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Terapia gênica com interferon-alfa no controle do câncer colorretal

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

Orientador: Prof. Dr. Ramon Kaneno

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Palavras-chave: Câncer colorretal; Células dendríticas; Interferon alfa; Terapia gênica.

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Resumo

O interferon alfa (IFN-α), um IFN do tipo I, se apresenta como uma citocina com grande potencial terapêutico, pois atua no combate direto às células tumorais, além de agir sobre a maturação de células dendríticas (DCs), que são células apresentadoras de antígenos profissionais e peças chave na elaboração da uma resposta antitumoral. Entretanto, a administração sistêmica de citocinas pode produzir toxicidade importante nos pacientes, de modo que a indução de sua produção *in situ* poderia representar uma forma de imunomodulação mais adequada. Assim, o objetivo deste estudo é verificar a ação de vetores lentivirais carregando o gene do IFN-α para transdução de células tumorais, permitindo assim a produção localizada de IFN-α, a fim de explorar, *in vitro*, seu potencial lítico e imunomodulatório sobre DCs. Vetores lentivirais carregando o gene do IFN-α humano (Lego-IFN) ou GFP (Lego-GFP) foram utilizados para a transdução *in vitro* de células de câncer colorretal. A transdução foi feita com diferentes multiplicidades de infecção (MOIs – 0.3, 1.0, 2.0, 4.0) para avaliarmos o efeito dose-dependente, seguido de co-cultura com DCs derivadas de monócitos de doadores saudáveis (DC-0.3, DC-1.0, DC-2.0, DC-4.0). Após 48h de co-cultura, as DCs foram avaliadas fenotípica e funcionalmente, através da análise dos marcadores de membrana por citometria de fluxo, capacidade de aloestimulação e de indução de linfócitos T citotóxicos (CTLs). Nós observamos que a transdução com Lego-GFP, mas não com Lego-IFN, aumentou a imunogenicidade das células tumorais, com aumento de expressão de CD54 e HLA-DR. A co-cultura de DCs com células tumorais transduzidas com Lego-IFN aumentou discretamente seu perfil de ativação, mas não seu potencial aloestimulatório *in vitro*. Observamos que linfócitos cultivados com DC-2.0 produziram níveis mais altos de IFN-γ, sugerindo a indução de um perfil Th1, enquanto que DC-GFP induziu maior produção de IL-10 e IL-4. DC-4.0 foi mais eficiente na geração de CTLs que o controle DC, entretanto, DC-GFP induziu ainda mais proliferação de linfócitos T $CD8^+$. O aumento de imunogenicidade das células tumorais e o potencial superior de geração de CTLs por Lego-GFP e DC-GFP exigem uma maior investigação.

Palavras-chave: câncer colorretal, citocinas, interferon alfa, células dendríticas

Abstract

Interferon alpha (IFN-α) is a type I IFN with great therapeutic potential, since it is able to directly fight tumor cells and enhance the maturation of dendritic cells (DCs), the main antigen-presenting cells, required for an effective antitumor response. However, the systemic administration of cytokines can induce severe collateral effects. Therefore, the induction of cytokine secretion *in situ* should represent a more adequate approach for cytokine-based immunotherapy. Thus, the goal of this study was to induce IFN-α secretion by colon cancer cells by transduction with a lentivirus vector carrying the human IFN-α gene, followed by analysis of its immunomodulatory potential over DCs. Transduction was made with different multiplicities of infection (MOIs – 0.3, 1.0, 2.0 and 4.0) to evaluate the dose-dependent effects. Such cells were co-cultured with monocyte-derived DCs from healthy donors (DC-0.3, DC-1.0, DC-2.0 and DC-4.0). Forty-eight hours later, DCs were evaluated for their phenotype (surface activation/maturation markers) by flow cytometry, their ability to induce allogeneic response in a mixed leukocyte reaction (MLR) and effectiveness to induce cytotoxic T cells. We observed that transduction with Lego-GFP, but not Lego-IFN, increased tumor cells' immunogenicity with up-regulation of the markers CD54 and HLA-DR. Co-culture of Lego-IFN-transduced tumor cells with DCs slightly enhanced their activation phenotype but not their potential to stimulate T cell proliferation *in vitro*. Furthermore, we observed that lymphocytes cultured with DC-2.0 produced higher levels of IFN-γ, suggesting an induction of Th1 profile on T cells, while DC-GFP induced more IL-10 and IL-4. Additionally, DC-4.0 was more efficient in generating cytotoxic T lymphocytes (CTLs) that the control DC, however DC-GFP induced even more $CD8^+$T cell proliferation. The enhancement of tumor cell immunogenicity and the superior induction of CTLs by Lego-GFP and DC-GFP require additional investigation.

Keywords: colorectal cancer, cytokines, interferon alpha, dendritic cells

Sumário

Capítulo I: Revisão da literatura

Interferons do tipo I

Os interferons (IFNs) foram descritos pela primeira vez em 1957, por Isaacs e Lindenmann, que investigavam o fenômeno de interferência viral, no qual a exposição a um vírus atenuado confere resistência contra infecções posteriores pelo mesmo vírus (1). Até então, acreditava-se que o bloqueio de receptores na membrana da célula alvo pelo vírus inativado era responsável por conferir resistência às células e que este era um fenômeno relativamente "passivo". Entretanto, Isaacs e Lindenmann não encontravam evidências de que o vírus atenuado estivesse *"impedindo a captura de vírus vivos pelas células (...) e que para estabelecer o fenômeno de interferência, mais de 4h de incubação eram necessárias"*. Em seus experimentos, os autores utilizaram pedaços da membrana corioalantóide de ovos de galinha cultivados *in vitro* tratados com o vírus *influenza* A atenuado pelo calor (o que abolia sua infectividade, mas não suas propriedades de interferência), para depois serem desafiados com vírus *influenza* A vivos. Observaram que o fenômeno de interferência acontecia de fato nas membranas infectadas, o que foi chamado de "interferência inicial", mas que, além disso, ao transferir as membranas para uma cultura fresca, livre de vírus, o novo sobrenadante também apresentava uma "interferência residual", de magnitude comparável à inicial. Este achado levou os autores a especularem que a própria membrana estava envolvida no fenômeno de interferência e não somente as partículas virais. Os autores cogitaram a possibilidade de que a membrana pré-imunizada produzia um "agente de interferência" caracterizado como *"uma macromolécula com propriedades distintas daquelas dos vírus* influenza *A atenuados".* Este agente foi chamado de interferon. Em humanos, existem 3 famílias distintas de IFNs. Os do tipo I, no qual se enquadram os IFNs α, β, ω e ε (2); tipo II, representado pelo IFN-γ (3) e tipo III, no qual estão os mais recentemente descobertos IFNs λ (4). Dada a amplitude do tema, a presente revisão é focada na biologia do IFN tipo I, com ênfase no papel do IFN-α na estimulação do sistema imune e no combate ao câncer.

IFNs do tipo I são produzidos por praticamente qualquer tipo celular em resposta a

uma gama de estímulos externos que muito frequentemente envolve a ativação de receptores de reconhecimento de padrão (PRR – *pattern recognition receptors*) por componentes e/ou produtos microbianos (5), além de componentes endógenos ectopicamente expressos, como RNA e DNA extracelulares (6). Os PRRs estão presentes na superfície, citoplasma e compartimentos endossomais das células. Na superfície, os receptores *toll-like* (TLR) reconhecem principalmente produtos microbianos, sendo que o TLR4 é o mais potente indutor de IFNs do tipo I mediante ativação por lipolisacarídeos (LPS) de origem bacteriana (5). O receptor RIG-I (*RNA helicases retinoic acid-inducible gene I*) é um dos principais receptores citosólicos de RNA e está intimamente ligado com a produção de IFNs do tipo I, assim como o receptor MDA5 (*melanoma differentiation-associated gene 5*) (5). Em compartimentos endossomais, o TLR3 reconhece RNA de fita dupla e TLR7 e 8 reconhecem RNA de fita simples (6). Dependendo da natureza do patógeno (como sua espécie e local de infecção) e da origem da célula alvo, os PRRs são diferencialmente ativados, na tentativa de montagem de uma resposta adequada ao tipo de infecção (7). Assim, ampliando o conceito primordial de indução de IFN tipo I por infecções virais, sabe-se hoje que muitos outros estímulos podem ser responsáveis pela indução dessa citocina.

A família de IFNs do tipo I compartilha o receptor IFNAR, que comanda a indução de diversos genes através da ativação de ISGF3 (*IFN-stimulated gene factor 3*) que induz a subsequente fosforilação de fatores de transcrição como STAT1 e 2 e IRF9 *(interferon regulatory factor 9)* (8), levando à sua ativação e entrada no núcleo, possibilitando a transcrição de genes IFN-respondedores, em sua maioria com papel antiviral, envolvidos em processos como síntese de proteínas, crescimento e sobrevivência celulares (9) (8).

TLRs e sinalização de IFNs do tipo I

Os TLRs são PRRs transmembrana do tipo I e respondem principalmente a estímulos microbianos, tendo importante função na indução de imunidade inata. Em mamíferos, a família dos TLRs consiste em 13 membros, sendo que os TLR1-9 são conservados entre

humanos e camundongos (9). Há uma estrutura básica entre os TLRs, composta por um ectodomínio N-terminal envolvido no reconhecimento de padrões moleculares patógeno-associados (PAMPs – *pathogen associated molecular patterns*), um domínio transmembrana do tipo I e um domínio toll/receptor de interleucina 1 (IL-1) (TIR) intracelular responsável pela transdução do sinal (6) (9).

Dependendo da sua localização celular e de seus ligantes, os TLRs podem ser subdivididos em 2 grupos: o primeiro, composto pelos TLRs1,2,4,5,6 e 11, é expresso na superfície das células e reconhecem principalmente componentes de membrana microbiana como lipídeos e proteínas; o segundo engloba os TLRs3, 7, 8 e 9, que estão localizados em vesículas citoplasmáticas e reconhecem material genético (6).

A ativação de TLR3 e TLR4 leva à indução direta do promotor do IFN-β, que desencadeia o recrutamento de proteínas adaptadoras, como TRIF (*TIR domain-containing adaptor protein inducing IFNβ*), que levam complexos sinalizadores até o núcleo, onde IRF3 (*interferon regulatory factor-3*) é ativado e promove resposta transcricional de IFN-β (9) (10). Na maioria dos casos, IRF3 e 7 são fundamentais para a produção de IFN-α/β (5). TLR4 responde à ligação com LPS bacteriano através de um complexo formado na superfície da célula com a proteína de diferenciação mielóide 2 (*MD2 - myeloid differentiation protein 2)* (11). O complexo TLR4-MD2 recruta moléculas adaptadoras que irão para o núcleo iniciar a transdução de sinal.

A produção de IFNs do tipo I também pode ser mediada pela ligação de TLR7, 8 e 9 e indução da molécula adaptadora MyD88, que é recrutada para o domínio TIR do receptor e orquestra a ativação do fator de transcrição IRF7 (12). TLR7 e 9 localizam-se no retículo endoplasmático de células não estimuladas e migram para as vesículas endolisossomais após ativação (13). TLR7 reconhece principalmente RNA retroviral de fita simples e é altamente expresso em DCs plasmocitóides (pDCs), capazes de liberar altas doses de IFNs do tipo I após infecção viral. Há uma alta expressão de TLR7 e 9 em pDCs (8). Nestas células, a ativação de TLR7 ocorre após internalização de partículas virais e direcionamento

para vesículas endolisossomais onde RNA de fita simples é reconhecido, iniciando a resposta antiviral (6). Além disso, TLR7 quando expresso em DCs convencionais (cDCs) e ativado por RNA bacteriano, também induz a produção de IFNs do tipo I (14). TLR8 reconhece RNA de fita simples e é expresso ubiquamente, especialmente em monócitos, e é ativado após infecção bacteriana (6). TLR9 reconhece DNA microbiano, que contém ilhas CpG não metiladas, forte indutoras de resposta imune (15).

A estimulação simultânea de dois ou mais TLRs, o que geralmente ocorre em situações de infecção, pode desencadear atividades sinérgicas, agonistas ou antagonistas entre os receptores (16). Foi demonstrado que na infecção por *Mycobacterium tuberculosis,* a ativação de TLR2 pela bactéria inibe a produção de IFNs do tipo I por TLR9, através da rápida degradação da molécula IRAK-1 (*IL-1R associated kinase 1*), necessária para a sinalização de IFNs do tipo I via MyD88 (16) (17).

RIG-I e sinalização de IFNs do tipo I

Os receptores RIG-I e RIG-I-*like* (RLR) reconhecem vírus em replicação no citoplasma, especialmente na fase inicial de infecção e são capazes de distinguir RNA invasor de RNA endógeno (18). A família dos RLRs compreende os receptores RIG-I, MDA5 e LGP2. Eles possuem uma estrutura em comum, o domínio helicase DExD/Hbox, cuja função é o desdobramento de RNA de fita dupla (19). Estes receptores também possuem o domínio de recrutamento e ativação de caspase (CARD), responsável direto pela indução de produção de INFs do tipo I (18). A ação dos RLRs está ligada à ubiquitinação de seus domínios. O domínio CARD dos receptores RIG-I necessita de ubiquitinação para que haja sinalização da produção de IFNs do tipo I. O mesmo ocorre com o receptor MDA5, porém sob controle de outros fatores de ubiquitinação. Além da regulação positiva de sua atividade, os RLRs também são negativamente controlados por ubiquitinação, evitando a ativação descontrolada destes receptores (18).

Os RLRs ainda possuem um domínio C-terminal que se liga a RNA de fital dupla. Na

presença de ATP, o domínio CARD interage com IPS-1 *(interferon β promoter stimulator-1),* uma molécula imprescindível para a sinalização de IFNs do tipo I mediante infecção viral (20), que se localiza na membrana externa da mitocôndria. MDA5 também sinaliza a produção de IFNs através de IPS-1, que ativa IRF3 e IRF7 (21).

RIG-I é essencial para a produção de IFNs do tipo I, além de outras citocinas pró inflamatórias, mediante infecção viral (18). Células que não possuem RIG-I tem produção deficiente de IFNs do tipo I frente à infecção com diversas famílias de vírus (22). Células com o gene do LGP2 nocauteado tem produção de IFNs diminuída, mas não totalmente bloqueada, sugerindo que este receptor coopera com RIG-I e MDA5 no reconhecimento de RNA viral (18).

Após ligação com RNA viral, RIG-I passa por uma mudança conformacional para expor seu domínio CARD, na presença de ATP. O domínio CARD interage com proteínas adaptadoras para a ativação de quinases de forma TRAF3 *(tumor necrosis fator receptor associated fator 3)*-dependente. Estas quinases fosforilam IRF3 e 7 (23), resultando na sua translocação para o núcleo e consequente transcrição de genes IFN-respondedores (24).

NLRs e sinalização de IFNs do tipo I

As proteínas NLRs *(nucleotide binding domain and leucina rich repeat)* são uma família de proteínas citosólicas implicadas na resposta inata frente a infecções bacterianas e indução de resposta anti-inflamatória e estão associadas à regulação de inflamassomas (25). A composição destas proteínas é relativamente comum a todos os membros e consiste em: um domínio amino-terminal EBD *(effector-binding domain)*, um domínio central NOD (nucleotide-binding oligomerization domain) responsável pela própria oligomerização e um domínio LRD *(carboxy-terminal ligand-recognition domain)* (26).

Os NLRs podem ser subdivididos em 2 categorias, NLRC e NLRP. Os NLRC possuem domínios CARD e compreendem NOD1 e NOD2, entre outros. Estes receptores estão envolvidos no reconhecimento citoplasmático de bactérias, mais especificamente de

fragmentos de peptideoglicanos. Diferentes peptideoglicanos ativam receptores NOD distintos, o que leva a diferentes vias de sinalização (27). O segundo grupo, NLRP, são proteínas com um domínio PYR *(pyrin)* (28). A ligação de NLRs por DAMPs *(danger-associated molecular patterns)* ou PAMPs leva à ativação de NF-κB *(nuclear fator kappa B)*, sinalização de MAPK ou inflamassomas (29).

O receptor NOD2 está envolvido na sinalização de IFNs do tipo I, especialmente de IFN-β, através do reconhecimento de RNA viral de fita simples e ativação de IRF3. Semelhantemente à sinalização por RIG-I, o domínio CARD de NOD 2 também necessita de ligação com IPS-1 para induzir a produção de INFs (30).

O papel do IFN-α na terapia antitumoral

O IFN-α é uma das citocinas mais amplamente exploradas no contexto da terapia sistêmica, como revisto por Dranoff (31). A atividade antitumoral relevante do IFN-α vem sendo amplamente testado para o tratamento de vários tipos de tumores (32). Por exemplo, em um ensaio clínico realizado com pacientes com melanoma, o tratamento com IFN-α2b em alta dosagem (1000 U/mL) produziu modulação positiva da ação de linfócitos T e células NK e aumento na expressão de MHC de classe II pelas células tumorais (33). Em outro ensaio do mesmo grupo, com pacientes de melanoma metastático, os autores observaram que o tratamento sistêmico com IFN-α2b reduziu a incidência de recidiva da doença (34).

O IFN-α também se mostrou eficaz no tratamento de tumores hematológicos, a exemplo da leucemia mielóide crônica, com uma melhora de sobrevida em 15% dos pacientes tratados com quimioterapia e a citocina, *versus* aqueles submetidos somente ao regime convencional de quimioterapia (35).

Medicamentos que têm como princípio ativo o IFN-α são atualmente comercializados por diversos laboratórios farmacêuticos. Em 1986 o FDA aprovou as drogas Intron A (IFN-α2b, Schering) e Roferon A (IFN-α2a, Hoffman-La Roche). Mais recentemente foram também aprovadas as formas peguiladas da citocina, que possuem meia vida mais longa no

organismo, a exemplo da droga Pegasys (PEG-IFN-α2a, Hoffman-La Roche), aprovada em 2002. Porém, assim como na terapia com IL-2, o uso sistêmico do IFN-α também oferece reações adversas ao paciente, incluindo manifestações físicas e psiquiátricas, fato que pode exigir a redução da dose, a implementação de intervalos no tratamento ou a interrupção do uso do medicamento.

Além de sua ação direta sobre as células tumorais, o IFN-α tem sido amplamente explorado pelo seu importante papel no desenvolvimento dirigido de DCs. Nesse sentido, foi relatado que PBMCs (monócitos obtidos a partir de sangue periférico) cultivados em meio rico em IFN-α e GM-CSF adquiriram características morfológicas de DCs, com alta expressão de MHC-I e -II, da molécula co-estimulatória B7 e do marcador CD40, além de se mostrarem boas apresentadoras de antígenos (36). Estudos *in vitro* demonstram a maior eficiência de DCs geradas na presença de IFN-α, em comparação com DCs geradas na ausência desta citocina, quanto à capacidade de sensibilizar linfócitos T $CD8^+$ com alta atividade citotóxica. Observou-se também geração de maior número linfócitos T $CD4^+$ secretores de IFN-γ e menor expansão de linfócitos T $CD4^+CD25^+Foxp3^+$ (regulatórios) (37). Tais resultados indicam que preparações de DCs utilizando IFN-α ou a presença da citocina no ambiente tumoral induzam DCs que apresentem resultados clínicos superiores aos observados até hoje.

O sucesso das vacinas de DCs diferenciadas a partir de monócitos é dependente da eficiência em se gerar DCs maduras para administração em animais portadores de tumor, sendo que a presença de IFN-α no meio de cultura dessas células (38-40), é capaz de direcionar essa maturação para que produzam IL-12 e adquiram o fenótipo α-DC1. Essas DCs, por sua vez, têm a habilidade de dirigir a maturação de linfócitos T para um fenótipo Th1, favorecendo a resposta antitumoral. A adição de prostaglandina E_2 (PGE_2) ao coquetel de citocinas usadas na preparação de vacinas de DCs tem o propósito de induzir a maturação destas células (37). Entretanto, esse mediador sozinho tende a reduzir a habilidade das DCs em desencadear um perfil de resposta do tipo Th1, com produção de

IFN-γ. A adição de IFN-α, por sua vez, promove a secreção de IFN-γ pelos linfócitos de modo dose-dependente, mesmo na presença de PGE_2 (41).

Em estudo clínico de fase I/II observou-se que a administração de vacinas terapêuticas de αDC-1 derivadas de monócitos do sangue a 22 pacientes com glioma maligno recorrente não provocou mortes ou efeitos tóxicos de nível 3 ou 4. Os autores demonstraram pela primeira vez a indução *in vivo* de linfócitos T $CD8^+$ contra 3 dos 4 peptídeos encontrados em gliomas (42). Em outro estudo, as αDC-1 foram geradas com o uso de um coquetel de citocinas contendo IFN-α e ácido poliosínico:policitidílico (poli I:C). Sua comparação com DCs convencionais mostra que as αDC-1 são mais hábeis em induzir a geração de linfócitos $CD4^+$ produtores de IFN-γ e que a capacidade de induzir a produção de IL-12 também é superior às DCs convencionais, com produção de níveis de IL-12 cerca de 100 vezes maiores do que de IL-10 (43).

O uso de vetores virais na terapia gênica

Entretanto, embora diferentes preparações de vacinas de DCs tenham demonstrado sua eficiência como vacina terapêutica, essa alternativa de tratamento continua a ter um custo extremamente elevado, dificultando seu uso como terapia corrente. Assim, ainda na tentativa de modular a resposta imune antitumoral utilizando o potencial imunomodulatório das citocinas e o papel central das DCs na elaboração desta resposta, as vacinas gênicas utilizando vetores virais têm sido cada vez mais investigadas no campo da imunoterapia antitumoral. Nesse aspecto, a versatilidade dos vetores virais permite customizar as vacinas, de forma que atinjam um alvo muito específico ou tenham ação localizada, minimizando os efeitos adversos comuns produzidos por terapias sistêmicas. Deste modo, alguns estudos têm relatado a inserção de genes codificadores de antígenos tumorais, visando potencializar a atividade das células apresentadoras de antígenos (44, 45). Outros grupos têm trabalhado com a inserção de genes codificadores de proteínas imunoestimulatórias, como as citocinas, desencadeando uma resposta imune direcionada(46), além da possibilidade de inserção de

genes codificadores de moléculas com ação lítica direta sobre as células tumorais (47).

Um aspecto importante na construção das vacinas gênicas com vetores virais é que não há necessidade de uso de células dos pacientes ou de qualquer material biológico específico que restrinja demasiadamente sua preparação em laboratório ou sua utilização terapêutica (47).

Muitos tipos de vetores virais têm sido avaliados para uso na imunoterapia, cujas características específicas devem atender a diferentes demandas ou estratégias moleculares. Os vetores adenovirais são provavelmente as ferramentas mais extensamente exploradas no campo da vetorologia aplicada à imunoterapia do câncer (48), porém outros vírus vêm apresentando ótima aplicabilidade na área e entre eles destacam-se os lentivírus. Os lentivírus são membros da família *Retroviridae* (sendo o vírus da imunodeficiência humana-1 [HIV-1] o mais bem estudado desta família) (49). Segundo revisto por Emeagi e colaboradores (49), os lentivírus possuem características que os tornam ótimos candidatos para aplicação na terapia gênica, como a) possibilidade de inserção de grande quantidade de material genético (~10 kilobases); b) os lentivírus selecionam locais seguros de integração com o genoma hospedeiro, o que proporciona uma expressão persistente do gene de interesse e aumenta sua segurança de uso; c) são capazes de transduzir células que se dividem e células quiescentes; d) em geral não há imunidade pre-existente a esses vetores (50) e eles desencadeiam resposta imune neutralizadora menos intensa quando comparada àquela induzida por vetores adenovirais, não comprometendo a expressão do gene de interesse [36]. Além disso, vetores derivados de HIV-1 de terceira geração tem pobre capacidade replicativa e contém apenas três (gag, pol e rev) dos nove genes do vírus (51), garantindo integração estável no genoma do hospedeiro e ausência de expressão de qualquer gene viral que não os transgenes (52).

Levando em conta estas informações, consideramos que a terapia gênica é uma importante estratégia imunoterapêutica a ser estudada, para o desenvolvimento de propostas que atendam à demanda crescente de pacientes com câncer em todo o mundo.

Considerando as DCs como elementos centrais da resposta imune antitumoral, nossa visão é de que a estimulação *in vivo* das DCs, através da administração de citocinas ou moduladores capazes de induzir a produção de mediadores como IL-12, IL-18, IFN-γ e IFN-α ou de inibir a geração *in situ* de citocinas regulatórias como IL-10, TFG-β e fatores angiogênicos, poderia ser uma abordagem economicamente viável. Além disso, visto que nesse caso não haveria restrição do tratamento pelo complexo principal de histocompatibilidade (MHC), nem pela disponibilidade de DCs (outra limitação técnica dessas vacinas), alternativas nessa linha teriam aplicabilidade mais ampla.

Objetivo

Assim a transdução de células tumorais *in situ* com gene de IFN-α seria capaz de induzir a síntese desta citocina no sítio tumoral, favorecendo a maturação de DCs produtoras de IL-12 e a consequente ativação de células Th1. Desse modo, o presente estudo teve por objetivo avaliar a viabilidade de uso de um lentivírus como vetor para a transferência do gene de IFN-α humano em células tumorais e o efeito da transdução sobre a capacidade de sensibilização e ativação de células dendríticas.

Referências bibliográficas

1. Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proceedings of the Royal Society of London Series B, Biological sciences. 1957;147(927):258-67.
2. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. Immunological reviews. 2004;202:8-32.
3. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Advances in immunology. 2007;96:41-101.
4. O'Brien TR, Prokunina-Olsson L, Donnelly RP. IFN-lambda4: the paradoxical new member of the interferon lambda family. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2014;34(11):829-38.
5. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. Nature reviews Immunology. 2015;15(2):87-103.
6. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology. 2010;11(5):373-84.
7. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nature reviews Immunology. 2006;6(9):644-58.
8. Uematsu S, Akira S. Toll-like receptors and Type I interferons. The Journal of biological chemistry. 2007;282(21):15319-23.
9. Perkins DJ, Vogel SN. Space and time: New considerations about the relationship between Toll-like receptors (TLRs) and type I interferons (IFNs). Cytokine. 2015;74(2):171-4.
10. Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, et al. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. Immunity. 2002;17(3):251-63.
11. Akashi-Takamura S, Miyake K. TLR accessory molecules. Current opinion in immunology. 2008;20(4):420-5.
12. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, et al. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. Nature immunology. 2004;5(10):1061-8.
13. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature. 2008;452(7184):234-8.
14. Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, et al. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nature immunology. 2009;10(6):587-94.
15. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
16. Liu YC, Simmons DP, Li X, Abbott DW, Boom WH, Harding CV. TLR2 signaling depletes IRAK1 and inhibits induction of type I IFN by TLR7/9. Journal of immunology. 2012;188(3):1019-26.
17. Simmons DP, Canaday DH, Liu Y, Li Q, Huang A, Boom WH, et al. Mycobacterium tuberculosis and TLR2 agonists inhibit induction of type I IFN and class I MHC antigen cross processing by TLR9. Journal of immunology. 2010;185(4):2405-15.
18. Kato H, Takahasi K, Fujita T. RIG-I-like receptors: cytoplasmic sensors for non-self RNA. Immunological reviews. 2011;243(1):91-8.
19. Yoneyama M, Fujita T. RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. Cytokine & growth factor reviews. 2007;18(5-6):545-51.
20. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nature immunology. 2005;6(10):981-8.
21. Kumar H, Kawai T, Kato H, Sato S, Takahashi K, Coban C, et al. Essential role of IPS-1 in innate immune responses against RNA viruses. The Journal of experimental medicine. 2006;203(7):1795-803.

22. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature. 2006;441(7089):101-5.
23. Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature. 2006;439(7073):204-7.
24. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature. 2005;434(7034):772-7.
25. Kufer TA, Sansonetti PJ. NLR functions beyond pathogen recognition. Nature immunology. 2011;12(2):121-8.
26. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nature reviews Immunology. 2003;3(5):371-82.
27. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nature immunology. 2003;4(7):702-7.
28. Claes AK, Zhou JY, Philpott DJ. NOD-Like Receptors: Guardians of Intestinal Mucosal Barriers. Physiology. 2015;30(3):241-50.
29. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like receptors in microbial recognition and host defense. Immunological reviews. 2009;227(1):106-28.
30. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, et al. Activation of innate immune antiviral responses by Nod2. Nature immunology. 2009;10(10):1073-80.
31. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nature reviews Cancer. 2004;4(1):11-22.
32. Tarhini AA, Gogas H, Kirkwood JM. IFN-alpha in the treatment of melanoma. J Immunol.189(8):3789-93.
33. Kirkwood JM, Richards T, Zarour HM, Sosman J, Ernstoff M, Whiteside TL, et al. Immunomodulatory effects of high-dose and low-dose interferon alpha2b in patients with high-risk resected melanoma: the E2690 laboratory corollary of intergroup adjuvant trial E1690. Cancer. 2002;95(5):1101-12.
34. Kirkwood JM, Strawderman MH, Ernstoff MS, Smith TJ, Borden EC, Blum RH. Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1996;14(1):7-17.
35. Interferon alfa versus chemotherapy for chronic myeloid leukemia: a meta-analysis of seven randomized trials: Chronic Myeloid Leukemia Trialists' Collaborative Group. Journal of the National Cancer Institute. 1997;89(21):1616-20.
36. Paquette RL, Hsu NC, Kiertscher SM, Park AN, Tran L, Roth MD, et al. Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells. Journal of leukocyte biology. 1998;64(3):358-67.
37. Gigante M, Mandic M, Wesa AK, Cavalcanti E, Dambrosio M, Mancini V, et al. Interferon-alpha (IFN-alpha)-conditioned DC preferentially stimulate type-1 and limit Treg-type in vitro T-cell responses from RCC patients. Journal of immunotherapy. 2008;31(3):254-62.
38. Giermasz AS, Urban JA, Nakamura Y, Watchmaker P, Cumberland RL, Gooding W, et al. Type-1 polarized dendritic cells primed for high IL-12 production show enhanced activity as cancer vaccines. Cancer immunology, immunotherapy : CII. 2009;58(8):1329-36.
39. Lee JJ, Foon KA, Mailliard RB, Muthuswamy R, Kalinski P. Type 1-polarized dendritic cells loaded with autologous tumor are a potent immunogen against chronic lymphocytic leukemia. Journal of leukocyte biology. 2008;84(1):319-25.
40. Yang DH, Kim MH, Lee YK, Hong CY, Lee HJ, Nguyen-Pham TN, et al. Successful cross-presentation of allogeneic myeloma cells by autologous alpha-type 1-polarized dendritic cells as an effective tumor antigen in myeloma patients with matched monoclonal immunoglobulins. Annals of hematology.90(12):1419-26.

41. Sakakibara M, Kanto T, Inoue M, Kaimori A, Yakushijin T, Miyatake H, et al. Quick generation of fully mature dendritic cells from monocytes with OK432, low-dose prostanoid, and interferon-alpha as potent immune enhancers. J Immunother. 2006;29(1):67-77.
42. Okada H, Kalinski P, Ueda R, Hoji A, Kohanbash G, Donegan TE, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. J Clin Oncol.29(3):330-6.
43. Hansen M, Hjorto GM, Donia M, Met O, Larsen NB, Andersen MH, et al. Comparison of clinical grade type 1 polarized and standard matured dendritic cells for cancer immunotherapy. Vaccine.31(4):639-46.
44. Odunsi K, Matsuzaki J, Karbach J, Neumann A, Mhawech-Fauceglia P, Miller A, et al. Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. Proceedings of the National Academy of Sciences of the United States of America.109(15):5797-802.
45. Hermans IF, Chong TW, Palmowski MJ, Harris AL, Cerundolo V. Synergistic effect of metronomic dosing of cyclophosphamide combined with specific antitumor immunotherapy in a murine melanoma model. Cancer research. 2003;63(23):8408-13.
46. Malvicini M, Rizzo M, Alaniz L, Pinero F, Garcia M, Atorrasagasti C, et al. A novel synergistic combination of cyclophosphamide and gene transfer of interleukin-12 eradicates colorectal carcinoma in mice. Clin Cancer Res. 2009;15(23):7256-65.
47. Eisenberger A, Elliott BM, Kaufman HL. Viral vaccines for cancer immunotherapy. Hematology/oncology clinics of North America. 2006;20(3):661-87.
48. de Gruijl TD, van de Ven R. Chapter six--Adenovirus-based immunotherapy of cancer: promises to keep. Advances in cancer research.115:147-220.
49. Emeagi PU, Goyvaerts C, Maenhout S, Pen J, Thielemans K, Breckpot K. Lentiviral vectors: a versatile tool to fight cancer. Current molecular medicine.13(4):602-25.
50. Kootstra NA, Verma IM. Gene therapy with viral vectors. Annual review of pharmacology and toxicology. 2003;43:413-39.
51. Manilla P, Rebello T, Afable C, Lu X, Slepushkin V, Humeau LM, et al. Regulatory considerations for novel gene therapy products: a review of the process leading to the first clinical lentiviral vector. Human gene therapy. 2005;16(1):17-25.
52. Miller AD, Miller DG, Garcia JV, Lynch CM. Use of retroviral vectors for gene transfer and expression. Methods in enzymology. 1993;217:581-99.

Capítulo II: manuscrito do trabalho experimental redigido de acordo com as normas da revista Cancer Immunology, Immunotherapy

Potential of IFN-α-producing tumor cells on the activation and maturation of dendritic cells

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Abstract: Interferon alpha (IFN-α) is a type I IFN with great therapeutic potential, since it is able to directly fight tumor cells and enhance the maturation of dendritic cells (DCs), the main antigen-presenting cells, required for an effective antitumor response. However, the systemic administration of cytokines can induce severe collateral effects. Therefore, the induction of cytokine secretion *in situ* should represent a more adequate approach for cytokine-based immunotherapy. Thus, the goal of this study was to induce IFN-α secretion by colon cancer cells by transduction with a lentivirus vector carrying the human IFN-α gene, followed by analysis of its immunomodulatory potential. Transduction was made with different multiplicities of infection (MOIs – 0.3, 1.0, 2.0 and 4.0) to evaluate the dose-dependent effects. Such cells were co-cultured with monocyte-derived DCs from healthy donors (DC-0.3, DC-1.0, DC-2.0 and DC-4.0). Forty-eight hours later, DCs were evaluated for their phenotype (surface activation/maturation markers) by flow cytometry, their ability to induce allogeneic response in a mixed leukocyte reaction (MLR) and effectiveness to induce cytotoxic T cells. We observed that transduction with Lego-GFP, but not Lego-IFN, increased tumor cells' immunogenicity. Co-culture of Lego-IFN-transduced tumor cells with DCs slightly enhanced their activation phenotype but not their potential to stimulate T cell proliferation *in vitro*. Furthermore, we observed that lymphocytes cultured with DC-2.0 produced higher levels of IFN-γ, suggesting an induction of Th1 profile on T cells, while DC-GFP induced more IL-10 and IL-4. Additionally, DC-4.0 was more efficient in generating cytotoxic T lymphocytes (CTLs) that the control DC, however DC-GFP induced even more CD8+T cell proliferation. The enhancement of tumor cell immunogenicity and the superior induction of CTLs by Lego-GFP and DC-GFP required additional investigation.

Keywords: colorectal cancer, cytokines, interferon, dendritic cells

Précis: Transduction with Lego-GFP, but not Lego-IFN, increased tumor cells' immunogenicity; Co-culture of Lego-IFN-transduced tumor cells with DCs slightly enhanced their activation phenotype but not their potential to stimulate T cell proliferation *in vitro*.

Abbreviations

7AAD – 7- aminoactinomycin D

ANOVA – analysis of variance

CCR – colorectal cancer

CD – cluster of differentiation

CFSE - carboxyfluorescein succinimidyl ester

CTLs – cytotoxic T lymphocytes

DCs – dendritic cells

DEX – dextramer

ELISA – enzyme linked immuno sorbent assay

FDA – U.S. Food and Drug Administration

GFP – green fluorescent protein

GM-CSF – granulocyte/macroohage colony stimulating factor

HLA – human leukocyte antigen

iDCs – immature dendrtitic cells

Influenza – FLU

IFN – interferon

IL – interleukin

MLR – mixed leukocyte reaction

MOI – multiplicity of infection

mDCs – mature dendritic cells

PBMCs – peripheral blood mononuclear cells

PE – phycoerytrin

PD-L1 – programed death ligand-1

PD-1 – programed cell death protein 1

PSA – prostatic specific antigen

SFFV - retroviral enhancer/promoter of spleen focus forming virus

Introduction

Cancer is a diverse group of diseases that have been acquiring an epidemiologic character due to several causes, which include aging of the population and higher exposure to risk factors associated with the contemporary lifestyle [1]. The Brazilian National Institute of Cancer (INCA) has estimated that in the year 2014, more than half a million new cases of cancer have emerged among the Brazilian population and that colorectal cancer (CCR) is one of the most frequent in the country [2]. These data are quite similar to those observed in other western countries as reported by [3].

Despite the extensive progress made in diagnostics and therapeutics, surgical intervention still figures as the main choice for the treatment of colorectal cancer patients [4]. However, this type of cancer usually presents metastatic complications, which could compromise the cure through surgery [5, 6]. Consequently, cytotoxic chemotherapy is very frequently employed in the treatment of inoperable tumors or as neoadjuvant or adjuvant therapy, administered before or after surgery, respectively. The traditional regimen of cytotoxic chemotherapy is based on the administration of maximum tolerable doses of chemotherapeutic drugs. Despite the massive attack against tumor cells, this therapeutic schedule causes severe side effects, including the suppression of innate and adaptive defense mechanisms, requiring a “resting period” between treatments for the normalization of the patient’s hematological parameters, which implies that there is a lapse of reduced serum levels of medication that could allow the selective growth of resistant tumor variants, favoring relapse of the disease. CCR has a high rate of relapsing and of metastatic disease, so immunotherapeutic approaches with selected cytokines can be important alternatives for combined use with conventional treatments.

Systemic administration of interleukin (IL)-2 was the first cytokine-based therapy approved by the FDA (*Food and Drug Administration*) for the treatment of renal cell carcinoma and metastatic melanoma, with 20% and 8% of patients presenting total or partial clinical response, respectively [7, 8]. However, treatment with systemic IL-2 induces variable

and severe toxicity among patients, most frequently with induction of a vascular permeability syndrome, among other hematological alterations [8].

Another example of cytokines used for cancer treatment is the interferon (IFN)-α, one of the most vastly explored cytokines for systemic therapy of cancer [9], since besides their antiviral properties, type I IFNs also show relevant antitumor activity [10].

IFN-α is currently being tested for the treatment of several malignancies, such as chronic myelogenous leukaemia, melanoma and renal cell carcinoma [9-11]. High dosage IFN-α2b in the treatment of melanoma induces positive modulation of T and NK cells activity, while enhancing tumor cell immunogenicity by up-regulating major histocompatibility complex (MHC) class II expression [12]. In another clinical trial, with metastatic melanoma patients, the authors observed that systemic treatment with IFN-α2b reduces relapsing rates [13].

IFN-α has also been vastly explored due to its important role in the differentiation and maturation of dendritic cells (DCs). Peripheral blood mononuclear cells (PBMCs) cultivated in the presence of IFN-α and GM-CSF display morphological characteristic equivalent of DCs, with high expression of MHC class I and II and activation markers CD40 and CD86, as well as increased antigen presenting capacity [14]. *In vitro* studies report that DCs that differentiate in the presence of IFN-α have better potential to stimulate CD8+ and CD4+ T cell, and lower ability to expand $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells than those cultivated without this cytokine [11].

The success of DC-based immunotherapies relies on the elaboration of proper maturation/activation protocols so that DC can fulfill their therapeutic role. The presence of IFN-α is able to drive DC maturation and activation, inducing them to produce high levels of IL-12 and acquire the α-DC1 phenotype [15] [16] [17]. These DCs, in turn, have the capacity to drive the activation of T cells to a Th1 profile, favoring the antitumor response. Such results indicate that DC differentiation protocols that include IFN-α or the presence of this cytokine in the tumor microenvironment might induce clinically superior DCs for their use in cancer immunotherapy.

However, one of the main limitations of IFN-α is the life-threatening side effects elicited by the systemic treatment, which often requires interruption or dose reduction [18].

Side effects include neurological and hematological toxicity, flu-like symptoms such as fever, chills, myalgia and headaches [19]. Chronic manifestations include autoimmune and dermatological disorders and diabetes. Moreover, in the systemic administration schedule, cytokine degradation occurs and a smaller concentration of the drug is able to reach its end-point [18].

Taking these points into account, we hypothesized that sustained secretion of IFN-α by tumor cells would be able to activate DCs and improve antitumor responsiveness, while avoiding the side effects produced by systemic therapy and the degradation of the cytokine in the blood, cytokine gene transfer therapy figures as an interesting alternative. Then, in this study we aimed to evaluate the use of a lentiviral vector for IFN-α gene transfer to tumor cells and their *in vitro* effect over the maturation and activation of DCs.

Materials and methods

Tumor cells

Human tumor cell line HCT 116 of colorectal carcinoma was maintained in complete culture medium [RPMI 1640 medium (Gibco) supplemented with1% HEPES (Sigma), 10% FBS (Fetal bovine serum), 1% sodium pyruvate, 1% non-essential aminoacids, 1%antibiotics and antimycotics (Life Technologies)] at 37^{o}C and under 5% CO_2 tension. Cells were detached by treatment with trypsin-EDTA solution (Gibco) and washed with culture medium before use in the proposed assays. Immunophenotyping of HCT 116 cells was performed by flow cytometry, before and after transduction, using fluorochrome-conjugated monoclonal antibodies for the markers CD54, CD63, CD81, HLA-DR and CD83 (BD Biosciences).

Lentiviral vector

The lentiviral vector encoding human IFN-α (Lego-IFN) and eGFP (enhanced green

fluorescent protein – Lego-GFP) were constructed at the Viral Vectors Laboratory, Center for the Translational Investigation in Oncology, São Paulo State Cancer Institute, with the collaboration of Dr. Bryan Strauss. A third generation lentiviral vector was used, Lego-iG2, which was kindly provided by Dr. Kristoff Webber, University Medical Center Hamburg-Eppendorf, Hamburg, Germany [20]. Construction, production and titration of the viral vector was carried in accordance with protocols previously established in this laboratory [21]. Briefly, Lego-iG2 contains the SFFV (retroviral enhancer/promoter of spleen focus forming virus) promoter, expressed ubiquitously by several cell types but especially by hematopoietic cells. Since HCT 116 cells have epithelial origin, our first concern was that they would not be susceptible to Lego-iG2, so in the transduction assays, we also used K562 cells, originated from chronic myeloid leukaemia for purposes of comparison.

Transduction of HCT 116 cells with the lentiviral vector, confirmation of transduction and determination of MOI curve

Transduction was carried for 6-8h and confirmed by detection of GFP expression by both flow cytometry and fluorescence microscopy. To determine the multiplicity of infection (MOI) curve, we quantified the percentage of GFP^+ cells by flow cytometry were used.

Enzyme linked immuno sorbent assay (ELISA) for quantification of IFN-α production by transduced HCT 116 cells

Supernatant of transduced HCT 116 cells was collected at different time points (24h, 48h and 72h) and quantified for IFN-α by ELISA (eBiosciences), according to the manufacturers' instructions.

Peripheral blood collection

One hundred milliliters of peripheral blood were collected from healthy donors to obtain both monocytes and lymphocytes. Monocytes were differentiated into dendritic cells (DCs) and

lymphocytes of one donor were used in the mixed leukocyte reactions. For the generation of cytotoxic T lymphocytes (CTLs), monocytes from male HLA-A02+ (human leukocyte antigen A02) donors were obtained by leukapheresis at the Hemotherapy and Hematology Unit of Hospital Samaritano, São Paulo-SP, Brazil. All donors were informed about the purposes of this study and have signed the Agreement Term. The study was approved by the Ethics Committee of the School of Medicine of Botucatu under the registry 531.857.

Generation of monocyte-derived DCs

Mononuclear cells (PBMCs) were isolated from peripheral blood from healthy donors by centrifugation on Ficoll gradient. PBMCs were then submitted to a centrifugation on Percoll gradient for the enrichment of the monocyte population. Monocytes were seeded on 6-well culture plates in serum-free AIM V culture medium (Gibco) and left for 1h30 to adhere on plastic. After Percoll separation, the pellet of total lymphocytes was collected and cryopreserved for posterior use. Adherent cells, mostly monocytes, were then cultivated in the presence of IL-4 and GM-CSF (at 50ng/mL, Peprotech) for differentiation into immature DCs (iDCs). IFN-a-producing HCT 116 cells were seeded in transwell inserts for 6-well plates (BD Biosciences; membrane porosity of 0,4μm) and cultivated for 24h. Lego-GFP-transduced HCT 116 cells were used as negative control. After that, the cells were transferred into another 6-well culture plate containing iDCs to induce their maturation (mDCs). As a positive control, DCs were treated with recombinant human IFN-α on day 5. On day 7, mDCs were used for the proposed assays and were phenotyped to evaluate the co-culture effects on DC maturation. The use of genetically modified cells was approved by the Internal Commission of Biosafety of the Biosciences Institute of Botucatu (CQB 164/02-01).

Immunophenotyping

HCT 116 cells were phenotyped before and after transduction using fluorochrome

conjugated monoclonal antibodies for the surface markers CD9, CD63, CD81, CD54, HLA-ABC, HLA-DR, CD40, CD80, CD86 and CD83 (BD Biosciences). DCs were stained for the surface markers CD1a, CD14, CD83, HLA-DR, CD86, CD80, HLA-ABC and PD-L1 (BD Biosciences), 48h after culture with transduced HCT 116 cells. DCs were initially gated based on morphology and then doublets, 7- aminoactinomycin D^+ (7AAD) and $CD11c^-$ events were excluded from analysis. Both the percentage of positive cells and the median fluorescence intensity of the markers were analyzed. Flow cytometry was performed in a FACSCanto™ II flow cytometer with FACSDiva software (BD Biosciences) and all of the analyses were performed using FlowJo software vX10.6 (Tree Stars Inc.).

Mixed leukocyte reaction (MLR)

On day 7 of culture (48h after culture with transduced HCT 116 cells), DCs were with allogeneic total lymphocytes were stained with carboxyfluorescein succinimidyl ester (CFSE) [22] at a 1:10 DC: lymphocyte ratio. Cultures were maintained for 5 days in "U"-bottomed 96-well culture plates and supernatants were collected and cryopreserved for cytokine quantification. Proliferation was observed by CFSE dilution within $CD8^+$ and $CD4^+$ populations by flow cytometry. Phenotyping was carried by staining with fluorochrome conjugated monoclonal antibodies for the molecules CD3, CD4, CD8, CD69 and PD-1.

Cytokine quantification

Supernatants of the MLR assays were collected for cytokine quantification by Cytometric Beads Array (CBA) using the kit Human Th1/Th2/Th17 (BD Biosciences) according to manufacturer's instructions. CBA was carried in a FACSCanto II flow cytometer (BD Biosciences) using the software FACSDiva (BD Biosciences) for acquisition of samples and FCAP Array software (BD Biosciences) for analysis.

Specific cytotoxic T lymphocyte (CTL) stimulation

HLA-A02$^+$ iDCs were differentiated as previously described and cultured with IFN-α-producing HCT 116 cells on day 5. Twenty-four hours later DCs were pulsed with HLA-A02-restricted *Influenza* peptide (FLU - GILGFVFTL). DCs were then cultured with autologous total lymphocytes for an additional 7 days. Every 2 days culture medium was supplemented with IL-7, IL-2 and IL-15 (5ng/mL, 25U/mL and 5ng/mL, respectively; Peprotech). On day 7, lymphocytes were stained with fluorochrome conjugated monoclonal antibodies for the surface markers CD3 and CD8, as well as with a FLU-specific HLA-A02 dextramer (DEX) conjugated with phycoerytrin (PE). Analysis of CTL generation was performed by quantification of the CD8$^+$DEX$^+$ population by flow cytometry. A prostatic specific antigen (PSA) -specific HLA-A02 DEX served as irrelevant antigen control of the experiment.

Statistical analysis

Statistical analysis was carried by one way analysis of variance test (ANOVA) followed by multiple comparison Tukey test. Differences were considered significant when the probability of error was lower that 5% ($p<0.05$). GraphPad Prism 5 software was used for analyses and graphic preparations.

Results

HCT 116 were successfully transduced by the lentiviral vector and expressed high levels of IFN-α

In order to show the effectivity of our gene transference approach, we used an empty vector encoding only GFP to transduce HCT 116 cells. This control vector was also used to determine the curve of MOI. Success of transduction was confirmed by detection of GFP expression on the target cells by flow cytometry (Fig. 1A-D) and fluorescence microscopy (Fig. 1E and F), with rates highly comparable to those of K562 (data not shown). GFP$^+$ cells

were detectable even with the lowest MOI of 0.1 (Fig 1A). The MOI curve shows that increase of MOI reflected in the increase in number of cells infected with at least one viral particle, until reaching a *plateau* in the MOI 10.0 (Fig. 1G). Higher MOIs induces strong decrease of cell numbers in the flow cytometric analyses, suggesting toxicity despite the efficiency of transduction (data not shown). IFN-α quantification of culture supernatants by ELISA revealed that even at the MOI of 0.3, Lego-IFN-transduced cells produced 944.35 pg/mL of IFN-α 72h after transduction (Table 1). Transduction with Lego-GFP yielded very little IFN-α production by 72h (124.10 pg/mL; Table 1).

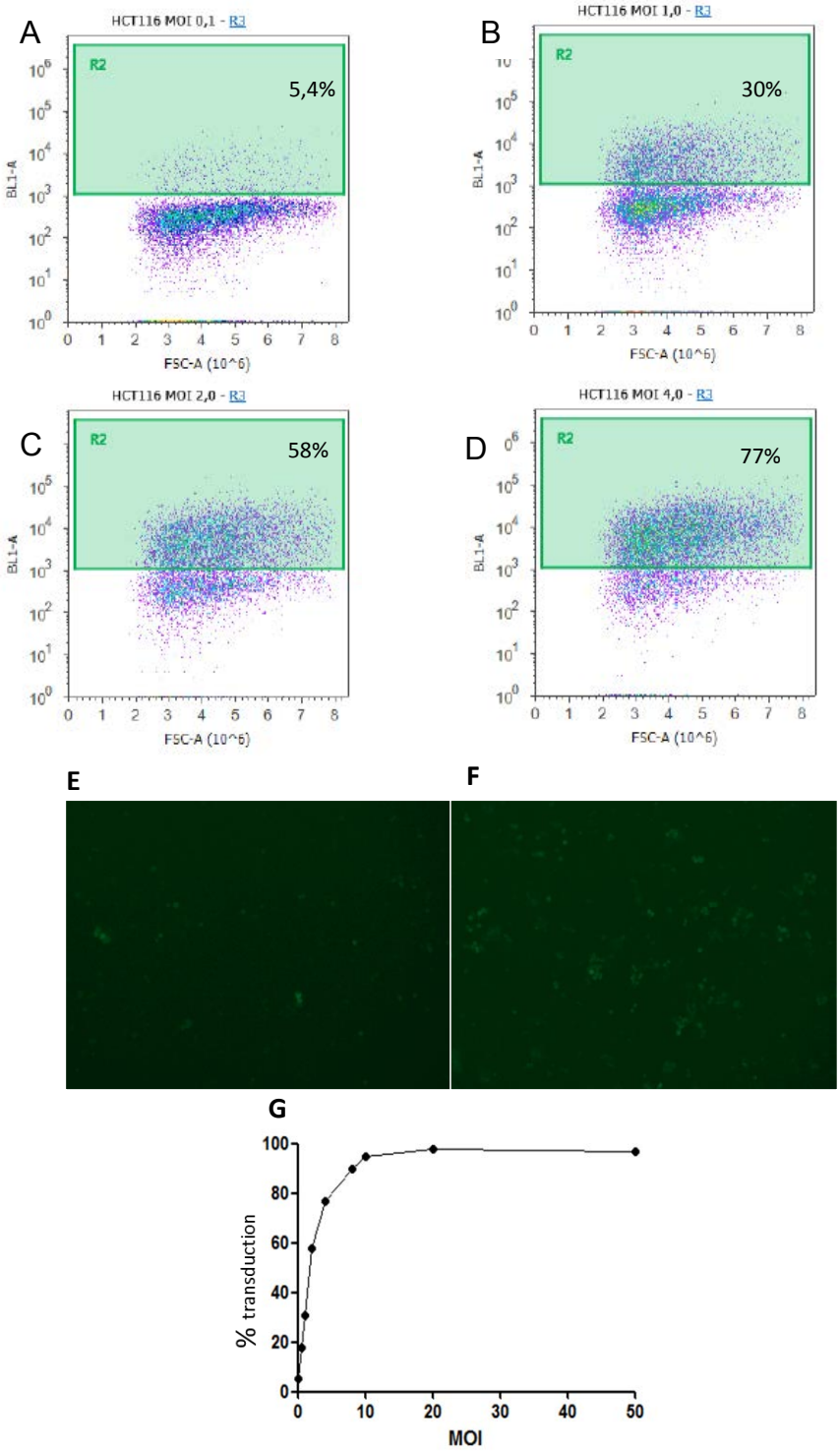

Fig. 1 – Success of transduction with Lego-GFP was demonstrated by quantification of GFP^+ cells by flow cytometry (Fig. 1 A-D) after transduction with MOIs 0.1, 1.0, 2.0 and 4.0, respectively, and by fluorescence microscopy (Fig. 1 E and F) of transduced HCT 116 cells showing GFP expression in the MOIs 2.0 and 4.0, respectively. (G) MOI curve showing that transduction rates reached a plateau after the MOI 10.0.

Table 1 – ELISA IFN-α quantification of the supernatants of HCT 116 cells transduced with Lego-IFN, Lego-GFP or non-transduced at different time points. WT (wild type); n.d. (not determined).

	Lego-IFN MOI 0.1	Lego-IFN MOI 3.0	Lego-GFP MOI 3.0	HCT 116 WT
24h	70.36 pg/mL	n.d.	n.d.	n.d.
48h	385.47 pg.mL	973.00 pg/mL		0
72h	944.35 pg/mL	n.d.	124.10 pg/mL	0

Transduction with Lego-GFP renders HCT 116 tumor cells more immunogenic

Post transduction phenotyping showed that Lego-GFP-transduced HCT 116 cells suffered a dramatic increase in the frequency of $CD54^+$ and $HLA\text{-}DR^+$. In addition, while Lego-IFN-transduced tumor cells had a slight decrease in the frequency of $CD63^+$ cells, that effect was not seen in Lego-GFP-transduced cells. Both Lego-IFN and Lego-GFP-transduced cells presented slight increase in the frequency of $CD83^+$ and decrease in $CD81^+$ cells.

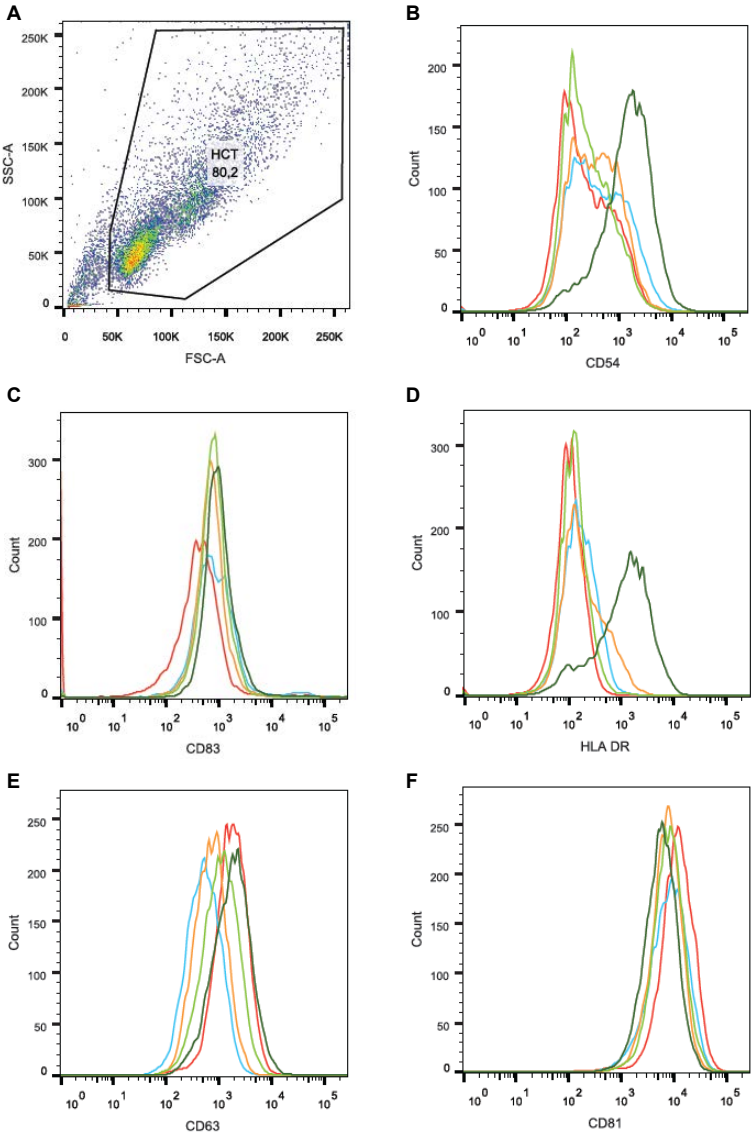

Fig.2 – Immunophenotyping of HCT 116 cells transduced with Lego-IFN and Lego-GFP, showing expression of CD54, CD83, HLA-DR, CD63 and CD81 in Lego-IFN-transduced cells (blue: MOI 1,0; orange: MOI 2,0; light green: MOI 4,0), Lego-GFP-transduced cells (dark green) and non-transduced cells (red). (A) Gating of HCT 116 cells based on morphology. Analysis CD54 (B), CD83 (C), HLA-DR (D) CD63 (E), and CD81 (F) in transduced and non-transduced cells.

Indirect co-culture with IFN-α-producing HCT 116 slightly increases the activation but not maturation of DCs

In order to verify whether IFN-α produced by HCT 116 cells would be able to modulate DC activation and maturation, the former were indirectly co-cultured with iDCs, using transwell inserts. After 48h, DCs were phenotypically analyzed by flow cytometry. The frequency of $CD1a^+$ cells, a typical marker for iDCs, was slightly lower in the groups DC-1.0 and DC-4.0, when compared to the control DC (Fig. 3A). Cells co-cultured with Lego-IFN-transduced tumor cells, significantly increased the frequency of $CD86^+$ cells (Fig. 2F). Frequency of HLA-DR^+ and $CD80^+$ cells were slightly up-regulated in comparison with DC-GFP (Fig. 3C and E). Interestingly, DC-GFP presented the lowest percentage of PD-$L1^+$ cells, which is an immunoregulatory marker, while IFN-α-stimulated DCs showed a slight up-regulation of positive cells for this marker (Fig. 3G).

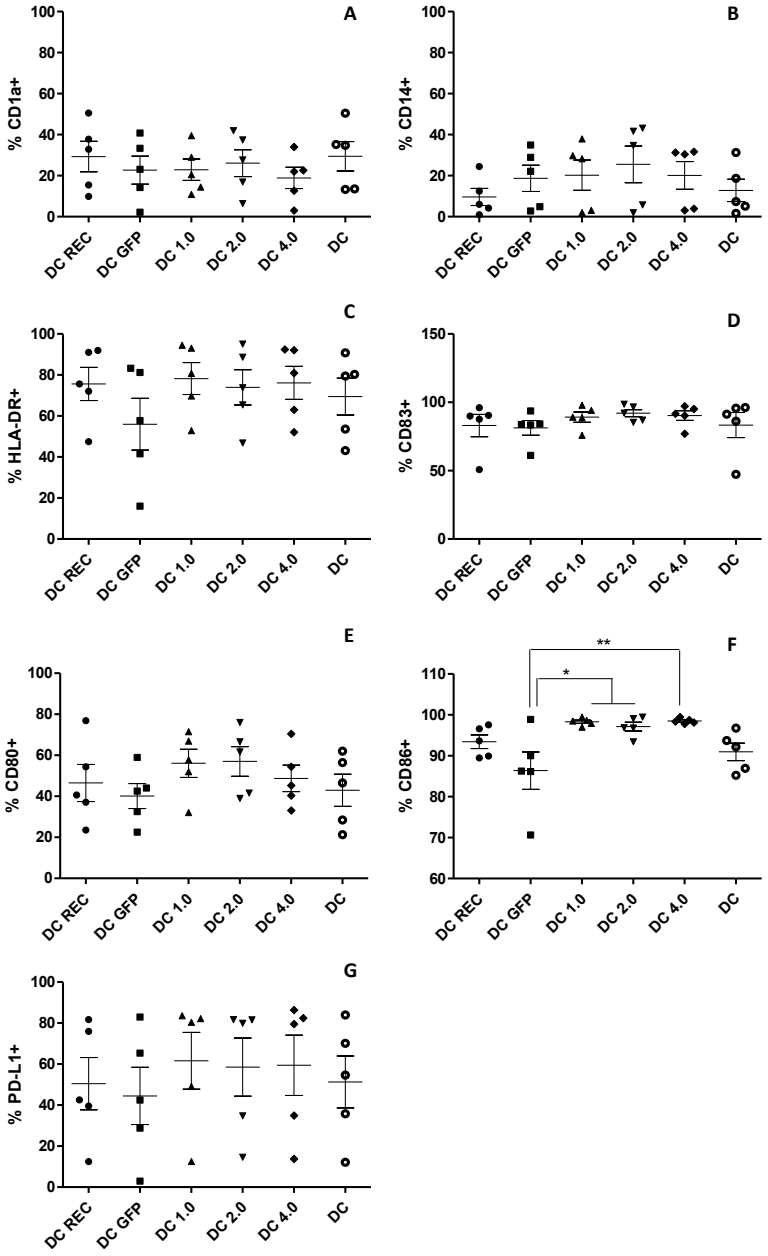

Fig. 3 – Immunophenotyping of monocyte-derived DCs co-cultured for 48h with IFN-α-producing HCT 116 cells (transduced with variable MOIs of Lego-IFN or with Lego-GFP). Data are expressed as mean ± SEM of positive cell frequency of five independent DC:lymphocyte combinations. *p<0.05

Indirect co-culture with Lego-IFN-transduced HCT 116 cells slightly decreases the allostimulatory potential of DCs

Given that the maturation of DCs was only slightly modulated by the co-culture with IFN-α-producing HCT 116 cells, we next sought to evaluate if the functional properties of DCs would be affected by transduced cell soluble products. For that purpose, DCs were tested in a MLR assay with CFSE stained lymphocytes. Proliferation rates were analyzed 5 days later. DC-4.0 induced slightly less $CD4^+$ and $CD8^+$ T cell proliferation in comparison with all other groups, especially the control group DC(Fig. 4A and B). We also observed that after the MLR assays, viability of lymphocytes stimulated by DC-4.0 was significantly lower than lymphocytes stimulated by DC-GFP and DC (Fig.4 C and D).

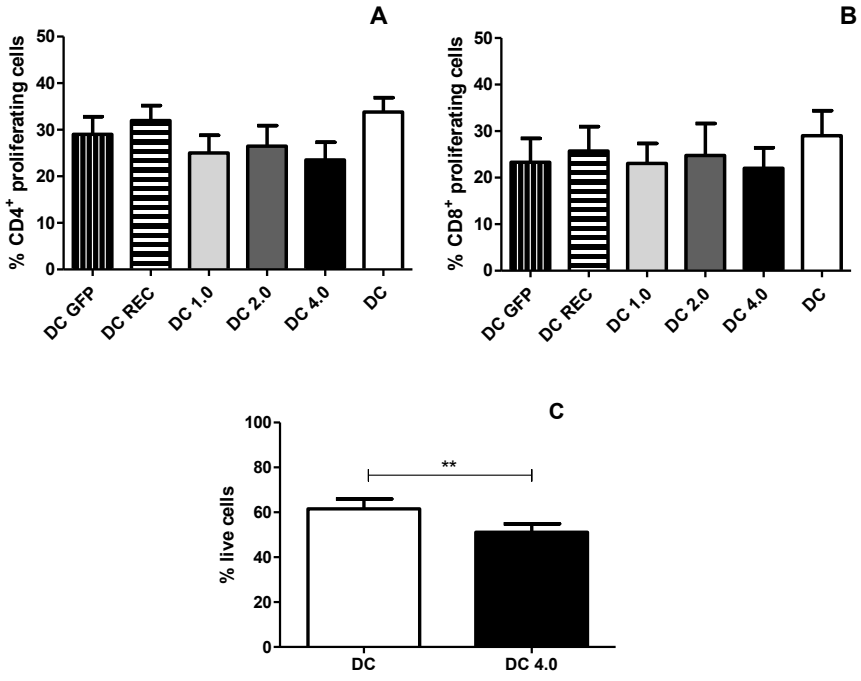

Fig. 4 – Mixed leukocyte reaction was carried out for 5 days, in a 1:10 DC:lymphocyte ratio. Proliferation rates of T lymphocytes were expressed as percentage of proliferating T lymphocytes (CFSE low events) within the (A) $CD4^+$ population and (B) $CD8^+$ population. Data are presented as mean ± SEM of five independent assays (C) viability of lymphocytes cultured with DC-4.0 in comparison with DC; **p=0.0015.

Indirect co-culture of transduced HCT 116 with DCs discretely improves their T lymphocyte activation potential

In order to further evaluate the effect of our DCs on T lymphocytes, the latter were phenotyped at the end of the allogeneic proliferation assays. Frequency and level of expression of the markers CD69 and PD-1 were analyzed within the proliferating subsets. Our data in Fig. 5 shows that exposition of DCs to Lego-IFN-transduced tumor cells do not influence the profile of proliferating lymphocytes obtained in the MLR assay.

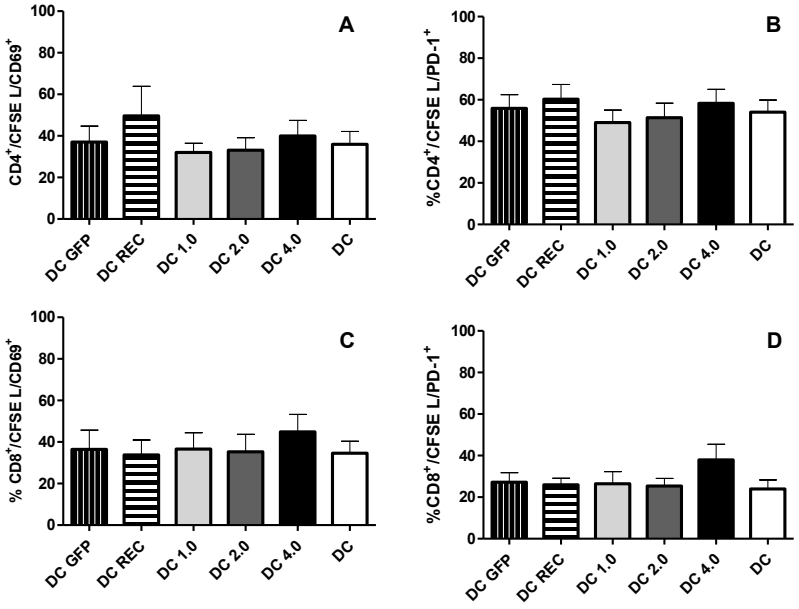

Fig. 5– Phenotypical analysis of activation markers on CD4+ and CD8+ lymphocytes expanded during MLR assay by flow cytometric analysis of the frequency of CD69+ (A, C) and PD-1+ (C,D) within proliferating $CD4^+$ and $CD8^+$ subsets, respectively. Data are expressed as mean ± SEM of five independent assays. ANOVA followed by Tukey-Kramer post-test.

T cells induced by DC-2.0 produced higher levels of IFN-γ

Cytokines produced by T lymphocytes on MLR assays were quantified by CBA technique, using the Human Th1/Th2/Th17 kit. Our results show that lymphocytes cultured with DC-2.0

produced slightly higher levels of IFN-γ (Fig. 6A). Interestingly, DC-GFP induced more IL-10 (Fig. 6C) while DCs cultured with Lego-IFN-transduced tumor cells tended to inhibit IL-4 production (Fig. 6E). No relevant changes were observed on TNF-α (Fig. 6B), IL-2 (Fig. 6F) and IL-17 production (data not shown).

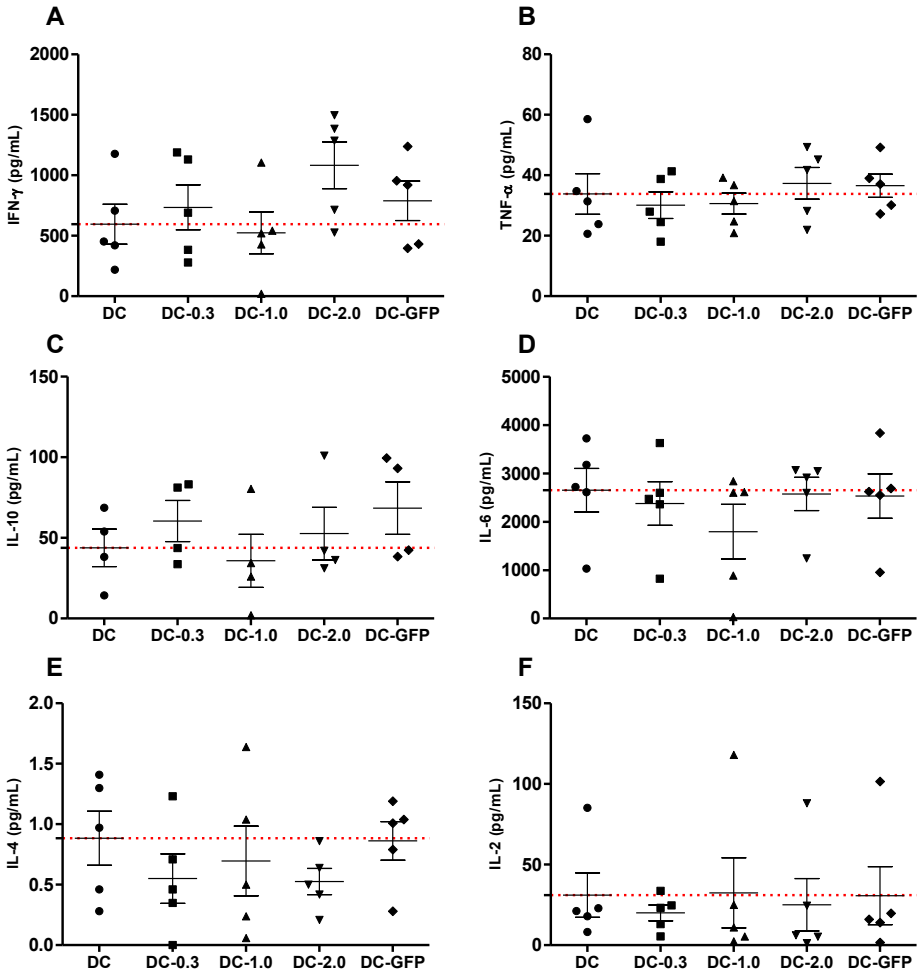

Fig. 6 – Analysis of Th1/Th2/Th17 profile cytokines by CBA technique. Data are expressed as mean and standard error of IFN-γ (A), TNF-α (B), IL-10 (C), IL-6 (D), IL-4 (E) and IL-2 (F) quantified in supernatants of five independent co-cultures. Red dotted line represents the mean of production of cytokine by the control group DC.

CTL generating potential of DCs is enhanced after co-culture with transduced HCT 116 cells

We next evaluated the ability of DCs cultured with Lego-transduced tumor cells to stimulate the generation of spcific CTLs durin a 7-day autologous culture of HLA-A02^{+} FLU peptide-pulsed DCs with autologous lymphocytes. The resulting CTLs were stained with CD8 fluorochrome-conjugated monoclonal antibody and a PE-conjugated FLU-specific HLA-A02 dextramer (DEX). A PE-conjugated PSA-specific HLA-A02 DEX was used as an irrelevant antigen control. Data were normalized in relation to the control group DC and are shown in Fig. 7. Co-culture with IFN-α-producing HCT 116 cells improved CTL generating potential of DC-4.0, however, DC-GFP showed an even higher T CD8^{+} stimulatory capacity.

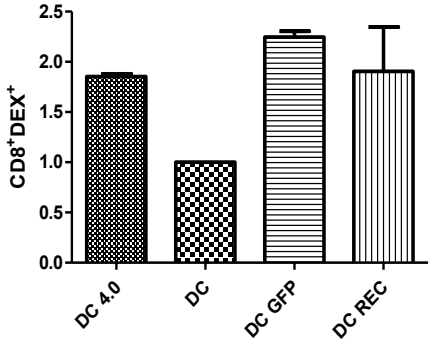

Fig. 7 - HLA-A2+ DC, stimulated with Lego-IFN-transduced HCT-116 cells were loaded with HLA-A02-restricted *Influenza* peptide (FLU - GILGFVFTL), following co-culture with autologous total lymphocytes for an additional 7 days (IL-2, IL-7 and IL-15 were added each 2 days) for generation of CTL. Specificity of resulting CD3+/CD8+ cells were tested on their ability to link FLU-specific HLA-A02 dextramer (DEX) conjugated with phycoerytrin, and analyzed by flow cytometry. A prostatic specific antigen (PSA)-specific HLA-A02 DEX served as irrelevant antigen control of the experiment (data not shown).

Discussion

One of the biggest challenges in DC-based immunotherapies is the effective activation of these cells *ex vivo*, since cancer patients-derived DCs present a tolerogenic profile and reduced capacity to generate antitumor responses, especially regarding CD4+ and CD8+ T cell activation [23] [24]. In addition, the costs of manipulating DCs ex vivo are high, which can translate into very expensive DC-based treatments. As an alternative, treatments for the modulation of DC activation and maturation *in situ* could represent a less costly approach. Then, in the present study we investigated the feasibility of use of a lentiviral vector for transferring the IFN-α gene into tumor cells and how these transduced cells interfere on DC maturation/activation features.

Firstly, we demonstrated that HCT-116 cells were efficiently transduced by Lego-IFN/Lego-GFP constructs, showing GFP fluorescence of these cells both by flow cytometry and fluorescence microscopy. The former technique was also used to determine the MOI curve, in order to establish the best work conditions. We next demonstrated the functional efficacy of this transduction approach, showing that Lego-IFN-transduced cells produced high levels of IFN-α, at both 24h and 72h marks, while Lego-GFP-transduced cells secrete just 1/7 of those levels and wild type non transduced cells did not produced this cytokine. This result indicates that *in vitro* IFN-α production was a consequence of transduction with specific gene and not only a natural response to a virus infection-like event. Of course, this phenomenon seems to be responsible for the IFN-α production by cells transduced by GFP-carrying vector, however the in much lower levels than those produced by Lego-IFN-transduced cells. We also verified that transduction with either vectors did not interfere with tumor cell viability (data not shown).

An interesting observation was that transduction with Lego-GFP induced phenotypical changes in tumor cells with increased expression of surface CD54 and HLA-DR, suggesting a cell modulation for a more immunogenic profile. CD54 or ICAM-1 (intercellular adhesion

molecule-1) is a co-stimulatory molecule especially important in mediating adhesion of CTLs to target cells through interaction with its ligand LFA-1 (lymphocyte function-associated antigen-1) [25] [26]. Although we have not investigated this point, increased expression of CD54 appears to facilitate the recognition of tumor cells by CTLs to perform cytolytic effect.

Increased expression of HLA-DR on tumor cells is also a relevant change induced by transduction with Lego-GFP. Although the main target of antitumor CTLs is represented by MHC class I molecules, recognition of peptides loaded on class II molecules by $CD4^+$ T lymphocytes is essential to the establishment of a complete protective response, since tumor-specific $CD4^+$ T helper cells are required for the development of a Th1-driven response. Then, increase of HLA-DR on tumor cells transduced with Lego-GFP could represent an increase in their immunogenicity. No relevant changes were observed on the expression of CD83 and CD63 on tumor cells. Although we have observed phenotypical changes suggesting increased immunogenicity of tumor cells, we chose to perform the co-culture assays using the transwell system, in order to evaluate the role of the putative IFN-α secreted by transduced cells. Therefore, further assays are necessary to investigate the effect of a direct interaction between tumor cells and DCs.

We next investigated how IFN-α production by transduced cells could change the phenotypic and functional features of DCs. Our data show that the supernatant of tumor cells transduced with Lego-GFP slightly reduced the frequency of cells expressing the maturation/activation markers HLA-DR and CD86. These results are compatible with higher levels of IL-10 found in the supernatant of T lymphocytes stimulated by these DCs (analyzed by CBA). This finding is in agreement with previous reports showing that tumor cells spontaneously secrete regulatory cytokines such as IL-10 and TGF-β [23]. In contrast, exposition of DCs to Lego-IFN-transduced tumor cells kept the same frequency of $HLA\text{-}DR^+$ and $CD86^+$ cells as those found in cultures stimulated with recombinant IFN-α (DC-REC). In fact, we observed that frequency of $CD86^+$ cells is significantly higher than in DC-GFP group, and slightly higher (non-significantly) than in DC REC and DC controls. Therefore, these

results suggest that in vitro production of IFN-α by transfected tumor cells is able to promote phenotypical changes on dendritic cells, as previously reported [11] [27] [28]. In contrast, we observed that Lego-IFN-transduced cells induced up-regulation of PD-L1frequency. PD-L1 is a molecule involved in the regulation of immune responses through inhibition of T cell activation and induction of IL-10 production. However, this molecule is also up-regulated in activated DCs as a regulatory mechanism of immune responses [29]. Since IFN-α-stimulated DCs also presented the highest frequency of $CD86^+$ cells, PD-L1 up-regulation in this case could represent a mechanism of natural contraction of the immune respose.

In the next step, we investigated whether these phenotypical changes on DC would be associated with functional changes of these cells, performing a MLR assay. We observed that DC-4.0 induced slightly, but not significantly lower levels of allogeneic T cell proliferation that DC-GFP and that IFN-stimulated DCs as a whole induced less proliferation that the control group DC. The lack of difference between the alloreactive responsiveness using DC exposed to Lego-IFN and Lego-GFP-transduced tumor cells may be due to an excessive level of IFN-α production by the former cells. This condition could be mimicking a state of chronic viral infection, leading to an excessive stimulation of lymphocytes resulting in exhaustion or death [30]. In fact, double checking the flow cytometric analysis of the MLR assays, we observed, by morphological changes, significantly higher numbers of dead cells in those samples where lymphocytes were co-cultured IFN-α-stimulated DCs, than in those cultured with DC. Furthermore, it has been shown that interaction of PD-L1 with the T cell regulatory receptor PD-1 induces T cell apoptosis [31]. Since IFN-α-stimulated DCs presented higher percentages of PD-L1, the up-regulation of this marker could be associated with the decreased viability of T cells. Therefore, we believe that our results represent not a fail of the vector construction, but rather the necessity of a better titration of DC:lymphocyte ratio for the MLR assays.

Furthermore, when we evaluated the phenotype of T cells after the MLR assay, we observed that DC-4.0 activated T cells more efficiently, which is shown by a slight increase in

the frequency of $CD4^+/CD69^+$ and $CD8^+/CD69^+$ T cells.

Cytokine quantification of T cells stimulated by DC-GFP revealed that these cells produced slightly higher levels of IL-10 and IL-4 and lower levels of IFN-γ in comparison with DC-2.0, suggesting that DC-GFP may be inducing a Th2 profile on T cells, as expected for wild type tumor cells. In contrast, DC-4.0 induced higher levels of IFN-γ production by T cells and that is in accordance with the establishment of a Th1 profile induced by IFN-α-treated DCs [11].

Finally, DC-4.0 showed an increased potential in stimulating specific $CD8^+$ T cells in comparison with control group DC, which has been consistently reported. However, DC-GFP was able to induce even more $CD8^+$ T cell stimulation, even though DC-4.0 and DC-GFP presented similar levels of HLA-ABC expression and frequency.

Conclusion

Taken together, our results allow us to conclude that Lego-IFN construct effectively transfers the IFN-α gene into tumor cells and such a process induces specific cytokine synthesis. In addition, IFN-α secreted by transduced tumor cells is able to increase the activation state of DCs, and slightly tune lymphocyte differentiation for a Th1 profile. The effect of this vector on allogeneic responsiveness and ability to induce the generation of antitumor CTL requires additional studies.

Bibliographic references

1. Goodson, W.H., 3rd, et al., *Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: the challenge ahead.* Carcinogenesis, 2015. **36 Suppl 1**: p. S254-96.
2. Silva, I.N.d.C.-J.A.G.d. *Estimativa 2014-Incidência de câncer no Brasil*. 2014; Available from: http://www.inca.gov.br/estimativa/2014/.
3. *American Cancer Society. Cancer Facts and Figures, 2013.* . 2013 Accessed on 15/03/2013.]; Available from: http://migre.me/f0kxi.
4. Soreide, K., et al., *Advances in the understanding and treatment of colorectal cancer.* Discov Med, 2011. **12**(66): p. 393-404.
5. Rodel, C., R. Hofheinz, and T. Liersch, *Rectal cancer: state of the art in 2012.* Curr Opin Oncol, 2012. **24**(4): p. 441-7.
6. Mazzolini, G., et al., *Immunotherapy and immunoescape in colorectal cancer.* World J Gastroenterol, 2007. **13**(44): p. 5822-31.
7. Kaufman, H.L., et al., *The Society for Immunotherapy of Cancer consensus statement on tumour immunotherapy for the treatment of cutaneous melanoma.* Nat Rev Clin Oncol, 2013. **10**(10): p. 588-98.
8. Rosenberg, S.A., et al., *Durability of complete responses in patients with metastatic cancer treated with high-dose interleukin-2: identification of the antigens mediating response.* Ann Surg, 1998. **228**(3): p. 307-19.
9. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nat Rev Cancer, 2004. **4**(1): p. 11-22.
10. Tarhini, A.A., H. Gogas, and J.M. Kirkwood, *IFN-alpha in the treatment of melanoma.* J Immunol, 2012. **189**(8): p. 3789-93.
11. Gigante, M., et al., *Interferon-alpha (IFN-alpha)-conditioned DC preferentially stimulate type-1 and limit Treg-type in vitro T-cell responses from RCC patients.* J Immunother, 2008. **31**(3): p. 254-62.
12. Kirkwood, J.M., et al., *Immunomodulatory effects of high-dose and low-dose interferon alpha2b in patients with high-risk resected melanoma: the E2690 laboratory corollary of intergroup adjuvant trial E1690.* Cancer, 2002. **95**(5): p. 1101-12.
13. Kirkwood, J.M., et al., *Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684.* J Clin Oncol, 1996. **14**(1): p. 7-17.
14. Paquette, R.L., et al., *Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells.* J Leukoc Biol, 1998. **64**(3): p. 358-67.
15. Giermasz, A.S., et al., *Type-1 polarized dendritic cells primed for high IL-12 production show enhanced activity as cancer vaccines.* Cancer Immunol Immunother, 2009. **58**(8): p. 1329-36.
16. Lee, J.J., et al., *Type 1-polarized dendritic cells loaded with autologous tumor are a potent immunogen against chronic lymphocytic leukemia.* J Leukoc Biol, 2008. **84**(1): p. 319-25.
17. Yang, D.H., et al., *Successful cross-presentation of allogeneic myeloma cells by autologous alpha-type 1-polarized dendritic cells as an effective tumor antigen in myeloma patients with matched monoclonal immunoglobulins.* Ann Hematol, 2011. **90**(12): p. 1419-26.
18. Paul, F., S. Pellegrini, and G. Uze, *IFNA2: The prototypic human alpha interferon.* Gene, 2015. **567**(2): p. 132-137.
19. Yamane, A., et al., *Interferon-alpha 2b-induced thrombocytopenia is caused by inhibition of platelet production but not proliferation and endomitosis in human megakaryocytes.* Blood, 2008. **112**(3): p. 542-50.

20. Weber, K., et al., *A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis.* Mol Ther, 2008. **16**(4): p. 698-706.
21. Strauss, B.E., et al., *A lentiviral vector with expression controlled by E2F-1: a potential tool for the study and treatment of proliferative diseases.* Biochem Biophys Res Commun, 2006. **348**(4): p. 1411-8.
22. Lyons, A.B., *Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution.* J Immunol Methods, 2000. **243**(1-2): p. 147-54.
23. Zou, W., *Immunosuppressive networks in the tumour environment and their therapeutic relevance.* Nat Rev Cancer, 2005. **5**(4): p. 263-74.
24. Zitvogel, L., A. Tesniere, and G. Kroemer, *Cancer despite immunosurveillance: immunoselection and immunosubversion.* Nat Rev Immunol, 2006. **6**(10): p. 715-27.
25. Marlin, S.D. and T.A. Springer, *Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1).* Cell, 1987. **51**(5): p. 813-9.
26. Segura, E., et al., *CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo.* J Immunol, 2007. **179**(3): p. 1489-96.
27. Luft, T., et al., *Type I IFNs enhance the terminal differentiation of dendritic cells.* J Immunol, 1998. **161**(4): p. 1947-53.
28. Hansen, M., et al., *Comparison of clinical grade type 1 polarized and standard matured dendritic cells for cancer immunotherapy.* Vaccine, 2013. **31**(4): p. 639-46.
29. Zou, W. and L. Chen, *Inhibitory B7-family molecules in the tumour microenvironment.* Nat Rev Immunol, 2008. **8**(6): p. 467-77.
30. Teijaro, J.R., et al., *Persistent LCMV infection is controlled by blockade of type I interferon signaling.* Science, 2013. **340**(6129): p. 207-11.
31. Blank, C., et al., *Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro.* Int J Cancer, 2006. **119**(2): p. 317-27.