40, constituting the bioactive form IL-12p70 [38]. Its main action is to induce the production of IFN-γ by CD4 + T cells (generating a Th1 response), NK and CD8+ T cells [50]. Also, increases the cytotoxic capacity, proliferation and migration of CD8+ T cells [50] and NK cells [104] favoring the effector phase of antitumor activity. In addition, IL-12 has anti-angiogenic activity, through the production of INF-γ, which in turn increases the production of interferon-γ inducible protein-10 (IP-10) [127], an inhibitor of neovascularization that blocks proliferation of endothelial cells [17,41], improving the anti-tumor activity.

TNF-α is a potent pro-inflammatory cytokine, produced mainly by macrophages, but it can also be produced by NK cells, B lymphocytes and DCs [103]. Among the sort of stimuli to induce TNF-α expression by DC are viral and bacterial infections, binding to CD40L, oxidative stress, heat shock proteins, HMGB1 and interaction with activated NK cells [125]. TNF-α has an autocrine function and affects DCs in several ways, for example, improving the viability and migration of Langerhans cells [24] by increasing MHC-II expression in DCs [131] and promoting their maturation/activation [84]. Most cell types can produce this cytokine in response to virus infection [37], but pDCs, which express high levels of TLR7/9 and IRF-7, are the main DCs that produce this cytokine [8,37].

IL-1 is released by macrophages, monocytes and DCs in response to TNF-α [77]. It can be found in three forms, IL-1α (intracellular), IL-1β (extracellular), and IL1-RA.

IL-1 α and β have pro-inflammatory effects due to the release of histamine by mast cells, causing vasodilation and signs of localized inflammation, in addition to having chemotactic activity on neutrophils. The third form IL-1RA, has inhibitory activity and acts by preventing the binding of IL-1α and β to their respective receptors [77]. IL-1 also stimulates the production of IL-6, another cytokine produced by DCs, macrophages, T cells, endothelial cells and fibroblasts [143], which induces the production of transforming growth factor (TGF)-β, and the the differentiation of naive CD4+ T cells in Th17 [66,143], stimulating the differentiation of follicular helper T cells and, thus, increasing the production of IL-21 [163].

In contrast to the mentioned cytokines, IL-10 is a cytokine that is known for its multiple inhibitory and immunoregulatory effects [119]. In fact, this cytokine can be considered a marker of suppressive DC because, in addition to its low capacity for presenting antigens, the DCs that produce this cytokine suppress the capacity of other DCs to do it [156]. IL-10 acts by negatively regulating the expression of inflammatory cytokines in Th1 cells, the expression of MHC-II and co-stimulatory molecules in APCs[51]. In addition, in conjunction with TGF-β, it stimulates the differentiation of regulatory T cells (Treg) via Foxp3[53], thus establishing an inverse correlation with IL-12 and limiting the antitumor response [113,114].

II. Dendritic cells-based anti-tumor vaccines

During the early stages of carcinogenesis, tumors have the ability to modify and silence the immune response, especially because they do not express relevant antigens to activate the immune system [88], some of them can produce   regulatory cytokines

[31,45,59]. So, the use of DC-based vaccines primarily intend to correct this state of anergy, reactivating the immune system and stimulating it to recognize tumor cells.

The first clinical trial of DC-based vaccines in immunotherapy was published in 1996 by Hsu et al. [52], in which four patients with follicular B-cell lymphoma received a series of three subcutaneous injections of DCs pulsed with antigens obtained from biopsies of the lymph nodes in conjunction with KLH (Keyhole limpet hemocyanin), a highly immunogenic protein. The authors observed that the four patients developed an immune response against KLH, one of which showed complete tumor regression and the other a partial regression [52].

Despite the large number of studies conducted for the development of therapeutic anti-tumor vaccines of DCs and that several medical centers around the world use these vaccines clinically, there is still no consensus protocol nor a gold-standard for sensitizing these cells. Some groups advocate the use of CD34+ hematopoietic progenitor cells differentiated *in vitro* in DC by culture with Flt3-L[34], but the most widely used protocol and with the largest volume of clinical data is based on the *in vitro* differentiation of monocytes isolated from mononuclear cells of peripheral blood (PBMC) obtained by leukopheresis [118]. These cell suspension, rich in monocytes, obtained by adhering to plastic or by selecting CD14+ is differentiated into immature DCs by the action of recombinant cytokines, classically GM-CSF and IL-4 [19] (Figure 2).
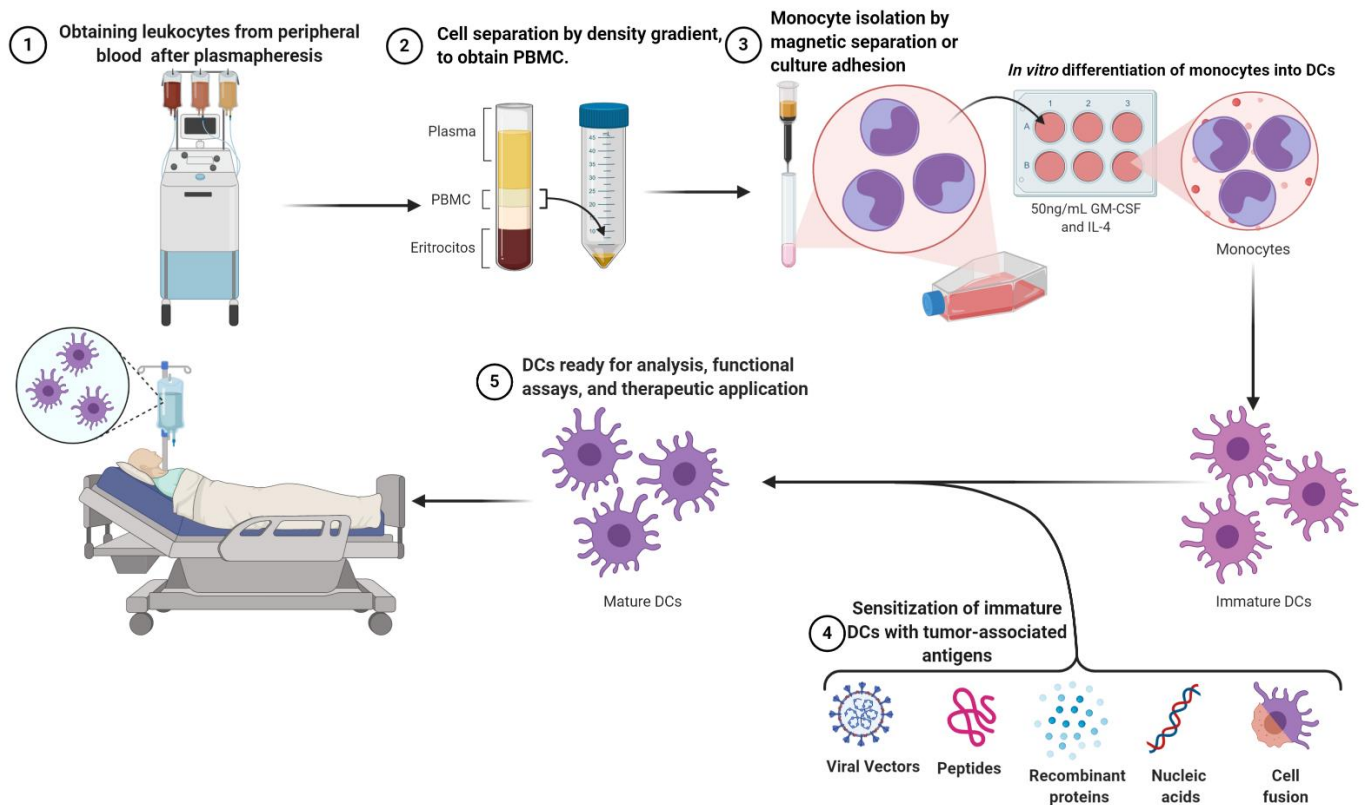
Figure 2. Basic protocol for *in vitro* differentiation of peripheral blood monocytes into dendritic cells. Leukocyte-rich samples from the patient, obtained by plasmapheresis, are submitted to separation by density gradient centrifugation to obtain mononuclear cells (PBMC). This suspension is then enriched to monocytes by adhesion to the plastic surface of culture plates, or by selection of CD14+ cells by magnetic separation or cell sorting. These cells are then cultured with recombinant GM-CSF and IL-4 for 5 days, to obtain the iDC. The maturation and activation of these cells is induced by exposure to tumor-associated antigens. This step can be optimized by the addition of different cytokines or growth factors. Two days later, these cells are activated and ready to be inoculated in patients.

Anguille et al. [5] compared the classic protocol described above with their own, in which IL-4 was replaced by IL-15 [5], followed by culture for 5 days and subsequent activation of maturation by the addition of a mix of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, PGE2) and a soluble cocktail of TRL 7/8. The authors found that the DCs in their protocol showed superior expression of CD83, CD40, and co-stimulation molecules (CD80, CD86) in addition to secreting more IFN-γ and IL-12p70. In another protocol, authors proposed to combine IL-4 with IL-3, instead of GM-CSF, based on the fact that monocytes have receptors for this interleukin and DCs use IL-3 to proliferate [82].

However, this combination generated DCs that produced less IL-12p70 and more IL-10 compared to those differentiated with GM-CSF or by co-culture with allogeneic CD4+ T cells, as well as less ability to induce IFN production-γ [32]. In addition, the use of pro-inflammatory cytokines such as TNF-α and IFN-γ to improve the activation of iDCs is an option to be considered, since the maturation stimulation is evidenced by the increased expression of surface markers such as CD83, CD86, HLA-DR, CD40 and CD25 [20,27].

In our previous studies we have shown that selected chemotherapeutic agents can also stimulates the maturation of DCs [95]. We observed that low non-cytotoxic concentrations of chemotherapeutic drugs such as vinblastine, paclitaxel, azacytidine, methotrexate and mitomycin C increase the expression of CD83, CD40, MHC-II and the production of IL-12p70 in DCs generated *in vitro* [63,129]. We have also observed that paclitaxel (PTX) and doxorubicin (DOX) in low concentrations increase the expression of the antigen processing machinery components, such as calmodulin, LMP2, LMP7, TAP1 and tapasin [62] by human colon cancer cells HCT-116.

The protocols for DCs sensitization may vary according to the nature of the antigen, with purified proteins, recombinant proteins, peptides, and nucleic acids being commonly used. Purified proteins and eventually peptides, are usually obtained by lysing tumor cells from the patient himself, aiming to obtain the largest variety of unique antigens of the individual to sensitize autologous DCs. The *rationale* of this approach is to enable the stimulation of a specific immunity to the repertoire of unknown antigens found in the patient's cancer [75]. In addition to its relatively low cost, the main advantages of using autologous tumor lysate are the absence of restriction by HLA and the short preparation time, compared to artificial strategies for the production of neoantigens, in contrast with known peptides that just can be loaded on specific class I molecules.

In our group, we used tumor cell lysates to assess whether the treatment of tumor cells with low concentrations of chemotherapeutic agents increases the immunogenicity of antigens expressed on the surface. For this, DCs generated *in vitro* were exposed to the tumor lysate of colorectal cancer cells HCT-116 pretreated with low concentrations of PTX or DOX. These DCs were then co-cultured with autologous lymphocytes for two weeks, and subsequently tested for the presence of specific HCT-116 cytotoxic cells. We found that DCs treated with this lysate induced cytotoxic T cells (CTL) with higher cytotoxic activity than DCs loaded with untreated tumor cell lysate [62]. Using this same protocol, in another study we observed, that the lysate of HCT-116 cells previously treated with 5-fluororacil and chloroquine (autophagy blocker) increases the expression of DC maturation markers (CD80, CD83, CD86 and HLA- DR), as well as its ability to stimulate the proliferation of TCD8+ and TCD4+ cells specific for colorectal cancer cells, in addition to increasing the expression of IFN-γ and decreasing the expression of IL-10 [161].

Tumor lysates have also been used in a phase I clinical study in pediatric patients with high-grade gliomas to sensitize DCs *in vitro*. These DCs were then used as therapeutic vaccines and, after the administration of nine doses, the patients showed peak production of IL-4, IL-6, IL-10 TNF-alpha and INF-γ on the 28th day, but without a clinical improvement directly related to the vaccine [71].

Recombinant proteins used on DCs sensitization protocols are usually chimeric or fused proteins with multiple epitopes that have the ability to stimulate the immune response more intensely, in addition to not being restricted by HLA, like some peptides. In general, these proteins are highly expressed in some types of cancer, such as the mannan-MUC1 fusion protein (MFP), present in several adenocarcinomas [72]. In a phase I clinical study, ten patients with different types of adenocarcinomas (lung, ovary,

kidney, breast, colon and stomach) with high expression of MUC1 were treated with autologous DCs pulsed with this fusion protein. After 3 monthly doses of the vaccine, it was observed an increased production of IFN-γ, and stabilization of the disease in those patients with ovarian and kidney progressive cancer, although high levels of anti-MUC1 antibodies were only observed in 3 patients [80].

Another recombinant protein to be considered is melanoma-associated antigen 3 (MAGE-A3), which is an antigen highly expressed in melanomas and non-small cell lung cancer, neuroblastomas, sarcomas, bladder cancer and hepatocarcinomas [116]. In a clinical study, tumor regression was observed in six melanoma patients treated with three doses of subcutaneous injections of a MAGE-A3 protein for three months [87]. In a subsequent study, this protocol was tested in 39 patients with metastatic melanoma in stages III and IV. Fourteen of them experienced fast regression of the disease just following the second dose [86]. These promising results stimulated researchers to create a recombinant protein called MAGE-A3 CI, synthesized by combining MAGE-A3 with adjuvant AS15 [68]. The anti-tumor and immune-stimulating activity of MAGE-A3 CI has been demonstrated in several studies, including a clinical trial, conducted in 71 patients with small cell lung cancer, in which anti-MAGE-A3 antibodies were detected in all subjects. In addition, more than half of the patients had elevated levels of specific TCD4+ and TCD8+ cells. In another study, 25 patients with stage IIB and IV MAGE-A3+ melanoma were treated with MAGE-A3 CI, administered intramuscularly (13 patients) or intradermally (12 patients), hoping to directly stimulate conventional DCs, dermal DCs and Langerhans. It was found that one patient in each group showed a specific proliferative response of TCD8+ lymphocytes against MAGE-A3. Also, four patients in the first group and seven in the other showed increased proliferation of TCD4+ lymphocytes. The proliferation of conventional DCs, dermal DCs and Langerhans cells

did not vary between administration routes, although the intradermal route produced a greater response of TCD4+. Finally, both pathways were well tolerated, and all patients generated antibodies specific for MAGE-A3 (NCT01425749) [132].

Viral transfection has also been used to sensitize DCs. For example, in the study by Di Nicola et al. the tyrosine kinase gene was inserted into the vaccine virus of Ankara (attenuated version of the poxvirus) aiming to transfect DCs with this gene, since tyrosine kinase is one of the tumor antigens associated with melanoma. This vaccine was tested in six patients with malignant melanoma in stages 3 and 4, with a gradual increase in TCD8+ specific cells for the tumor antigen and an increase in IFN-γ in the bloodstream of patients from the 5th day after injection [29]. Another virus used as a transfection vector is the adenovirus, more specifically the virus with defective replication that encodes the MART-1, a melanoma antigen. This is an immunogenic antigen, normally expressed in normal pigment cells, which is exacerbated in malignant cells, including melanomas. This virus was used to sensitize DCs of 14 patients with metastatic melanoma, aiming to activate MART-1-specific T CD8+ and CD4+ cells. After administering three doses of 10^7 DCs, authors observed an increase in serum levels of IFN-γ as well as in the number of circulating MART-1-specific CD8+ T cells, compatible with the stabilization of the disease in four out of 14 patients, and with the increased mean survival time from 27 to 42 months in other 4 patients [16].

DCs can also be sensitized with artificial vectors, such as artificial adjuvant vector cells (AAV), which are fibroblasts artificially expressing α-galactosylceramide (α-GalCer), a structure morphologically similar to CD1d, able to stimulate invariant NK cells (iNK). This NK subset is characterized by highly expressing the invariant αβ chains of the T cell receptor (TCR) and producing IL-4 and IFN-γ, being able to stimulate a Th1 response [14]. Unlike conventional T cells, which recognize peptides linked to MHC

molecules, iNK cells recognize glycolipids linked to non-polymorphic MHC class I molecules, called CD1d [67]. When iNK recognize the AAVs they destroy these cells and, in addition, they activate DCs that phagocytize the cellular fragments of these vectors [39]. In an experimental *in vivo* trial, the group observed an increase in IFN-γ and a reduction in tumor mass [40], while in the phase I/II clinical study, 17 out of 18 patients with advanced lung cancer treated with AAVs had increased production of IFN-γ, with increased survival of 18 months higher than the control patients [93].

The transfection of nucleic acids with non-viral vectors and their subsequent protein expression in DCs has also been widely used. The technique consists in isolating genetic material from tumor cells, joining them to a non-viral transfection vehicle that introduces this information into the DCs, which can later express the corresponding proteins for antigenic presentation, allowing tumor antigens (exogenous), usually presented in association with MHC class II molecules, are now presented in the context of class I molecules (after cytosolic processing)[90]. In our previous studies, we transfected iDC with total RNA from HCT-116 colon cancer cells, previously treated with 5-fluorouracil (5-FU). We observed that these DCs showed increased expression of maturation and co-stimulation markers (CD83, HLA-DR, CD80 and CD86), compared to DCs transfected with RNA from untreated tumor cells. In addition, we observed a functional improvement in DCs, evidenced by the increased capacity to induce the proliferation of allogeneic T cells, improved generation of HCT-116-specific CD8+ T cells, and increased *in vitro* production of IFN-γ (116). In a similar study, using an animal model, we observed that mice treated with DCs sensitized with total RNA from tumor cells exposed to 5-FU, significantly reduced the growth of tumor cells implanted subcutaneously [25]. In a phase II clinical study, 43 patients with castration-resistant prostate cancer, previously treated with docetaxel, were treated with autologous DC

vaccines transfected with mRNA encoding prostate specific antigen (PSA) and prostate acid phosphatase (PAP). The authors observed that the mean survival time and progression-free survival time slightly increased compared with patients treated with docetaxel alone, with 78% of patients showing a specific response to PSA and PAP [65].

Another method used to sensitize DCs, with very promising results, is the creation of hybridomas composed of DCs and tumor cells. This allows DCs to be exposed to a wide range of tumor-associated antigens (ATTs) from autologous tumor cells [142] and to process them endogenously and present peptides simultaneously through MHC class I and II, resulting in the activation of both TCD4 + and TCD8+ cells [64]. To demonstrate this, Pinho and Barbuto et al. used breast cancer cells that constitutively express a cytomegalovirus (CMV) antigen and fused them by electrofusion with moDCs. The resulting hybrid cells expressed CD80, CD86, CD83, HLA-DR and MHC-I in higher levels than the unfused DCs. In addition, hybrid DCs induced the expansion of CMV-specific cytotoxic T lymphocytes (CTL) and a high production of IFN-γ when exposed to CMV+ tumor cells [108]. Clinical trials using this technique in cancer patients have had similarly promising results, for instance in patients with kidney or metastatic breast cancer. Hybrid DCs were inoculated in 23 patients, with stabilization of the disease observed in five patients with breast cancer and five with renal cancer. The disease regressed almost completely in two of the patients with an intense reduction in tumor masses. In addition, in most patients, a large number of CD4+ and CD8+ T cells expressing IFN-γ were found [9]. In another phase I/II study, DCs generated *in vitro* were fused with glioma cells from 32 patients with gliobastoma and inoculated intradermally in their cervical region. The patients were divided into 2 groups, one consisting of patients with recurrent temozolomide-resistant gliobastoma (n=10), and the other with recently diagnosed patients (untreated patients; n=22). Therapy was well tolerated in both groups,

and in those temozolomide-resistant patients the average of progression-free survival and overall survival were 10.3 and 18.0 months. In contrast, in the recently diagnosed patients, these times were much higher, with averages of 18.3 and 30.5 months, respectively. In both groups, results were superior to those of conventional temozolomide therapy [117]. In addition, in the resistant group, hybrid DCs have been shown to have specific immune responses against chemoresistance-associated antigens (WT-1, gp-100 and MAGE-A3) highly expressed in temozolomide-resistant gliomas [4].

Thus, although there is no standard protocol for the preparation DCs for clinical use, all vaccine proposals have their low toxicity as a very positive common point. According to most reports, the most common side effects in clinical trials were grade 1 or 2 events, including fever, pain and inflammation at the injection site [6], enabling safe use in different types of cancer, as reported below.

1. Prostate cancer

Prostate cancer is the second most prevalent cancer in men, with an estimated incidence of 65,840 cases in 2020 in Brazil [55]. For diagnosis, prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) are used, both almost exclusively expressed in prostate epithelial cells and increased in cancer tissues, therefore, both represent the most commonly used serum markers for diagnosis and for monitoring prostate cancer [158]. Most patients are diagnosed when the tumor is still delimited by the organ, so radical prostatectomy, radiotherapy and chemotherapy are effective modalities of treatment in such cases [78]. However, almost 30% of patients experience cancer recurrence [153], motivating the search for therapeutic vaccines. One of the first attempts of anti-tumor immunization was based on the affinity of PSA peptides to HLA-A2 [101]. It has been demonstrated the possibility of simultaneous induction of specific cytotoxic T cells for different epitopes, by combining several known PSA peptides obtained from

patient samples, linked by an oligopeptide. However it depends on the patient's HLA panel for effectiveness [23]. The first results of autologous DC vaccines were hopeful, since a decrease in serum PSA levels was observed without side effects [159].

Years later, in 2010, the FDA would approve the first therapeutic vaccine based on DCs, the Sipuleucel-T, after 4 years of clinical studies. This vaccine is composed of autologous DC originated from monocytes obtained by leukopheresis and sensitized with a recombinant fusion protein of GM-CSF and PAP. In the first two phases of clinical studies involving 31 patients with metastatic prostate cancer, side effects were not reported, and a decrease in serum PSA levels was also found [133]. Then, in the phase III randomized clinical trial, 127 patients with metastatic prostate cancer were evaluated. After 36 months of treatment 34% of the patients were alive, compared with 11% in the control group, who had average survival of only 4.5 months. The mean T cells counting was eight times higher in treated patients than the controls [135]. These results were so well received that their use as therapy in conjunction with other first line chemotherapy [111,134] (NCT01981122) and radiotherapy [147] (NCT01818986) also had remarkable results. Most current clinical studies work in the same way, always using DC-based vaccines in combination with other antineoplastic agents, including immunotherapeutics such as Nivolumab or Pembrolizumab (PD-1 blockers) (NCT03525652, NCT03600350, NCT04090528) [155].

2. Breast cancer

Breast cancer represents approximately 25% of all cancers in the world [130]. In 2020, 66,280 new cases of breast cancer are estimated in Brazil. In addition, the risk of this disease in women is estimated between 10% and 12.8% [55]. The prognostic markers most commonly used for the diagnosis of this disease are estrogen hormone receptors (ER), progesterone (PR) and human epidermal growth factor receptor (HER2), which are

usually expressed on the surface of breast cancer cells [57,105]. In recent years, mucin 1 (MUC1) has been added to the marker panel, as it is overexpressed in patients with poor prognosis [60]. The main methods used in therapy against breast cancer are surgery, radiotherapy, chemotherapy and hormonal therapy [91]. The most widely used DC vaccine against breast cancer was Lapuleucel-T, which uses a principle similar to the Sipuleucel-T, with HER2 recombined to the adjuvant GM-CSF. It was observed that the 18 patients reaceiving this DCs tolerated the therapy very well, with no adverse effects and without increasing the T lymphocyte counting [106]. However, one of the disadvantages of this vaccine was that its effectiveness depends on the expression of HER2 or MUC-1 in the cancerous tissues of patients [89]. Therefore, in patients whose tumor has low expression of these markers, it has been proposed to use DC sensitized *in vitro* with autologous tumor lysates, resulting in high production of specific CTL and production of IFN-γ *in vitro* [28], as well as proliferation of T CD4+ and TCD8+ cells [42]. Currently, clinical trials use DC vaccines pulsed with HER-2 peptide with neoadjuvant chemotherapy (NCT03387553, NCT02063724, NCT02061423) and new approaches like the use of neoantigens (synthetic peptides formed from patient biopsies) to pulse autologous DC (NCT04105582).

3. Colorectal cancer

The most recent statistics indicate that colorectal cancer (CRC) is the third most common cancer in the world [130] and will affect more than 40,990 people by the end of 2020 in Brazil [55]. Conventional therapy for patients with CRC is based on total or partial colectomy, usually followed by adjuvant or neoadjuvant chemotherapy [15]. Although 80% of CRC patients treated with surgery and chemotherapy have a mostly favorable response [144], a recent study shows that 50% of CRC cases treated with the mentioned procedure have tumor recurrence [126]. Under this premise, the use of

immunotherapy as a complementary treatment to combat this disease was evaluated. In the past decade, there have been relatively few studies of DC vaccines applied to CRC compared to other cancers.

The three largest completed studies were randomized phase II studies performed on patients with colorectal cancer who underwent surgical metastasis resection, followed by DC vaccination. In chronological order, the first involved ten patients with CRC and liver metastases treated with three doses of vaccines of DCs pulsed *in vitro* with CEA peptide (carcinoembryonic antigen), intradermally and intravenously before resection of liver metastases. Liver biopsies from seven out of 10 patients revealed a high number of CEA-specific T cells with production of large amounts of IFN-γ. Another interesting finding was the presence of CEA-specific T cells in lymph nodes close to liver metastases in one patient, but not in peripheral blood [76]. In the second study, 26 patients with liver metastases from CRC, received randomly DC vaccines previously activated by the soluble CD40 ligand (CD40L), with a five-year follow-up. When CD40L binds CD40 on the surface of DCs, it stimulates the production of IL-12, activating T cells towards the Th1 profile [83]. *In vitro* analysis showed that, although the addition of CD40L did not increase the expression of MHC I or II in DC, it increased the expression of CD86. Unfortunately, these findings were not sufficient to improve the patient's immune response or recurrence-free survival (RFS), which was 58% in the first year and 38% in 5 years. Thirty-three percent of treated patients had a favorable response against the tumor, one week after vaccination, with increased levels of IFN-γ, and these same patients had a longer time to recurrence (63%) in 5 years than those with low IFN-γ levels (18%). Unfortunately, these findings were not significantly better than those observed in the control group [10].

In the third study, two types of treatment were applyied, the first using DCs treated *ex vivo* with a viral vector encoding CEA and MUC1 called "PANVAC" (DC/PANVAC) and the second directly injecting PANVAC together with GM-CSF (PANVAC-P) in 76 patients with metastatic CRC. The results show that the RFS was similar between the two groups (47% DC/PANVAC vs 55% PANVAC-P). A 2-year overal survival increased in both groups, achieving being 94% of patients in the DC/PANVAC group and 87% of patients in the PANVAC-P group. Finally, the specific response of T cells against CEA was similar in both groups [92]. The results indicate that the best results are achieved with the use of DCs vaccines as complementary postoperative therapy. Since the tumors are different in each patient, the use of lysates obtained from tumor biopsies as a source of the unique tumor antigens of these patients would increase their efficiency (NCT02919644, NCT04147078).

4. Other types of cancer

The clinical efficiency of DC-based vaccines has been tested in all types of cancer at different stages [43,150], among them, one that has shown promising results was melanoma. In the phase II study by Boudewijns et al. [13] patients with melanoma in stage III (n=22) and IV (n=32) were included. These patients received three doses of DC vaccines transfected with gp100 mRNA and tyrosinase (tumor antigens highly expressed in melanoma) treated or not with cisplatin [26]. The best results were observed in patients treated with DCs transfected in combination with cisplatin, showing an increase in T CD8+ cells in 56% of patients in phase IV compared to the isolated application of DCs (40%). However, there was no significant difference between the protocols applied to patients in phase III. In another randomized study, still in progress, 144 patients with high-risk melanoma in stages III and IV were treated with pre-sensitized DCs with autologous tumor lysate for 18 months. Preliminary results show that the application of

of sensitized DCs significantly improves the disease-free survival time (51.8% vs 27.1% of control group) and the overall survival (92.9% vs 70.3%). Stage IV patients showed a 49.7% increase in free survival compared to 29.4% for placebo control, and stage III patients showed a 68.6% increase compared to 9.4% of placebo (NCT02301611).

Similar results were observed in the treatment of glioblastoma, one of the cancers with the highest rate of complications in surgery [58], indicating that the use of therapeutic DC vaccines is one of the alternatives to be considered for the treatment of this disease. In the randomized, phase II study with placebo by Wen et al., 124 patients with newly diagnosed glioblastoma were divided into 2 groups, the first (n=81) treated with DCs sensitized with tumor antigens expressed in glioblastoma (AIM2, MAGE1, TRP-2, HER2, IL-13Rα2 and gp100) and the second (n=43) treated with placebo. Patients received four DCs applications per week for 12 months, which increased the overall survival time by 2.2 months and the progression-free survival time by 2 months compared to the placebo group [154].

Since conventional glioblastoma therapy consists of resection of the tumor (when possible) followed by radiotherapy and temozolomide, there is a low overall survival of 15 months on average [140]. In a phase II study with 32 newly diagnosed patients with low residual tumor volume after resection, a vaccine for DCs pulsed with autologous tumor lysate was combined with conventional therapy. DCs treatment started before radiotherapy and continued during treatment with temozolomide. The results showed a median overall survival of 23.4 months, a time almost nine months longer than that observed in patients treated with only conventional therapy. In addition, the authors observed an increase in the proliferation of TCD8+ cells, and the production of IFN-α and IL-12 after vaccination in 11 patients [56].

Some forms of leukemia have also been investigated for the feasibility of using DC vaccines, such as acute myeloid leukemia (AML)[100]. In a phase II study, the effect of DC vaccines transfected with WT1 mRNA, an antigen overexpressed in AML[110], was investigated in 30 geriatric patients with a very high risk of relapse. The anti-leukemic response was evidenced by the decrease in the blast counting in peripheral blood and stabilization of red bone marrow in 13 patients, four of them also stabilized the disease. The overall survival rate was higher in the 13 patients who responded to treatment, compared to those who did not respond (53.8% vs. 25.0%). To demonstrate a correlation between clinical response and immune stimulation caused by DC vaccination, the frequency of cytotoxic T cells and production of cytokines were evaluated. The authors observed an increased proliferation of WT1-specific TCD8+ cells and an increased production of IFN-γ and TNF-α in all patients, especially in those who positively responded to treatment [7].

WT1 is also overexpressed in acute lymphoid leukemia (ALL) [148], and its peptides have been used to sensitize DCs for the treatment of ALL in pediatric patients. In the clinical case reported by de Saito et al. a 15-year-old girl received a hematopoietic stem cell transplant and 14 doses of allogeneic DCs-vaccine, sensitized with WT1 antigen (the donor was her sister with compatible HLA). The data show that WT1-specific TCD8+ cells were detected since the seventh vaccine application and the proliferation of those cells increased following subsequent vaccinations. In addition, authors observed the remission of blasts in the peripheral blood after the fourth dose of vaccine. which was mantained for 44 months [120].

III.   Conclusion

These data allow us to conclude that DC-based anti-tumor vaccines are safe, and their production is feasible in many medical centers. Compared to other more invasive protocols that involve serious side effects, the use of DCs as therapeutic vaccines rarely induces side effects when combined with other therapies. This feature allows these therapeutic vaccines to be used as complementary therapy for cancer patients, especially in those with difficult surgical excision or for prophylaxis of relapsing diseases. Although the clinical use of these vaccines is not definitive, mainly due to the large variety of protocols, which makes difficult to standardize it in a scale, finding new ways to increase the immunogenicity of tumor antigen sources and new combinatorial therapies with DCs remain a challenge for making the clinical application of these vaccines more efficient and feasible.

IV.   References

1.   Abbas A.K., Lichtman A.H. and Pillai S. Chapter 6: Antigen Processing and Presentation to T Lymphocytes. Cellular and molecular immunology, 2017.
2.   Abbas A.L., Andrew. Chapter 1: Properties and Overview of Immune Responses. Cellular and Molecular Immunology 8th Edition, 2017.
3.   Agrawal S., Reemtsma K., Bagiella E., Oluwole S.F. and Braunstein N.S. Role of TAP-1 and/or TAP-2 antigen presentation defects in tumorigenicity of mouse melanoma. Cellular immunology 228(2):130-137, 2004.
4.   Akasaki Y., Kikuchi T., Homma S., Koido S., Ohkusa T., Tasaki T., Hayashi K., Komita H., Watanabe N., Suzuki Y., Yamamoto Y., Mori R., Arai T., Tanaka T., Joki T., Yanagisawa T. and Murayama Y. Phase I/II trial of combination of temozolomide chemotherapy and immunotherapy with fusions of dendritic and glioma cells in patients with glioblastoma. Cancer Immunology, Immunotherapy 65(12):1499-1509, 2016.
5.   Anguille S., Smits E.L., Cools N., Goossens H., Berneman Z.N. and Van Tendeloo V.F. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. Journal of translational medicine 7:109, 2009.
6.   Anguille S., Smits E.L., Lion E., van Tendeloo V.F. and Berneman Z.N. Clinical use of dendritic cells for cancer therapy. The Lancet Oncology 15(7):e257-e267, 2014.
7.   Anguille S., Van de Velde A.L., Smits E.L., Van Tendeloo V.F., Juliusson G., Cools N., Nijs G., Stein B., Lion E., Van Driessche A., Vandenbosch I., Verlinden A., Gadisseur A.P., Schroyens W.A., Muylle L., Vermeulen K., Maes M.-B., Deiteren K., Malfait R., Gostick E.,

Lammens M., Couttenye M.M., Jorens P., Goossens H., Price D.A., Ladell K., Oka Y., Fujiki F., Oji Y., Sugiyama H. and Berneman Z.N. Dendritic cell vaccination as postremission treatment to prevent or delay relapse in acute myeloid leukemia. Blood 130(15):1713-1721, 2017.

8. Asselin-Paturel C., Brizard G., Chemin K., Boonstra A., O'Garra A., Vicari A. and Trinchieri G. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. J Exp Med 201(7):1157-1167, 2005.

9. Avigan D., Vasir B., Gong J., Borges V., Wu Z., Uhl L., Atkins M., Mier J., McDermott D., Smith T., Giallambardo N., Stone C., Schadt K., Dolgoff J., Tetreault J.-C., Villarroel M. and Kufe D. Fusion Cell Vaccination of Patients with Metastatic Breast and Renal Cancer Induces Immunological and Clinical Responses. Clinical Cancer Research 10(14):4699, 2004.

10. Barth R.J., Jr., Fisher D.A., Wallace P.K., Channon J.Y., Noelle R.J., Gui J. and Ernstoff M.S. A randomized trial of ex vivo CD40L activation of a dendritic cell vaccine in colorectal cancer patients: tumor-specific immune responses are associated with improved survival. Clinical cancer research : an official journal of the American Association for Cancer Research 16(22):5548-5556, 2010.

11. Bevan M.J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med 143(5):1283-1288, 1976.

12. Blum J.S., Wearsch P.A. and Cresswell P. Pathways of antigen processing. Annual review of immunology 31:443-473, 2013.

13. Boudewijns S., Bloemendal M., de Haas N., Westdorp H., Bol K.F., Schreibelt G., Aarntzen E., Lesterhuis W.J., Gorris M.A.J., Croockewit A., van der Woude L.L., van Rossum M.M., Welzen M., de Goede A., Hato S.V., van der Graaf W.T.A., Punt C.J.A., Koornstra R.H.T., Gerritsen W.R., Figdor C.G. and de Vries I.J.M. Autologous monocyte-derived DC vaccination combined with cisplatin in stage III and IV melanoma patients: a prospective, randomized phase 2 trial. Cancer immunology, immunotherapy : CII 69(3):477-488, 2020.

14. Brennan P.J., Brigl M. and Brenner M.B. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nature Reviews Immunology 13(2):101-117, 2013.

15. Brenner H., Kloor M. and Pox C.P. Colorectal cancer. The Lancet 383(9927):1490-1502, 2014.

16. Butterfield L.H., Comin-Anduix B., Vujanovic L., Lee Y., Dissette V.B., Yang J.-Q., Vu H.T., Seja E., Oseguera D.K., Potter D.M., Glaspy J.A., Economou J.S. and Ribas A. Adenovirus MART-1-engineered autologous dendritic cell vaccine for metastatic melanoma. Journal of immunotherapy (Hagerstown, Md. : 1997) 31(3):294-309, 2008.

17. Campanella G.S.V., Colvin R.A. and Luster A.D. CXCL10 Can Inhibit Endothelial Cell Proliferation Independently of CXCR3. PLoS One 5(9):e12700, 2010.

18. Carbone F.R. and Heath W.R. Cross-Priming: Its Beginnings. The Journal of Immunology 185(3):1353, 2010.

19. CECHIM G. and CHIES J.A.B. In vitro generation of human monocyte-derived dendritic cells methodological aspects in a comprehensive review %J Anais da Academia Brasileira de Ciências. 91, 2019.

20. Chabot V., Martin L., Meley D., Sensebé L., Baron C., Lebranchu Y., Dehaut F. and Velge-Roussel F. Unexpected impairment of TNF-α-induced maturation of human dendritic cells in vitro by IL-4. Journal of translational medicine 14:93-93, 2016.

21. Clayton K., Vallejo A.F., Davies J., Sirvent S. and Polak M.E. Langerhans Cells—Programmed by the Epidermis. 8(1676), 2017.

22. Collin M. and Bigley V. Human dendritic cell subsets: an update. Immunology 154(1):3-20, 2018.

23. Correale P., Walmsley K., Zaremba S., Zhu M., Schlom J. and Tsang K.Y. Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide. Journal of immunology (Baltimore, Md. : 1950) 161(6):3186-3194, 1998.

24. Cumberbatch M., Griffiths C.E., Tucker S.C., Dearman R.J. and Kimber I. Tumour necrosis factor-alpha induces Langerhans cell migration in humans. The British journal of dermatology 141(2):192-200, 1999.

25. de Camargo M.R., Gorgulho C.M., Rodrigues C.P., Penitenti M., Frederico J.C.L., Rodrigues M.A.M. and Kaneno R. Low Concentration of 5-Fluorouracil Increases the Effectiveness of Tumor RNA to Activate Murine Dendritic Cells. Cancer biotherapy & radiopharmaceuticals 32(8):302-308, 2017.

26. de Vries T.J., Smeets M., de Graaf R., Hou-Jensen K., Bröcker E.B., Renard N., Eggermont A.M., van Muijen G.N. and Ruiter D.J. Expression of gp100, MART-1, tyrosinase, and S100 in paraffin-embedded primary melanomas and locoregional, lymph node, and visceral metastases: implications for diagnosis and immunotherapy. A study conducted by the EORTC Melanoma Cooperative Group. The Journal of pathology 193(1):13-20, 2001.

27. Decker W.K., Li S., Xing D., Robinson S.N., Yang H., Steiner D., Komanduri K.V., Bollard C.M. and Shpall E.J. Deficient T(H)-1 responses from TNF-alpha-matured and alpha-CD40-matured dendritic cells. Journal of immunotherapy (Hagerstown, Md. : 1997) 31(2):157-165, 2008.

28. Delirezh N., Moazzeni S.M., Shokri F., Shokrgozar M.A., Atri M. and Kokhaei P. Autologous dendritic cells loaded with apoptotic tumor cells induce T cell-mediated immune responses against breast cancer in vitro. Cellular immunology 257(1-2):23-31, 2009.

29. Di Nicola M., Carlo-Stella C., Mortarini R., Baldassari P., Guidetti A., Gallino G.F., Del Vecchio M., Ravagnani F., Magni M., Chaplin P., Cascinelli N., Parmiani G., Gianni A.M. and Anichini A. Boosting T Cell-Mediated Immunity to Tyrosinase by Vaccinia Virus-Transduced, CD34⁺-Derived Dendritic Cell Vaccination. Clinical Cancer Research 10(16):5381, 2004.

30. Díaz-Rodríguez Y., Cordeiro P., Belounis A., Herblot S. and Duval M. In vitro differentiated plasmacytoid dendritic cells as a tool to induce anti-leukemia activity of natural killer cells. Cancer immunology, immunotherapy : CII 66(10):1307-1320, 2017.

31. Domínguez-Soto A., Sierra-Filardi E., Puig-Kröger A., Pérez-Maceda B., Gómez-Aguado F., Corcuera M.T., Sánchez-Mateos P. and Corbí A.L. Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. Journal of immunology (Baltimore, Md. : 1950) 186(4):2192-2200, 2011.

32. Ebner S., Hofer S., Nguyen V.A., Fürhapter C., Herold M., Fritsch P., Heufler C. and Romani N. A novel role for IL-3: human monocytes cultured in the presence of IL-3 and IL-4 differentiate into dendritic cells that produce less IL-12 and shift Th cell responses toward a Th2 cytokine pattern. Journal of immunology (Baltimore, Md. : 1950) 168(12):6199-6207, 2002.

33. Farkas L., Beiske K., Lund-Johansen F., Brandtzaeg P. and Jahnsen F.L. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. The American journal of pathology 159(1):237-243, 2001.

34. Fay J.W., Palucka A.K., Paczesny S., Dhodapkar M., Johnston D.A., Burkeholder S., Ueno H. and Banchereau J. Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed CD34(+) progenitor-derived dendritic cells. Cancer immunology, immunotherapy : CII 55(10):1209-1218, 2006.

35. Ferlazzo G., Klein J., Paliard X., Wei W.-Z. and Galy A. Dendritic Cells Generated From CD34+ Progenitor Cells With flt3 Ligand, c-Kit Ligand, GM-CSF, IL-4, and TNF-α Are

Functional Antigen-Presenting Cells Resembling Mature Monocyte-Derived Dendritic Cells. Journal of Immunotherapy 23(1), 2000.

36. Fernandes R.K., Bachiega T.F., Rodrigues D.R., Golim M.d.A., Dias-Melicio L.A., Balderramas H.d.A., Kaneno R. and Soares Â.M.V.C. Paracoccidioides brasiliensis interferes on dendritic cells maturation by inhibiting PGE2 production. PLoS One 10(3):e0120948-e0120948, 2015.

37. Fitzgerald-Bocarsly P. and Feng D. The role of type I interferon production by dendritic cells in host defense. Biochimie 89(6-7):843-855, 2007.

38. Floss D.M., Moll J.M. and Scheller J. IL-12 and IL-23-Close Relatives with Structural Homologies but Distinct Immunological Functions. Cells 9(10), 2020.

39. Fujii S.-I. and Shimizu K. Immunotherapy with artificial adjuvant vector cells: Harnessing both arms of the immune response. Oncoimmunology 2(4):e23432-e23432, 2013.

40. Fujii S., Shimizu K., Kronenberg M. and Steinman R.M. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nature immunology 3(9):867-874, 2002.

41. Gao N., Liu X., Wu J., Li J., Dong C., Wu X., Xiao X. and Yu F.-S.X. CXCL10 suppression of hem- and lymph-angiogenesis in inflamed corneas through MMP13. Angiogenesis 20(4):505-518, 2017.

42. Gao Y., Chen X., Gao W., Yang Y., Ma H. and Ren X. A new purification method for enhancing the immunogenicity of heat shock protein 70-peptide complexes. Oncol Rep 28(6):1977-1983, 2012.

43. Garg A.D., Vara Perez M., Schaaf M., Agostinis P., Zitvogel L., Kroemer G. and Galluzzi L. Trial watch: Dendritic cell-based anticancer immunotherapy. Oncoimmunology 6(7):e1328341-e1328341, 2017.

44. Goldberg A.C. and Rizzo L.V. MHC structure and function   antigen presentation. Part 1 %J Einstein (São Paulo). 13:153-156, 2015.

45. Gonzalez H., Hagerling C. and Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. Genes Dev 32(19-20):1267-1284, 2018.

46. Goodbourn S., Didcock L. and Randall R.E. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. 81(10):2341-2364, 2000.

47. Granot T., Senda T., Carpenter D.J., Matsuoka N., Weiner J., Gordon C.L., Miron M., Kumar B.V., Griesemer A., Ho S.H., Lerner H., Thome J.J.C., Connors T., Reizis B. and Farber D.L. Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. Immunity 46(3):504-515, 2017.

48. Hancharou A.Y., Chyzh K.A., Titov L.P., Soroka N.F. and DuBuske L.M. Characterization Of Plasmacytoid Dendritic Cells From Blood Of Patients With Systemic Lupus Erythematosus. Journal of Allergy and Clinical Immunology 129(2):AB163, 2012.

49. Henrickson S.E., Perro M., Loughhead S.M., Senman B., Stutte S., Quigley M., Alexe G., Iannacone M., Flynn M.P., Omid S., Jesneck J.L., Imam S., Mempel T.R., Mazo I.B., Haining W.N. and von Andrian U.H. Antigen availability determines CD8^{+} T cell-dendritic cell interaction kinetics and memory fate decisions. Immunity 39(3):496-507, 2013.

50. Henry C.J., Ornelles D.A., Mitchell L.M., Brzoza-Lewis K.L. and Hiltbold E.M. IL-12 produced by dendritic cells augments CD8+ T cell activation through the production of the chemokines CCL1 and CCL17. Journal of immunology (Baltimore, Md. : 1950) 181(12):8576-8584, 2008.

51. Howes A., Gabryšová L. and O'Garra A. Role of IL-10 and the IL-10 Receptor in Immune Responses. Reference Module in Biomedical Sciences. Elsevier, 2014.

52. Hsu F.J., Benike C., Fagnoni F., Liles T.M., Czerwinski D., Taidi B., Engleman E.G. and Levy R. Vaccination of patients with B–cell lymphoma using autologous antigen–pulsed dendritic cells. Nature Medicine 2(1):52-58, 1996.

53. Hsu P., Santner-Nanan B., Hu M., Skarratt K., Lee C.H., Stormon M., Wong M., Fuller S.J. and Nanan R. IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via

STAT3 and Foxo1. Journal of immunology (Baltimore, Md. : 1950) 195(8):3665-3674, 2015.

54. Huysamen C. and Brown G.D. The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. FEMS Microbiol Lett 290(2):121-128, 2009.

55. INCA. Estimativa 2020: incidência de câncer no Brasil: Instituto Nacional de Câncer José Alencar Gomes da Silva/ Ministério da Saúde.; 2019. 120 p.

56. Inogés S., Tejada S., de Cerio A.L.-D., Gállego Pérez-Larraya J., Espinós J., Idoate M.A., Domínguez P.D., de Eulate R.G., Aristu J., Bendandi M., Pastor F., Alonso M., Andreu E., Cardoso F.P. and Valle R.D. A phase II trial of autologous dendritic cell vaccination and radiochemotherapy following fluorescence-guided surgery in newly diagnosed glioblastoma patients. Journal of translational medicine 15(1):104-104, 2017.

57. Iqbal B. and Buch A. Hormone receptor (ER, PR, HER2/neu) status and proliferation index marker (Ki-67) in breast cancers: Their onco-pathological correlation, shortcomings and future trends. 9(6):674-679, 2016.

58. Jackson C., Westphal M. and Quiñones-Hinojosa A. Complications of glioma surgery. Handbook of clinical neurology 134:201-218, 2016.

59. Jarnicki A.G., Lysaght J., Todryk S. and Mills K.H. Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. Journal of immunology (Baltimore, Md. : 1950) 177(2):896-904, 2006.

60. Jing X., Liang H., Hao C., Yang X. and Cui X. Overexpression of MUC1 predicts poor prognosis in patients with breast cancer. Oncol Rep 41(2):801-810, 2019.

61. Jongbloed S.L., Kassianos A.J., McDonald K.J., Clark G.J., Ju X., Angel C.E., Chen C.J., Dunbar P.R., Wadley R.B., Jeet V., Vulink A.J., Hart D.N. and Radford K.J. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 207(6):1247-1260, 2010.

62. Kaneno R., Shurin G.V., Kaneno F.M., Naiditch H., Luo J. and Shurin M.R. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. Cellular Oncology 34(2):97-106, 2011.

63. Kaneno R., Shurin G.V., Tourkova I.L. and Shurin M.R. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. Journal of translational medicine 7:58, 2009.

64. Koido S. Dendritic-Tumor Fusion Cell-Based Cancer Vaccines. Int J Mol Sci 17(6):828, 2016.

65. Kongsted P., Borch T.H., Ellebaek E., Iversen T.Z., Andersen R., Met Ö., Hansen M., Lindberg H., Sengeløv L. and Svane I.M. Dendritic cell vaccination in combination with docetaxel for patients with metastatic castration-resistant prostate cancer: A randomized phase II study. Cytotherapy 19(4):500-513, 2017.

66. Korn T., Bettelli E., Oukka M. and Kuchroo V.K. IL-17 and Th17 Cells. Annual review of immunology 27:485-517, 2009.

67. Krovi S.H. and Gapin L. Invariant Natural Killer T Cell Subsets—More Than Just Developmental Intermediates. 9(1393), 2018.

68. Kruit W.H., Suciu S., Dreno B., Mortier L., Robert C., Chiarion-Sileni V., Maio M., Testori A., Dorval T., Grob J.J., Becker J.C., Spatz A., Eggermont A.M., Louahed J., Lehmann F.F., Brichard V.G. and Keilholz U. Selection of immunostimulant AS15 for active immunization with MAGE-A3 protein: results of a randomized phase II study of the European Organisation for Research and Treatment of Cancer Melanoma Group in Metastatic Melanoma. J Clin Oncol 31(19):2413-2420, 2013.

69. Langerhans P. Ueber die Nerven der menschlichen Haut. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin 44(2):325-337, 1868.

70. Lanier L.L., O'Fallon S., Somoza C., Phillips J.H., Linsley P.S., Okumura K., Ito D. and Azuma M. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. Journal of immunology (Baltimore, Md. : 1950) 154(1):97-105, 1995.

71. Lasky J.L., 3rd, Panosyan E.H., Plant A., Davidson T., Yong W.H., Prins R.M., Liau L.M. and Moore T.B. Autologous tumor lysate-pulsed dendritic cell immunotherapy for pediatric patients with newly diagnosed or recurrent high-grade gliomas. Anticancer research 33(5):2047-2056, 2013.

72. Lau S.K., Weiss L.M. and Chu P.G. Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. Am J Clin Pathol 122(1):61-69, 2004.

73. Leal Rojas I.M., Mok W.-H., Pearson F.E., Minoda Y., Kenna T.J., Barnard R.T. and Radford K.J. Human Blood CD1c(+) Dendritic Cells Promote Th1 and Th17 Effector Function in Memory CD4(+) T Cells. Front Immunol 8:971-971, 2017.

74. Leone P., Shin E.-C., Perosa F., Vacca A., Dammacco F. and Racanelli V. MHC Class I Antigen Processing and Presenting Machinery: Organization, Function, and Defects in Tumor Cells. JNCI: Journal of the National Cancer Institute 105(16):1172-1187, 2013.

75. Lesimple T., Neidhard E.-M., Vignard V., Lefeuvre C., Adamski H., Labarrière N., Carsin A., Monnier D., Collet B., Clapisson G., Birebent B., Philip I., Toujas L., Chokri M. and Quillien V. Immunologic and Clinical Effects of Injecting Mature Peptide-Loaded Dendritic Cells by Intralymphatic and Intranodal Routes in Metastatic Melanoma Patients. Clinical Cancer Research 12(24):7380, 2006.

76. Lesterhuis W.J., de Vries I.J., Schuurhuis D.H., Boullart A.C., Jacobs J.F., de Boer A.J., Scharenborg N.M., Brouwer H.M., van de Rakt M.W., Figdor C.G., Ruers T.J., Adema G.J. and Punt C.J. Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests. Annals of oncology : official journal of the European Society for Medical Oncology 17(6):974-980, 2006.

77. Lie P.P.Y., Cheng C.Y. and Mruk D.D. The biology of interleukin-1: emerging concepts in the regulation of the actin cytoskeleton and cell junction dynamics. Cellular and molecular life sciences : CMLS 69(4):487-500, 2012.

78. Litwin M.S. and Tan H.J. The Diagnosis and Treatment of Prostate Cancer: A Review. Jama 317(24):2532-2542, 2017.

79. Liu C., Lou Y., Lizée G., Qin H., Liu S., Rabinovich B., Kim G.J., Wang Y.H., Ye Y., Sikora A.G., Overwijk W.W., Liu Y.J., Wang G. and Hwu P. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. The Journal of clinical investigation 118(3):1165-1175, 2008.

80. Loveland B.E., Zhao A., White S., Gan H., Hamilton K., Xing P.-X., Pietersz G.A., Apostolopoulos V., Vaughan H., Karanikas V., Kyriakou P., McKenzie I.F.C. and Mitchell P.L.R. Mannan-MUC1–Pulsed Dendritic Cell Immunotherapy: A Phase I Trial in Patients with Adenocarcinoma. Clinical Cancer Research 12(3):869, 2006.

81. Lundberg K., Albrekt A.-S., Nelissen I., Santegoets S., de Gruijl T.D., Gibbs S. and Lindstedt M. Transcriptional profiling of human dendritic cell populations and models--unique profiles of in vitro dendritic cells and implications on functionality and applicability. PLoS One 8(1):e52875-e52875, 2013.

82. Lutz M.B. IL-3 in dendritic cell development and function: a comparison with GM-CSF and IL-4. Immunobiology 209(1-2):79-87, 2004.

83. Ma D.Y. and Clark E.A. The role of CD40 and CD154/CD40L in dendritic cells. Seminars in immunology 21(5):265-272, 2009.

84. Maney N.J., Reynolds G., Krippner-Heidenreich A. and Hilkens C.M.U. Dendritic cell maturation and survival are differentially regulated by TNFR1 and TNFR2. Journal of immunology (Baltimore, Md. : 1950) 193(10):4914-4923, 2014.

85.   Manfred B. Lutz N.R. Cytokines Produced by Dendritic Cells. Handbook of Dendritic Cells, 2006, pp. 355-383.

86.   Marchand M., van Baren N., Weynants P., Brichard V., Dréno B., Tessier M.H., Rankin E., Parmiani G., Arienti F., Humblet Y., Bourlond A., Vanwijck R., Liénard D., Beauduin M., Dietrich P.Y., Russo V., Kerger J., Masucci G., Jäger E., De Greve J., Atzpodien J., Brasseur F., Coulie P.G., van der Bruggen P. and Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 80(2):219-230, 1999.

87.   Marchand M., Weynants P., Rankin E., Arienti F., Belli F., Parmiani G., Cascinelli N., Bourlond A., Vanwijck R., Humblet Y. and et al. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. Int J Cancer 63(6):883-885, 1995.

88.   McGranahan N., Rosenthal R., Hiley C.T., Rowan A.J., Watkins T.B.K., Wilson G.A., Birkbak N.J., Veeriah S., Van Loo P., Herrero J. and Swanton C. Allele-Specific HLA Loss and Immune Escape in Lung Cancer Evolution. Cell 171(6):1259-1271.e1211, 2017.

89.   Miglietta F., Dieci M.V., Griguolo G., Guarneri V. and Conte P.F. Chemotherapy for advanced HER2-negative breast cancer: Can one algorithm fit all? Cancer treatment reviews 60:100-108, 2017.

90.   Mitchell D.A. and Nair S.K. RNA-transfected dendritic cells in cancer immunotherapy. The Journal of clinical investigation 106(9):1065-1069, 2000.

91.   Moo T.-A., Sanford R., Dang C. and Morrow M. Overview of Breast Cancer Therapy. PET Clin 13(3):339-354, 2018.

92.   Morse M.A., Niedzwiecki D., Marshall J.L., Garrett C., Chang D.Z., Aklilu M., Crocenzi T.S., Cole D.J., Dessureault S., Hobeika A.C., Osada T., Onaitis M., Clary B.M., Hsu D., Devi G.R., Bulusu A., Annechiarico R.P., Chadaram V., Clay T.M. and Lyerly H.K. A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. Ann Surg 258(6):879-886, 2013.

93.   Motohashi S., Nagato K., Kunii N., Yamamoto H., Yamasaki K., Okita K., Hanaoka H., Shimizu N., Suzuki M., Yoshino I., Taniguchi M., Fujisawa T. and Nakayama T. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. Journal of immunology (Baltimore, Md. : 1950) 182(4):2492-2501, 2009.

94.   Muntjewerff E.M., Meesters L.D. and van den Bogaart G. Antigen Cross-Presentation by Macrophages. Front Immunol 11:1276, 2020.

95.   Nars M.S. and Kaneno R. Immunomodulatory effects of low dose chemotherapy and perspectives of its combination with immunotherapy. Int J Cancer 132(11):2471-2478, 2013.

96.   Nestle F.O., Conrad C., Tun-Kyi A., Homey B., Gombert M., Boyman O., Burg G., Liu Y.J. and Gilliet M. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med 202(1):135-143, 2005.

97.   Nizzoli G., Krietsch J., Weick A., Steinfelder S., Facciotti F., Gruarin P., Bianco A., Steckel B., Moro M., Crosti M., Romagnani C., Stolzel K., Torretta S., Pignataro L., Scheibenbogen C., Neddermann P., De Francesco R., Abrignani S. and Geginat J. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood 122(6):932-942, 2013.

98.   Noubade R., Majri-Morrison S. and Tarbell K.V. Beyond cDC1: Emerging Roles of DC Crosstalk in Cancer Immunity. Front Immunol 10:1014-1014, 2019.

99.   O'Keeffe M., Mok W.H. and Radford K.J. Human dendritic cell subsets and function in health and disease. Cellular and molecular life sciences : CMLS 72(22):4309-4325, 2015.

100. Oliva E.N., Franek J., Patel D., Zaidi O., Nehme S.A. and Almeida A.M. The Real-World Incidence of Relapse in Acute Myeloid Leukemia (AML): A Systematic Literature Review (SLR). Blood 132(Supplement 1):5188-5188, 2018.

101. Olson B.M., Frye T.P., Johnson L.E., Fong L., Knutson K.L., Disis M.L. and McNeel D.G. HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase. Cancer immunology, immunotherapy : CII 59(6):943-953, 2010.

102. Panda S.K., Kolbeck R. and Sanjuan M.A. Plasmacytoid dendritic cells in autoimmunity. Current opinion in immunology 44:20-25, 2017.

103. Parameswaran N. and Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot Gene Expr 20(2):87-103, 2010.

104. Parihar R., Dierksheide J., Hu Y. and Carson W.E. IL-12 enhances the natural killer cell cytokine response to Ab-coated tumor cells. The Journal of clinical investigation 110(7):983-992, 2002.

105. Parise C.A. and Caggiano V. Breast Cancer Survival Defined by the ER/PR/HER2 Subtypes and a Surrogate Classification according to Tumor Grade and Immunohistochemical Biomarkers. Journal of Cancer Epidemiology 2014:469251, 2014.

106. Peethambaram P.P., Melisko M.E., Rinn K.J., Alberts S.R., Provost N.M., Jones L.A., Sims R.B., Lin L.R.C., Frohlich M.W. and Park J.W. A phase I trial of immunotherapy with lapuleucel-T (APC8024) in patients with refractory metastatic tumors that express HER-2/neu. Clinical cancer research : an official journal of the American Association for Cancer Research 15(18):5937-5944, 2009.

107. Pfeifer J.D., Wick M.J., Roberts R.L., Findlay K., Normark S.J. and Harding C.V. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature 361(6410):359-362, 1993.

108. Pinho M.P., Sundarasetty B.S., Bergami-Santos P.C., Steponavicius-Cruz K., Ferreira A.K., Stripecke R. and Barbuto J.A. Dendritic-tumor cell hybrids induce tumor-specific immune responses more effectively than the simple mixture of dendritic and tumor cells. Cytotherapy 18(4):570-580, 2016.

109. Powell J.D. and Delgoffe G.M. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. Immunity 33(3):301-311, 2010.

110. Rampal R. and Figueroa M.E. Wilms tumor 1 mutations in the pathogenesis of acute myeloid leukemia. Haematologica 101(6):672-679, 2016.

111. Rini B.I., Weinberg V., Fong L., Conry S., Hershberg R.M. and Small E.J. Combination immunotherapy with prostatic acid phosphatase pulsed antigen-presenting cells (provenge) plus bevacizumab in patients with serologic progression of prostate cancer after definitive local therapy. Cancer 107(1):67-74, 2006.

112. Riol-Blanco L., Sánchez-Sánchez N., Torres A., Tejedor A., Narumiya S., Corbí A.L., Sánchez-Mateos P. and Rodríguez-Fernández J.L. The Chemokine Receptor CCR7 Activates in Dendritic Cells Two Signaling Modules That Independently Regulate Chemotaxis and Migratory Speed. The Journal of Immunology 174(7):4070, 2005.

113. Rivas J.R., Alhakeem S.S., Eckenrode J.M., Zhang Y., Collard J.P., Hildebrandt G.C., Fleischman R.A., Chen L., Thorson J.S., Leggas M. and Bondada S. Enhancing Anti-Tumor Immunity and Responses to Immune Checkpoint Blockade By Suppressing Interleukin-10 in Chronic Lymphocytic Leukemia. Blood 134(Supplement_1):5486-5486, 2019.

114. Rivas J.R., Alhakeem S.S., Liu Y., Eckenrode J.M., Marti F., Collard J.P., Zhang Y., Shaaban K.A., Muthusamy N., Hildebrandt G.C., Fleischman R.A., Chen L., Thorson J.S., Leggas M. and Bondada S. Interleukin-10 suppression enhances T-cell antitumor immunity and responses to checkpoint blockade in chronic lymphocytic leukemia. bioRxiv:2020.2007.2015.204560, 2020.

115. Rock K., Gamble S. and Rothstein L. Presentation of exogenous antigen with class I major histocompatibility complex molecules. 249(4971):918-921, 1990.

116. Roeder C., Schuler-Thurner B., Berchtold S., Vieth G., Driesch P., Schuler G. and Lüftl M. MAGE-A3 is a frequent tumor antigen of metastasized melanoma. Archives of dermatological research 296(7):314-319, 2005.

117. Rønning P.A., Helseth E., Meling T.R. and Johannesen T.B. A population-based study on the effect of temozolomide in the treatment of glioblastoma multiforme. Neuro Oncol 14(9):1178-1184, 2012.

118. Sabado R.L., Balan S. and Bhardwaj N. Dendritic cell-based immunotherapy. Cell Research 27(1):74-95, 2017.

119. Sabat R., Grütz G., Warszawska K., Kirsch S., Witte E., Wolk K. and Geginat J. Biology of interleukin-10. Cytokine & growth factor reviews 21(5):331-344, 2010.

120. Saito S., Yanagisawa R., Yoshikawa K., Higuchi Y., Koya T., Yoshizawa K., Tanaka M., Sakashita K., Kobayashi T., Kurata T., Hirabayashi K., Nakazawa Y., Shiohara M., Yonemitsu Y., Okamoto M., Sugiyama H., Koike K. and Shimodaira S. Safety and tolerability of allogeneic dendritic cell vaccination with induction of Wilms tumor 1-specific T cells in a pediatric donor and pediatric patient with relapsed leukemia: a case report and review of the literature. Cytotherapy 17(3):330-335, 2015.

121. Sallusto F. and Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179(4):1109-1118, 1994.

122. Salmond R.J., Emery J., Okkenhaug K. and Zamoyska R. MAPK, phosphatidylinositol 3-kinase, and mammalian target of rapamycin pathways converge at the level of ribosomal protein S6 phosphorylation to control metabolic signaling in CD8 T cells. Journal of immunology (Baltimore, Md. : 1950) 183(11):7388-7397, 2009.

123. Schreibelt G., Klinkenberg L.J.J., Cruz L.J., Tacken P.J., Tel J., Kreutz M., Adema G.J., Brown G.D., Figdor C.G. and de Vries I.J.M. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. Blood 119(10):2284-2292, 2012.

124. Schulz O., Diebold S.S., Chen M., Naslund T.I., Nolte M.A., Alexopoulou L., Azuma Y.T., Flavell R.A., Liljestrom P. and Reis e Sousa C. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 433(7028):887-892, 2005.

125. Serbina N.V., Salazar-Mather T.P., Biron C.A., Kuziel W.A. and Pamer E.G. TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. Immunity 19(1):59-70, 2003.

126. Sevá-Pereira G., Cypreste R.N., Oliveira Filho J.J., Moraes S.P.d. and Tarabay P.B. Recurrence pattern of rectal cancer after surgical treatment. Analysis of 122 patients in a tertiary care center %J Journal of Coloproctology (Rio de Janeiro). 38:18-23, 2018.

127. Sgadari C., Angiolillo A.L. and Tosato G. Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10. Blood 87(9):3877-3882, 1996.

128. Shen L., Sigal L.J., Boes M. and Rock K.L. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity 21(2):155-165, 2004.

129. Shurin GV T.I., Kaneno R, et al. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. The Journal of Immunology (183):137-144, 2009.

130. Siegel R.L., Miller K.D. and Jemal A. Cancer statistics, 2020. CA: A Cancer Journal for Clinicians 70(1):7-30, 2020.

131. Simmons D.P., Wearsch P.A., Canaday D.H., Meyerson H.J., Liu Y.C., Wang Y., Boom W.H. and Harding C.V. Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing. Journal of immunology (Baltimore, Md. : 1950) 188(7):3116-3126, 2012.

132. Slingluff C.L., Jr., Petroni G.R., Olson W.C., Smolkin M.E., Chianese-Bullock K.A., Mauldin I.S., Smith K.T., Deacon D.H., Varhegyi N.E., Donnelly S.B., Reed C.M., Scott K., Galeassi N.V. and Grosh W.W. A randomized pilot trial testing the safety and immunologic effects of a MAGE-A3 protein plus AS15 immunostimulant administered into muscle or into dermal/subcutaneous sites. Cancer immunology, immunotherapy : CII 65(1):25-36, 2016.

133. Small E.J., Fratesi P., Reese D.M., Strang G., Laus R., Peshwa M.V. and Valone F.H. Immunotherapy of Hormone-Refractory Prostate Cancer With Antigen-Loaded Dendritic Cells. Journal of Clinical Oncology 18(23):3894-3903, 2000.

134. Small E.J., Lance R.S., Gardner T.A., Karsh L.I., Fong L., McCoy C., DeVries T., Sheikh N.A., GuhaThakurta D., Chang N., Redfern C.H. and Shore N.D. A Randomized Phase II Trial of Sipuleucel-T with Concurrent versus Sequential Abiraterone Acetate plus Prednisone in Metastatic Castration-Resistant Prostate Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 21(17):3862-3869, 2015.

135. Small E.J., Schellhammer P.F., Higano C.S., Redfern C.H., Nemunaitis J.J., Valone F.H., Verjee S.S., Jones L.A. and Hershberg R.M. Placebo-Controlled Phase III Trial of Immunologic Therapy with Sipuleucel-T (APC8015) in Patients with Metastatic, Asymptomatic Hormone Refractory Prostate Cancer. Journal of Clinical Oncology 24(19):3089-3094, 2006.

136. Souza-Fonseca-Guimaraes F., Young A., Mittal D., Martinet L., Bruedigam C., Takeda K., Andoniou C.E., Degli-Esposti M.A., Hill G.R. and Smyth M.J. NK cells require IL-28R for optimal in vivo activity. Proc Natl Acad Sci U S A 112(18):E2376-E2384, 2015.

137. Steinman R.M., Adams J.C. and Cohn Z.A. Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. J Exp Med 141(4):804-820, 1975.

138. Steinman R.M., Lustig D.S. and Cohn Z.A. Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. J Exp Med 139(6):1431-1445, 1974.

139. Steinman R.M. and Witmer M.D. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc Natl Acad Sci U S A 75(10):5132-5136, 1978.

140. Stupp R., Mason W.P., van den Bent M.J., Weller M., Fisher B., Taphoorn M.J., Belanger K., Brandes A.A., Marosi C., Bogdahn U., Curschmann J., Janzer R.C., Ludwin S.K., Gorlia T., Allgeier A., Lacombe D., Cairncross J.G., Eisenhauer E. and Mirimanoff R.O. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England journal of medicine 352(10):987-996, 2005.

141. Sunshine G.H., Katz D.R. and Feldmann M. Dendritic cells induce T cell proliferation to synthetic antigens under Ir gene control. J Exp Med 152(6):1817-1822, 1980.

142. Takakura K., Kajihara M., Ito Z., Ohkusa T., Gong J. and Koido S. Dendritic-tumor fusion cells in cancer immunotherapy. Discov Med 19(104):169-174, 2015.

143. Tanaka T., Narazaki M. and Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 6(10):a016295-a016295, 2014.

144. Tebbutt N.C., Cattell E., Midgley R., Cunningham D. and Kerr D. Systemic treatment of colorectal cancer. European journal of cancer (Oxford, England : 1990) 38(7):1000-1015, 2002.

145. Theisen D. and Murphy K. The role of cDC1s in vivo: CD8 T cell priming through cross-presentation. F1000Research 6:98, 2017.

146. Toebak M.J., Gibbs S., Bruynzeel D.P., Scheper R.J. and Rustemeyer T. Dendritic cells: biology of the skin. Contact dermatitis 60(1):2-20, 2009.

147. Twardowski P., Wong J.Y.C., Pal S.K., Maughan B.L., Frankel P.H., Franklin K., Junqueira M., Prajapati M.R., Nachaegari G., Harwood D. and Agarwal N. Randomized phase II trial of sipuleucel-T immunotherapy preceded by sensitizing radiation therapy and

sipuleucel-T alone in patients with metastatic castrate resistant prostate cancer. Cancer treatment and research communications 19:100116, 2019.

148. Uckun F.M. and Qazi S. Wilms' tumor gene (<i>WT1</i>) is strongly expressed in high-risk subsets of pediatric acute lymphoblastic leukemia. Cancer Drug Resistance 1(4):250-265, 2018.

149. Valladeau J. and Saeland S. Cutaneous dendritic cells. Seminars in immunology 17(4):273-283, 2005.

150. Versteven M., Van den Bergh J.M.J., Marcq E., Smits E.L.J., Van Tendeloo V.F.I., Hobo W. and Lion E. Dendritic Cells and Programmed Death-1 Blockade: A Joint Venture to Combat Cancer. Front Immunol 9:394, 2018.

151. Vieira I.R., Fernandes R.K., Rodrigues D.R., Gorgulho C.M., Kaneno R. and Soares Â.M.V.C. TLR9 stimulation induces increase in fungicidal activity of human dendritic cells challenged with Paracoccidioides brasiliensis. Medical Mycology 56(7):911-915, 2018.

152. Volpi C., Fallarino F., Pallotta M.T., Bianchi R., Vacca C., Belladonna M.L., Orabona C., De Luca A., Boon L., Romani L., Grohmann U. and Puccetti P. High doses of CpG oligodeoxynucleotides stimulate a tolerogenic TLR9–TRIF pathway. Nature Communications 4(1):1852, 2013.

153. Ward J.F., Blute M.L., Slezak J., Bergstralh E.J. and Zincke H. The long-term clinical impact of biochemical recurrence of prostate cancer 5 or more years after radical prostatectomy. The Journal of urology 170(5):1872-1876, 2003.

154. Wen P.Y., Reardon D.A., Armstrong T.S., Phuphanich S., Aiken R.D., Landolfi J.C., Curry W.T., Zhu J.-J., Glantz M., Peereboom D.M., Markert J.M., LaRocca R., Rourke D.M., Fink K., Kim L., Gruber M., Lesser G.J., Pan E., Kesari S., Muzikansky A., Pinilla C., Santos R.G. and Yu J.S. A Randomized Double-Blind Placebo-Controlled Phase II Trial of Dendritic Cell Vaccine ICT-107 in Newly Diagnosed Patients with Glioblastoma. Clinical Cancer Research 25(19):5799, 2019.

155. Westdorp H., Creemers J.H.A., van Oort I.M., Schreibelt G., Gorris M.A.J., Mehra N., Simons M., de Goede A.L., van Rossum M.M., Croockewit A.J., Figdor C.G., Witjes J.A., Aarntzen E.H.J.G., Mus R.D.M., Brüning M., Petry K., Gotthardt M., Barentsz J.O., de Vries I.J.M. and Gerritsen W.R. Blood-derived dendritic cell vaccinations induce immune responses that correlate with clinical outcome in patients with chemo-naive castration-resistant prostate cancer. Journal for ImmunoTherapy of Cancer 7(1):302, 2019.

156. Wilson E.B., Kidani Y., Elsaesser H., Barnard J., Raff L., Karp C.L., Bensinger S. and Brooks D.G. Emergence of distinct multiarmed immunoregulatory antigen-presenting cells during persistent viral infection. Cell Host Microbe 11(5):481-491, 2012.

157. Wu J., Li S., Yang Y., Zhu S., Zhang M., Qiao Y., Liu Y.-J. and Chen J. TLR-activated plasmacytoid dendritic cells inhibit breast cancer cell growth in vitro and in vivo. Oncotarget 8(7):11708-11718, 2017.

158. Xu H., Wang F., Li H., Ji J., Cao Z., Lyu J., Shi X., Zhu Y., Zhang C., Guo F., Fang Z., Yang B. and Sun Y. Prostatic Acid Phosphatase (PAP) Predicts Prostate Cancer Progress in a Population-Based Study: The Renewal of PAP? Disease Markers 2019:7090545, 2019.

159. Xue B.H., Zhang Y., Sosman J.A. and Peace D.J. Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen. The Prostate 30(2):73-78, 1997.

160. Ye Y., Gaugler B., Mohty M. and Malard F. Plasmacytoid dendritic cell biology and its role in immune-mediated diseases. Clin Transl Immunology 9(5):e1139-e1139, 2020.

161. Zamame Ramirez J.A., Romagnoli G.G., Falasco B.F., Gorgulho C.M., Sanzochi Fogolin C., Dos Santos D.C., Junior J.P.A., Lotze M.T., Ureshino R.P. and Kaneno R. Blocking drug-induced autophagy with chloroquine in HCT-116 colon cancer cells enhances DC maturation and T cell responses induced by tumor cell lysate. Int Immunopharmacol 84:106495-106495, 2020.

162. Zhang J.G., Czabotar P.E., Policheni A.N., Caminschi I., Wan S.S., Kitsoulis S., Tullett K.M., Robin A.Y., Brammananth R., van Delft M.F., Lu J., O'Reilly L.A., Josefsson E.C., Kile B.T.,

Chin W.J., Mintern J.D., Olshina M.A., Wong W., Baum J., Wright M.D., Huang D.C., Mohandas N., Coppel R.L., Colman P.M., Nicola N.A., Shortman K. and Lahoud M.H. The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. Immunity 36(4):646-657, 2012.

163. Zhou L., Ivanov, II, Spolski R., Min R., Shenderov K., Egawa T., Levy D.E., Leonard W.J. and Littman D.R. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nature immunology 8(9):967-974, 2007.