IGOR DE LUNA VIEIRA

Avaliação dos efeitos autócrino e parácrino da terapia gênica com adenovírus portador de IFNβ e sua relação com IFNAR1 no melanoma e microambiente murino

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IGOR DE LUNA VIEIRA

Avaliação dos efeitos autócrino e parácrino da terapia gênica com adenovírus portador de IFNβ e sua relação com IFNAR1 no melanoma e microambiente murino

Tese apresentada a Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Oncologia

Orientador: Dr. Bryan Eric Strauss

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DEDICATÓRIA

Aos grandes pensadores e aos meus familiares.

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Ad-GFP	-	Vetor adenoviral portador do gene GFP
Ad-mIFN	-	Vetor adenoviral portador do gene Interferon beta murino
Ad-p19	-	Vetor adenoviral portador do gene p19-ARF murino
ANG-1	-	Angiopoetina - 1
ANOVA	-	Análise de variância
ATCC	-	do Inglês American Type Culture Collection
B16	-	Linhagem de melanoma murino
B16.B2	-	linhagem B16 *knockout* para IFNAR1
B16.G	-	linhagem B16 GFP positiva, fluorescente verde
BAK	-	proteína pró apoptótica da família Bcl-2
BAX	-	proteína pró apoptótica da família Bcl-2
bFGF1	-	Fator de crescimento fibroblastico basal 1
BRAF	-	gene produtor da proteína B-raf
BSA	-	albumina
CO_2	-	Dióxido de carbono
CRISPR/Cas9	-	do inglês Clustered Regularly Interspaced Short Palindromic Repeats
DISC	-	complexo indutor de sinal de morte
DMEM	-	Meio de cultura Dulbecco’s Modified Eagle
DNA	-	Ácido Desoxirribonucleico
E. coli	-	Escherichia coli
EDTA	-	ácido etilenodiamino tetra-acético
ELISA	-	do inglês “Enzyme Linked ImmunonoSorbent Assay
FADD	-	Proteína associada ao Fas com domínio de morte
FAS	-	Receptor de fator de necrose tumoral
FasL	-	Ligante do Faz indutor de apoptose pertencente ao TNF
FBS	-	Soro fetal bovino
FDA	-	Intituição americada de controle de saúde pública
Fig	-	Figura
GAPDH	-	Gene Glyceraldehyde 3-phosphate dehydrogenase
GFP	-	Proteína verde fluorescente
I.P.	-	iodeto de propídio

IFN	-	Interferon
IFNAR1	-	Receptor 1 do interferon alpha e beta
IFNAR2	-	Receptor 2 do interferon alpha e beta
IFNα	-	Interferon alpha
IFNβ	-	Interferon beta
IFNγ	-	Interferon gama
IFNλ	-	Interferon Lambda
IL-06	-	Interleucina 6
IL-08	-	Interleucina 8
IL-24	-	Interleucina 24
IRF-7	-	fator de regulação do interferon 7
IRF-9	-	fator de regulação do interferon 9
IRFs	-	fatores de regulação do interferon
ISG3	-	fator de estimulação do gene Interferon 3
ITRs	-	inverted terminal repeats
JAK1	-	janus quinase 1
MAPK	-	Proteína mitogena ativada por quinases
MHC	-	complexo de histocompatibilidade de classe 1
mL	-	mililitro
MOI	-	multiplicidade de infecção
MT	-	Microambiente tumoral
NF1	-	proteína neurofibromina 1
ng	-	nanogramas
NIH-3T3	-	Linhagem de fibroblastos murina
NIH-3T3.A1	-	linhagem NIH-3T3 *knockout* para IFNAR1
NIH-3T3.G	-	linhagem NIH-3T3 GFP positiva, fluorescente verde
NRAS	-	NRAS proto-oncogene
ºC	-	graus celsius
$p14^{Arf}$	-	do inglês ARF tumor suppressor humano
$p19^{Arf}$	-	do inglês ARF tumor suppressor murino
PBS	-	Tampão fosfato-salino
PCR	-	técnica de transcrição reversa seguida de reação em cadeia da polimerase
PFA 4%.	-	Paraformadeído 4%

pg	-	picogramas
RGD	-	Tripeptídeo arginina-glicina-ácido aspártico
rIFNβ	-	proteína recombinante do Interferon beta
RNA	-	Ácido ribonucleico
RPMI	-	Meio de cultura Roswell Park Memorial Institute 1640
sgRNAs	-	RNA-guia
STAT-1	-	Ativador transcricional 1
STAT-2	-	Ativador transcricional 2
T4 PNK	-	enzima T4 polynucleotide kinase
tEnd	-	Linhagem endotelial murina
tEnd.A5	-	linhagem tEnd *knockout* para IFNAR1
tEnd.TO	-	linhagem tEnd *tomato* posiva, fluorescente vermelha
TNF	-	fator de necrose tumoral
TNF-α	-	fator de necrose tumoral alpha
TRAIL	-	Ligante indutor de apoptose relacionado ao TNF
TU	-	Particular infectantes
TYK2	-	tirosina quinase 2
VEGF	-	Fator de crescimento de endotelio vascular
VEGF-A	-	Fator de crescimento de endotelio vascular A
VEGF-C	-	Fator de crescimento de endotelio vascular C

RESUMO

Vieira VL. *Avaliação dos efeitos autócrino e parácrino da terapia gênica com adenovírus portador de IFNβ e sua relação com IFNAR1 no melanoma e microambiente murino* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2021.

O câncer é uma doença complexa, frequentemente sem cura e que está presente em todas as populações e em todas as idades. O câncer gera enormes perdas econômicas e emocionais, sendo que seus tratamentos atuais são caros e, tipicamente, pouco efetivos em casos avançados. O melanoma é um câncer de pele agressivo na sua evolução e frequentemente mortal para os hospedeiros, que apresentam a doença metastática, essa doença ainda possui poucos tratamentos efetivos. Frente a esta situação, é necessário a criação e desenvolvimento de novas abordagens com maior eficiência no tratamento do câncer. O Interferon beta (IFNβ) é uma citocina imuno-estimuladora com efeito anti-tumoral conhecido, porém sua aplicação sistêmica é tóxica. Nosso grupo propõe o uso de terapia gênica utilizando vetores adenovirais portadores do transgene terapêutico IFNβ (Ad-mIFN), assim, proporciona a produção do IFNβ de modo localizado e transiente, o que pode conferir um escape do efeito tóxico sistêmico. Os dados do grupo mostram que, em modelos murinos, a terapia gênica com Ad-mIFN *in vitro* resulta em morte celular e no modelo *in situ* induz retardo na progressão tumoral do melanoma. A investigação nesta tese teve como objetivo estudar os efeitos autócrinos e parácrinos nas células do microambiente e tumorais do melanoma após terapia gênica com IFNβ e a influência do seu receptor IFNAR1 neste contexto. Para investigar a influência da sinalização do IFNβ nos efeitos autócrinos e parácrinos, realizou-se o *knockout* do IFNAR1 em células da B16 e do microambiente (tEnd, NIH3T3) utilizando a tecnologia de CRISPR/Cas9. Os resultados mostraram que células transduzidas pelo Ad-mIFN necessitam da produção e liberação do IFNβ para a ativação da via do IFNα/β, pela ligação com os receptores IFNAR1/IFNAR2 e finalmente induzir a célula a morte, mecanismo conhecido como autócrino. As células transduzidas pelo Ad-mIFN também induzem efeito parácrino por inibir as células próximas, pelo mecanismo parecido com o autócrino, mas direcionado a outras células próximas. As células *knockout* para o IFNAR1 ficaram bloqueadas de receber os efeitos autócrinos e parácrinos do Ad-mIFN e da proteína recombinante do IFNβ (rIFNβ), porém mesmo sem o IFNAR1 elas

continuam produzindo o efeito parácrino. Esses dados foram observados em células tumorais e células endoteliais e de fibroblastos. Outro estudo *in vitro* da terapia com Ad-mIFN e Ad-p19 na angiogênese utilizando células endoteliais, descobriu uma linhagem endotelial não responsiva ao tratamento com Ad-mIFN e a proteína IFNβ sozinha, porém essas células sofriam inibição quando colocadas em ensaios de efeito parácrino pela linhagem de melanoma transduzida com o Ad-mIFN. A observação desse fenômeno sugere a possibilidade da existência de outros fatores além do IFNβ, produzidos pelas células de melanoma transduzidas com Ad-mIFN, que estariam envolvidos no efeito parácrino. Ensaios de expressão gênica da linhagem de melanoma transduzida pelo Ad-mIFN mostraram aumento significativo de TRAIL, que pode ser um candidato para estar envolvido no efeito parácrino. Juntos, estes resultados indicam que a presença de IFNAR1 não é essencial para produção dos fatores necessários para induzir o efeito parácrino, porem a presença de IFNAR1 é necessário para a célula sofrer este efeito. Futuramente, as linhagens deficientes em IFNAR1 podem ser avaliadas em ensaios *in vivo* para revelar o papel do IFNAR1 nos efeitos autócrinos e parácrinos entre células tumor e seu microambiente após tratamento, incluindo com Ad-mIFN.

Descritores: Interferon beta; Terapia genética; Melanoma; Microambiente tumoral; Receptor de interferon alfa e beta; Morte celular.

ABSTRACT

Vieira VL. *Evaluation of the autocrine and paracrine effects of IFNβ gene therapy with adenovirus and its relationship with IFNAR1 in melanoma and microenvironment of the murine model* [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2021.

Cancer is a complex disease, often without cure, which is present in all populations and at all ages. Cancer generates enormous economic and emotional losses, and its current treatments are expensive and, typically, ineffective in advanced cases. Melanoma is an aggressive skin cancer in its evolution and often deadly for the hosts that present metastatic disease since few effective treatments are available. Faced with this situation, it is necessary to create and develop new approaches with greater efficacy in the treatment of cancer. Interferon beta (IFNβ) is an immunostimulatory cytokine with a known anti-tumor effect, but its systemic application is toxic. Our group proposes the use of gene therapy using adenoviral vectors that carry the therapeutic transgene IFNβ (Ad-mIFN), thus providing the production of IFNβ in a localized and transient way, which should avoid the systemic toxic effect. Our studies have shown that, in murine models, gene therapy with Ad-mIFN *in vitro* results in cell death and, in the *in situ* model, delays the progression of melanoma tumors. The research performed in this thesis aimed to study the autocrine and paracrine effects in the microenvironment and tumor cells of melanoma after IFNβ gene therapy and the role of the IFNAR1 receptor. To investigate the influence of IFNβ signaling on autocrine and paracrine effects, we performed IFNAR1 knockout on B16 and microenvironment cells (tEnd, NIH3T3) using the CRISPR / Cas9 technology. The results showed that cells transduced by Ad-mIFN require the production and release of IFNβ for activation of the IFNα/β pathway, by binding with the IFNAR1/IFNAR2 receptors and finally inducing cell death, a mechanism known as autocrine. The cells transduced by Ad-mIFN also induce a paracrine effect by inhibiting nearby cells, by a mechanism similar to the autocrine but directed at other nearby cells. The cells with IFNAR1 knockout were blocked from receiving the autocrine and paracrine effects of Ad-mIFN and recombinant IFNβ protein (rIFNβ), however even without IFNAR1 they continue to produce the paracrine effect. These data were observed in tumor cells and endothelial and fibroblast cells. Another *in vitro* study of therapy with Ad-mIFN and Ad-p19 in angiogenesis identified an

endothelial cell line unresponsive to treatment with Ad-mIFN and the IFNβ protein alone, however these cells suffered inhibition when tested for the paracrine effect provided by the melanoma cell line transduced with Ad-mIFN. The observation of this phenomenon suggests the existence of factors other than IFNβ, produced by melanoma cells transduced with Ad-mIFN, which would be involved in the paracrine effect. Gene expression assays of the melanoma lineage transduced by Ad-mIFN showed a significant increase in TRAIL, which may be a candidate for involvement in the paracrine effect. Together, these results indicate that the presence of IFNAR1 is not essential for the production of the factors necessary to induce the paracrine effect, however the presence of IFNAR1 is necessary for the cell to suffer this effect. In the future, IFNAR1-deficient strains can be evaluated in *in vivo* assays to reveal the role of IFNAR1 in the autocrine and paracrine effects between tumor cells and their microenvironment after treatment, including with Ad-mIFN.

Descriptors: Interferon-beta; Genetic therapy; Melanoma; Tumor microenvironment; Receptor, interferon alpha-beta; Cell death.

1

Organização da tese

ORGANIZAÇÃO DA TESE

A tese foi organizada em capítulos, para um melhor entendimento da linha de pesquisa desenvolvida. Os trabalhos publicados ou em processo de publicação apresentados na tese não apresentam sequência cronológica, porém eles exibem uma sucessão lógica.

No **Capítulo 02**, é apresentada uma revisão publicada na revista *Clinics*, sobre o tema de estudo do grupo. A revisão descreve a tecnologia de terapia gênica como uma imunoterapia para o câncer. Nessa abordagem de terapia o grupo utilizou vetores virais portadores de genes terapêuticos para o tratamento do câncer. O foco desta terapia gênica consiste em entregar os genes terapêuticos Interferon beta e $p19^{Arf}$ ($p19^{Arf}$ murino, $p14^{Arf}$ humano ou canino) em modelos murinos, humanos e caninos utilizando principalmente vetores adenovirais. Na revisão são discutidos a combinação terapêutica e seus resultados promissores, entretanto o Ad-mIFN sozinho é capaz de induzir os efeitos anti-tumorais de modo eficiente e promissor em diversos modelos.

No **Capítulo 03**, é mostrado o trabalho em andamento, onde são explorados os papéis do IFNAR1 utilizando células de melanoma e do microambiente *knockout* para esse receptor no tratamento com Ad-mIFN. Os resultados elucidaram os mecanismos dos efeitos autócrino e parácrino após tratamento com Ad-mIFN, com a demonstração da importância da produção do IFNβ e da presença do IFNAR1 para ativação da via do IFNα/β e sucesso do tratamento.

No **Capítulo 04**, exibe-se o trabalho de investigação do efeito parácrino do tratamento com Ad-mIFN. O trabalho foi publicado na revista *Journal of Interferon and Cytokine Research* em abril de 2019. Neste trabalho, além de demonstrar o efeito parácrino inibidor de células endoteliais, as investigações descobriram uma linhagem que não responde ao tratamento com Ad-mIFN e nem a proteína recombinante do Interferon beta (rIFNβ), porém a linhagem é inibida pelo efeito parácrino induzido por células B16 transduzidas pelo Ad-mIFN. Essa observação sugere a possibilidade da participação de outros fatores, além do IFNβ, envolvidos no efeito parácrino do tratamento com o Ad-mIFN.

O **Capítulo 05** apresenta a discussão final da tese referente à evolução dos trabalhos realizados publicados ou em processo de finalização.

2

Perspectivas para a imunoterapia do câncer mediada por transferência gênica de p19Arf e Interferon beta

Perspectives for canccer immunotherapy mediated by p19Arf plus Interferon-beta gene transfer

Bryan E. Strauss, Gissele Rolemberg Oliveira Silva, Igor de Luna Vieira,
Otto Luiz Dutra Cerqueira, Paulo Roberto Del Valle,
Ruan Felipe Vieira Medrano e Samir Andrade Mendonca

Artigo de revisão publicado na revista Clinics em Maio de 2018

RESUMO

Os tratamentos com imunoterapias do câncer estão em grande evidência nos últimos anos, porém as melhorias tecnológicas das subáreas dessa modalidade de terapia não estão sendo exploradas, como o desenvolvimento de novos métodos de entrega e a combinação de terapias que atuam em múltiplos alvos na imunidade do câncer. O trabalho do nosso grupo tem como objetivo o uso de tecnologias em transferência gênica para promoção de morte celular e estímulo imune. Foram trabalhados com combinação de transferência gênica com os cDNAs de $p14^{Arf}$ (p19Arf murino) e Interferon beta. Os dados mostraram que o modelo induz morte celular por ativação do sistema imune resultando em efeito anti-tumoral nos modelos murinos de melanoma e câncer de pulmão. Frente aos resultados promissores alcançados até o momento, o grupo de estudo pretende investir esforços no desenvolvimento do modelo translacional e pré-clínico de tratamento imunoterápico anti-tumoral com terapia gênica utilizando IFNβ e ARF. Nessa revisão, são mostrados as perspectivas e desafios enfrentados. A evolução contou também com o uso de tumores humanos e células imunes para verificar a resposta observada no modelo murino. Dentro das pesquisas, também utilizaram-se modelos clinicamente relevantes como a indução de tumores espontâneos em animais e o modelo xenoenxerto derivado de pacientes humanos. Buscou-se combinar o modelo de imunoterapia com outros tratamentos como a quimioterapia ou o *checkpoint blockade,* com objetivo de reduzir as doses e aumentar a eficiência final. O estudo é realizado no Instituto do Câncer do Estado de São Paulo que possui estrutura necessária para o estudo translacional e multidisciplinar deste método.

CLINICS

REVIEW ARTICLE

Perspectives for cancer immunotherapy mediated by p19Arf plus interferon-beta gene transfer

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While cancer immunotherapy has gained much deserved attention in recent years, many areas regarding the optimization of such modalities remain unexplored, including the development of novel approaches and the strategic combination of therapies that target multiple aspects of the cancer-immunity cycle. Our own work involves the use of gene transfer technology to promote cell death and immune stimulation. Such immunogenic cell death, mediated by the combined transfer of the alternate reading frame (p14ARF in humans and p19Arf in mice) and the interferon-β cDNA in our case, was shown to promote an antitumor immune response in mouse models of melanoma and lung carcinoma. With these encouraging results, we are now setting out on the road toward translational and preclinical development of our novel immunotherapeutic approach. Here, we outline the perspectives and challenges that we face, including the use of human tumor and immune cells to verify the response seen in mouse models and the incorporation of clinically relevant models, such as patient-derived xenografts and spontaneous tumors in animals. In addition, we seek to combine our immunotherapeutic approach with other treatments, such as chemotherapy or checkpoint blockade, with the goal of reducing dosage and increasing efficacy. The success of any translational research requires the cooperation of a multidisciplinary team of professionals involved in laboratory and clinical research, a relationship that is fostered at the Cancer Institute of Sao Paulo.

KEYWORDS: Melanoma; Immunotherapy; Immunogenic Cell Death; Translational Medicine; Viral Vectors.

INTRODUCTION

The progression of cancers depends, in part, on the ability of tumor cells to escape immunosurveillance (1). Tumor cells accomplish this by a variety of mechanisms, collectively termed immunoediting, which include the hijacking of signaling events to promote an immunosuppressive microenvironment and selecting tumor cells that are no longer recognized by the immune system (2,3). The goal of immunotherapy is to enhance, if not reboot, the cancer-immunity cycle, starting with tumor cell killing by an immunogenic mechanism, enhancing the function of antigen-presenting cells (APCs) and stimulating cytolytic and helper T (Th) cell responses, thus completing the cycle (4). Each round through the immunity cycle may amplify the antitumor immune response since the killing of the tumor cells may release additional antigens that would then contribute to broaden the T cell repertoire.

Immunotherapies that directly target tumor cell killing typically rely on the induction of immunogenic cell death (ICD). Such therapeutic approaches include treatment with certain chemotherapeutic agents, such as anthracyclines and oxaliplatin, or the application of oncolytic viruses. These agents promote the release of tumor antigens in conjunction with the release of signals that activate APCs, including ATP, high mobility group box-1 (HMGB1) and interferon-β (IFNβ), and the exposure of calreticulin on the surface of tumor cells (5). Alternatively, tumor vaccines may provide one or more critical antigens and may even deliver *ex vivo*-modified dendritic cells (DCs) to stimulate an antitumor immune response. Checkpoint blockade typically relies on the use of monoclonal antibodies to reverse the negative regulation of T cells due to their expression of inhibitory molecules, such as cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) or programmed cell death protein 1 (PD-1) (4). Alternatively, adoptive cell transfer (ACT) can be used, where autologous T cells may be selected/modified *ex vivo*, expanded, and returned to the patient to induce tumor cell killing. In particular, chimeric antigen receptor (CAR) T cells have recently gained much attention, especially for the treatment of B cell leukemias (6) and lymphomas (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm 581216.htm). Since each of these immunotherapies targets particular points in the cancer-immunity cycle, their combination may also lead to an even greater efficacy (4).

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The promise of immunotherapies has justifiably gained considerable attention over the past few years. However, the role of gene transfer/gene therapy in this setting has not yet been fully explored. For example, oncolytic viruses induce cell killing as a result of viral replication leading to cell lysis but do not necessarily encode a transgene, such as granulocyte macrophage-colony stimulating factor (GM-CSF). The application of oncolytic viruses is often referred to as virotherapy, a close cousin of gene therapy, but not a gene transfer approach per se. In fact, Imlygic (talimogene laherparepvec, T-Vec, an oncolytic herpes virus encoding GM-CSF) was considered a first in-class product when it was approved by the U.S. Food and Drug Administration (FDA) (7). The recently approved CAR T cell approaches Kymriah and Yescarta for the treatment of B cell leukemia and some large B cell lymphomas, respectively (6), (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm581216.htm) have been classified by the FDA as cell-based gene therapies; that is, the gene transfer aspect is not performed directly in the patient, but a vector encoding the CAR is applied to T cells *ex vivo*.

To the best of our knowledge, no *in situ* cancer gene therapy approach that acts as an inducer of ICD, characterized by the release of ATP, calreticulin and HMGB1, has been described to date. Nevertheless, gene therapy approaches that induce an immune response are known. The transfer of the thymidine kinase (TK) gene derived from the herpes simplex virus by means of nonreplicating adenoviral vectors (Ad-TK) has been extensively explored (8). In transduced tumor cells, TK, in conjunction with cellular enzymes, converts prodrugs (ganciclovir, valacyclovir, acyclovir) into their active forms to block DNA replication and induce cell death. The Ad-TK approach, also termed gene-mediated cytotoxic immunotherapy (GMCI), is known not only for the associated bystander effect but also for its ability to stimulate an antitumor immune response (8). Several clinical trials are being or have been performed, including a phase III trial for the treatment of high-grade glioma, where time to death, but not overall survival, was increased (9).

Also in development are approaches that combine GMCI with other therapeutics that boost the antitumor response, including the association of Ad-TK with FMS-like tyrosine kinase 3 ligand (FLT3L) gene transfer (10). A particularly interesting approach is the use of Toca 511 (vocimagene amiretrorepvec), a nonlytic, replicating retroviral vector that spreads among tumor cells for the delivery of the cytosine deaminase (CD) gene, which converts 5-fluorocytosine into 5-fluorouracil (5FU) and has been shown stimulate antitumor immune responses (11). In a phase I trial, compared to an external control, Toca 511 significantly improved the overall survival of patients with high-grade glioma (12). While a variety of gene transfer approaches can be considered immunotherapies, further improvements may result in more robust responses in a larger number of patients.

A role for the p19Arf and IFNβ gene transfer in cancer immunotherapy

Our work has focused on cancer gene therapy using adenovirus-mediated gene transfer to elicit both cell death and activation of an immune response against tumors. Here, we will provide an overview of one immunotherapeutic approach that utilizes a specialized vector to deliver the cDNA encoding the alternate reading frame (ARF; p14ARF in humans and p19Arf in mice) and IFNβ proteins to cancer cells. Our group is also developing additional modalities described elsewhere, including a review in this issue of Clinics, which involve the use of our specialized vector for the transfer of the cDNA encoding the tumor suppressor p53. The following discussion will provide an overview of the development of our gene transfer approach and of the evidence suggesting that the transfer of p19Arf and IFNβ indeed acts as an immunotherapy in mouse models of melanoma and lung cancer.

The antitumor activities of p53 are frequently related to its role as a regulator of transcription of a variety of target genes, which in turn direct cell death, inhibit the cell cycle and DNA repair, and block angiogenesis, among others (13,14). Although quite complex, key regulators of p53 include the human homolog of murine double minute-2 (HDM2), which directs p53 for degradation, and p14ARF, which disrupts the interaction between MDM2 and p53, thus freeing p53 to act (15). Despite variable reports, published data indicate that up to 90% of melanomas retain p53 in the wild-type form (16). However, p53 is essentially dormant due to the loss of agonistic or gain in antagonistic factors, including the lack of p14ARF in 50% and the overexpression of HDM2 in 56% of melanomas (16-18). We reason that the endogenous wild-type p53 (p53wt) may be activated in response to gene transfer and that the activated p53, a powerful transcription factor, may be harnessed to not only act as a tumor suppressor but also drive the expression of the transgene encoded by the gene transfer vector as described below. Thus, we expect to establish dynamic interactions among the gene transfer vector, the therapeutic gene(s) and endogenous p53.

Our gene transfer platform involves a nonreplicating, recombinant, serotype 5 adenovirus (Ad5) in which transgene expression is controlled by a p53-responsive promoter, PGTxβ, called PG for simplicity. The chimeric promoter includes 13 copies of a p53-responsive element (PG), a TATA box (Tx) and the first intron of the rabbit β-globin gene (β), as previously detailed (19). The PG promoter can be used to drive the expression of the p53 cDNA, establishing a positive feedback mechanism that is initiated due to leaky transcription even in p53-null cells (20,21). Alternatively, the PG promoter may be employed to drive the transcription of any gene of choice as long as p53 is present in the cell. Expression from the PG promoter is 5 to 7 times higher than that seen from typical, constitutive promoters, such as the cytomegalovirus (CMV) immediate early promoter enhancer or the retroviral long terminal repeat (LTR) (19-21). In fact, we have developed three viral platforms, namely, retrovirus, adenovirus and adeno-associated virus, in which transgene expression is dependent on p53 (19,22,23). Such vectors are expected to have utility in not only models of cancer gene therapy but also other conditions that involve cellular stress, such as hypoxia (22,24).

After developing the PG vectors, our next task was to use these to deliver the p19Arf cDNA and assess whether endogenous p53 could be activated. Indeed, the transfer of p19Arf, but not p53, resulted in increased p53 activity in B16 mouse melanoma cells (with endogenous p53wt). However, we noted that this activation was accentuated when gene transfer was combined with chemotherapeutic agents, resulting in increased cell death both *in vitro* and *in vivo* (25).

Since we wished to maximize cell death and induce immune stimulation, we decided to perform simultaneous transfer of p19Arf and IFNβ because the p53/Arf and type I interferon (IFNα/β) pathways have been indicated to cooperate.

The presence of p53 has been shown to increase cell death and minimize viral replication in cells treated with recombinant type I interferon protein (rIFN) (26). Interestingly, one report form the literature suggests that ARF, not p53, is actually the critical factor that mediates the apoptotic response to rIFN (27). Such interactions are to be expected since both type I IFN and the p53/Arf pathways play important roles in regulating cell death (28). Indeed, we noted that compared to single gene transfer, the combined transfer of p19Arf and IFNβ (p19Arf + IFNβ) mediated by our p53-responsive Ad5 vector resulted in enhanced B16 cell death regardless of *in vitro* or *in vivo* application. In a mouse model of *in situ* gene therapy (that is, injection of viral particles directly into the tumor mass), only the combined gene transfer approach was associated with extensive tumor cell killing and prolonged survival, although IFNβ gene transfer could also delay tumor progression (29). From this study, we learned that the combined, but not individual, gene transfer was especially effective for the induction of cell death.

In a later study, we examined both the critical aspects of the adenovirus-mediated gene transfer and the specific mechanism of cell death in response to p19Arf and IFNβ gene transfer. In this work, improvements to vector design involved the use of the RGD tripeptide included in the knob domain of the adenovirus fiber protein, thus broadening the spectrum of cells that may be transduced since virus-cell interaction depends on integrins but not the coxsackievirus and adenovirus receptor (30-32). We also explored the use of a single vector for the simultaneous transfer of the p19Arf and IFNβ cDNA, finding that both co-transduction and IRES-mediated expression of two proteins from a single transcript were equally effective (32). As expected, combined, but not single, gene transfer resulted in enhanced killing of B16 cells. However, we uncovered an important bystander effect, where the presence of exogenous p19Arf was shown to sensitize the melanoma cells to secreted IFNβ produced by neighboring cells that had received the IFNβ vector. In addition, we provided functional and molecular evidence indicating that the presence of the adenoviral vector itself was important for mediating the antiviral response that contributed to cell killing. In other words, gene transfer was more effective than the pharmacological activation of the p53/Arf and type I IFN pathways (32).

The mechanism of cell death was also explored, revealing that apoptosis was not responsible for cell death in response to combined gene transfer, since caspase activity was not required for cell killing. Instead, we observed that combined, but not individual, gene transfer activated critical mediators of necroptosis. Moreover, only combined gene transfer was associated with the presentation of all three classic markers of ICD, namely, calreticulin, ATP and HMGB1. As expected, involvement of the p53 pathway was revealed by elevated levels of p53 target genes (p21/Cdkn2a, Puma, Phlda2), as well as p53 itself, when B16 cells were treated with combined gene transfer both *in vitro* and *in vivo*. Interestingly, combined gene transfer *in vivo* in a model of *in situ* gene therapy was associated with vesicular and perinuclear staining of LC3β, suggesting a potential role for autophagy in our model. Microarray analysis of B16 cells after *ex vivo* gene transfer revealed increased expression of genes associated with the immune response, p53 activity, cell death and antiviral response and decreased expression of cell cycle-related transcripts. Therefore, the multimodal cell death mechanism was consistent with necroptosis associated with the release of ICD markers and an antiviral response (31,32).

The cellular response to p19Arf + IFNβ gene transfer shows that our approach is effective at killing tumor cells by a mechanism that is expected to promote an antitumor immune response. In another study using the B16 mouse melanoma cell line, several parameters of this immune response were revealed. For example, B16 cells were transduced *ex vivo*, and then the dying cells were applied as a prophylactic vaccine in syngeneic immunocompetent mice. Later, naïve B16 cells were implanted in the opposite flank, representing a tumor challenge. While vaccinations involving IFNβ gene transfer alone or in combination with p19Arf were effective in slowing challenge tumor growth, only the combined approach was significantly associated with increased survival (33). With optimization, tumor progression at the vaccination site could be completely eliminated in both immunocompetent C57BL/6 or T cell-deficient BALB/c nude mice (both endowed with high activity of natural killer (NK) cells); however, this protection was lost in innate and adaptive immunodeficient NOD-SCID mice, suggesting a critical involvement of NK cells. Gene expression analysis revealed substantial upregulation of Ulbp1, IL-16, Killer/DR5 and FAS/Apo1, which are critical factors for the NK cell response, albeit only in association with the combined gene transfer approach. Evidence for a *de facto* immune response was revealed by the depletion of either $CD4^+$ or $CD8^+$ T cell populations, which reversed the protective effect of the prophylactic vaccine. Moreover, the vaccination approach promoted a Th1 cytokine profile. Strikingly, the optimized prophylactic vaccination protocol provided significant protection against tumor growth even when the challenge was applied 70 days post-vaccination. Alternatively, subcutaneous Tm1 tumors were first established before the application of a therapeutic vaccine consisting of dying Tm1 cells in response to *ex vivo* gene transfer and resulted in a significant reduction of tumor progression only in response to the combined p19Arf + IFNβ treatment (33). In other words, our gene transfer approach with p19Arf + IFNβ can be classified as immunotherapy.

Although the vaccination approach involving p19Arf + IFNβ gene transfer provided evidence for an effective antitumor immune response, we also wished to explore whether *in situ* cancer gene therapy using our vectors can serve as immunotherapy. To this end, we first confirmed the induction of ICD in Lewis lung carcinoma (LLC, mouse lung carcinoma, p53wt) cells only in response to the combined gene transfer (34). We then showed that a prophylactic vaccination model using LLC cells in C57BL/6 mice was especially effective in slowing challenge tumor growth when the vaccine cells had been treated with the combination of p19Arf + IFNβ. *In situ* gene therapy showed that both IFNβ alone and in combination with p19Arf could significantly delay tumor progression, although the association of cisplatin with gene therapy resulted in nearly complete tumor inhibition only in the presence of p19Arf + IFNβ. Indeed, *in situ* gene therapy followed by subcutaneous injection of naïve LLC cells resulted in greatly enhanced inhibition of the challenge tumor when the primary tumor had been treated with p19Arf + IFNβ rather than IFNβ alone. In addition, microarray analysis of tumors treated with *in situ* gene therapy revealed a gene signature consistent with an immune response and chemotactic enrichment, suggesting the presence of neutrophils and $CD8^+$ T cells. In one study, the presence of infiltrating neutrophils was confirmed, and depletion of

the granulocyte population reversed the benefit of *in situ* gene therapy (34). In this study, we revealed, among other findings, that our *in situ* gene transfer approach initiates an antitumor immune response and can be considered immunotherapy.

Taken together, these results reveal that the p19Arf + IFNβ gene transfer strategy promotes an immune response that includes the participation of NK cells, neutrophils, a Th1 response and, importantly, $CD4^+$ and $CD8^+$ T cells (31,35). The cellular response to combined gene transfer has been shown to induce ICD and, specifically in the case of B16, cell death consistent with necroptosis (31,35). While the studies performed to date have been interesting, some developments remain before we can affirm that our approach indeed holds promise for clinical application.

CHALLENGES AND PERSPECTIVES FOR THE P19ARF + IFNβ IMMUNOTHERAPY

As described in the following sections, we are looking forward to the next phase of our investigation where we face the challenge of further developing our approach and incorporating more clinically relevant models. In other words, we are poised to advance on our journey along translational research (Figure 1). We are concerned with the interactions of the p19Arf + IFNβ gene transfer approach with respect to

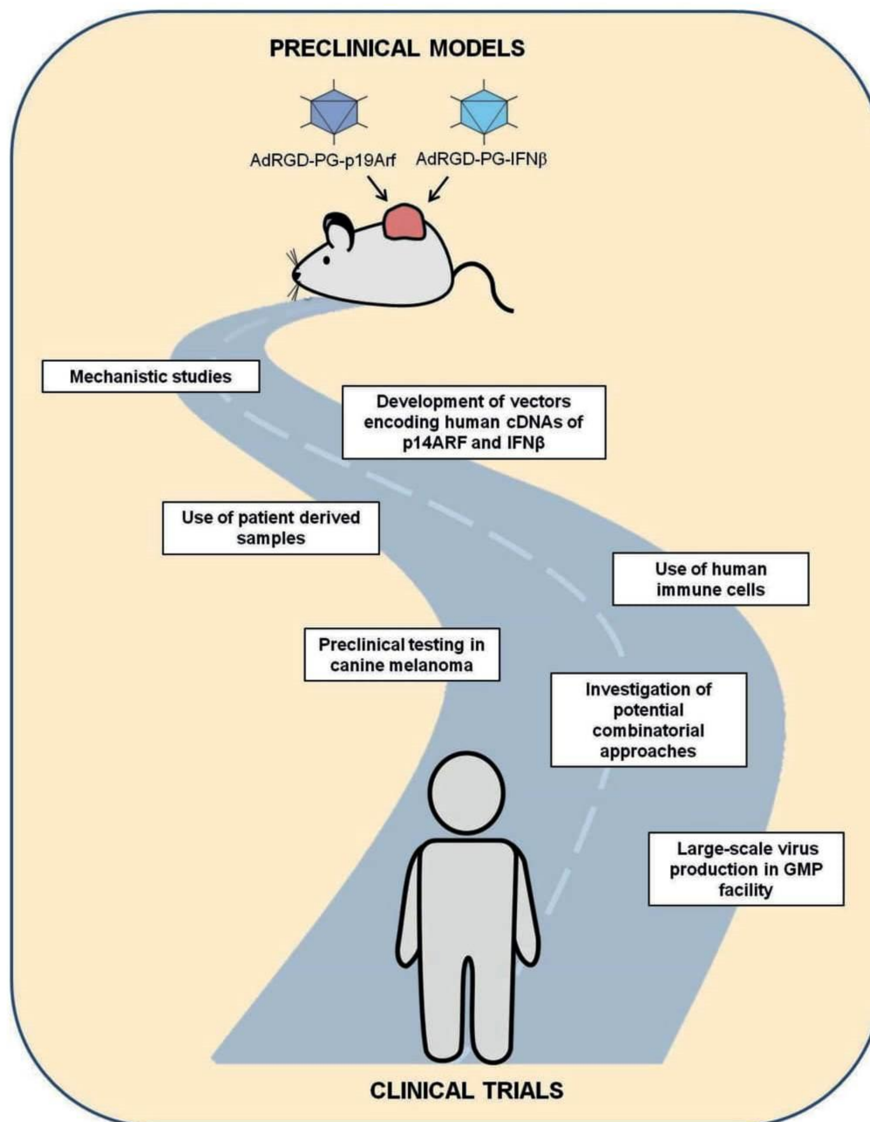

Figure 1 - Schematic representation of the challenges and perspectives of continuing along the road of translational research. Critical milestones include testing our approach in human cells, including patient-derived tumor and immune cells, and models of spontaneous tumors, such as canine melanoma. In addition, the combination of our approach with chemotherapy or other immunotherapies may enhance efficacy. Virus production following good manufacturing practices (GMP) is critical for providing not only larger quantities of vectors but also biological agents of pharmaceutical quality.

not only the tumor cell response but also the impact on the tumor microenvironment. While the use of mouse models aids in such studies, especially when the immune response is to be examined, it is critical to determine the behavior of human tumor cells and even the activation of human immune cells upon p14ARF + human IFNβ (hIFNβ) gene transfer. Another critical step towards the preclinical evaluation of our immunotherapeutic approach involves the use of alternative models of tumor treatment.

Potential impact of p19Arf + IFNβ on tumor angiogenesis

Since both the p53/Arf and IFN pathways are known to impact the tumor microenvironment, the study of such interactions may show the potential of our gene therapy approach in inhibiting angiogenesis and possibly metastasis. Tumor cells, nontumor cells, and noncellular components are partners working together for the survival of solid cancers (36). Nontumor cells are important components of the microenvironment, promoting tumorigenesis through a variety of cell types and mechanisms (37). The network of blood vessels is a critical component of the tumor microenvironment and provides oxygen, nutrients, immune surveillance and a route for metastasis, essentially fueling tumorigenesis (38). Failure of many cancer treatments can be explained, in part, by mechanisms that include poor biodistribution of drugs and an unfavorable tumor microenvironment (39). Thus, the impact of novel treatments on the tumor microenvironment must not be overlooked.

In addition to the well-known tumor suppressor activity of p53, its influence can reach beyond the single tumor cell (40). Depending on whether it is wild type or mutant, the p53 protein can modulate the extracellular matrix (41,42) and induce the secretion of proinflammatory proteins (43-45) and lactate, resulting in the acidification of the tumor boundary (46,47). p53 also coordinates the crosstalk between cancer and noncancer cells (48,49).

A p53-dependent and independent role for p19Arf in the angiogenic switch has been shown to accelerate tumor growth (50). Alternatively, p14ARF can sequester hypoxia-inducible factor 1α (HIF-1α), thus inhibiting HIF-1 transactivation in a p53-independent manner (51). Notably, p14ARF has been shown to suppress angiogenesis by blocking the translation of the vascular endothelial growth factor (VEGF)-A transcript (52). Thus, Arf can play a role in inhibiting tumor angiogenesis.

Type I IFNs are also recognized for their ability to block angiogenesis (28). For example, IFNβ has been shown to upregulate inducible nitric oxide synthase (iNOS), thus blocking angiogenesis and tumor progression in a xenograft model of human prostate carcinoma (53). IFNα and IFNβ have also been shown to block the production of essential mediators of angiogenesis, including basic fibroblast growth factor (bFGF), VEGF and interleukin (IL)-8 (28). Thus, each of the central players in our gene transfer approach may negatively impact tumor angiogenesis, but this point remains to be experimentally tested.

Gearing up for the study of p14ARF + hIFNβ gene transfer in human cells

Despite good results in preclinical models, many promising candidate drugs do not successfully cross the bridge between the bench and bedside (54). As discussed above, the combined p19Arf + IFNβ gene transfer approach triggered cell death mechanisms with remarkable immunogenic features, leading to reduced tumor burden and increased survival in animal models, which are desirable characteristics for any cancer therapeutics in development. Although our gene transfer approach seems promising, the translation of these results from mouse models to human cells represents a major step forward toward clinical relevance.

Mice and humans share many functional gene sequences but greatly diverge with respect to transcriptional regulation (55). Reliance on mouse models to predict human physiology and response to therapy is severely limited, and a critical step in closing this gap is the inclusion of studies performed in human cells. While patient-derived xenograft (PDX) models may be preferred (56), the use of established cell lines comes with ease of use and a wealth of knowledge in the literature about their genotype and response to various treatments. For example, a variety of human melanoma cell lines with distinct genotypes, especially with respect to p53, are widely available.

Nonetheless, the transition to human cell lines comes with an additional task with respect to our gene transfer approach. The cellular response to IFNβ is species specific; that is, mouse cells treated with human IFNβ, or vice versa, will produce distinct antiproliferative responses (57). Thus, we have to be particularly careful to match the cDNA encoded in the vectors with the species being treated. The assays described for B16 and LLC cells above used only mouse cDNA. To initiate tests in human cells, a new set of vectors harboring human protein coding sequences must be constructed. Human cDNA (p14ARF and hIFNβ) encoded by the p53-responsive adenoviral vectors and applied to human melanoma cell lines creates the opportunity to test the induction of cell death, cooperation between the p53/Arf and IFN pathways and the mechanism of cell death. Since human melanoma cell lines are known to harbor either wild-type or mutant p53, we would also have the opportunity to explore the specific role of p53 in our gene transfer approach. For this, an additional set of vectors encoding human cDNA, with expression under the control of the constitutive CMV promoter, will be required.

The new vector constructs encoding human cDNA with either constitutive or p53-dependent expression will enable a deeper investigation of the molecular basis of the approach, including their behavior in cell culture and immunodeficient animals, the study of the contribution of p53 to the cellular response, and, possibly, their combined use with pharmacotherapeutic approaches.

Seeking clinical relevance: use of patient-derived tumor samples

For decades, the number of established cell lines from tumor tissues has sharply increased, and this has undoubtedly resulted in many advances in the understanding of oncogenic mechanisms and the discovery of new targets for therapeutic interventions (58,59). As mentioned above, our own work has frequently employed established cell lines. During the isolation of *ex vivo* immortalized cell lines from normal tissues, some homeostasis mechanisms, such as cell cycle control, need to be inhibited, either by mutations of key genes or addition of growth factors in the culture medium. In comparison, cell lines derived from tumor tissues already exhibit these mechanisms, although often in a deregulated fashion (60,61). In addition to being cultured *in vitro*,

these cells can be inoculated into immunocompromised animals to generate xenografts, which may be either heterotopic or orthotopic, providing the opportunity to study these cells *in vivo* (62).

While models based on human cell lines in monolayer cultures and their corresponding xenografts are widely used in cancer research, many limitations exist regarding this approach. Human cancers are known to have extremely heterogeneous cell populations comprising both neoplastic cells and stromal cells that actively contribute to tumor progression (63-66). Additionally, samples derived from different patients have unique sets of mutations, leading to differences in several signaling pathways and individual series of carcinogenic events (67). The establishment and propagation of these cell lines over time imply that only certain subpopulations will be selected, and the resulting clonality may not be representative of the original tumor heterogeneity (67,68). Therefore, the number of effective therapeutic tools that emerge from studies using cultured patient-derived cells is still small (59,69).

In contrast, PDXs involve the implantation of tumor tissues from patients directly into immunocompromised recipient mice (70). Unlike cancer cell lines, PDX models do not require *in vitro* culture steps, thus avoiding the selection of distinct cell populations and the exclusion of stromal components. The *in situ* physiological conditions encountered by these grafted cells in host mice, such as oxygen pressure, nutrients and metabolites, are similar to those found at the site of the patient's primary tumor. Experimental data suggest that overall genomic and gene expression profiles of PDXs are representative of the patients of origin and are stable throughout sequential *in vivo* generations (71).

Taking this into account, the establishment of each PDX is individually performed, preserving intra- and intertumor heterogeneity and providing a clear advantage when used for oncologic drug discovery and preclinical development (72). Evidence in the literature indicates success in predicting personalized anticancer treatments previously investigated using PDX models (73-75). Through the PDX approach, mutations in melanoma have been mapped in more detail, which provides complementary diagnostic data with greater predictive power to indicate the therapeutic approach to be adopted (76,77).

Another important feature recapitulated by the PDX model is the maintenance of a three-dimensional (3D) arrangement. Cells grown on a flat surface differ greatly from their 3D counterparts *in vivo* because of reduced cell-cell and extracellular matrix-cell interactions, a limitation already encountered with the traditional model of cancer cell lines (78-80). However, the culture model in 3D environments represents an intermediate approach between traditional cell culture and animal models, since it largely recapitulates the architecture of tissues *in vivo* (81-83).

Given that samples from patients are generally scarce and difficult to obtain, the use of 3D culture models enable the use of traditional cancer cell lines, prior to PDX models, for the standardization of viral infection kinetics, pharmacokinetics, molecular biology, biochemistry and imaging techniques. In addition, 3D cultures are less costly and labor-intensive than animal studies and should be prioritized at a preliminary stage. Experimental data show that the pattern of gene expression, mRNA splicing, intracellular signaling, cytoskeletal organization and secretion profile of cells grown in 3D cultures more closely resemble what is observed *in vivo* than when grown in two-dimensional (2D) cultures (84-88).

Initially, 3D cell cultures were derived from breast development studies and breast cancer models (89). Later, these methods were adapted for a variety of cells derived from different organ systems (90). Melanoma spheroids grafted in collagen gel matrix reproduce the architecture of a tumor encountered *in vivo*, including a gradient of oxygen and nutrients and a hypoxic and necrotic central zone (90,91). In this model, tumor heterogeneity is recreated in a manner similar to that found in patients, where only cells growing at the periphery have differential activity of the extracellular signal-regulated kinase (ERK). Consequently, small-molecule inhibitors of the mitogen-activated protein kinase (MAPK) pathway do not affect cells in the core of the spheroid (92,93). This observation highlights important implications that must be considered for the translation of novel targets for future therapies, both in terms of molecular responses and biodistribution of therapeutic agents, including adenoviral vectors.

Another important consequence of cell culture in a 3D environment is the enrichment of 'cancer stem cell' subpopulations (94). Cancer stem cells represent rare subpopulations that have important characteristics including the capacity for autorenewal, tumor initiation and increased resistance to chemotherapy. Evidence suggests that cancer stem cells are the main cell type responsible for metastasis and tumor repopulation after debulking in response to chemotherapy (95-98). In 3D models of melanoma, a valuable strategy for obtaining, and consequently studying, this subpopulation is to culture melanocytes over extended periods of time in modified culture media (99). The use of these strategies contributes to physiologically relevant findings and greater chances of success in clinical trials.

Ex vivo models for the activation of human immune cells

Since our strategy relies on not only the induction of tumor cell death but also the potential modulation of the tumor microenvironment, especially the tumor immune environment, it is crucial to investigate this interaction. As long as we use syngeneic mouse models, such assays are readily available. However, the transition to human models, including established cell lines or primary tumor samples, gives rise to a critical issue when considering tumor-immune interactions. *In vivo* models where human cells are implanted in mice create a situation where immunodeficient animals are necessary to minimize species incompatibility as described above, although this also means that the immune response cannot be evaluated. While mouse models with a humanized immune system are available, they are quite complex and associated with limitations in the emulated immune response (100). Alternatively, *in vitro* models permit the evaluation of specific aspects of the interaction between human immune cells and human tumor cells that have undergone some manipulation, such as gene transfer. Although such *ex vivo* models still do not reproduce all of the facets of treating a patient, they can reveal important aspects of the impact of treatments on the microenvironment, thereby opening a window to visualize important steps of immune activation.

In terms of the cancer-immunity cycle, our approach targets the first step, that is, the killing of tumor cells while releasing antigens and critical factors that activate DCs. As described above, we can transfer p14ARF and hIFNβ cDNA

to established cell lines or primary samples. To model DC activation, we isolate mononucleated cells from blood and culture them with GM-CSF and IL-4 to induce the differentiation of monocytes to DCs. At that point, it is possible to modulate the maturation of the DCs by co-culture with previously treated tumor cells. A mature DC is expected to express co-stimulatory CD80, CD86, CD83 and MHC-II molecules. The state of maturation is thought to be dependent on the kind, duration and intensity of stimuli given to differentiated DCs

In addition to the characterization of mature DCs based on their immunophenotype, it is crucial to assess their ability to activate naïve T cells, as DCs are known to induce the T cell activation, marked by increased proliferation and change in differentiation markers such as CD4, CD8, Foxp3, RORγt, GATA-3 and T-bet. The activated T cells can then be co-cultured with tumor cells in vitro to induce tumor cell cytolysis and T cell proliferation. Ultimately, these activated T cells can be transplanted into immunosuppressed tumor-bearing mice used to generate the mature DCs to enable the assessment of T cell homing to the tumor and the induction of tumor cell death. Thus, the cancer-immunity cycle can be explored, although some adaptations may be necessary.

A critical issue when using *ex vivo* models of DC and T cell activation is the donor source used to acquire these cells. To minimize immune differences, the tumor, DC and T cells should come from the same patient. Nevertheless, DCs from cancer patients have been shown to generate a T cell response associated with increased regulatory T (Treg) cells (101). Moreover, the fusion of DCs with cancer cells has been shown to be superior to simple mixing of DCs with tumor cells in a vaccine model (102). Thus, the experimental design may greatly influence the result obtained with respect to both DC and T cell activation and functional assessment.

Beyond the mouse: preclinical testing of gene therapy in canine melanoma

Few animal models recapitulate the entire cancer developmental process, starting with benign neoplasms, progressing to primary tumors and giving rise to metastases. Most murine models of human cancer involve the investigation of certain aspects of the complex interactions among the tumor, host and therapeutic modalities. As described above, we face difficult choices between established mouse or human cell lines and patient-derived cells since each of these cells has a different genetic profile. In addition, we encounter a serious limitation when human cells are implanted in immunodeficient mice. However, the study of cancer in dogs represents an important strategy in translational oncology. Spontaneous cancer can develop in dogs due to their relatively long life expectancy and exposure to environmental conditions that are similar to those experienced by humans (103,104). Dogs also have a complex immune system, including mature immune development accompanied by typical Treg cell responses (105).

In dogs, melanoma is the malignancy most commonly found on the digits and in the oral cavity. Canine and human melanomas share histological and biological similarities, including cell morphology, disease progression and response to therapies. Similar to human melanomas, canine melanomas are chemoresistant tumors (106).

Although not completely understood, the pathogenesis of canine melanoma involves the loss of function of tumor suppressor proteins, such as PTEN and p16/INK4a, common alterations that contribute to the origin of this cancer in both dogs and humans (107). BRAF is mutated in more than 56% of human cutaneous melanoma (108), but low rates of mutations of this gene are described in canine (109) and human mucosal melanoma (110). Both canine and human mucosal melanoma show the activation of cancer-related signaling pathways, such as AKT and MAPK, and are sensitive to the inhibition of these pathways (111).

These biological similarities between canine and human tumors may be responsible for the observed concordance in treatment responses. In veterinary medicine, many of the chemotherapy protocols are based on those used to treat humans (112). Moreover, sequencing of the canine genome was recently completed, and assay reagents and platforms are now commercially available, creating an opportunity to investigate tumor biology and drug response in dogs (113). Therefore, spontaneously occurring tumors in dogs may be considered preclinical models.

Various clinical trials of cancer therapies performed in dogs complement the use of the typically used murine cancer models for the development of new treatments (105,114-116). For example, gene therapy using herpes simplex virus TK and canine IFNβ combined with a subcutaneously delivered cellular vaccine expressing human IL-2 and GM-CSF significantly prolonged disease-free and overall survival while maintaining the quality of life of dogs with melanoma (117). In another study, the administration of the adenovector CD40L in stage III canine oral melanoma resulted in complete remission with 2 intratumor injections before cytoreductive surgery (118).

For both veterinary patients and their owners, this is an opportunity to participate in clinical trials of experimental therapies. However, for humans, these trials in veterinary patients are considered preclinical tests. This situation is referred to as a co-clinical trial, where the same drug is tested in both human and canine patients who have the same tumor type or mutation spectrum (105). With regards to gene therapy, co-clinical trials provide a unique opportunity to reveal mechanisms of pathogenesis and to identify correlations between outcomes in canines and humans.

Clinical trials in pets must be performed with informed consent of their owners and must be approved by an accredited animal care institute. The scientific motivation and translational study must be balanced in relation to the ethical and clinical perceptions of animal care. In general, pet dogs are treated in studies with designs that are similar to those used in clinical trials in human. However, the historical conventions of phase I, II and III studies may be less rigid while focus is maintained on developing the technical and biological aspects of the treatment strategy (119).

In the future, it is reasonable to expect clinical researchers will view naturally occurring cancers in dogs and other animals as complementary models to the translational study of new therapies. This approach may provide early toxicity detection, optimization of clinical trial design, reduction of costs and improvement in the future care of both canine and human cancer patients.

Potential combinatorial approaches involving the p19Arf + IFNβ immunotherapy

Even though the experimental models described above can provide an opportunity for us to investigate vectors

encoding p14ARF and IFNβ under conditions that more closely resemble human cancer, it is reasonable to presume that future translational approaches will most likely be applied in combination with other treatment modalities, a practice well established in the clinic for chemo, radio and targeted therapies (120) and notably underscored by the combined use of CTLA-4 and PD-1 checkpoint blockers (121).

Indeed, in view of the synergistic results obtained with dual CTLA-4 and PD-1 blockade in melanoma, their combined use was rapidly approved by the FDA and has highlighted the capability of targeting multiple immune pathways to provide benefit for patients who otherwise would not respond to cancer immunotherapy (122). Combinatorial approaches are also expected to circumvent acquired immunological resistance mechanisms and fuel the field of immunotherapy to move steadily forward (122).

However, caution is warranted, as, along with improved therapeutic results, there is a price to pay when targeting multiple checkpoint modulators, with an increase in autoimmunity and a series of immune-related adverse events (123). Although in the case of p19Arf + IFNβ gene transfer, we have not observed any signs of an autoimmune response, such as vitiligo in the melanoma model, we cannot rule out the possibility that such responses may be generated. New therapies, including ours, will require careful optimization and development to avoid these toxicities while still inducing antitumor immunity.

In fact, combinatorial strategies employing current and novel immunotherapeutic modulators and their mechanisms of action are being intensively studied in both preclinical and clinical settings (122). Furthermore, regarding the potential combinatorial approaches for our vectors, the immunomodulatory functions exerted by IFNβ are likely a critical target to be exploited. For example, in melanoma, a type I IFN signature correlated with $CD8^+$ T cell infiltration and IFNβ production by tumor-associated DCs has been shown to be critical for mediating antitumor immunity (124) This function may prove to be particularly useful for the so-called cold tumors, which, in response to β-catenin signaling, lack the production of CCL4 and fail to recruit $CD103^+$ DCs and produce IFNβ within the tumor microenvironment and, consequently, are deprived of the CXCL9/10 chemokines that drive T cell influx (125). Accordingly, these noninflamed tumors represent a challenge that current immunotherapeutic strategies have not yet been able to successfully target. Moreover, type I IFNs are notorious for increasing the expression of MHC-I molecules on tumor cells (126). Therefore, if we were to consider the disruption in the MHC-I pathway as a hallmark of immune evasion and therapy inefficacy (127), the use of IFNβ, especially under conditions where its expression levels and dynamics are controlled, could render tumor cells visible to the immune system, avoiding primary resistance to subsequent combinatorial immunotherapies.

In addition to these immunomodulatory functions, type I IFNs can promote different inhibitory mechanisms to regulate the amplitude and duration of the response, including the production of the indoleamine 2,3-dioxygenase (IDO) enzyme and, of special interest to our group, the expression of PD-L1 in both tumors and tumor-infiltrating immune cells (28,126). Although these inhibitory pathways may seem as an impediment to our therapeutic approach at first, we hypothesize that they actually create a potential opportunity for us to combine checkpoint modulators with our treatment regimen and ensure effective targeting of the IFNβ-mediated immunity cycle.

Combinatorial approaches can also augment cell death or even modulate pathways involved in ICD, potentially circumventing intrinsic defects or genetic alterations that may affect the ability of treated cells to elicit optimal antitumor immunity upon p19Arf + IFNβ gene transfer. For example, the use of other ICD inducers, such as doxorubicin and mitoxantrone chemotherapy, could be an interesting approach to induce caspase 3 activity along with the p19Arf + IFNβ necroptotic cell death and to change a resistant/non-ICD scenario to a *bona fide* ICD or remediate the capability of some agents to trigger ER stress responses, autophagy-dependent accumulation of ATP and HMGB1 release from the nucleus (128).

Our gene transfer approach promotes cell death and immune stimulation, thus creating a variety of opportunities for us to explore and optimize various aspects, including the development of vaccination strategies and the direct application of our vectors into the tumor mass. Nevertheless, many key aspects remain to be investigated, most importantly, the use of patient-derived tumor and immune cells to validate the evidence gathered from mouse models and the exploration of tumor responses in alternative models, such as spontaneously arising cancers in dogs. In terms of technological development, the production of viruses of pharmaceutical quality, that is, by following good manufacturing practices (GMP), is a critical step that is not currently available in Brazil. Nonetheless, the know-how required to conduct preclinical studies and clinical trials is readily available in our community and may be further supported by international collaborations.

The translational road ahead of us is long but certainly exciting. The goal of translational medicine is to evaluate therapeutic strategies that are successfully developed in preclinical models in clinical trials performed in humans. In addition, although we envision it as a straightforward path, the translational road should be seen as a two way road, with exchange of information between the bench and bedside, and vice versa. In this regard, it is critical that a multidisciplinary team of basic and clinical scientists work together to ensure that a clinically relevant and viable approach is developed as we progress along the road of translational research.

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AUTHOR CONTRIBUTIONS

Strauss BE, Silva GR, Vieira IL, Cerqueira OL, Del Valle PR, Medrano RF and Mendonça SA wrote and reviewed the text. Medrano RF designed the figure.

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3

A influência do IFNAR1 nos efeitos autócrinos e parácrinos após terapia gênica com adenovírus portador de Interferon beta

Trabalho em andamento

RESUMO

O melanoma é um câncer de pele, considerado agressivo na sua evolução e frequentemente mortal para o hospedeiro que apresenta a doença metastática. O melanoma possui tratamento caro e frequentemente pouco efetivo em casos avançados. A terapia gênica é uma forma de tratamento alternativa que exibe uma nova esperança de tratamento para o melanoma. Essa terapia alternativa ao câncer pode ser entendida como uma plataforma de vetores, onde diferentes genes terapêuticos são inseridos e formam um vetor terapêutico. O grupo do Laboratório de Vetores Virais desenvolve novas terapias gênicas para o câncer, utilizando vetores adenovirais portadores do Interferon beta (Ad-mIFN) para o melanoma. O Interferon beta (IFNβ) é uma citocina, inicialmente conhecida por seus efeitos antivirais, possui amplo efeito citotóxico e ação antitumoral. Os resultados prévios do grupo com uso do Ad-mIFN, mostraram efeitos antitumorais promissores pela indução de morte celular, retardo no crescimento tumoral e ativação do sistema imune. Neste projeto foi investigado o papel do IFNAR1 nas células do melanoma e células do microambiente após tratamento com o Ad-mIFN. Realizou-se o *knockout* do IFNAR1 pela técnica CRISPR/Cas9 nas linhagens de melanoma B16, células endoteliais tEnd e células de fibroblastos NIH-3T3 (novas linhagens B16.B2, tEnd.A5 e 3T3-NIH.A1, respectivamente). Os clones *knockout* para o IFNAR1 não responderam ao tratamento com o Ad-mIFN. A partir desse efeito, sugere-se que o mecanismo de morte do tratamento ocorre pela ligação do IFNβ secretado que se liga ao IFNAR1. Foi criado um modelo co-cultura para avaliar o efeito parácrino do tratamento. Nos ensaios de co-cultura foi possível observar que as células tratadas com o vetor Ad-mIFN induzem efeito parácrino que inibiu as células próximas e que a ausência do IFNAR1 nas células produtoras de IFNβ não interfere na indução do efeito parácrino. As células *knockout* também não respondem ao efeito parácrino. A expressão gênica das linhagens transduzidas com o Ad-mIFN causou aumento da expressão de TRAIL e de genes da assinatura gênica da via do IFNα/β como STAT-1, STAT-2, IRF-7 e IRF-9. As células *knockout* não apresentaram indução dos genes estudados. Desta forma, sugere-se que a presença do IFNAR1 é de extrema importância nas terapias com IFNβ e a sua ausência bloqueia os efeitos do tratamento direto e parácrino do tratamento em células tumorais e do microambiente.

ABSTRACT

Melanoma is a type of skin cancer, considered aggressive in its evolution and often deadly to the host who has metastatic disease. Melanoma generates economic and emotional loss, and its current treatment is expensive and often ineffective in advanced cases. Alternative therapies for melanoma may represent a new hope for treatment. Gene therapy, an alternative therapy to cancer with promising results, can be understood as a vector platform, where different therapeutic genes are inserted and applied in the tumor tissue or cells of interest in order to eliminate the cancer cells. Our group develops gene therapies for cancer, using adenoviral vectors carrying Interferon beta (Ad-mIFN) and validated models of murine melanoma. Interferon beta (IFNβ) is a cytokine, initially known for its antiviral effects, has wide cytotoxic and cytostatic effects, including antitumor activity. The previous results of our research group using Ad-mIFN showed promising antitumor effects, such as the induction of cell death, delayed tumor growth and activation of the immune system. In this project, we investigated the role of IFNAR1 in melanoma tumor cells and representative cells of the tumor microenvironment after treatment with Ad-mIFN. We performed IFNAR1 knockout by the CRISPR/Cas9 technique on the B16 melanoma line, tEnd endothelial cells and NIH-3T3 fibroblast cells (new lines B16.B2, tEnd.A5 and 3T3-NIH.A1, respectively). Clones without IFNAR1 did not respond to treatment with Ad-mIFN. From this effect, we suggest the mechanism of treatment death occurs by binding the secreted IFNβ that binds to IFNAR1. We used a co-culture model to assess the paracrine effect of the treatment. In the co-culture assays it was possible to observe that the cells treated with the Ad-mIFN vector induce a paracrine effect that inhibited the nearby cells and that the absence of IFNAR1 in the IFNβ producing cells does not interfere in the induction of the paracrine effect. Knockout cells also do not respond to the paracrine effect. Gene expression of strains transduced with Ad-mIFN increased the TRAIL expression and IFN gene pathway signature genes such as STAT-1, STAT-2, IRF-7 and IRF-9. The knockout cells showed no induction of the studied genes. Thus, we suggest that the presence of IFNAR1 is extremely important in therapies with IFNβ and its absence blocks the effects of direct and paracrine treatment of treatment on tumor cells and the microenvironment.

INTRODUÇÃO

Câncer e o melanoma

O câncer foi uma das maiores causas de morte no Brasil em 2020, estando atrás apenas de acidentes em geral, doenças cardiovasculares e doenças respiratórias. São esperados mais de 309 mil novos casos de câncer para o ano de 2021 no Brasil e esse número só tende a aumentar (INCA 2021). O câncer não é somente uma doença, ele acarreta enormes gastos em saúde pública e implica em vários problemas sociais.

Dentre a vasta gama de canceres, o melanoma se destaca como um dos mais agressivos dos canceres de pele, sozinho ele soma mais de 80% das mortes do grupo. O melanoma geralmente é resistente às terapias convencionais como quimioterapia e radioterapia o que o torna ainda mais mortal (Fischer et al. 2018).

O melanoma tem origem grega (*melas* "negro" e *oma* "tumor") e sua primeira descrição aconteceu por relatos de Hipócrates a mais de 2500 anos atrás (Rebecca, Sondak and Smalley 2012). O melanoma tem ocorrência relacionada à exposição à luz ultravioleta e histórico familiar da doença. A grande maioria dos casos está presente na população caucasiana (Cho and Chiang 2010), origina-se a partir de melanócitos que, após transformação, se adaptam a diversos microambientes e se tornam altamente invasivos, o que contribui para seu alto potencial metastático e alta mortalidade (Satyamoorthy and Herlyn 2002).

Os esforços da pesquisa identificaram mutações frequentes na Proteína-quinases ativadas por mitógenos (MAPK) e genes como BRAF, NRAS e NF1**.** Os alvos terapêuticos do melanoma relacionados a inibidores de BRAF, como Vemurafenib, Dabrafenib e Trametinib foram aprovados pela FDA como tratamento deste câncer (Luke et al. 2017)**.**

Microambiente tumoral

A formação do câncer é um processo complexo que engloba uma vasta combinação de eventos. Dentro os eventos necessários, pode-se ressaltar o processo de carcinogênese que inicia o processo tumoral e a associação com células não tumorais, conhecidas como células do microambiente tumoral (MT) (Fischer et al. 2005).

O MT de tumores sólidos é geralmente constituído por células endoteliais, pericitos, adipócitos, fibroblastos e as células do sistema imune como linfócitos, neutrófilos, natural killers, eosinófilos, basófilos, macrófagos e células dendríticas. Essas células fornecem as estruturas essenciais para a manutenção e crescimento do tumor, como os vasos sanguíneos que auxiliam o transporte de nutrientes e metabólitos, os fibroblastos que são responsáveis pela produção da matriz extracelular, adipócitos com funções endócrinas e as células do sistema imunes com funções diversas como produção de fatores de crescimento tumoral, **Fig 01**, (Stockmann et al. 2008, Zonneville et al. 2018, Thijssen et al. 2018, Wu et al. 2019, Liu et al. 2019, Lei et al. 2020)**.**

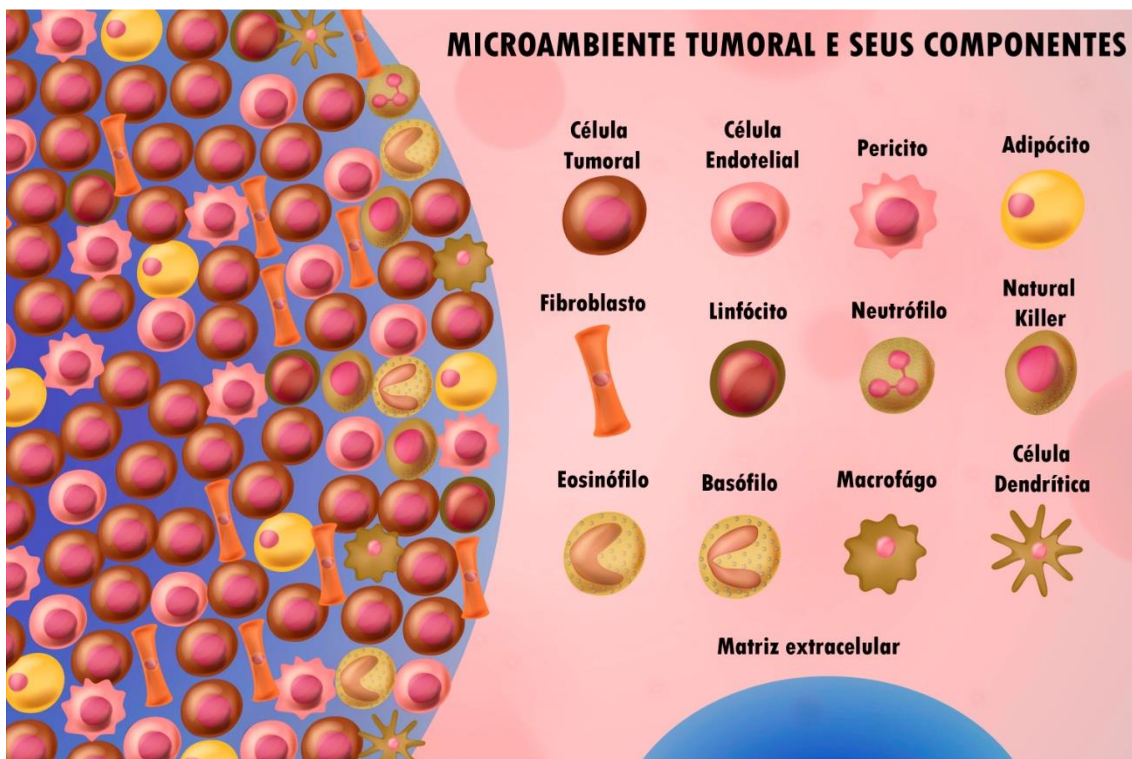

Figura 1 - Microambiente tumoral. A ilustração representa o microambiente tumoral juntamente com seus componentes agrupados ao lado esquerdo, como as células endoteliais, Pericitos, Adipócitos, Fibroblastos, Linfócitos, Neutrófilos, Células Natural Killer, Eosinófilos, Basófilos, Macrofágos, Células Dendríticas e a Matriz extracelular. A direita encontram-se os componentes do microambiente tumoral separados.

As células do MT podem representar grande parte do total de células encontradas nos tumores sólidos e devido à suas funções essenciais, o microambiente tumoral é considerado um alvo promissor no tratamento em câncer (Roma-Rodrigues et al. 2019).

Terapia Gênica

Sendo que o câncer é uma doença diversa e complexa, faz-se necessário uma abordagem terapêutica que atinge um ou mais alvos moleculares chaves para matar a célula tumoral ou redirecionar a resposta do organismo contra o tumor. Dentro dessa visão, a terapia gênica representa uma estratégia promissora para tratamento do câncer, e esta abordagem vêm crescendo nos últimos anos.

A terapia gênica propõe introduzir material genético nas células indivíduos ou tecidos com objetivo de tratamento ou prevenção de doenças. Esse processo pode ser feito pela introdução de genes e/ou sequências não-codificantes com intuito de alterar o fenótipo da célula alvo. Esta terapia pode ser realizada por diversas ferramentas, denominadas como vetores recombinantes, que podem ser virais, não virais ou oligonucleotídeos sozinhos (Dunbar et al. 2018, George 2017).

No início, a terapia gênica tinha como foco as doenças monogênicas, com o avançar do tempo, sua abrangência foi ampliada para diversos tipos de doenças, incluindo o câncer, que hoje representa a maior parcela de ensaios clínicos estudados em terapia gênica (Miller 1992, Smith and Blomberg 2017).

O adenovírus é um dos vetores mais usados em terapia gênica, ele é conhecido por seu tropismo natural para o epitélio respiratório, entretanto é capaz de transduzir vários tipos celulares em qualquer etapa do ciclo celular. Este vetor pode carregar genes exógenos terapêuticos e sua maquinaria viral não insere permanentemente seu material genético nas células transduzidas, sua presença epissomal é conhecida como transiente ou temporária. Devido as suas características, o adenovírus é considerado um bom modelo para pesquisa, vacinas e terapias do câncer (Haddada, Cordier and Perricaudet 1995, Wold and Toth 2013).

O adenovírus é um vírus de formato icosaédrico com capsídio não envelopado portador de um DNA linear de fita dupla. O capsídeo icosaédrico é formado por 12 cópias da proteína trimérica hexon, cada um dos seus vértices com a proteína base pentônica e a proteína fibra que possui uma porção globular (Knob) na extremidade C-terminal. O seu genoma varia entre 26 e 40 Kb com extremidades de origem de replicação chamadas ITRs (inverted terminal repeats), outras partes desse genoma podem se dividir em genes de fase inicial (*early*) e fase tardia (*late*). Os vetores

adenovirais recombinantes usados na pesquisa possuem deleções na região *early*, responsáveis pela replicação do vírus e também por iniciar boa parte das respostas imunes em células infectadas (Warnock, Daigre and Al-Rubeai 2011)**.** Os vetores adenovirais se ligam aos receptores, internalizam nas células por endocitose direcionadas ao endossomo, onde perdem as proteínas da fibra e escapam do endossomo. Após escape do endossomo e com suas partes desmontadas ela OU ELE? direciona seu DNA ao núcleo, onde permanece como DNA extra cromossomal, esse movimento resulta na expressão transiente dos genes terapêuticos (Horwood et al. 2002).

Interferon

O Interferon foi descoberto na década de 50 como uma proteína secretada em resposta a infecção por vírus (ISAACS and LINDENMANN 1957, NAGANO and KOJIMA 1954). Desde 1986, o Interferon tem aprovação pra alguns tipos de canceres como leucemia, linfoma, melanoma, entre outros (Conlon, Miljkovic and Waldmann 2019).

A família do Interferon é dividida em Tipo I (IFNα/β), Tipo II (IFNγ) e Tipo III (IFNλ). O Tipo I é considerado a maior família e mais estudada, e seus membros são expressos numa ampla gama de células. A família Tipo I também é conhecida por ser responsável pelos efeitos anti-virais mais robustos (ISAACS and LINDENMANN 1957, ISAACS, LINDENMANN and VALENTINE 1957, Stark et al. 1998).

Os IFNα/β têm uma ampla variedade de atividades biológicas que incluem propriedades antivirais, antiproliferativas, citotoxicidade, indução de morte diretamente ou por ativação de células imunes (Células dendríticas, Células T, Natural killers e Monócitos), e também potencializam a expressão do complexo de histocompatibilidade de classe 1 (MHC) (Yang et al. 2004, Greiner et al. 1987, Pestka 2007). O tratamento com IFNα/β pode induzir os genes pró apoptóticos (Caspases, TRAIL, BAK e BAX) e regular negativamente os genes pró-mitóticos como o fator de crescimento de endotélio vascular (VEGF) (Indraccolo 2010).

A ativação da via clássica dos IFNα/β ocorre quando o IFN-α ou IFN-β se liga com o par de receptores cognatos, IFNAR1 e IFNAR2 na membrana das células, resultando na dimerização entre os dois receptores e ativação da via *(Constantinescu et*

al. 1994, Müller et al. 1994, Platanias 2005). Recentemente, foi descrito que a degradação do IFNAR1 e ausência da assinatura da via do IFNα/β são observadas em células tumorais e do estroma no tumor colorretal e esses fenômenos estão diretamente ligados à formação de um ambiente imunossupressor pela diminuição do infiltrado leucocitário (Katlinski et al. 2017, Araya and Goldszmid 2017). Baixos níveis da proteína IFNAR1 nas células tumorais ou nas células estromais também são observados na clínica médica, como um prognóstico ruim (Araya and Goldszmid 2017). Os receptores cognatos possuem um domínio que ativa tirosina quinase 2 (TYK2) e janus quinase 1 (JAK1) nas porções intracelulares da IFNAR1 e IFNAR2, respectivamente (Darnell, Kerr and Stark 1994, Yang et al. 1996). A ligação do IFNα/β nos receptores causa auto-fosforilização e ativação do JAK1, que ativa a família de proteínas tradutoras de sinal e ativadoras de transcrição STATs (Stark et al. 1998, Darnell et al. 1994, Fasler-Kan et al. 1998, Meraz et al. 1996). A ativação dos STATs faz com que estas proteínas formem heterodímeros que após agrupamento se conectam aos fatores de regulação do interferon, IRFs, e formam o complexo, conhecido como fator de estimulação do gene Interferon 3 (ISG3), que se transloca para dentro do núcleo e se liga aos sítios específicos do DNA conhecidos como elementos responsivos a estimulação do interferon (ISREs) (Platanias and Fish 1999, Platanias 2005, Darnell et al. 1994, Stark et al. 1998, Aaronson and Horvath 2002) **Fig 02**.

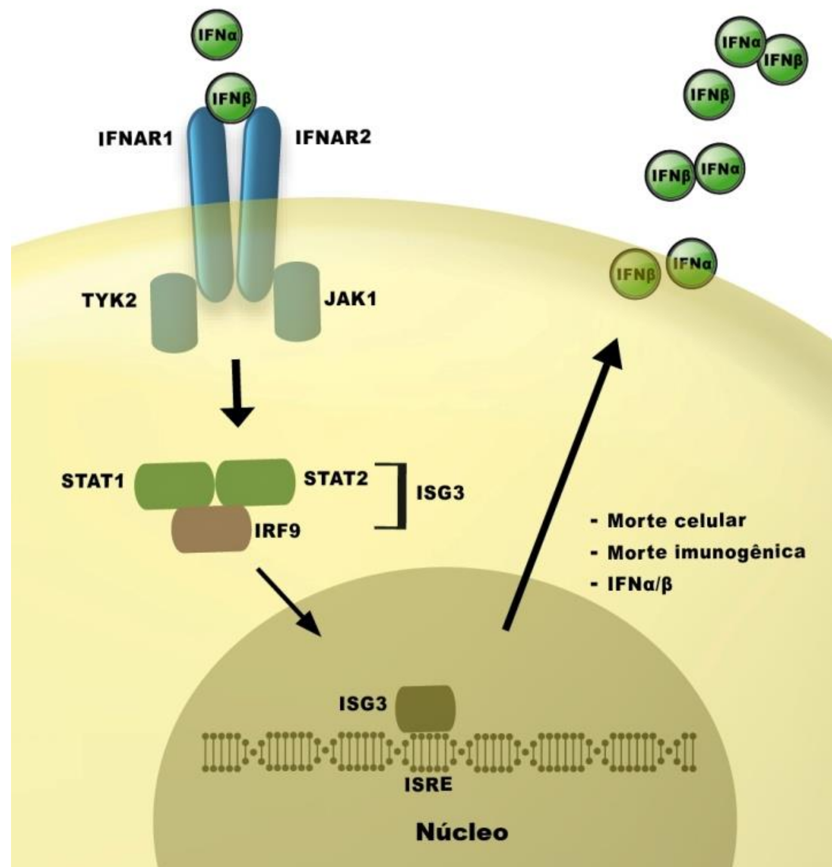

Figura 2 - Via do Interferon Tipo I. Descrição da ativação da via do Interferon tipo I pela ligação da proteína IFNα ou IFNβ nos dois receptores cognatos IFNAR1 e IFNAR2, seguido da autofosforilação e ativação dos JAKs. Forma-se a ativação clássica do JAK/STAT com ativação dos STATs que se ligam com o IRF9 e estruturam o complexo ISGF3, que se transloca para o núcleo e se liga a regiões específicas do DNA conhecidas como ISREs. A ligação com ISREs induz expressão de genes responsivos ao Interferon relacionados à morte celular, indução de morte imunogênica e produção de IFNα/β.

A ativação da via de IFNα/β pode modular a expressão de uma ampla variedade de genes, podendo chegar até 1000 genes modulados. A modulação gênica do IFNα/β estimula eventos biológicos diversos como efeitos anti-virais, antiproliferativo, citotóxicos e imunomoduladores (Stark et al. 1998, Kalie et al. 2008). O mecanismo antiproliferativo do Interferon é conhecido desde a década de 70 e este atua como sensibilizador via proteína associada ao FAS com domínio de morte (FADD) e a Caspase-8 (Balachandran et al. 2000). Outros mecanismos antiproliferativos incluem a supressão do fator de transcrição de ciclinas, E2F-1(Erickson et al. 1999, Einat, Resnitzky and Kimchi 1985). O IFNα/β também induz a via extrínseca de apoptose pela indução de genes como fator de necrose tumoral (TNF), o receptor e ligante de Fas (Fas/FasL) e o ligante indutor de apoptose relacionado ao TNF (TRAIL) (LeBlanc and

Ashkenazi 2003, Ashkenazi and Dixit 1999). Os ligantes indutores de morte induzidos por Interferon se ligam a receptores específicos e formam um complexo indutor de sinal de morte (DISC), que por sua vez se liga às caspases ativadas (Schoggins et al. 2011, Apelbaum et al. 2013).

Dentro da ampla gama de efeitos possíveis do IFNα/β, o seu tratamento induz efeitos antitumorais e o uso de reguladores negativos de STATs ou IRFs são associados ao aumento da progressão tumoral. Esses resultados demonstram que o IFNα/β pode ser usado no tratamento do câncer (Gastl and Huber 1988, Levy and Darnell 2002, Schindler, Levy and Decker 2007). Por outro lado, o uso do IFNβ pode apresentar efeitos adversos quando aplicados sistemicamente no tratamento do melanoma, esses efeitos podem variar de sintomas comuns de uma gripe até sintomas mais complicados como neuropsicopatias (Rubin et al. 2012).

Os resultados publicados demonstraram que a terapia gênica com adenovírus portadores de IFNβ induzem morte celular devido aos efeitos citotóxicos do IFNβ em ensaios *in vitro*. O tratamento também mostrou aumento da morte celular em tumores sólidos, ativação do sistema imune e aumento de vida dos camundongos (Medrano et al. 2016, Catani et al. 2016, Merkel et al. 2013, Hunger, Medrano and Strauss 2017a). Desta forma, o intuito do projeto é investigar o papel do IFNAR1 nas células de melanoma e do microambiente tumoral após tratamento com Ad-mIFN.

OBJETIVOS

Objetivo geral

Investigar o papel do IFNAR1 após terapia gênica com adenovírus portadores do gene terapêutico IFNβ em células do microambiente tumoral e do melanoma murino

Objetivos específicos

- Usar a tecnologia de CRISPR/Cas9 para eliminar expressão do IFNAR1 em células B16 e tEnd

- Avaliar a clonogenicidade e morte celular após tratamento com Interferon Beta recombinante (rIFNβ) ou Ad-mIFN em clones celulares com *knockout* para o IFNAR1

- Investigar o efeito parácrino do tratamento com Ad-mIFN em co-culturas com os clones celulares com *knockout* para o IFNAR1

- Avaliar a expressão de genes da via de Interferon na linhagem tEnd, B16 e B16 *knockout* para IFNAR1 para revelar a assinatura gênica após tratamento com Ad-mIFN

MATERIAL E MÉTODOS

Linhagens celulares

Foram utilizadas as linhagens de melanoma B16F10 (B16, ATCC CRL-6475), a linhagem de células endoteliais murinas tEnd, cedida pela pesquisadora Dr. Cristina Maria Fernandes do Instituto Butantan e a linhagem de fibroblastos NIH-3T3 (3T3, ATCC-CRL-1658). A linhagem HEK293A (*Human Embrionic Kidney 293* Invitrogen, Carlsbad, CA, USA) foi usada para produção dos vetores adenovirais recombinantes.

Cultivo celular

As linhagens celulares B16 e NIH-3T3 foram cultivadas em meio Roswell Park Memorial Institute 1640 Medium (RPMI) e a linhagem HEK293A cultivada em meio Dulbecco's Modified Eagle (DMEM), todos acrescidos de 10% de FBS e 1% de Antibiotic-Antimytotic (todos os reagentes do Life Technologies). Todas as linhagens foram mantidas a 37ºC em atmosfera úmida com 5% de CO_2.

Produção dos vetores adenovirais – Amplificação do título viral

De acordo com o protocolo de amplificação viral desenvolvido pela pesquisadora Alinge Hunger (Hunger et al. 2017b), as células HEK293A foram infectadas com os vetores adenovirais para a produção do lisado. Este lisado bruto, contendo as partículas virais, foi aplicado em gradiente de iodixanol e sujeito a ultracentrifugação. Após a centrifugação, formou-se uma banda clara que foi removida e aplicada em colunas PD-10 contendo resina Sephadex™ G-25 (GE, Cat. Nº 52-1308-00 BB), a partir do qual o vírus foi eluído e aliquotado. Posteriormente, as alíquotas foram armazenadas a -80ºC e o estoque de vírus purificado foi titulado biologicamente com o kit Adeno-X Rapid Titer (Clontech).

Vetores Adenovirais

Para realizar a transferência gênica nas células, utilizaram-se vetores adenovirais sorotipo 5, não replicativo que contêm a fibra do capsídeo modificada com a inserção do tripeptídeo RGD, que proporciona a transdução de um amplo espectro de células alvo (Mizuguchi et al. 2001). Todos os vetores adenovirais que foram utilizados no projeto foram desenvolvidos durante a pós-graduação da aluna Aline Hunger Ribeiro (Hunger et al. 2017b).

- AdRGD-PG-IFN (Ad-mIFN): Vetor usado para expressão da proteína IFNβ murina.
- AdRGD-PG-eGFP (Ad-GFP): Vetor controle que expressa eGFP (proteína verde fluorescente).

Transdução adenoviral

Para transduzir as células, os vírus foram diluídos em meio de cultura específicos para cada tipo de linhagem e colocados juntos com as células. O tempo de incubação com os vetores e o MOI (multiplicidade de infecção) foi realizado de acordo com o experimento.

Citometria de fluxo

Detecção de células GFP positivas

As células ($2x10^4$) foram coletadas após 48 horas do tratamento com o vetor Ad-GFP (MOI 500), fixadas com PFA 4% e analisadas por citometria de fluxo (Attune, Life Technologies) para detecção de células fluorescência GFP.

Marcação com anticorpo IFNAR1

As células foram coletadas usando PBS 1X/EDTA 15 mM e mantidas em gelo em todo o processo de marcação. As células foram incubadas com PBS 1X/3% BSA por uma hora, marcadas com anticorpo primário anti-IFNAR1 diluído PBS 1X/3% BSA em por 30 minutos, lavadas com PBS 1X/3% BSA e por último marcadas com anticorpo secundário Alexa-647 diluído em PBS 1X/3% BSA por 30 minutos. Após processo de

marcação, as células foram lavadas com PBS 1X e analisadas por citometria de fluxo para detecção da fluorescência referente ao anticorpo secundário. Com intuito de compararmos as células marcadas com uma linhagem ausente para o IFNAR1, foram utilizadas células marcadas somente com o anticorpo secundário.

Morte celular com marcador iodeto de propídio (I.P.)

As células aderidas juntamente com as células que estavam no sobrenadante foram coletadas e fixadas com etanol 70%. Após processo de fixação, as células foram marcadas com I.P. por 15 minutos a 37°C, lavadas com PBS 1X e analisadas por citometria de fluxo para detecção da população sub-G1/hipodiplóide.

ELISA para detecção de Interferon Beta

As células da linhagem B16 foram transduzidas por 48 horas com MOI de 500, o meio condicionado foi retirado, centrifugado para separar os restos celulares do meio, este foi congelado a -80 °C até o momento da execução da técnica. Os ensaios foram realizados segundo protocolo do fabricante (LEGEND MAZX MOUSE IFNβ - BioLegend) e a absorbância (450nm) foi determinada pelo leitor de microplacas Victor^{Tm} (Perkin-Elmer-EUA).

Edição gênica do IFNAR1 pela tecnologia CRISPR/Cas9

A técnica de edição gênica por CRISPR/Cas9 usa uma endonuclease guiada por um RNA previamente desenhado, que cliva o DNA da célula hospedeira e induz mutações indel por deleção ou adição de nucleotídeos. No projeto foi utilizado o sistema CRISPR Tipo II, derivado da bactéria *Streptococcus pyogenes,* com a endonuclease Cas9.

O primeiro passo para realização da técnica foi o desenho *in silico* dos pares de RNA-guia (sgRNA), com sequências de 20 nucleotídeos como homologia para o primeiro exon do IFNAR1, localizados próximos ao códon de iniciação. O site https://zlab.bio/guide-design-resources foi usado para desenhar os sgRNAs Top e Botton referentes as melhores localizações para serem editadas no éxon 1, **Tabela 01**.

Tabela 1 - Descrição dos *primers* utilizados na construção dos plasmídeo CRISPR/Cas9.

	Top (5' – 3')	Botton (5' – 3')
sgRNA1	CACCG**AAGACGATGCTCGCTGTCGT**	AAAC**ACGACAGCGAGCATCGTCTT**C
sgRNA2	CACCG**GGGTAGCACCCAAGGCGCCC**	AAAC**GGGCGCCTTGGGTGCTACCC**C

Os insertos foram sujeitos a fosforilação com a enzima T4 polynucleotide kinase (T4 PNK) (Thermo Fisher Scientific) e passaram por processo de anelamento. Após preparação dos insertos sgRNAs, estes foram clonados no vetor pSpCas9(BB)-2A-Puro (PX459) V2.0.

Os plasmídeos foram inseridos em bactérias *E. coli* competentes Stbl3, por eletroporação. As bactérias eletroporadas foram semeadas em solução LB Agar acrescido de ampicilina, onde somente as bactérias que tiveram eficiência na inserção do plasmídeo foram coletadas e os plasmídeos funcionais foram isolados pelo kit Plasmid Miniprep (Qiagen).

As células foram transfectadas com os plasmídeos (500 ng) utilizando o reagente FuGENE (Promega) e incubadas por 72 horas. Após incubação, foi acrescentado o antibiótico Puromicina (200ng/mL) nos poços até sobrarem somente células resistentes ao antibiótico. As células remanescentes foram separadas em clones de células únicas em placas de 96 poços.

Sequenciamento Sanger

O sequenciamento foi realizado para identificar as edições gênicas no DNA dos clones que tiveram edição no gene IFNAR1 pela tecnologia de CRISPR/Cas9. Desenhou-se um par de *primers* (**Tabela 02**) para a região do primeiro éxon onde foram desenhados os os gRNA. Os *primers* foram validados pelo site https://www.ncbi.nlm.nih.gov/tools/primer-blast/.

Tabela 2 - Descrição das sequencias dos primers utilizados no sequenciamento.

	Forward (5' – 3')	Reverse (5' – 3')
Primers	CTGCCCAGAGGTAGTCTCCA	CTCCCCTTAGTGGGCATTTCT

O DNA genômico dos clones foi extraído pelo kit PureLink Genomic DNA (Invitrogen) e utilizado para reação de PCR com o par de *primers* desenhado na **Tabela 02.** O produto do PCR foi verificado para 500 pares de base por gel de 1% de agarose. Os produtos do PCR verificados foram purificados pelo kit ExoSAP-IT Express (Thermo Fisher Scientific) ou purificados do gel de agarose pelo kit QIAprep Spin Miniprep Kit (Quiagen). Após obter o produto do PCR purificado, preparraram-s as amostras para o sequenciamento com o kit BigDye Terminator (Thermo Fisher Scientific) e precipitaram-se os produtos na placa para o sequenciamento. O sequenciamento foi realizado pelo Método Sanger no equipamento ABI 3730 DNA Analyzer (Thermo Fisher Scientific), oferecido pela Rede Premium da FMUSP. As análises foram realizadas pelos programas BioEdit Sequence Alignment Editor e pelo site de análise https://ice.synthego.com/#/.

Co-cultura

Para executar o ensaio parácrino em co-cultura, as células do projeto foram transduzidas com vetores lentivirais LEGO-iG2 (eGFP, fluorescente verde) e LEGO-iT2 (*tomato*, fluorescente vermelho) (MOI 1) e foram selecionados clones 100% fluorescentes. Os vetores lentivirais integram seu material genético no DNA do hospedeiro, tornando as células permanentemente editadas.

No ensaio foram realizados a co-cultura entre uma linhagem fluorescente e outra sem fluorescência, onde apenas uma foi tratada.

As células foram tratadas com os vetores adenovirais (MOI 500) por 6 horas, lavadas com PBS 1X coletadas e co-cultivadas com as células não tratadas e incubadas por 72 horas. Após incubação, os poços foram fotografados pelo microscópio de

fluorescência invertido EVOS FL (Thermo Fisher Scientific) e as células foram coletadas e fixadas com PFA 4% e quantificadas por citometria de fluxo.

RT-PCR

As células foram transduzidas com os vetores Ad-GFP ou Ad-mIFN (MOI 500) em placas de 6 poços por 48 horas, coletadas e lisadas com Trizol (Thermo Fisher Scientific). A extração do RNA das soluções com Trizol foi realizada pela adição de clorofórmio, separação da fase aquosa por centrifugação, e isolamento do RNA por outra centrifugação com etanol absoluto, por fim, o precipitado foi dissolvido em água ultra-pura livre de endonucleases. A pureza e qualidade do RNA foi medida por espectometria e pela observação das duas bandas (18S e 28S) em gel de 1% de agarose. Os *primers* foram desenhados e validados pelo site https://www.ncbi.nlm.nih.gov/tools/primer-blast/, **Tabela 03**. Utilizou-se o GADPH como normalizador. A reação foi realizada pelo Kit SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (Thermo Fisher Scientific). As reações foram corridas no equipamento 7500, pelo Software da máquina, version 2.05 (Thermo Fisher Scientific). O método $2^{-\Delta\Delta Ct}$ foi utilizado para quantificação e os resultados foram normalizados pelas células tratadas com vetor Ad-eGFP.

Tabela 3 - Descrição das sequencias primers utilizados no ensaio de RT-PCR.

Gene	***Primers Forward* (5' – 3')**	***Primers Reverse* (5' – 3')**
VEGF-A	AGTCTACAAAAAGCACCCCGC	CTCCTAGGCCCCTCAGAAGT
VEGF-C	CCCCAAACCAGTCACAATCAG	TGTCTTGTTAGCTGCCTGAC
ANG-1	GCTGGTGAAGAGTCCCAACTAC	GATGCTACTTATTTTGCCCGC
bFGF1	AAGCCCAAACTGCTCTACTG	CCCTTTATATACACTTCGCCCG
IL-06	TCCAGTTGCCTTCTTGGGAC	AGTCTCCTCTCCGGACTTGT
IL-08	CATCTTCGTCCGTCCCTGTG	TGGGACTGCTATCACTTCCTTT
IL-24	TTAGGACCCTAGCAGGAGCA	CTCTTGACCCTCAAGCCCTG
TNF-α	AGGCTGTCGCTACATCACTG	CTCTCAATGACCCGTAGGGC
TRAIL	TGAAGAGGTGACTTTGAGAACCT	AGCTGCCACTTTCTGAGGTC
IFNAR1	ACACCCTAAAGTGGAGCAGC	TTCGCCTCGTCTTTTGTTCG
IFNAR2	ACCGTCTGCTTTTGATGGGT	AGAGGGTGTAGTTAGCGGGT
STAT-1	ATTCACCTATGAGCCCGACC	TCGTTCTACCACGAAGGAGC
STAT-2	AATTTGGGACTTCGGCTTCTTG	ACATGTAACTCCTCTGTCACTG
IRF-1	AAAGTCCAAGTCCAGCCGA	GTCCGGGCTAACATCTCCAC
IRF-7	CTGAGCGAAGAGAGCGAAGA	TCCCGGCTAAGTTCGTACAC
IRF-9	GCCCTCAACAAGAGTTCCGA	TGGTTCCGTGGTTGGTTAGG
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTC

Análise estatística

As análises foram feitas pelo software GraphPad Prism. Os testes utilizados nos experimentos foram ANOVA, seguido de Tukey. Os dados foram considerados significativos quando $p<0,05$.

RESULTADOS

Produção e validação dos vetores

O projeto começou com a produção dos vetores adenovirais não replicativos, Ad-GFP (vetor controle) e Ad-mIFN (vetor objeto do estudo). Esta etapa foi realizada através da transdução da linhagem produtora de adenovírus, HEK293A. Os vetores foram coletados, purificados por gradiente de Iodixanol e titulados. Os títulos biológicos variaram de 1.10^{10} a 3.10^{10} TU/mL.

Após a produção dos vetores adenovirais, es[illegible] foram submetidos a testes de va[illegible]ão quanto à produção dos transgenes. Células [illegible] foram transduzidas com MOI 500, dos vetores Ad-GFP ou Ad-mIFN e incubadas por 48 horas. Após incubação, as células e o meio de cultura condicionado, foram coletados e analisados por citometria de fluxo para detecção de células GFP positivas e o meio foi avaliado para detecção da proteína IFNβ por ELISA. O ensaio de citometria mostrou 36% de células de células GFP positivas e o ensaio de ELISA, detectou concentração no limite (500pg/mL) de detecção do equipamento de leitura do ELISA de IFNβ no meio das células transduzidas pelo vetor Ad-mIFN, **Fig. 03 A e B.**

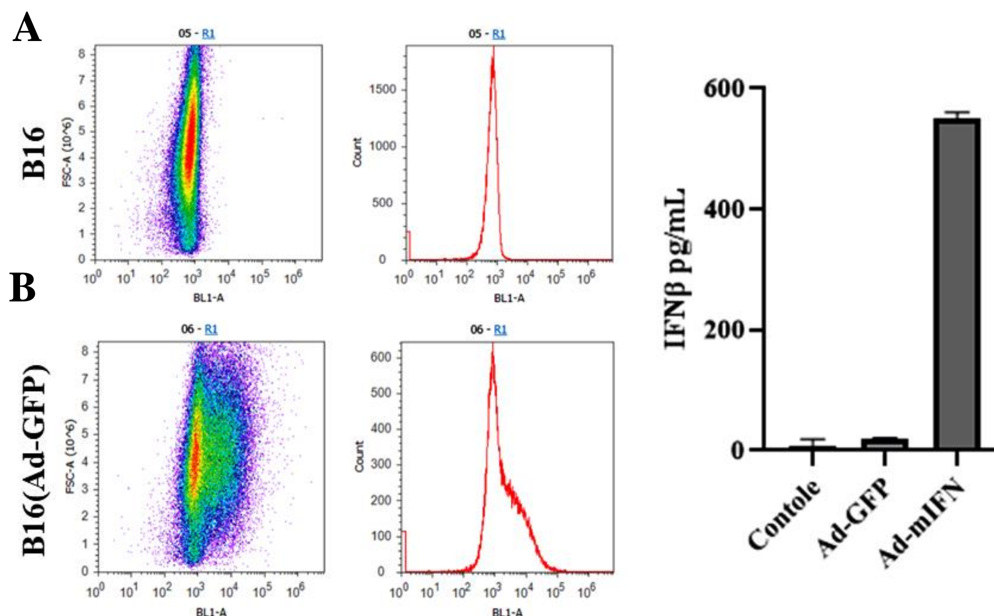

Figura 3- Validação de transdução pelos vetores Ad-GFP e Ad-mIFN. As células B16 foram transduzidas com MOI 500 dos vetores e incubadas por 48 horas, o meio condicionado foi coletado e as células foram fixadas com PFA 4%. (A) Os gráficos de pontos e histograma mostram as células GFP positivas por citometria de fluxo para detecção

de células positivas para eGFP. (B) Ensaio de ELISA para detecção de Interferon Beta (IFNβ) no meio condicionado das células.

Edição gênica do *IFNAR1* pela tecnologia CRISPR/Cas9

A construção do vetor plasmídial CRISPR/Cas9 para edição gênica do *IFNAR1*, foi realizada em colaboração com o aluno de doutorado, Ricardo Cesar Cintra. Células B16, tEnd e NIH3T3 foram transfectadas com os plasmídeos construídos contendo o gRNA para o IFNAR1, após transfecção, as células foram selecionadas por resistência a puromicina e as células sobreviventes foram separadas em clones de células únicas em placas de 96 poços. Os clones celulares foram avaliados para a detecção da proteína de membrana IFNAR1 por citometria de fluxo e sequenciados para detecção de mutações indel na região gênica do IFNAR1, **Figs 04, 05 e 06**. Os clones, B16.B2 originários da linhagem de melanoma B16, o clone tEnd.A5 provindo da linhagem de células endoteliais tEnd e o clone NIH3T3.A1 vindo da linhagem NIH3T3, passaram por um processo criterioso de seleção de células e prosseguiram para os ensaios do projeto.

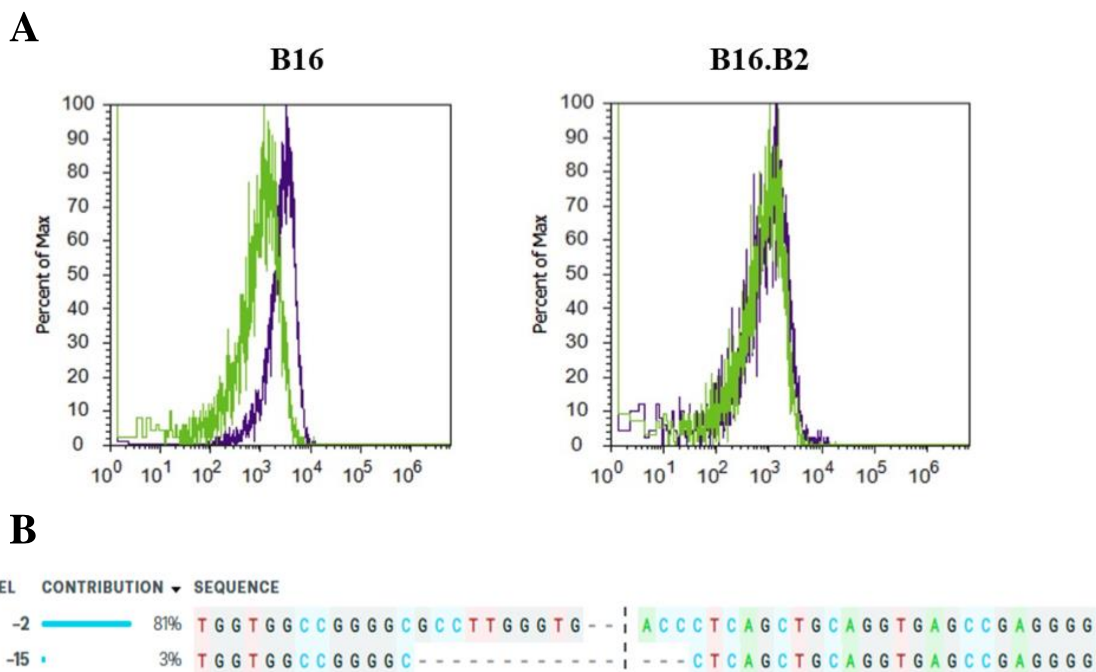

Figura 4 - Validação da edição gênica do IFNAR1 pela tecnologia CRISPR/Cas9 da B16.B2. (A) Citometria de fluxo para detecção de IFNAR1 na membrana das células B16 (parental) e B16.B2 (editada para o IFNAR1). As células foram coletadas frescas e marcadas com anticorpo primário anti-IFNAR1 e anticorpo secundário Alexa-647 (pico roxo) como controle, ou somente com o anticorpo secundário (pico verde). (B) Análise *in silico* do

sequenciamento Sanger realizado pelo programa ICE v2 CRISPR Analysis Tool com a distribuição dos nucleotídeos do sequenciamento do clone B16.B2 comparado com sequência nucleotídica das células parentais B16 na região da edição gênica do IFNAR1.

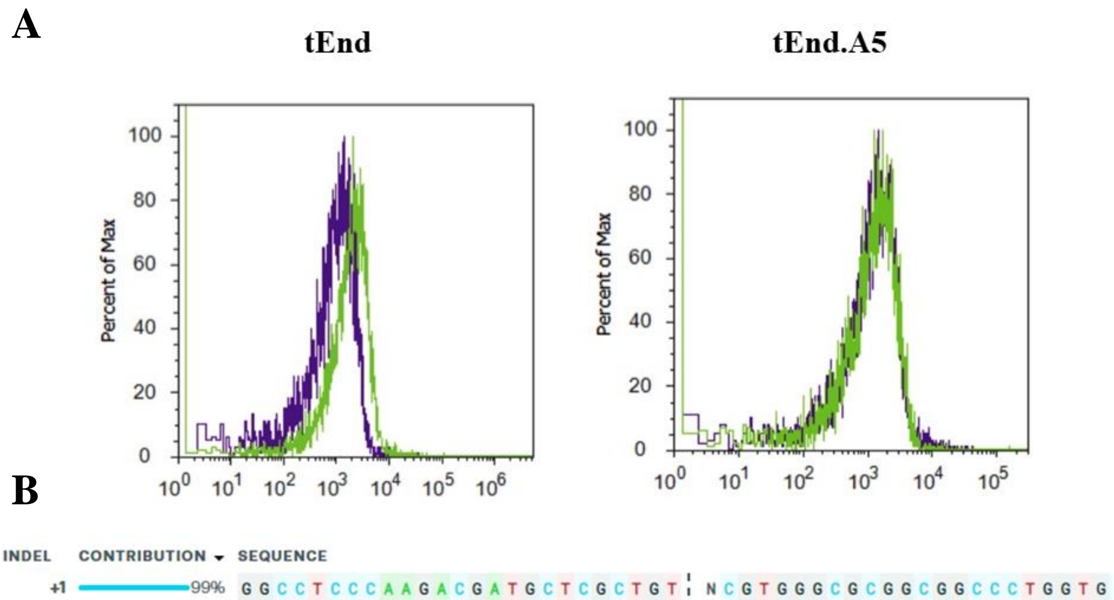

Figura 5 - Validação da edição gênica do IFNAR1 nas células da tEnd.A5. (A) Citometria de fluxo para detecção de IFNAR1 na membrana das células tEnd (parental) e tEnd.A5 (editada para o IFNAR1). As células foram coletadas frescas e marcadas com anticorpo primário anti-IFNAR1 e anticorpo secundário Alexa-647 (pico roxo) como controle, ou somente com o anticorpo secundário (pico verde). (B) Análise *in silico* do sequenciamento Sanger realizado pelo programa ICE v2 CRISPR Analysis Tool com a distribuição dos nucleotídeos do sequenciamento do clone tEnd.A5 comparado com a sequência nucleotídica das células parentais tEnd na região da edição gênica do IFNAR1.

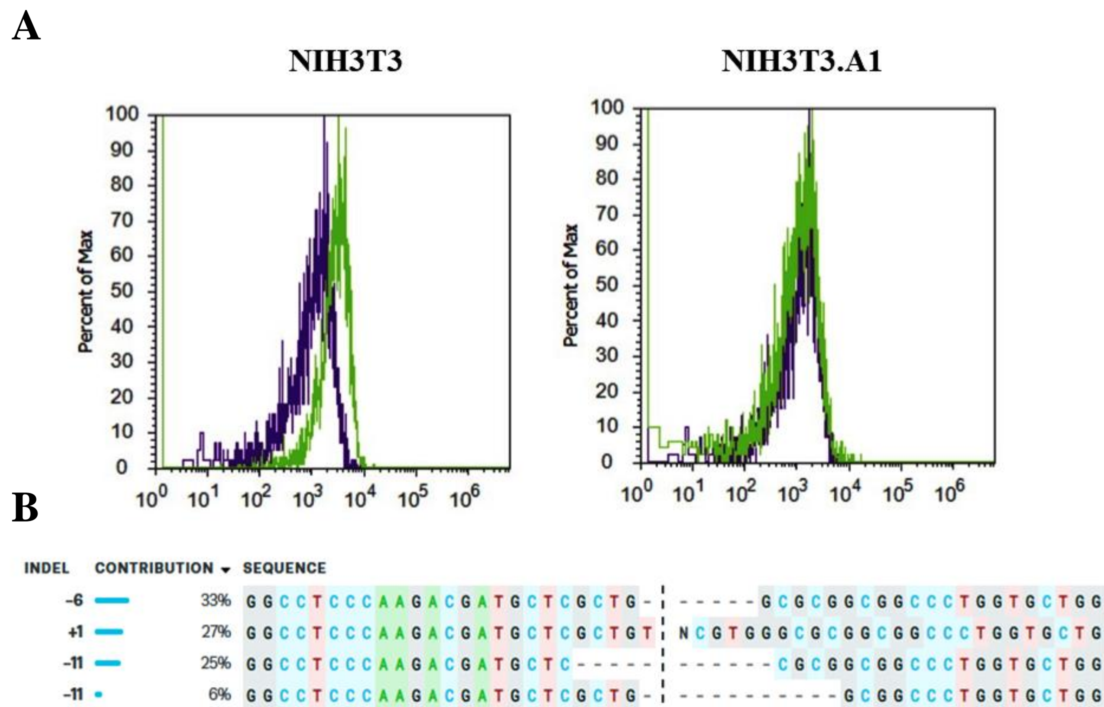

Figura 6 - Validação da edição gênica do IFNAR1 nas células da NIH3T3.A1. (A) Citometria de fluxo para detecção de IFNAR1 na membrana das células NIH3T3 (parental) e NIH3T3.A1 (editada para o IFNAR1). As células foram coletadas frescas e marcadas com anticorpo primário anti-IFNAR1 e anticorpo secundário Alexa-647 (pico roxo) como controle, ou somente com o anticorpo secundário (pico verde). (B) Análise *in silico* do sequenciamento Sanger realizado pelo programa ICE v2 CRISPR Analysis Tool, com a distribuição dos nucleotídeos do sequenciamento do clone NIH3T3.A1 comparado com a sequência nucleotídica das células parentais NIH3T3, na região da edição gênica do IFNAR1.

Avaliação da produção de IFNβ após transdução com o vetor Ad-mIFN no clone *knockout* para o IFNAR1

A linhagem B16 e o clone B16.B2 foram transduzidos com vetores Ad-mIFN, incubadas e os meios de cultura foram avaliados quanto a produção de IFNβ secretada. O ensaio, demonstrou alta produção da proteína IFNβ nas células B16 e o clone B16.B2 transduzidas, **Fig 07**.

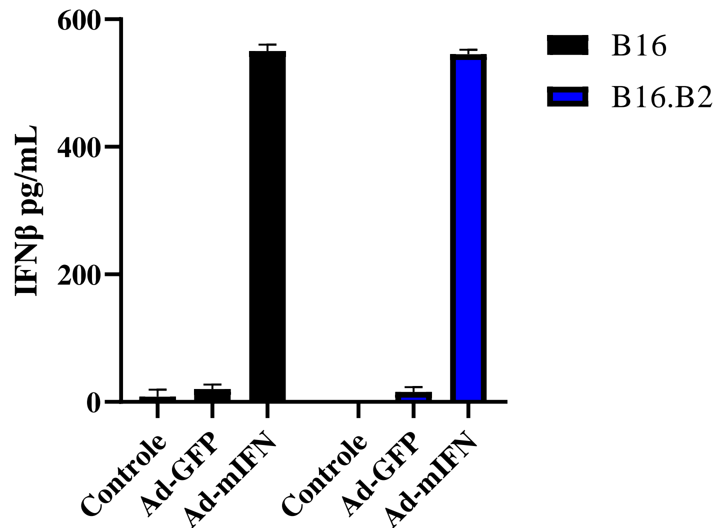

Figura 7 - Ensaio de ELISA para detecção de IFNβ. As células foram transduzidas (MOI 500) e incubadas por 48 horas, o meio foi centrifugado e depois analisado utilizando kit LEGEND MAZX MOUSE IFNβ - BioLegend e a absorbância (450nm) foi determinada pelo leitor de microplacas Victor^{Tm} (Perkin-Elmer-EUA). Ensaio realizado duas vezes.

Avaliação de clonogenicidade das células *knockout* para o IFNAR1 após transdução com o vetor Ad-mIFN

As células da B16 e B16.B2 foram transduzidas por 6 horas, lavados com PBS 1X para retirada dos vetores, coletadas, plaqueadas em placas de 100mm e incubadas por 10 dias. Após incubação, as colônias foram fixadas e coradas, **Fig 08 A**. A quantificação das colônias foi realizada com uso do programa ImageJ. Os resultados mostraram que o clone *knockout* para IFNAR1 não sofreu efeito de diminuição na quantidade de clones pelo tratamento com vetor Ad-mIFN como demonstrado pela linhagem B16, **Fig 08 B.**

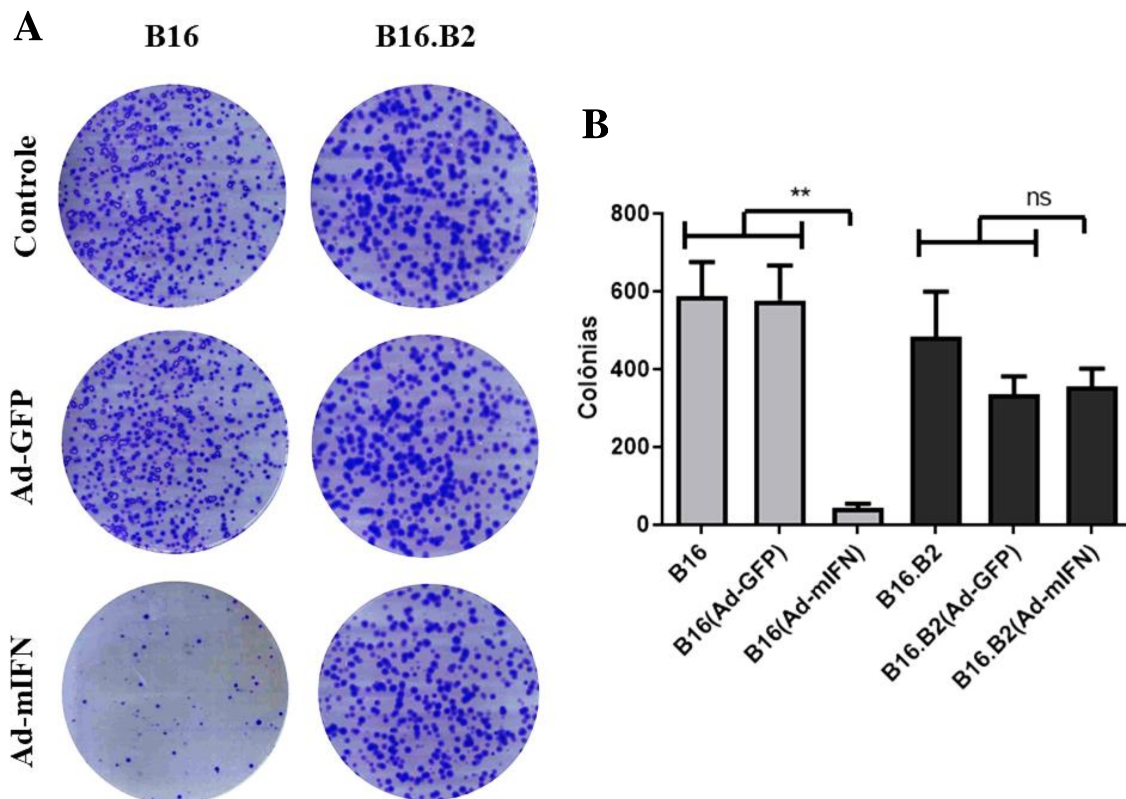

Figura 8 - Ensaio clonogênico das células B16.B2 após tratamento com Ad-mIFN. As células da B16 e B16.B2 foram transduzidas por 6 horas, lavadas com PBS 1X para total remoção dos vetores adenovirais, plaqueadas (1000 células) em placas de 100mm e cultivadas por 10 dias. Após o período do cultivo as colônias foram fixadas, coradas com Violeta de Metila e quantificadas com uso do programa ImageJ. (A) As imagens mostram as fotos tiradas das colônias nas placas de 100mm após 10 dias de incubação. (B) Quantificação das colônias da B16 e B16.B2. O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste One-Way Analysis of Variance, seguido do teste de Tukey, **p<0,01.

Avaliação de morte celular das células *knockout* do IFNAR1 após tratamento com Ad-mIFN

As linhagens de melanoma B16, B16.B2 e de fibroblastos NIH3T3 e NIH3T3.A1 foram transduzidas com o vetor Ad-mIFN, incubadas por 72 horas, marcadas com I.P. (Iodeto de propídeo) e analisadas por citometria para detecção de células hipodiplóides. Os resultados mostraram que os clones com *knockout* para IFNAR1 não tiveram indução de morte celular quando tratados com Ad-mIFN, diferente das linhagens não editadas que tiveram aumento significante de morte celular, **Figs 09 e 10**.

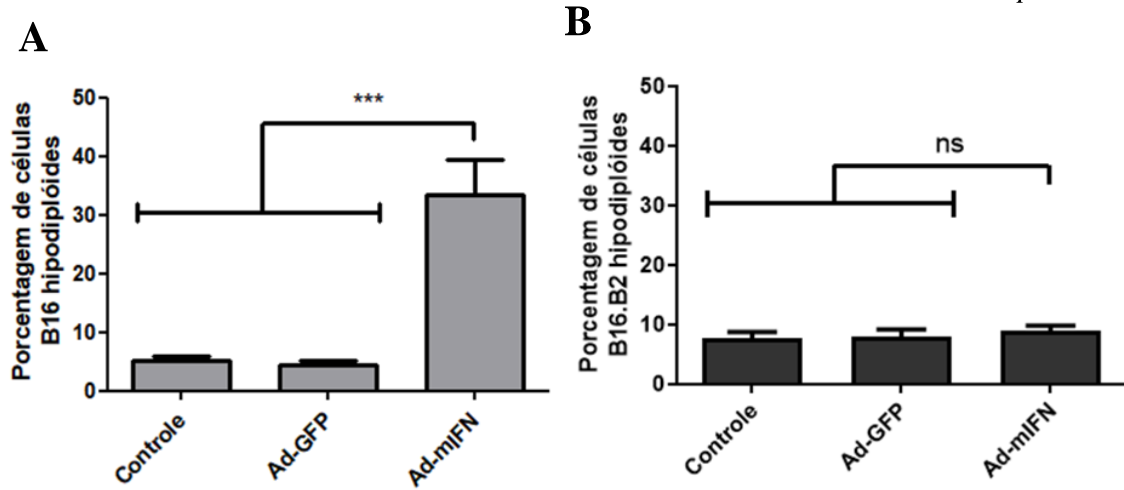

Figura 9 - Avaliação de morte celular das células B16 e B16.B2 após tratamento com Ad-mIFN. As células foram transduzidas com MOI 500 e incubadas por 72 horas. Após incubação, as células foram coletadas, fixadas, marcadas com P.I e analisadas por citometria de fluxo para identificação de células hipodiplóides. (A) Linhagem B16. (B) Clone B16.B2 (*knockout* para o IFNAR1). O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste One-Way Analysis of Variance, seguido do teste de Tukey, ***p<0,001.

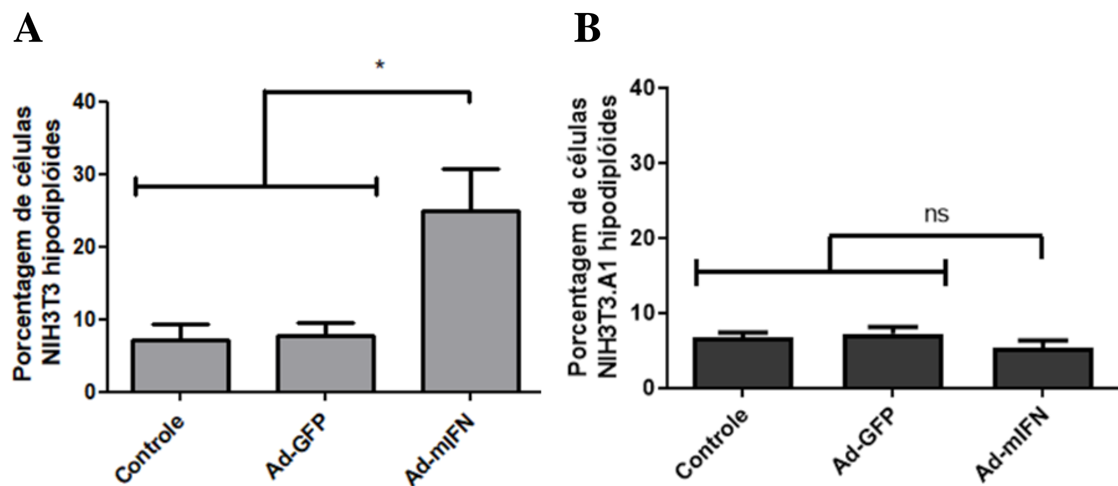

Figura 10 - Avaliação de morte celular das células da NIH3T3 e NIH3T3.A1 após tratamento com Ad-mIFN. As células foram transduzidas com MOI 500 e incubadas por 72 horas. Após incubação, as células foram coletadas, fixadas, marcadas com P.I e analisadas por citometria de fluxo para identificação de células hipoplóides. O ensaio foi realizado em triplicata biológica. (A) Linhagem NIH3T3. (B) Clone NIH3T3.A1 (*knockout* para o IFNAR1). A análise estatística foi realizada com o teste One-Way Analysis of Variance, seguido do teste de Tukey, *p<0,05.

Avaliação de morte celular das células ***knockout* do IFNAR1** após tratamento com rIFNβ

A linhagem B16 e o clone B16.B2 foram tratados com Interferon beta recombinante (rIFNβ) por 72 horas, coletados, marcados com I.P. e analisados por citometria de fluxo para detecção da porcentagem de células hipodiplóides. Os resultados demonstraram que as células B16.B2, não respondem ao tratamento com rIFNβ, diferente das células B16 que mostraram aumento significativo de morte celular, **Fig 11.**

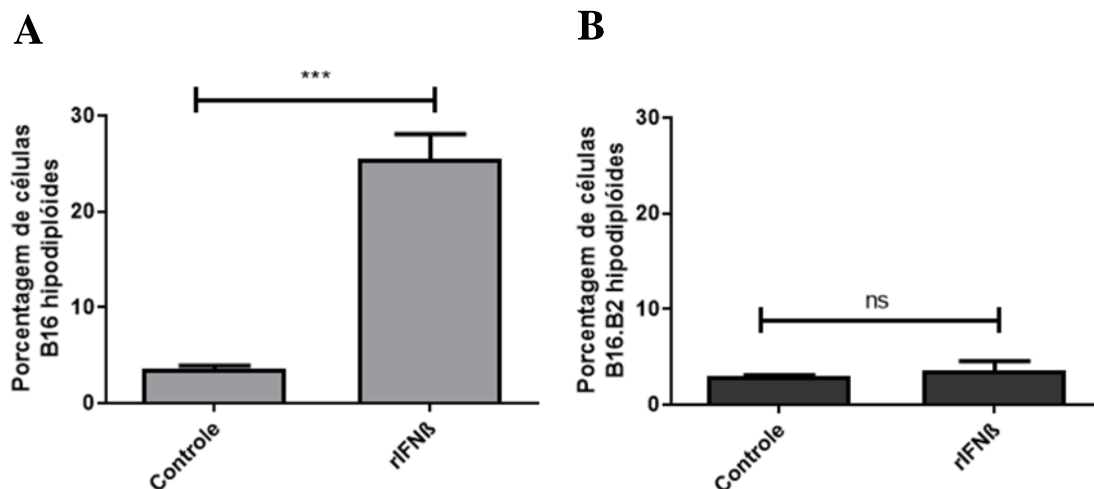

Figura 11 - Avaliação de morte celular das células B16 e do clone B16.B2 após tratamento com rIFNβ. As células foram tratadas com rIFNβ (200 pg/mL) e incubadas por 72 horas. Após incubação, as células foram coletadas, fixadas, marcadas com P.I e analisadas para hipoploidia. O ensaio foi realizado em triplicata biológica. (A) Linhagem B16 e (B) o clone B16.B2. A análise estatística foi realizada com o teste-t de Student não pareado, ***P<0,0001.

Validação do modelo parácrino de co-cultura

Para avaliar o efeito parácrino induzido pelo vetor Ad-mIFN, foi necessário modificar as nossas células permanentemente e torna-las identificáveis na co-cultura. As células foram previamente transduzidas por lentivírus carreadores de genes de proteínas fluorescentes eGFP (Verde fluorescente) e *tomato* (Vermelho fluorescente). Os vetores lentivirais inserem seu material genético nas células hospedeiras, tornando-as modificadas permanentemente (Perry and Rayat 2021). Após transduzirmos nossas células com os vetores lentivirais, selecionamos os clones B16.G, fluorescente verde derivado da linhagem de melanoma B16; tEnd.TO fluorescente vermelho derivado da

linhagem endotelial tEnd e a NIH3T3.G, fluorescente verde derivado da linhagem de fibroblastos NIH3T3. Para o melhor entendimento das diferentes características das nossas células, listamos abaixo as células usadas no ensaio de co-cultura de acordo com as suas características, **Tabela 04**.

Tabela 4 - Descrição das características das células utilizadas no ensaio de co-cultura.

Nomenclatura	Características
B16	Linhagem de melanoma murino
B16.G	B16 GFP positiva, fluorescente verde
B16.B2	B16 *knockout* para IFNAR1
tEnd	Linhagem endotelial murina
tEnd.TO	tEnd *tomato* posiva, fluorescente vermelha
tEnd.A5	tEnd *knockout* para IFNAR1
NIH-3T3	Linhagem de fibroblastos murina
NIH-3T3.G	NIH-3T3 GFP positiva, fluorescente verde
NIH-3T3.A1	NIH-3T3 *knockout* para IFNAR1

Com as células fluorescentes em mãos, prosseguimos com a validação do modelo de efeito parácrino. Co-cultivamos as células com e sem fluorescência por 72 horas, coletados as células e quantificamos ambas pelas suas fluorescências **Fig 12 A e B**. Com a técnica de citometria de fluxo foi possível separar as populações com e sem fluorescência e quantifica-las. Durante a execução dessa validação, enfrentamos uma barreira, co-cultivar células GFP fluorescentes com células não fluorescentes, porém transduzidas pelo vetor Ad-GFP. Neste caso em específico, ambas as células co-cultivadas apresentaram fluorescência GFP, porém as células não fluorescentes transduzidas pelo vetor Ad-GFP apresentaram baixa intensidade de fluorescência se comparado com as células B16.G, desse modo foi possível separar e identificar as células em citometria, **Fig 12 C.**

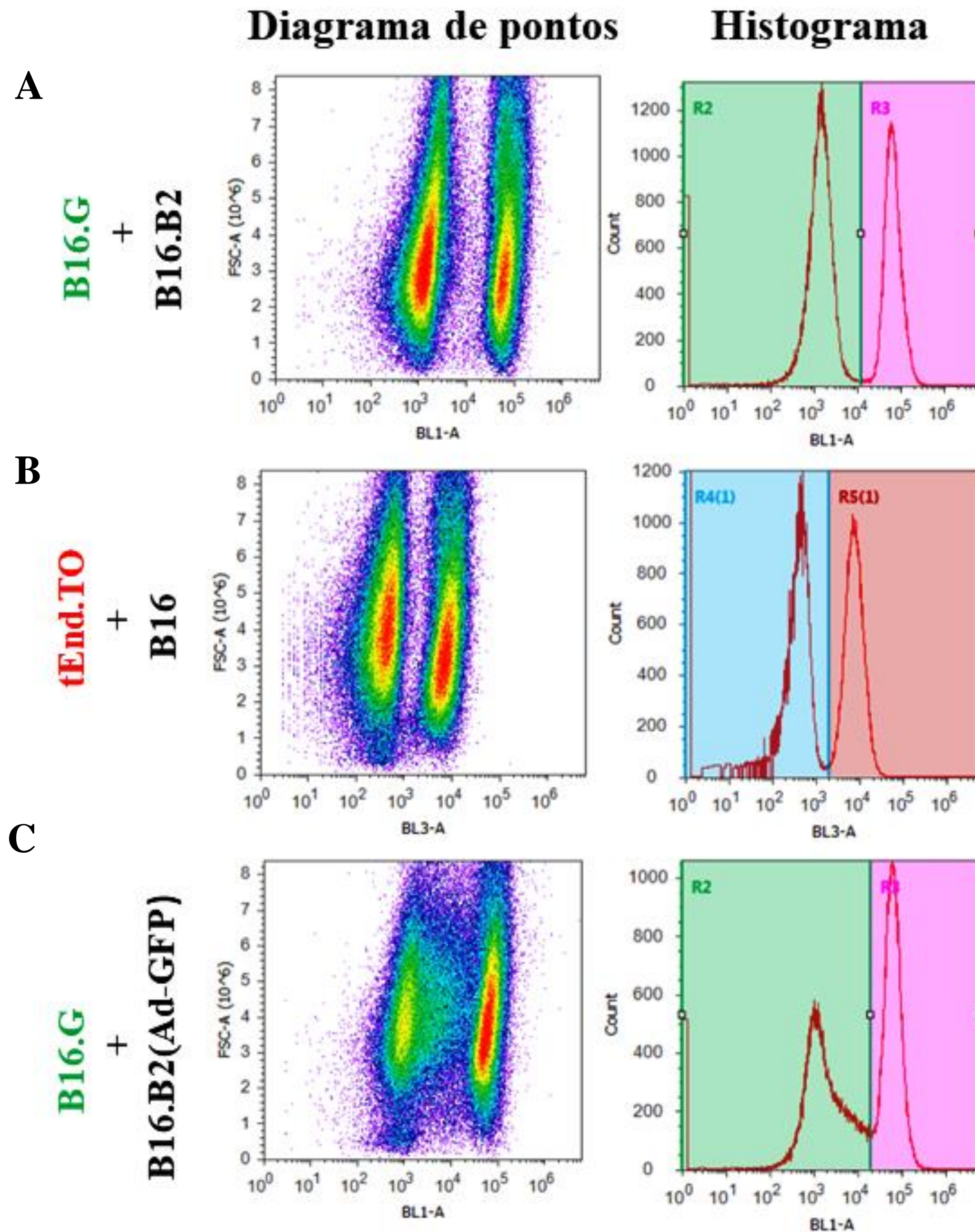

Figura 12 - Validação do modelo de co-cultura por quantificação de células por citometria de fluxo. Para realizar o ensaio de co-cultura, as células foram transduzidas por vetores lentivirais portadores de transgenes de proteínas fluorescentes eGFP (Verde) e *Tomato* (Vermelho) para obter clones com fluorescência permanente. Isolaram-se clones das linhagens transduzidas com alta fluorescência e sem a perda das características originais das células. (A) Citometria de fluxo para detecção de fluorescência verde para as linhagens B16.G (positiva para GFP, fluorescente verde) e o clone B16.B2 (sem fluorescência). (B) Citometria de fluxo para detecção de fluorescência vermelha das linhagens tEnd.TO (*Tomato* positiva, fluorescente vermelha) e o clone B16.B2 (sem fluorescência). (C) Citometria de fluxo para detecção de fluorescência verde para as linhagens B16.G (positiva para GFP, fluorescente verde) e o clone B16.B2 transduzido pelo vetor Ad-GFP.

Ensaio de efeito parácrino entre células tratadas com Ad-mIFN

Após validar o modelo, partimos para o primeiro ensaio de co-cultura com objetivo de avaliar o efeito parácrino induzido pelas células B16 transduzidas pelo vetor Ad-mIFN direcionado para outras células não tratadas. As células B16 foram transduzidas, co-cultivadas com outras células não transduzidas (NIH-3T3.G, tEnd.TO e B16) por 72 horas, coletadas e quantificadas por citometria de fluxo. A quantificação das células não transduzidas demonstrou que a co-cultura de células B16 sem tratamento ou transduzida pelo vetor controle Ad-GFP não afetou as células B16, NIH-3T3.G e tEnd.TO. Entretanto, as células que foram co-cultivadas com a linhagem B16 transduzida pelo vetor Ad-mIFN sofreram diminuição significativa, demonstrando efeito parácrino **Fig 13.**

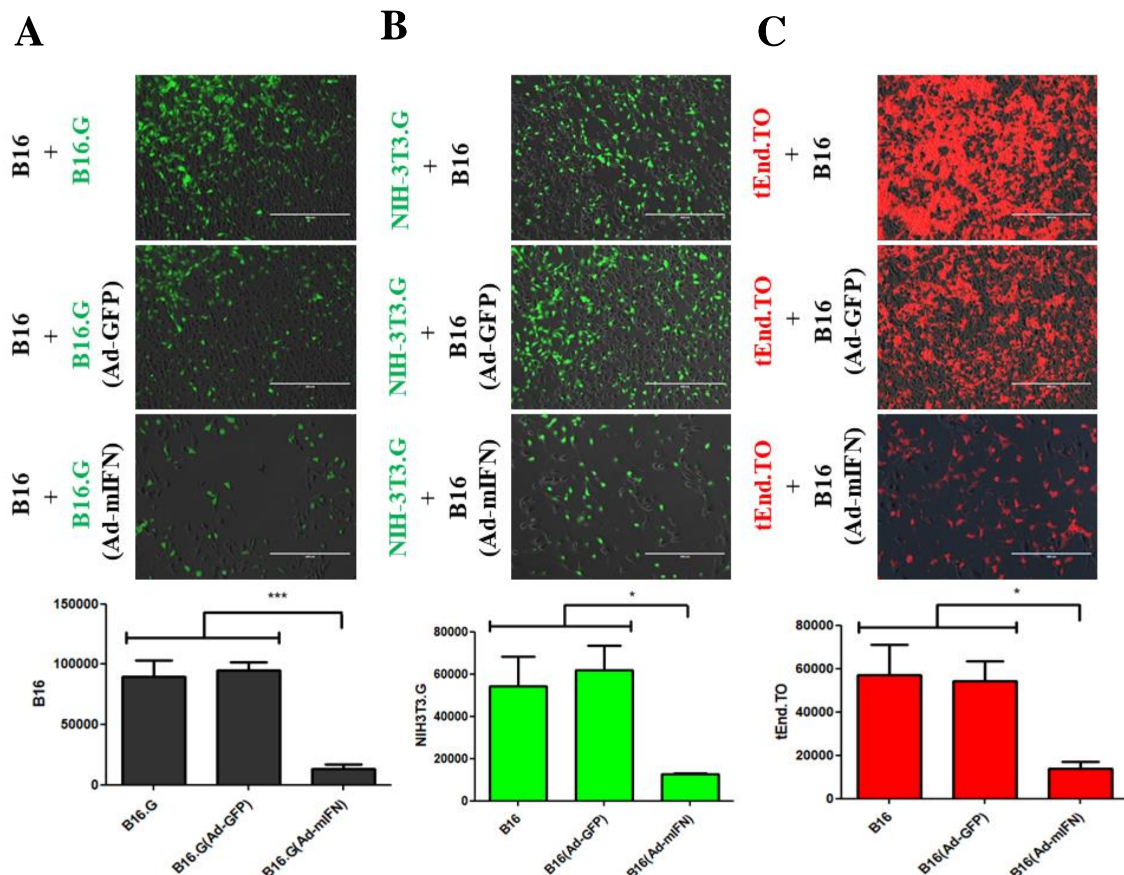

Figura 13 - Ensaio de co-cultura entre as células da B16(selvagem) transduzidas pelo vetor Ad-mIFN com as linhagens(selvagens) de microambiente e a própria B16 não transduzidas. As células B16 ou B16.G foram transduzidas com MOI 500 por 6 horas, lavadas para total remoção dos vetores, coletadas e co-cultivadas com as células da NIH3T3.G, tEnd.TO e B16 não transduzidas por 72 horas. Após período de incubação, os poços foram fotografados e as células aderidas foram coletadas, fixadas e quantificadas por citometria de fluxo. (A) Co-cultura entre a linhagem B16 (*wild type*) e a B16.G (GFP positiva, fluorescente verde) transduzida pelos vetores Ad-GFP ou

Ad-mIFN. (B) Co-cultura entre a linhagem NIH3T3.G (GFP positiva, fluorescente verde) e a linhagem B16 (*wild type*) transduzida pelos vetores Ad-GFP ou Ad-mIFN. (C) Co-cultura entre a linhagem tEnd.TO (*tomato* positiva, Fluorescente vermelha) e a linhagem B16 (*wild type*) transduzida pelos vetores Ad-GFP ou Ad-mIFN. Abaixo das fotos, encontram-se os gráficos com a contagem de células não transduzidas da co-cultura. O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste One-Way Analysis of Variance, seguido do teste de Tukey, ***$p<0,001$e *$p<0,05$.

Ensaio de efeito parácrino com células *knockout* para o IFNAR1 tratadas com Ad-mIFN

Frente aos dados anteriores afirmativos para indução de efeito parácrino da B16 transduzida pelo Ad-mIFN. Investigamos a linhagem B16.B2 (*knockout* para IFNAR1) no papel da célula produtora e receptora do efeito parácrino após transdução pelo vetor Ad-mIFN.

As células da B16.B2 foram transduzidas e co-cultivadas com as células fluorescentes B16.G, NIH-3T3.G e tEnd.TO. As fotos tiradas e a quantificação de células por citometria de fluxo após 72 horas, mostraram que o clone B16.B2 transduzido pelo vetor Ad-mIFN induz efeito parácrino observado pela inibição de células, **Fig 14**. O resultado do ensaio mostrou que mesmo com a ausência de IFNAR1 as células transduzidas mantiveram a indução do efeito parácrino.

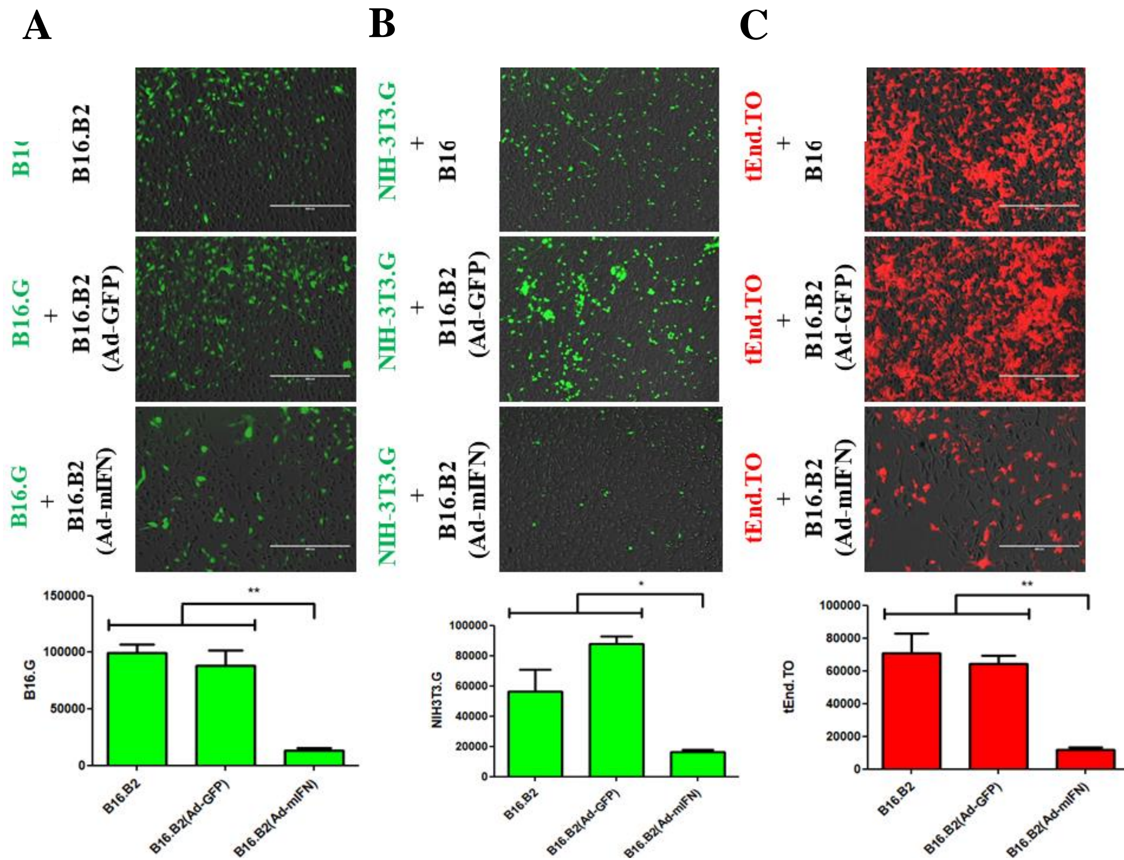

Figura 14 - Ensaio de co-cultura entre a linhagem B16.B2 (*knockout* para IFNAR1) transduzida pelo vetor Ad-mIFN com as células (selvagens) B16.G, NIH3T3.G e tEnd.TO. As células B16.B2 foram transduzidas com MOI 500 por 6 horas, lavadas para total remoção dos vetores, coletadas e co-cultivadas com outras linhagens B16.G, NIH3T3.G e tEnd.TO por 72 horas. Após período de incubação da co-cultura, os poços foram fotografados e as células aderidas foram coletadas, fixadas e quantificadas por citometria de fluxo. O ensaio foi realizado em triplicata biológica. (A) Co-cultura entre a linhagem B16.G (GFP positiva, fluorescente verde) e a B16.B2 transduzida pelos vetores Ad-GFP ou Ad-mIFN. (B) Co-cultura entre a linhagem NIH3T3.G (GFP positiva, fluorescente verde) e a B16.B2 transduzida pelos vetores Ad-GFP ou Ad-mIFN. (C) Co-cultura entre a linhagem tEnd.TO (*tomato* positiva, Fluorescente vermelha) e a B16.B2 transduzida pelos vetores Ad-GFP ou Ad-mIFN. Abaixo das fotos, encontram-se os gráficos com a contagem de células não transduzidas da co-cultura. O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste One-Way Analysis of Variance, seguido do teste de Tukey, $p<0,01$ e $*p<0,05$.

Após avaliar o papel da presença do IFNAR1 na indução de efeito parácrino, investigamos o papel do IFNAR1 nas células receptoras do efeito parácrino. A as células da B16.G foram transduzidas com vetor Ad-mIFN e co-cultivados com as células knockout para IFNAR1, B16.B2 e tEnd.A5 por 72 horas. As células foram fotografadas e quantificadas por citometria de fluxo. Os resultados demonstraram que os clones B16.B2 e tEnd.A5 não apresentaram diferença significativa na quantidade de

células quando co-cultivadas com a B16.G transduzida com o vetor Ad-mIFN, **Fig 15.** A perda do IFNAR1 bloqueou as células de sofrerem o efeito parácrino.

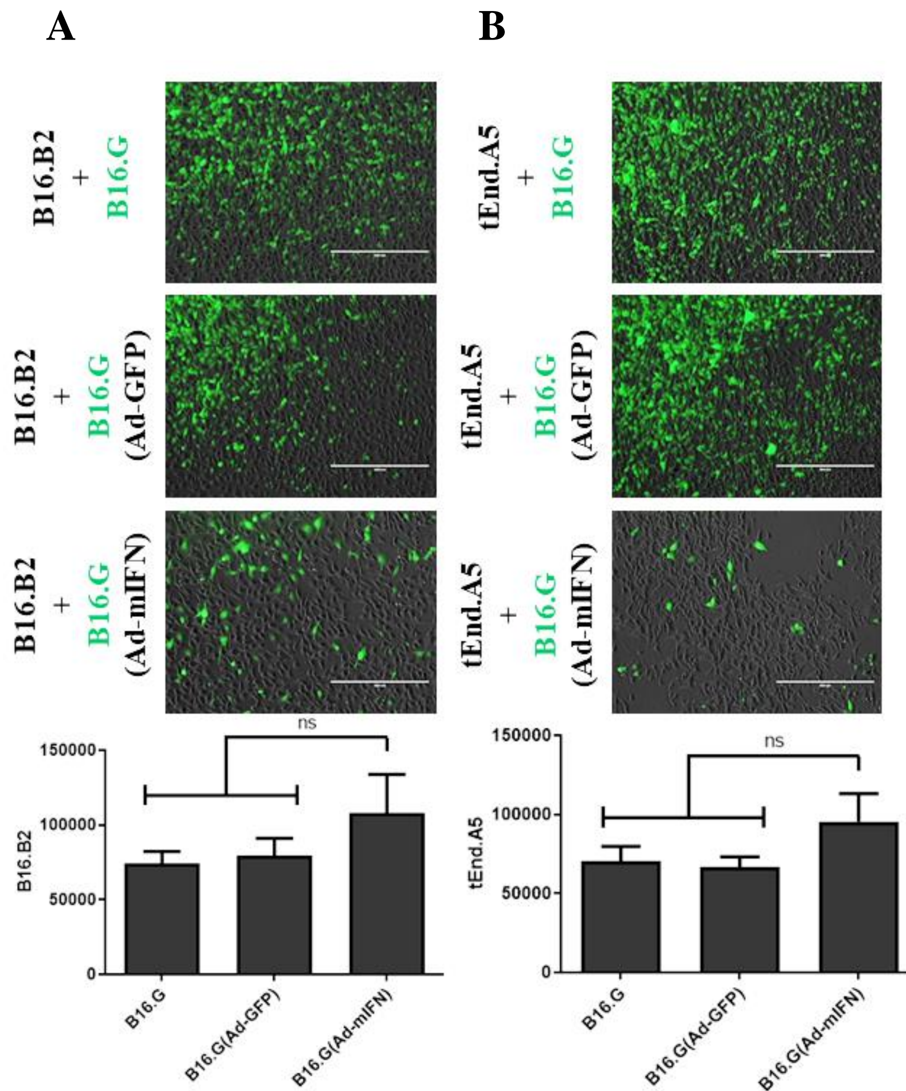

Figura 15 - Ensaio de co-cultura entre as células da B16.G (selvagem) transduzidas pelo vetor Ad-mIFN com as células de microambiente tEnd.A5 (knockout para IFNAR1) e tumorais B16.B2 (knockout para IFNAR1). As células B16.G foram transduzidas com MOI 500 por 6 horas, lavadas para total remoção dos vetores, coletadas e co-cultivadas com as células *knockout* para IFNAR1, B16.B2 ou tEnd.A5 por 72 horas. Após período de incubação da co-cultura os poços foram fotografados, as células aderidas foram coletadas, fixadas e quantificadas por citometria de fluxo. (A) Co-cultura entre a linhagem B16.G (GFP postiva, fluorescente verde) transduzida pelos vetores e a B16.B2 sem tratamento. (B) Co-cultura entre a linhagem B16.G (GFP postiva, fluorescente verde) transduzida pelos vetores e a tEnd.A5 sem tratamento. Abaixo das fotos, encontram-se os gráficos com a contagem de células não transduzidas da co-cultura. O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste One-Way Analysis of Variance, seguido do teste de Tukey.

Para finalizar os ensaios de co-culturas, foi investigado o papel da proteína IFNβ isolada na indução de efeito parácrino. Realizou-se a co-cultura entre as células B16.G e B16.B2, ambos incubadas e tratados com a rIFNβ por 72 horas. Ao final do tratamento, foram tiradas fotos e as células quantificadas por citometria de fluxo, **Fig 12**. Os resultados demonstram que houve inibição da células B16.G, porém as células B16.B2 não sofreram. O rIFNβ sozinho na co-cultura é capaz de inibir somente as células com o IFNAR1 e não afeta as células *knockout* para o IFNAR1.

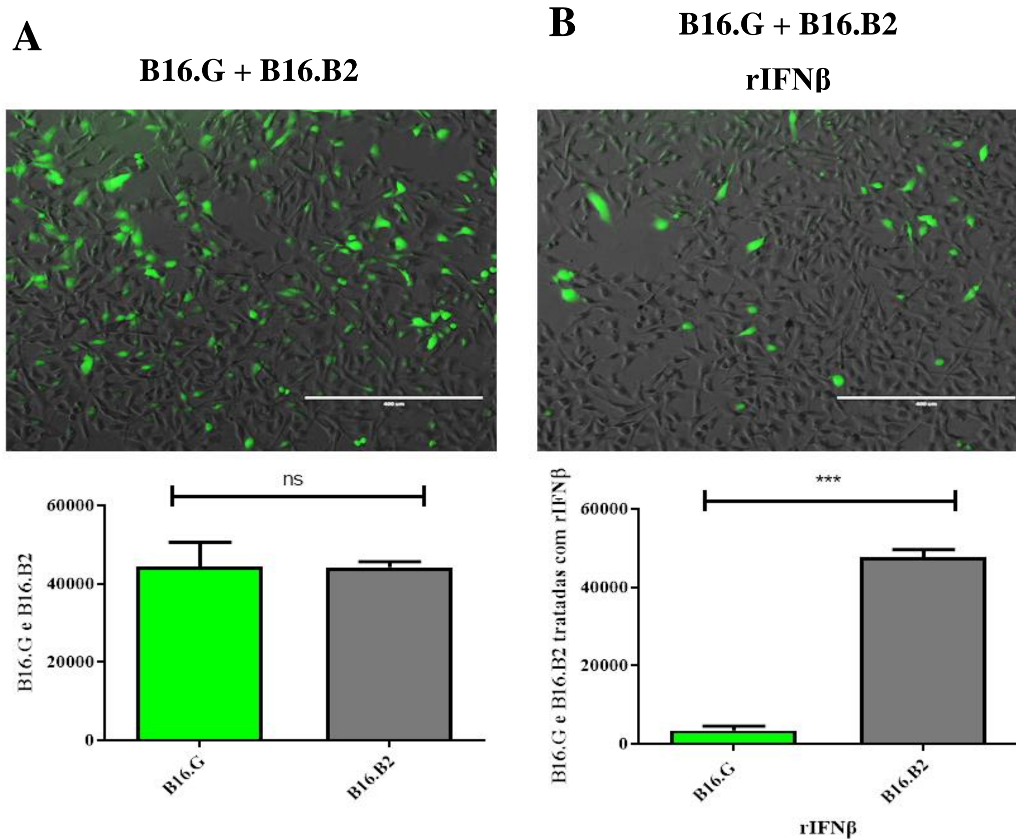

Figura 16 - Ensaio de co-cultura entre a B16.G (selvagem) e a B16.B2 (knockout para IFNAR1), ambas tratadas com rIFNβ. As células B16.G e B16.B2 foram co-cultivadas, tratadas com rIFNβ (200 pg/mL) e incubadas por 72 horas. Após período de incubação da co-cultura, os poços foram fotografados e as células aderidas foram coletadas, fixadas e quantificadas por citometria de fluxo. (A) Co-cultura entre a linhagens B16.G (fluorescente verde) e B16.B2, ambas sem tratamento. (B) Co-cultura entre a linhagens B16.G (fluorescente verde) e B16.B2 ambas tratadas com rIFNβ. O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste-t de student não pareado, ***P<0,0001; ** P<0,05; *P<0,05.

Avaliação da expressão gênica após tratamento com Ad-mIFN em células com e *knockout* para o IFNAR1

Avaliou-se o nível de mRNA de alguns genes cuja expressão poderia ser modulada pelo tratamento com o IFNβ. O ensaio foi realizado com genes de fatores de crescimento, citocinas e genes relacionados à via do Interferon nas linhagens B16 e B16.B2 após transdução com vetor Ad-mIFN. Os resultados mostraram as células da B16 mostrou aumento significativo do gene de TRAIL e de genes da assinatura gênica da ativação da via de Interferon , incluindo STAT-1, STAT-2, IRF-7 e IRF-9, **Fig 17 A**. As células da B16.B2 não apresentaram nenhuma alteração significativa dos genes estudados, **Fig 17 C**.

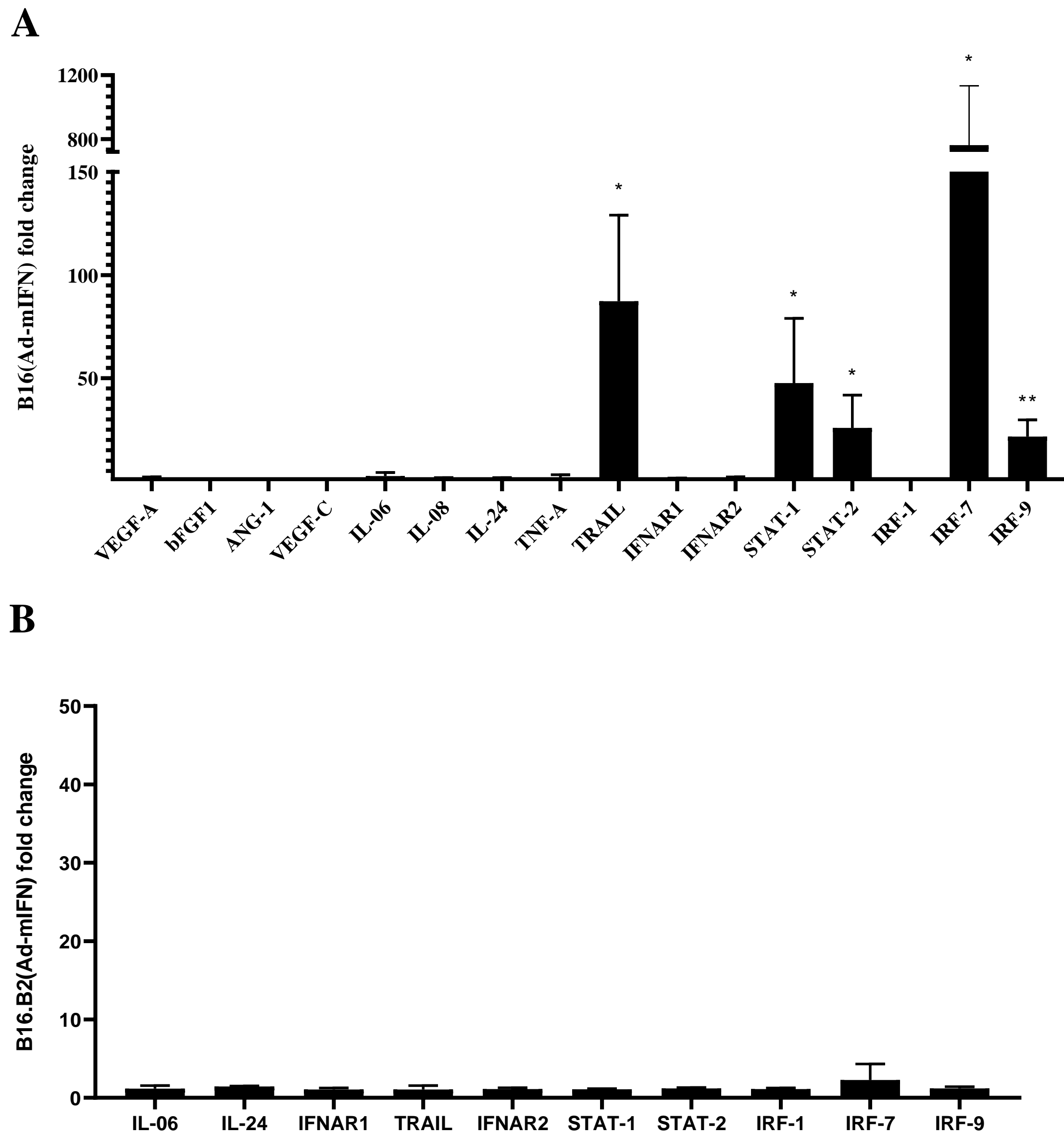

Figura 17 - Avaliação de expressão gênica nas linhagens B16 e B16.B2 transduzidas pelo vetor Ad-mIFN. As células foram transduzidas com MOI 500 por 48 horas, coletadas e tiveram seu RNA extraído e purificado. A reação de expressão foi realizada com Kit SuperScript™ III One-Step RT-PCR System no equipamento ABI 7500 Real Time System. O ensaio foi realizado em triplicata biológica. O perfil de expressão gênica foi examinado nas linhagens (A) B16 e (B) B16.B2. Os valores de *fold change* em comparação com GAPDH foram calculados pelo método $2^{-\Delta\Delta Ct}$ e normalizados pela amostra da B16 transduzida pelo vetor Ad-GFP (fold change das células transduzidas pelo vetor Ad-mIFN / fold change das células transduzidas pelo vetor Ad-GFP). O ensaio foi realizado em triplicata biológica. A análise estatística foi realizada com o teste-t de student não pareado, ** P<0,05 e *P<0,05.

DISCUSSÃO

A via do Interferon é intensivamente investigada há aproximadamente 70 anos, sendo possível compreender que ela vai além dos efeitos antivirais inicialmente descobertos. Hoje, o IFNα/β é amplamente estudado devido aos seus efeitos antitumorais, porém ele pode apresentar efeito tóxico se administrado sistemicamente. Apesar do seu efeito tóxico, seu uso é aprovado pela FDA como terapia antitumoral com resultados promissores (Conlon et al. 2019, Pestka 2007).

Faz-se necessário desenvolver estratégias terapêuticas para o IFNα/β, que minimizem seu efeito tóxico sistêmico. Neste contexto, aparecem as terapias gênicas como uma forma alternativa de terapia pro câncer, as quais procuram solucionar algumas barreiras especificas. Essa terapia já possui aprovação para uso ou está com status avançado de aprovação nos Estados Unidos para algumas doenças como hemofilia, desordens oculares e neurodegenerativas e o linfoma (Dunbar et al. 2018). O melanoma consta com a aprovação da viroterapia com Herpes vírus tipo 1 (HSV-1) atenuado conhecida como Talimogene laherparepvec (T-Vec). Além dessa viroterapia aprovada, existem outros 6 ensaios clínicos com terapia gênica para o melanoma em andamento (Hromic-Jahjefendic and Lundstrom 2020, Conry et al. 2018).

A nossa terapia gênica é uma tecnologia antitumoral promissora, pois apresenta um modelo de terapia com atuação local e transiente capaz de escapar ou minimizar o efeito tóxico sistêmico do IFNβ. O vetor adenoviral possui características específicas para o escape do efeito tóxico do IFNβ, como forte capacidade de transdução em diferentes tipos de células e produção temporária do transgene terapêutico. O adenovírus também não integra seu DNA ao do hospedeiro, o que elimina sua presença no tecido com o passar do tempo (Lee et al. 2017).

Pesquisas recentes mostraram a importância da presença contínua de IFNAR1 no ambiente tumoral, tanto nas células tumorais como nas células do microambiente tumoral. Baixos níveis da proteína IFNAR1 no ambiente tumoral observados na clínica médica foram identificados como um prognóstico ruim para o paciente (Araya and Goldszmid 2017). Outro trabalho publicado recentemente descreveu que a degradação do IFNAR1 e ausência da assinatura da via do IFNα/β (expressão aumentada de genes da via do Interferon) podem ser observadas em células tumorais e do microambiente. A

modulação do IFNAR1 e do IFNα/β está diretamente ligada à formação de um ambiente imunossupressor, o que demonstra sua importância no contexto de terapia (Katlinski et al. 2017).

Para investigarmos o papel do IFNAR1 após a terapia gênica com adenovírus portadores de IFNβ em células do melanoma e do microambiente tumoral, foi necessária a obtenção de clones *knockout* para o IFNAR1. Realizamos a construção dos clones B16.B2, tEnd.A5 e 3T3.A1 de células de melanoma, endoteliais e de fibroblastos, respectivamente. Usamos a tecnologia do CRISPR/Cas9 em parceria com o aluno de doutorado Ricardo Cintra.

Antes de começar os experimentos, validamos os vetores Ad-GFP e Ad-mIFN, que demonstraram sua capacidade na transdução e produção das proteínas eGFP e IFNβ, respectivamente.

Iniciamos a investigação comparando a resposta das células de melanoma B16 e de fibroblastos 3T3-NIH com seus respectivos clones *knockout* para o IFNAR1 após tratamento com Ad-mIFN. Nossos dados mostraram que a falta do receptor bloqueia a resposta do tratamento com Ad-mIFN, somente as células que possuem o IFNAR1 respondem com morte ao tratamento. O mesmo resultado foi observado quando as células foram tratadas com o rIFNβ. Ambas as células, com e sem o IFNAR1, produzem e secretam o IFNβ após transdução com o Ad-mIFN, embora somente as células que possuem o IFNAR1 respondem com morte celular ao tratamento do Ad-mIFN. Esses resultados sugerem que o tratamento com o Ad-mIFN induz morte celular pela ativação da via do Interferon após ligação do IFNβ produzido e secretado por ela mesma que se liga ao IFNAR1 e IFNAR2, **Fig. 18**. A ativação da via de IFN modula a expressão gênica e estimula eventos biológicos com efeitos antiproliferativos e citotóxicos associado à FAS, Caspase-8 e supressão da ciclina E2F-1 (Balachandran et al. 2000, Erickson et al. 1999, Kalie et al. 2008).

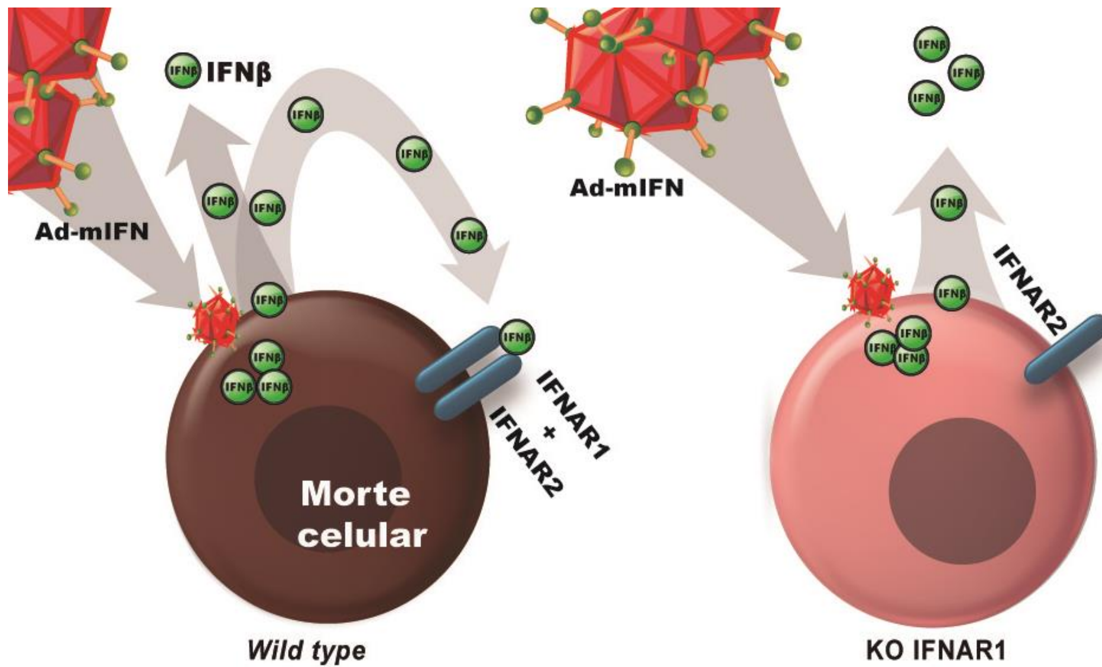

Figura 18 - Representação do mecanismo de efeito de morte nas células wild type transduzidas pelo vetor Ad-mIFN. O tratamento com Ad-mIFN induz a produção e secreção de IFNβ, que se liga a dupla de receptores IFNAR1 e IFNAR2 e ativa via do Interferon levando a morte celular. O mecanismo de morte celular não foi observado nas células *knockout* para o IFNAR1.

A terapia gênica com vetores adenovirais tem como objetivo forçar as células transduzidas a produzir os transgenes do vetor, no nosso caso o IFNβ que pode atuar tanto de modo autócrino como parácrino (Gautier et al. 2005, Song et al. 2015).

Como visto no ensaio de ELISA para o IFNβ, as células transduzidas pelo nosso vetor Ad-mIFN produzem e secretam o IFNβ no meio, o que indica uma possível atuação de efeito parácrino.

Frente aos nossos dados e as informações da literatura, decidimos investigar o efeito parácrino do nosso tratamento em células do melanoma e do microambiente. Utilizamos o ensaio de cocultura com células de diferentes fluorescências para avaliarmos o efeito parácrino do tratamento. Primeiro, a linhagem B16 foi transduzida com o Ad-mIFN e cocultivada com as duas linhagens de microambiente e outra B16 sem tratamento. Os dados mostraram que a linhagem de melanoma transduzida induz efeito parácrino de inibição em todas as linhagens que foram cocultivadas. O mesmo resultado foi observado quando colocamos a linhagem de melanoma *knockout* para o IFNAR no papel de produtora do efeito parácrino, porém, quando colocamos as

linhagens *knockout* no papel de receptoras do efeito parácrino, não foi observado efeito parácrino inibitório. Mesmo sem o IFNAR1, as células produzem o IFNβ e induzem efeito parácrino, entretanto a falta do IFNAR1 bloqueia a recepção do efeito parácrino.

De acordo com as observações dos ensaios de cocultura com linhagens *wild type* para o IFNAR1 mostraram que a as células do melanoma transduzida com o vetor Ad-mIFN induzem efeito parácrino inibidor de células do microambiente e até mesmo nas demais células do melanoma. Após verificarmos a presença do efeito parácrino após o tratamento com o Ad-mIFN, inserimos os clones *knockout* para o IFNAR1 no mesmo modelo, porém inserimos as células nas posições de produtor e receptor do efeito parácrino. Os resultados mostraram que os clones *knockout* para o IFNAR1 mantêm a indução do efeito parácrino, contudo bloqueiam a recepção desse efeito, **Fig 19.**

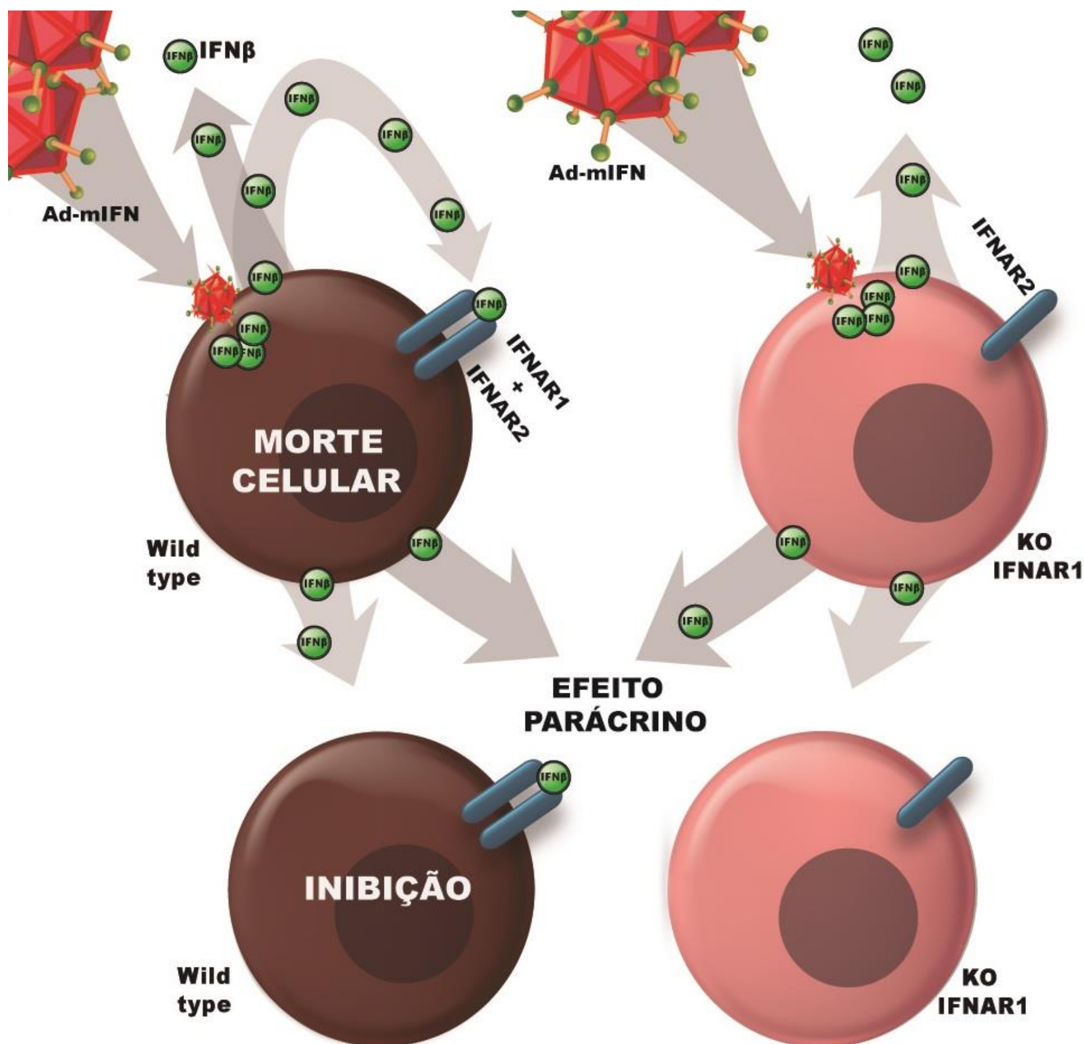

Figura 19 - Representação do mecanismo de efeito parácrino do tratamento com o vetor Ad-mIFN em células selvagens e knockout para o IFNAR1. O tratamento com Ad-mIFN em células com e sem o IFNAR1 induz efeito

parácrino de inibição de células que possuem o IFNAR1. O efeito tem relação direta com a produção de IFNβ e ativação da via de IFN pela ligação do IFNβ na dupla de receptores IFNAR1 e IFNAR2.

No último ensaio parácrino, utilizamos o rIFNβ como tratamento da cocultura entre a linhagem B16 *wild type* e outra *knockout* para o IFNAR1. Somente a linhagem que possuía o IFNAR1 foi afetada pelo tratamento. Esse ensaio demonstrou mais uma vez a importância do IFNAR1 para o sucesso do tratamento com Ad-mIFN e a forte dependência da produção e secreção do IFNβ para o sucesso do tratamento. Então, podemos concluir que ativação da via IFNβ é fundamental para o efeito parácrino. A ausência de IFNAR1 foi correlacionada à resistência ao efeito parácrino neste modelo. Esse modelo implica que o efeito não envolve outros fatores além do IFNβ, ou pelo menos estes outros fatores não teriam um papel marcante neste modelo.

As células transduzidas pelo vetor Ad-mIFN se transformaram fontes de produção do IFNβ e por consequência o efeito parácrino, resultando na morte de células do MT, incluindo fibroblastos e células endoteliais, conforme demonstrado nos resultados desta tese. Esse fenômeno pode explicar parte dos mecanismos que ocorrem no ambiente tumoral onde foi administrado o Ad-mIFN, para o sucesso do tratamento em camundongos com melanoma realizado em trabalhos anteriores (Medrano et al. 2016, Merkel et al. 2013, Hunger et al. 2017a).

No último ensaio do projeto, foi avaliado a expressão gênica de fatores de crescimento, genes da assinatura gênica do IFNβ e outros genes conhecidos por serem alterados pela via do IFNβ em células B16 *wild type* e *knockout* para o IFNAR1 após tratamento com o Ad-mIFN. Nas células *wild type*, os resultados mostraram que o Ad-mIFN induz aumento da expressão dos genes conhecidos da assinatura gênica do IFNβ como os STATs, IRF7 e do fator TRAIL. Entretanto, as células B16 *knockout* para IFNAR1 não apresentaram indução de expressão em nenhum dos genes estudados, confirmando a ausência de sinalização na via IFNα/β, conforme esperado. Os resultados sugerem que o tratamento nas células *wild type* produz o IFNβ que se liga aos receptores, ativa a via de IFN e induz aumento dos genes citados anteriormente. As células *knockout* para IFNAR1, mesmo produzindo o IFNβ, não têm ativação da via do IFNα/β e não apresentam modulação de genes relacionados (Araya and Goldszmid 2017).

Paralelamente ao efeito parácrino relacionado à produção de IFNβ pelas células, os dados de aumento da expressão de TRAIL podem estar envolvidos no efeito parácrino devido às características antitumorais desse fator (Kalliolias and Ivashkiv 2016, von Karstedt, Montinaro and Walczak 2017).

Este trabalho contribui para o entendimento do mecanismo molecular de morte celular que ocorre nas células tumorais e células do microambiente após terapia gênica com IFNβ utilizando vetores adenovirais. Além disso, fornece informações relevantes sobre a importância do IFNAR1 no ambiente tumoral frente a terapias que utilizem o IFNβ. O desenvolvimento de drogas ou terapias alternativas para o câncer é de grande importância devido à complexidade dessa doença, porém faz-se necessário investigar os mecânicos moleculares dessas terapias para sua melhor utilização.

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4

Os efeitos da transferência gênica de IFNβ versus a exposição da secretomica produzida por células do melanoma tratadas com terapia gênica com IFNβ e p19Arf em células endoteliais murinas

Distinct Roles of Direct Transduction Versus Exposure to the Tumor Secretome on Murine Endothelial Cells After Melanoma Gene Therapy with Interferon-β and p19Arf

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RESUMO

A vasculatura tumoral tem função essencial no desenvolvimento e manutenção do tumor. Devido a essa importância, as células endoteliais (CEs) da vasculatura, podem ser consideradas como um alvo terapêutico de grande interesse. Neste trabalho, foi avaliado os efeitos anti-angiogênicos da terapia gênica com Interferon beta (IFNβ) e Ad-p19Arf. Os dois genes terapêuticos são conhecidos pelos seus efeitos anti-tumorais e anti-angiogênicos. Os trabalhos anteriores do nosso grupo mostraram que a combinação dos genes terapêuticos na transferência gênica reduz a progressão tumoral por indução de morte celular e estímulo de resposta imune. Neste trabalho, abordamos o efeito da terapia gênica com adenovírus portadores de IFNβ e p19Arf em CEs e o possível efeito parácrino produzido por células de melanoma tratadas pelo mesmo tratamento. Os resultados mostraram que tratamento com Ad-p19 na tEnd inibiu a proliferação, formação de tubos, migração e aumentou a expressão de genes relacionados a via de p53, porem estas células tratadas com vetor Ad-mIFN não apresentaram resposta. Para avaliarmos o efeito parácrino direcionado as CEs, realizamos dois ensaios para esse fim. O primeiro ensaio foi a co-cultura entre as linhagens B16 previamente transduzidas com o Ad-mIFN e o uso do meio condicionado da B16 transduzida para tratarmos as CEs. Nos dois casos as CEs que foram co-cultivadas com a B16 transduzida pelo Ad-mIFN ou receberam o meio condicionado da B16 transduzida pelo Ad-mIFN, tiveram aumento significativo na morte celular. Utilizamos a proteína do IFNβ recombinante (rIFNβ) para tratarmos as células e os resultamos mostraram que a linhagem tEnd não responde ao rIFNβ, mostrando que existem fatores adicionais produzidos pela linhagem tumoral B16 transduzida pelo vetor Ad-mIFN que induz a morte celular da tEnd. Neste trabalho, nós demonstramos que as células endoteliais tEnd podem ser inibidas pelo tratamento direto com o vetor Ad-p19 e pelo efeito parácrino produzido pelas células tumorais B16 transduzidas pelo vetor Ad-mIFN. O tratamento com terapia gênica com o uso de vetores adenovirais portadores de p19Arf e IFNβ pode conferir efeito anti-angiogênico no tumor.

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Distinct Roles of Direct Transduction Versus Exposure to the Tumor Secretome on Murine Endothelial Cells After Melanoma Gene Therapy with Interferon-β and p19Arf

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Tumor vasculature plays a central role in tumor progression, making it an attractive therapeutic target. In this study, we explore the antiangiogenic potential of our melanoma gene therapy approach combining interferon β (IFNβ) and p19Arf gene transfer. Since these proteins are modulators of tumor vasculature, we explore the impact of IFNβ and p19Arf gene transfer on murine endothelial cells (tEnd). Adenovirus-mediated gene transfer of p19Arf to tEnd cells inhibited proliferation, tube formation, migration, and led to increased expression of genes related to the p53 cell death pathway, yet IFNβ gene transfer had no significant impact on tEnd viability. Alternatively, tEnd cells were exposed to the factors generated by transduced B16 (mouse melanoma) cells using either coculture or conditioned medium. In either case, transduction of B16 cells with the IFNβ vector, whether alone or in combination with p19Arf, resulted in endothelial cell death. Strikingly, treatment of tEnd cells with recombinant IFNβ did not induce death, demonstrating that additional factors produced by B16 cells contributed to the demise of tEnd cells. In this work, we have shown that our melanoma gene therapy strategy produces desirable negative effects on endothelial cells, possibly correlating with antiangiogenic activity.

Keywords: angiogenesis, endothelial cells, melanoma, interferon beta, p19Arf

Introduction

Melanoma is the most aggressive and deadliest form of skin cancer (Siegel and others 2014). The etiology of melanoma involves environmental, phenotypic, and genetic phenomena (Gandini and others 2005; Higgins and others 2015) and the tumor itself is composed of transformed melanocytes and a variety of stromal cells that provide support and even promote progression (Villanueva and Herlyn 2008). Particularly important are the tumor blood vessels that control the supply of nutrients (Dudley 2012), maintain blood flow, and regulate leukocyte trafficking, including recruitment of myeloid cells needed to promote angiogenesis (Schmid and Varner 2007). The blood vessels are composed of smooth muscle cells, pericytes, and endothelial cells, which are especially active in the amplification of cancer growth and its spread (Michiels 2003). Because the endothelial cells play such a critical role, we have chosen these as the subject of our study aimed at further developing a cancer gene therapy approach.

We have employed interferon β (IFNβ) and p19Arf for cancer gene therapy due to their multiple antineoplastic activities. IFNβ is a cytokine well known for its role in antiviral defense, immune modulation, and induction of cell death (Chawla-Sarkar and others 2003; Schoggins and Rice 2011; Medrano and others 2017). The p19Arf tumor suppressor protein (p19Arf in mice, p14Arf in humans) is best known for its role in preventing the interaction of p53 with MDM2, resulting in p53 activation and accumulation (Kamijo and others 1998; Zhang and others 1998; Milojkovic and others 2013). In fact, critical interaction between the IFN and p53/Arf pathways has been shown to aid antiviral defense and cell killing (Takaoka and others 2003; Sandoval and others 2004).

In addition, IFNβ and p19Arf each carry out antiangiogenic functions. IFNβ acts on endothelial cells, reducing the production of proangiogenic factors and inducing cell death (Bracarda and others 2010). Angiogenesis is negatively modulated by p19Arf through p53 dependent and independent mechanisms (Teodoro and others 2007;

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Kawagishi and others 2010; Ulanet and Hanahan 2010). Whether individually or in tandem, we expect that the use of IFNβ and p19Arf would impact the tumor vasculature by inhibiting angiogenesis and, possibly, destroying existing vessels.

Melanomas typically retain p53 in the wild-type form (Giglia-Mari and Sarasin 2003), possibly serving as a molecular target for treatment. To this end, we have developed adenoviral vectors wherein high-level transgene expression is controlled by p53 in a nonreplicating Ad5 vector (Bajgelman and Strauss 2008) or Ad5 with arginylglycylaspartic acid modification (Hunger and others 2017). We have used these vectors to transfer the p19Arf and IFNβ genes resulting in enhanced melanoma cell death *in vitro* and *in vivo* (Merkel and others 2013), induction of a protective immune response in models of melanoma and lung carcinoma (Catani and others 2016; Medrano and others 2016), and a strong bystander effect between treated and untreated melanoma cells (Hunger and others 2017). However, our previous studies did not include evaluation of the tumor vasculature upon treatment with our gene therapy approach.

In this study, we show that AdRGD-PG (nonreplicating Ad5 with RDG tripeptide modification and transgene expression under the control of p53) expressing p19Arf inhibits migration, tube formation, and cell proliferation and also induces cell death consistent with apoptosis upon transduction of the murine endothelial cell line (tEnd). Exposure of tEnd to the factors produced by B16 cells after their transduction with the adenoviral vector encoding IFNβ revealed a paracrine effect that resulted in endothelial cell death. Collectively these data indicate that our vectors have a desirable negative impact on endothelial cell activity consistent with the inhibition of angiogenesis upon melanoma gene therapy.

Materials and Methods

Cell lines

The murine melanoma cell line B16F10 (B16) was kindly provided by Dr. Roger Chammas, Instituto do Câncer do Estado de São Paulo, Faculdade de Medicina, Universidade de São Paulo, SP, Brazil; tEnd was kindly provided by Dr. Cristina Maria Fernandes, Instituto Butantã, SP, Brazil; and HEK293A (adenovirus-transformed human embryonic kidney) cell line was obtained commercially (Invitrogen Life Technologies, Carlsbad, CA). The B16 and tEnd cell lines were maintained in RPMI (Life Technologies) and HEK293 line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies) plus 5% fetal bovine serum (FBS; Life Technologies) and 1% antibiotic-antimytotic (Invitrogen Life Technologies), maintained at 37°C in a humidified atmosphere with 5% CO_2.

Production and transduction of adenoviral vectors

The AdRGD-PG vectors (nonreplicating Ad5 with RGD fiber modification and transgene expression under the control of a p53-responsive promoter, termed PG) encoding enhanced green fluorescent protein (GFP), p19Arf, or murine IFNβ have been described in detail elsewhere (Hunger and others 2017). AdRGD-CMVLacZ (Mizuguchi and others 2001) was kindly provided by Dr. Hiroyuki Mizuguchi (Osaka University, Japan). The adenoviruses were produced in the HEK293A cell line and purified by an iodixanol ultracentrifugation as described previously (Peng and others 2006). The viruses were titrated with Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA) and this biological titer was used when calculating the multiplicity of infection (MOI). For transduction, the MOI indicated in each assay was applied in DMEM or RPMI medium with 5% FBS and 1% antibiotic-antimytotic and maintained at 37°C at 5% CO_2 atmosphere.

X-Gal staining

tEnd cells were plated in 6 well dishes, 1×10^5 cells/well, transduced with the Ad-LacZ vector at an MOI of 200 and incubated for 24 h. After this period, the cells were fixed with 2% paraformaldehyde/0.2% glutaraldehyde for 5 min. For X-Gal staining, the cells were first washed with 0.1 M phosphate buffer (pH 7.3) supplemented with 2 mM $MgCl_2$, and then immersed in X-Gal staining buffer (0.1 M phosphate buffer, pH 7.3, supplemented with 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/mL of X-Gal) and incubated at 37°C for 16 h.

Flow cytometric detection of GFP positive cells

B16 and tEnd cells were seeded in 6 well dishes, 5×10^4 cells/well, transduced with Ad-GFP at an MOI of 200, and incubated for 24 h. After harvesting the cells, they were analyzed for GFP expression by flow cytometry (Attune; Invitrogen Life Technologies). The percentage of GFP positive cells was determined by the Attune software.

Immunofluorescence detection of p19Arf

B16 and tEnd cells were seeded onto 13 mm round glass coverslips, 5×10^4 cells/well, in 24-well dishes, transduced the next day with adenoviral vectors at an MOI of 200, and fixed with methanol 24 h later. Then, cells were blocked with bovine serum albumin (Sigma-Aldrich, St. Louis, MO), probed with a rabbit polyclonal antibody for p19Arf (AB-1; Cal-Biochem, San Diego, CA), followed by an Alexa488-labeled antirabbit secondary antibody (Molecular Probes, Eugene, OR). Hoechst 33258 (20 μg/mL; Molecular Probes) was used for nuclear staining. Cells were visualized by fluorescent microscopy (EVOS FL; Invitrogen Life Technologies).

Enzyme-linked immunosorbent assay for detection of IFNβ

Fifty thousand B16 or tEnd cells were transduced with the adenoviral vectors at an MOI of 200, and after incubation for 24 h the supernatants were collected. Detection of IFNβ was performed by ELISA (PBL Biomedical™, New Brunswick, NJ) following the manufacturer's protocol and using a Victor™ plate reader (Perkin-Elmer, Waltham, MA) where absorbance was measured at 450 nm.

Wound healing assay

The tEnd cells were seeded, 1×10^4 cells in 12-well plates, and 24 h later a pipette tip was used to create a wound in the confluent monolayer cells and transduction was performed using an MOI of 100 and cells were incubated in the

presence of Mitomycin C (20 μg/mL). Photos were taken at 0 h (time of wounding and transduction) and 48 h later. Pictures were taken using an EVOS FL microscope (Invitrogen Life Technologies). Calculations were performed as follows: $[(M^0 - M^{48})/M^0]*100 = X$, where M^0 is the area of the wound at time 0, M^{48} is the area 48 h later, and X is the resulting percentage of wound closure (area filled) due to migration.

Tubulogenesis assay

The tEnd cells were previously transduced using an MOI of 200 and incubated for 6 h. Then they were harvested, counted, and 1×10^4 cells/well were seeded in 24-well plates containing basic Matrigel (BD Biosciences, San Jose, CA). Photographs were taken 48 h after seeding of the cells and the number of tubes was quantified. Tubes were defined as closed tubes formed by >2 cells.

Clonogenic assay

The tEnd cells were transduced with the adenoviral vectors (MOI 200) and incubated for 6 h. Then, cells were harvested, counted, and 1×10^3 cells were seeded per 100 mm plate and incubated for 10 days. For the clonogenic assay with conditioned medium, 500 tEnd cells were seeded with conditioned medium in 6-well plates, and incubated for 10 days. After incubation time, cells were fixed with acetic acid–methanol (5%:95%) solution and stained with Trypan Blue. Colonies were quantified manually.

Cell viability assay

For the MTT assay, 1×10^3 cells were plated in 96-well dishes. The next day, they were transduced (MOI 200), then incubated for 48 h before determination of cell viability. Plates were incubated with 25 μL of MTT solution [5 mg/mL in 1×phosphate-buffered saline (PBS)], 37°C during 4 h. The dish was then removed and the precipitate solubilized by the addition of 100 μL lysis buffer (20% sodium dodecyl sulfate in 50% dimethylformamide (DMF)/2% acetic acid, pH adjusted to 4.7) before analysis using Victor™ plate reader (Perkin-Elmer) at 570 nm.

Identification of hypodiploid, Annexin-V, and caspase 3/7 positive cells

B16 and tEnd cells were transduced with the viral vectors at an MOI of 200 or treated with medium containing recombinant interferon β (rIFNβ, 10 U/mL; Millipore, Temecula, CA, USA) and incubated for 48 h. Alternatively, cells were plated and the following day incubation in the presence of conditioned medium was initiated and then maintained for 48 h. For quantification of hypodiploid cells, cells were harvested, fixed with 70% ethanol, and stained with propidium iodide. For identification of phosphotydilserine exposed at the cell surface, fresh cells were labeled with Annexin-V-Alexa488 (Invitrogen Life Technologies) immediately upon harvesting. In this case, the Ad-LacZ vector was used as a control. For identification of caspase 3/7 activity, fresh cells were treated with CellEvent Caspase 3/7 Detection Reagent (Invitrogen Life Technologies). All analyses were performed by flow cytometry and associated software (Attune; Invitrogen Life Technologies).

Coculture assay

We first modified tEnd cells with the Lego-iT2 lentiviral vector (kindly provided by Kristoffer Rieken, University Medical Center Hamburg, Germany), which offers constitutive expression of the dTomato fluorescent protein to generate the tEndTO cell line. Cells were transduced with Lego iT2 vectors at an MOI of 1 in the presence of 8 μg/mL polybrene and expanded to a 100 mm dish. Then clones with the highest fluorescence levels were screened to isolate a cell line with fluorescence detectable in 100% of the cells. For the assay, 2×10^4 B16 cells were transduced (MOI 300), incubated for 6 h, and then washed to remove virus particles. We then seeded 1×10^4 tEndTO cells with the previously transduced B16 cells. The 2 types of cells were cocultured for 72 h. Photographs (EVOS FL; Invitrogen Life Technologies) were taken at 24 and 72 h after seeding. Quantification of each cell type was aided by use of the Image J program. Results from each time point in each independent experiment were normalized using the following formula: $N_T/N_C = V$, where N_T is the average number of treated cells per field, N_C is the average number of control cells per field, and V is the resulting value.

Production of conditioned medium

To produce conditioned medium, 5×10^4 B16 cells were transduced (MOI 300) and incubated for 6 h and then washed 4 times with 1×PBS to remove virus particles. Fresh culture medium was then added and cells were incubated for 48 h. Then, medium was collected, centrifuged to separate out cellular debris, and stored at −80°C. The conditioned medium derived from B16 cells was named cm-B16 (non-transduced control), cm-B16-GFP (control transduced with Ad-GFP or Ad-LacZ), cm-B16-IFN (transduced with Ad-IFN), cm-B16-p19 (transduced with Ad-p19), and cm-B16-IFN+p19 (cotransduced with Ad-IFN and Ad-p19).

Reverse transcriptase-quantitative polymerase chain reaction analysis

The tEnd cells were plated in 6 well dishes, 5×10^4 cells/well, and transduced with the adenoviral vectors (MOI 200). After 24 h, total RNA was isolated using Trizol (Invitrogen Life Technologies) following the fabricant's instructions. Concentration of RNA was measured by absorbance at 260 nm. Quality of RNA was assessed by protein and salt concentration (absorbance 280 nm) and by visualizing the 18S and 28S ribosomal RNA bands in a 1% agarose gel. Primers (Supplementary Table S1) were designed and then analyzed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). We used β-actin as the reference gene. Primer efficiency was assessed and confirmed to be nearly 100%. Extracted RNA was reverse transcribed using random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Reaction conditions were 100 ng cDNA, 12.5 pmol each primer, and 8 μL of Syber Green PCR Master Mix (Invitrogen Life Technologies) in a final volume of 20 μL. Amplification was performed using denaturation at 95°C for 1 min and extension at 72°C for 1 min, and samples were analyzed by the 7500 Fast Software, version 2.05 (Applied Biosytems/Invitrogen Life Technologies). The $2^{-\Delta\Delta Ct}$ method was used for quantification and data

are presented as fold change in expression ($\log_2$) as compared with tEnd transduced with the GFP vector.

Statistical analysis

Data are expressed as the mean ± SD from at least 3 experiments. Statistical analysis was carried out using analysis of variance (ANOVA) followed by Tukey's test, significant at a level of $P < 0.05$. Analysis was performed by using GraphPad Prism 5.0.

Results

Validation of adenoviral vectors and efficiency of transduced cells

Expression of the GFP, LacZ, IFNβ, and p19Arf transgenes upon transduction with the recombinant adenoviral vectors was analyzed by fluorescence microscopy, staining with X-Gal, ELISA, or immunofluorescence, respectively (Supplementary Fig. S1). These assays demonstrate reliable expression of the transgenes when tEnd (mouse immortalized endothelial cells) or B16 (mouse melanoma cells) were transduced with our adenovectors. As seen in Supplementary Fig. S1, tEnd and B16 were transduced at similar levels (74.8% and 64.3%, respectively) as seen by detection of exogenous p19Arf. These data indicate that transduction of endothelial or melanoma cells with our RGD-modified vectors results in reliable gene transfer and transgene expression.

p19Arf inhibits angiogenic characteristics of transduced tEnd cells

We next transduced tEnd cells with our recombinant adenoviruses and analyzed indicators of angiogenic potential, specifically migration and tube formation. In the migration assay, the wound was formed and immediately followed by transduction and treatment with Mitomycin C (0 h). We observed significantly reduced migration when the tEnd cells had been transduced with Ad-p19 alone or in combination with Ad-IFN (Fig. 1A, B). In the tubulogenesis assay, cells were transduced with the vectors, incubated for 6 h, and then plated in wells containing Matrigel. After 48 h of incubation, the cells were photographed and the number of tubes formed was quantified. tEnd cells transduced with Ad-p19 alone or in combination with Ad-IFN showed significantly fewer tubes (Fig. 1C, D).

Transduction of tEnd with Ad-p19 triggers cell death

The reduction in angiogenic potential already described may be due, in part, to alterations in cell proliferation and viability. To test this possibility, tEnd cells were transduced with an MOI of 200 before colony formation, viability, cell cycle, and apoptosis were evaluated. Only combined Ad-p19 and Ad-IFN gene transfer significantly reduced clonogenicity and viability (MTT assay) of tEnd cells, although a tendency was noted with individual Ad-p19, but not Ad-IFN, treatment (Fig. 2A–C). Interestingly, Ad-p19 either alone or in combination with Ad-IFN strongly induced accumulation of hypodiploid (sub-G1), Annexin-V positive cells, and activation of caspases 3/7 (Fig. 2D–F), indicating that Ad-p19 was sufficient to induce cell death by a mechanism consistent with an apoptosis. As expected, p19Arf gene transfer, but not IFNβ, was associated with the activation of p53-responsive genes, such as *Cdkn1a* (*p21*), *Mdm2*, and *Puma* (*Bbc3*) (Supplementary Fig. S2). These assays suggest that Ad-p19 is the dominant factor for inducing tEnd cell death upon transduction.

Coculture of tEnd cells with transduced B16 melanoma cells reveals critical role of IFNβ

Up to this point in our study we have shown that direct transduction of tEnd cells with an adenoviral vector encoding p19Arf results in cell death, revealing a possible benefit of our melanoma gene therapy approach. We next examined whether the factors produced by the transduced tumor cells would have an impact on the endothelial cell line. For this, we used a coculture assay since this is a relevant means of simulating the interaction of different types of cells in the tumor microenvironment (Danhier and others 2012). To differentiate the 2 cell lines in the same plate, we labeled tEnd cells with constitutive expression of the dTomato fluorescent protein, generating the tEndTO cell line. In this assay, B16 cells were transduced (MOI 300, 6 h), washed, collected, and 1×10^4 of these cells were plated together with an equal number of tEndTO cells and observed for up to 72 h (Fig. 3A). Interestingly, the tEndTO population was reduced in the presence of B16 cells treated with Ad-IFN, whether alone or in combination with Ad-p19 (Fig. 3B). Consistent with our previous findings (Merkel and others 2013; Medrano and others 2016; Hunger and others 2017), transduction of B16 cells inhibits their proliferation when one or both of the genes were applied (Fig. 3C). This coculture assay shows that the secretome produced by B16 cells upon transduction with Ad-IFN, but not Ad-p19, can negatively impact the population of tEnd cells.

Conditioned medium derived from B16 transduced with the Ad-IFN vector induces tEnd cell death

Next, we used the conditioned medium derived from B16 cells (cm-B16) transduced with the recombinant adenoviral vectors for the cultivation of tEnd cells and examined the impact of the tumor secretome on these endothelial cells. The clonogenic assays showed that tEnd cells treated with cm-B16-IFN or cm-B16-IFN+p19 significantly reduced the number of colonies formed (Fig. 4A, B). In the cell viability, cell cycle, and apoptosis analyses, tEnd cells were treated with conditioned medium for 48 h and then the tests were performed. The 3 tests showed that conditioned medium from cm-B16-IFN and cm-B16-IFN+p19 decreased viability, increased the population of hypodiploid, Annexin-V labeled cells, and cells with caspase 3/7 activity (Fig. 4C–F, respectively). Note that the use of Ad-p19 did not seem to influence these assays, suggesting a critical role for IFNβ. These data suggest that the conditioned medium containing the secretome from B16 transduced with the Ad-IFN vector induces tEnd cell death by a mechanism consistent with apoptosis.

rIFNβ is not an inducer of tEnd cell death

Since the conditioned medium including IFNβ induced death of tEnd cells, we examined whether rIFNβ would be

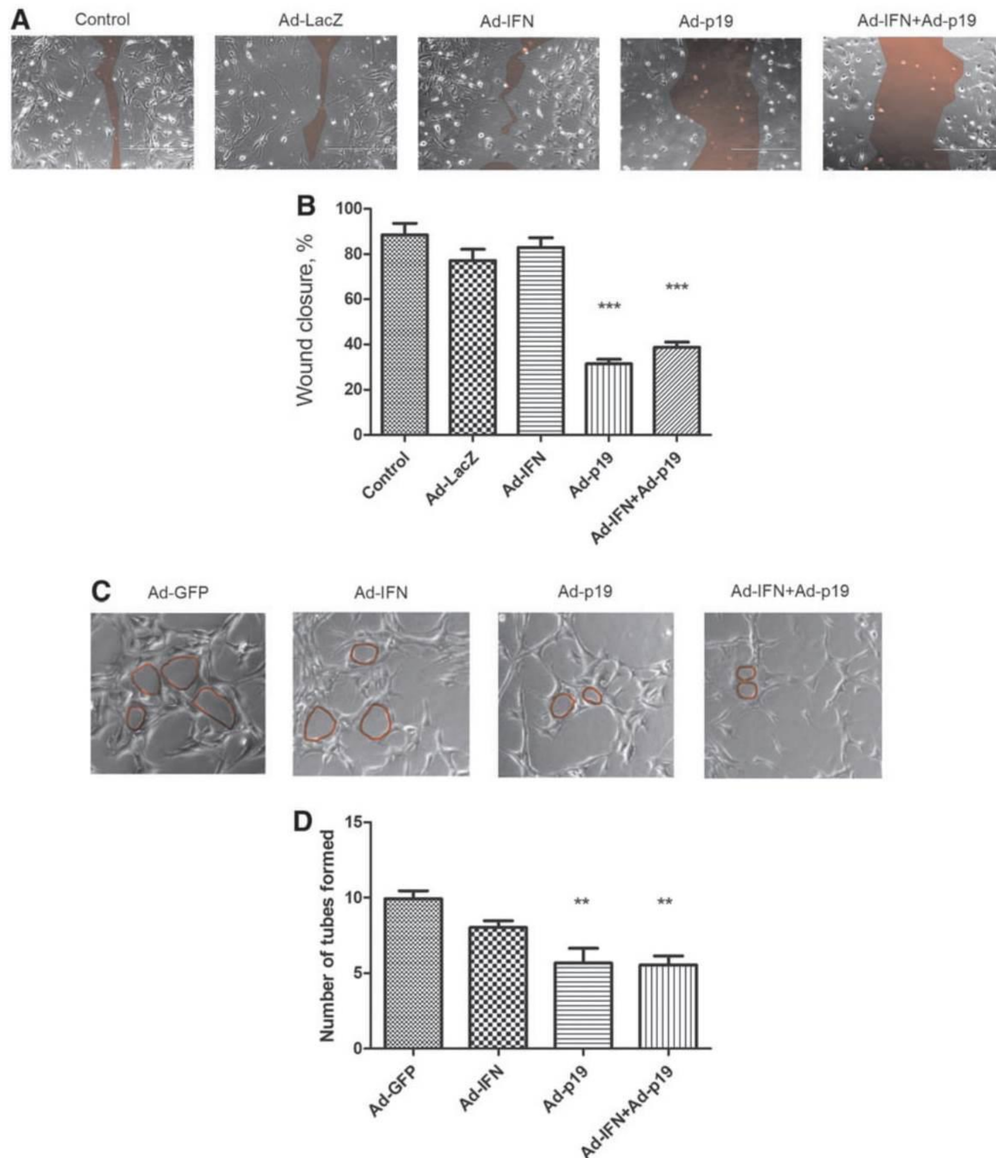


FIG. 1. Analysis of angiogenic characteristics after transduction of enthelial cells. **(A, B)** A monolayer of tEnd cells was scratched with a pipette tip, transduced (MOI 100), and treated with Mitomycin C (antiproliferative) at time 0 h. Photomicrographs (EVOS FL, 10×objective) were taken 48 h later. **(C, D)** tEnd cells were transduced (MOI 200) and incubated for 6 h before seeding on a basement of matrigel and incubation for 48 h before taking photomicrographs (EVOS FL, 10×objective) and manually counting the number of tubes formed. Results represent the average and SD from 3 independent experiments. $^{**}P<0.01$; $^{***}P<0.001$; ANOVA followed by Tukey's multicomparison post-test. ANOVA, analysis of variance; MOI, multiplicity of infection.

sufficient to bring about the same effect. We treated B16 and tEnd cells with rIFNβ 10 U/mL for 48 h before analyzing the cellular response. The percentage of hypodiploid, Annexin-V-labeled cells, and cells with activated caspases 3/7 was significantly increased only for B16, but not for tEnd, in the presence of rIFNβ (Fig. 5). This finding raises the intriguing possibility that one or more factors produced by B16 cells in addition to IFNβ are necessary for the killing of tEnd cells, as shown in Fig. 6.

Discussion

This study reveals the response of endothelial cells to our combined IFNβ+p19Arf gene transfer approach for the treatment of melanoma. In this study, we employed our AdRGD-PG vectors, containing the RGD-modified fiber and where transgene expression is controlled by p53, to transduce endothelial cells directly and observed that Ad-p19, but not Ad-IFN, was essential for impeding the angiogenic

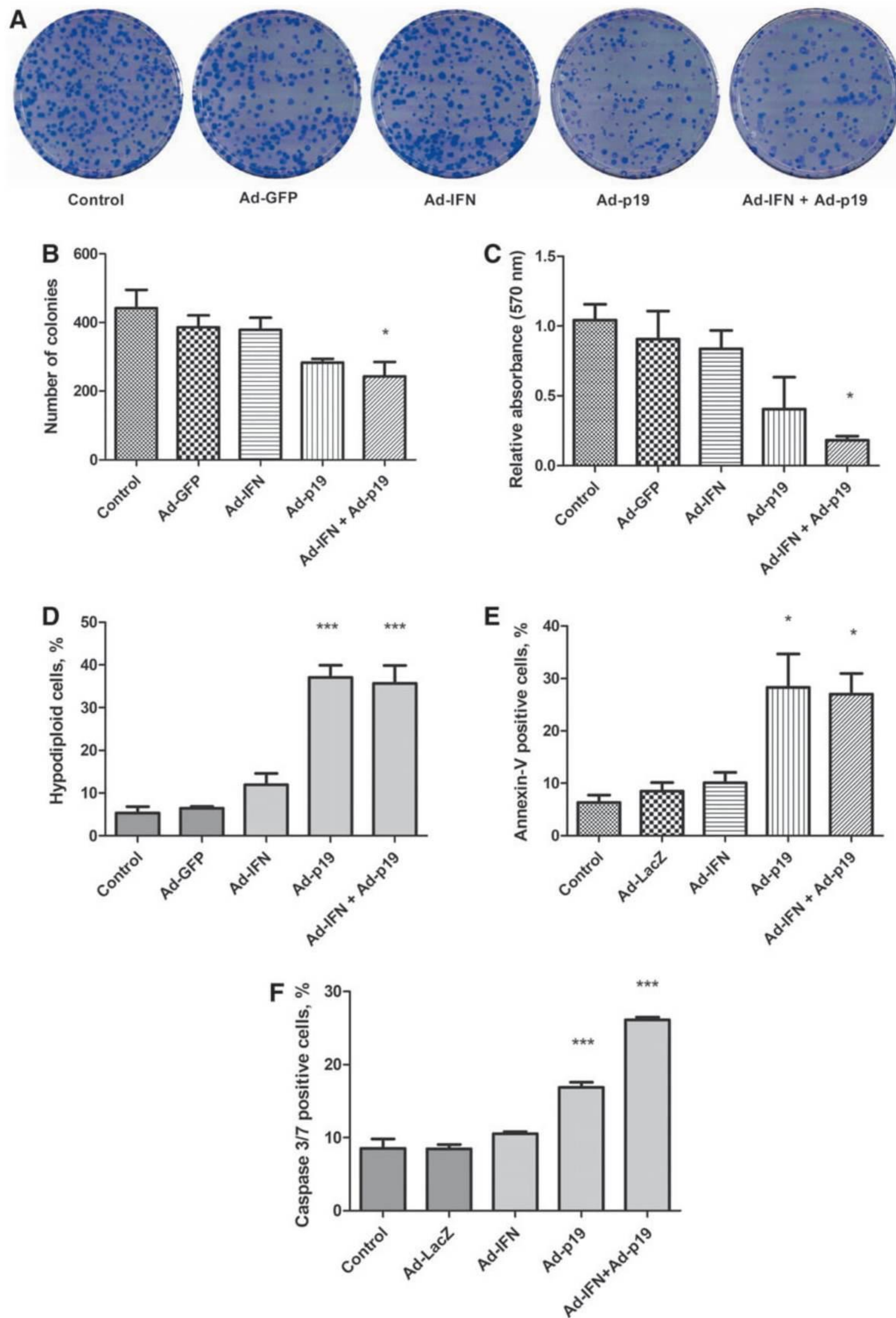


FIG. 2. Assessment of viability and cell death upon transduction of tEnd cells. **(A, B)** For this clonogenic assay, tEnd cells were transduced (MOI 200), and 6 h later, 1×10^3 cells were seeded in 10 cm plates, incubated for 10 days, fixed, and stained with Trypan Blue before manually counting clearly visible colonies. Plates were scanned (Multifunction Printer LaserJet, HP) to produce these images. **(C)** Viability assay wherein tEnd cells were transduced (MOI 200) and incubated for 48 h before MTT solution was added and analyzed at 570 nm. **(D)** tEnd cells were transduced (MOI 200), incubated for 48 h, fixed with 70% ethanol, and stained with PI before quantification of the hypodiploid cell population by flow cytometry. **(E)** For evaluation of cell death, tEnd cells were transduced (MOI 200) and incubated for 48 h before staining with Annexin-V/Alexa488. **(F)** Cytometry detection of tEnd activated caspases 3/7 after 48 h transduction (MOI 200). Results represent the average and SD from 3 independent experiments. *$P < 0.05$; ***$P < 0.001$; ANOVA followed by Tukey's multicomparison post-test. PI, propidium iodide.

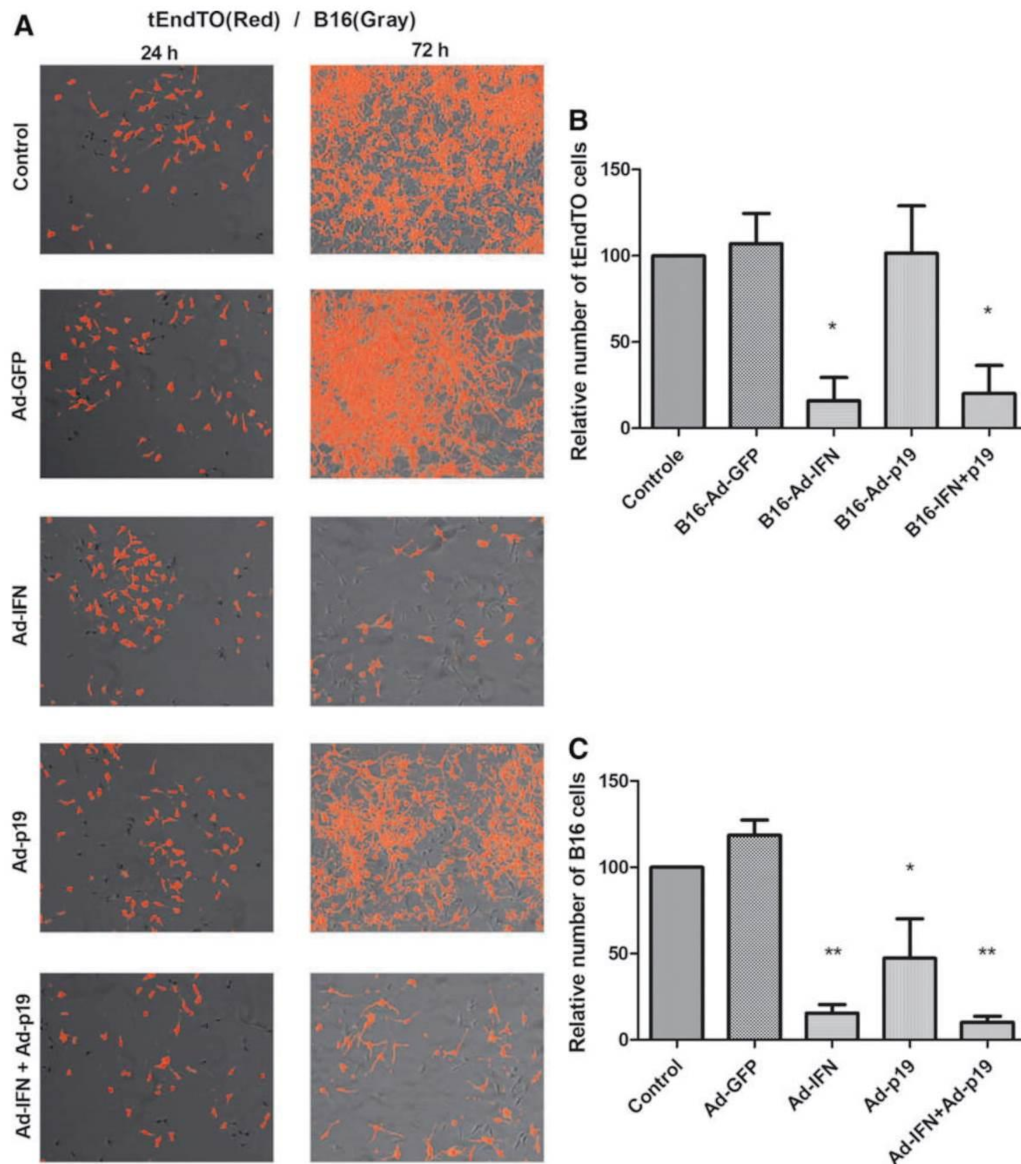
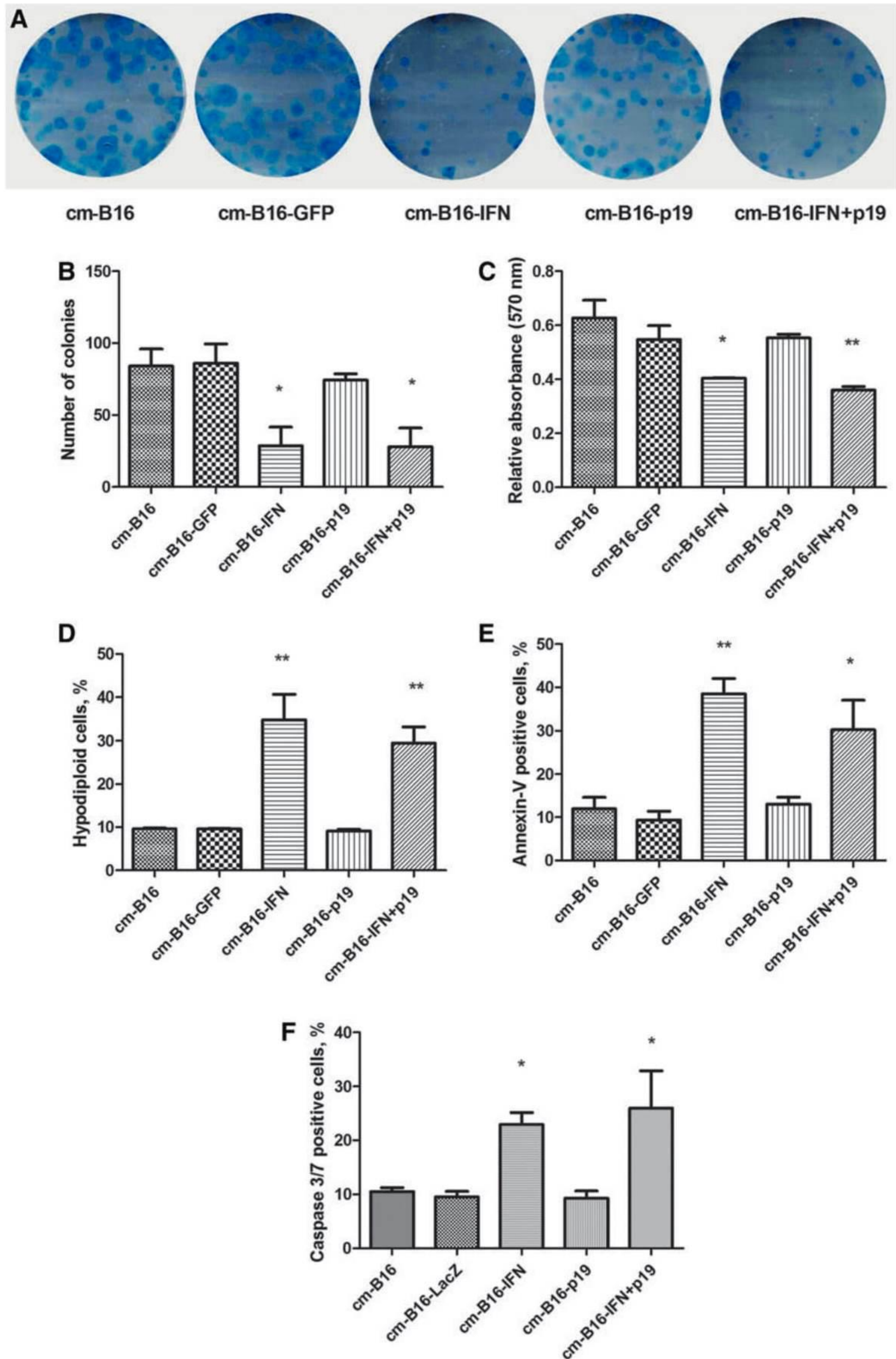


FIG. 3. Coculture of tEndTO with previously transduced B16 cells. B16 cells were transduced (MOI 300), incubated for 6 h, washed, collected, and plated together with tEndTO cells at equal proportions. The coculture was incubated for up to 72 h. **(A)** Fluorescence microscopy reveals the red tEndTO cells and the unmarked B16 cells (EVOS FL, 10× objective). Alterations in the cell populations were quantified by counting **(B)** marked tEndTO cells and **(C)** unmarked B16 cells at 72 h. Quantification was performed using Image J software. Results were normalized to the control condition and represent the average and SD from 3 independent experiments. $*P<0.05$; $**P<0.01$; ANOVA followed by Tukey's multicomparison post-test.

FIG. 4. Conditioned medium derived from transduced B16 cells was used for the cultivation of tEnd cells. B16 was transduced (MOI 300) with the adenoviral vectors, incubated for 6 h, washed, and fresh medium was added. After incubation for 48 h, the conditioned medium was collected and stored at −80°C before use. The conditioned medium was named according to the transgene: cm-B16 (nontransduced), cm-B16-GFP, cm-B16-IFN, cm-B16-p19, and cm-B16-IFN+p19. **(A, B)** For the clonogenic assay, 300 tEnd cells were seeded with conditioned medium in 6-well plates and incubated for 10 days. Then cells were fixed and stained. Plates were scanned to produce these images (Multifunction Printer LaserJet, HP) and clearly visible colonies were counted. **(C)** In this viability assay, tEnd cells were treated with conditioned medium for 48 h before MTT solution was added and analyzed at 570 nm. **(D)** The hypodiploid cell population was revealed after tEnd cells were cultivated in the presence of conditioned medium for 48 h, fixation, staining with PI, and then analysis by flow cytometry. **(E, F)** Apoptotic cells were assessed by flow cytometry after cultivation of tEnd cells in conditioned medium for 48 h and staining with Annexin-V and activated caspases 3/7, respectively. Results represent the average and SD from 3 independent experiments. $*P<0.05$; $**P<0.01$; ANOVA followed by Tukey's multicomparison post-test. GFP, green fluorescent protein; IFN, interferon.


A
cm-B16
cm-B16-GFP
cm-B16-IFN
cm-B16-p19
cm-B16-IFN+p19
B
Number of colonies
C
Relative absorbance (570 nm)
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F
Caspase 3/7 positive cells, %
cm-B16-LacZ

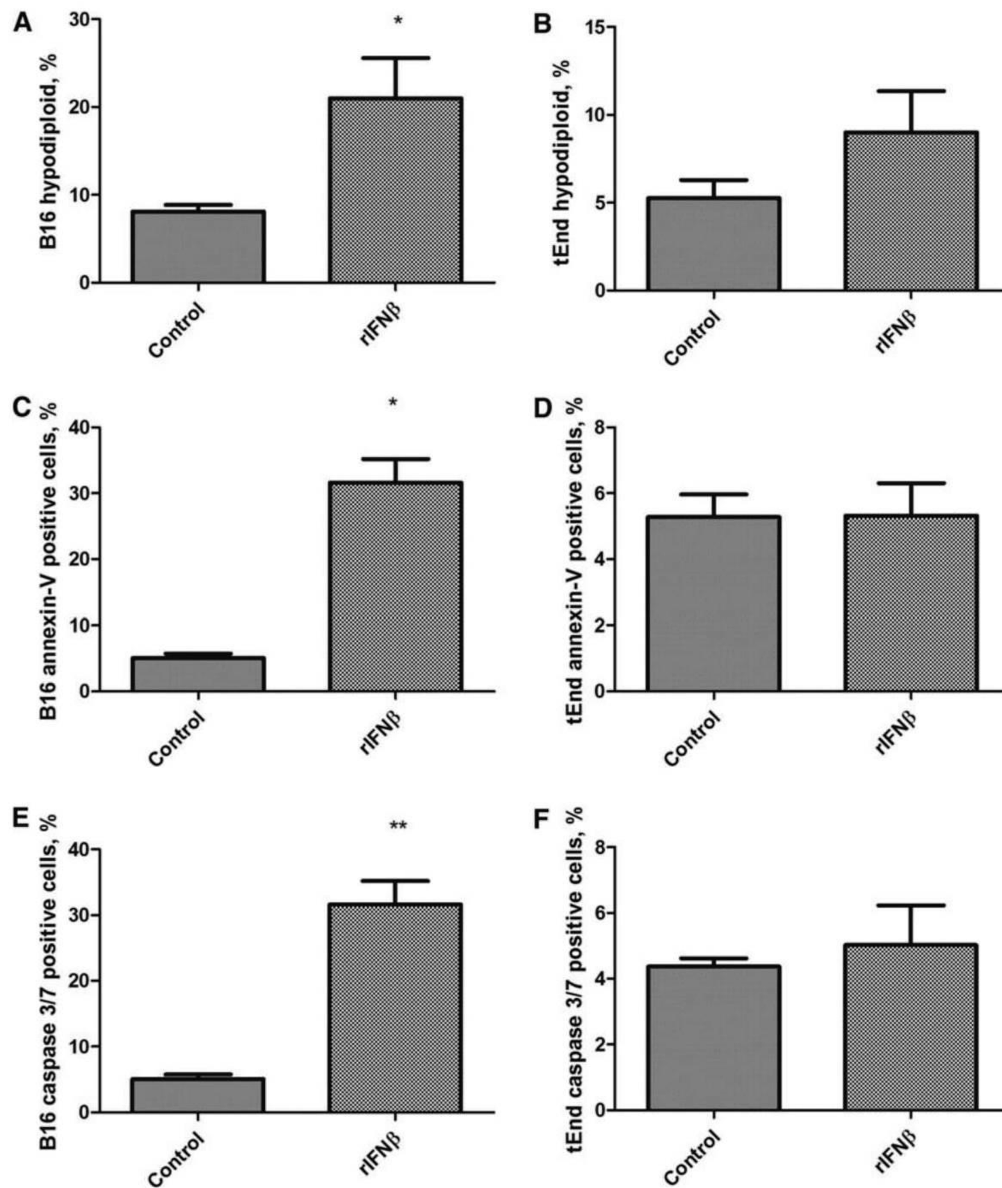

FIG. 5. rIFNβ is a cell death inducer for B16 but not for tEnd cells. B16 and tEnd cells were incubated with rIFNβ 10 U/mL for 48 h. After treatment, cells were collected, fixed, and stained with PI **(A, B)**, or fresh cells were stained with Annexin-V **(C, D)** or caspases 3/7 activity was determined **(E, F)**. Treated cells were analyzed by flow cytometry. Results represent the average and SD from 3 independent experiments. $*P<0.05$; $**P<0.01$; ANOVA followed by Tukey's multicomparison post-test. rIFNβ, recombinant IFNβ.

potential of tEnd cells and for the induction of their death. Alternatively, the secretome produced by B16 melanoma cells upon transduction with the adenoviral vectors also led to the death of tEnd cells; however, in this case B16 transduced with Ad-IFN was essential for inducing this response. Interestingly, rIFNβ was not sufficient to induce death in tEnd cells, but this response was not seen when treating B16 cells under the same conditions. We suspect that the secretome may contain important factors in addition to IFNβ, since direct transduction of tEnd with the vector encoding IFNβ had little impact. In either case, we postulate that our gene therapy approach would reduce tumor angiogenesis, by either direct or indirect signaling.

Adenoviral vectors have been widely used for cancer gene therapy due to their relative stability, efficient gene transfer *in vivo*, and broad tropism in terms of cell type (Crystal 2014; Kasala and others 2014). Even so, transduction by serotype 5 adenovirus (Ad5) relies on the expression of a specific cellular receptor [coxsackievirus and adenovirus receptor (CAR)], thus tropism may be considered limited if

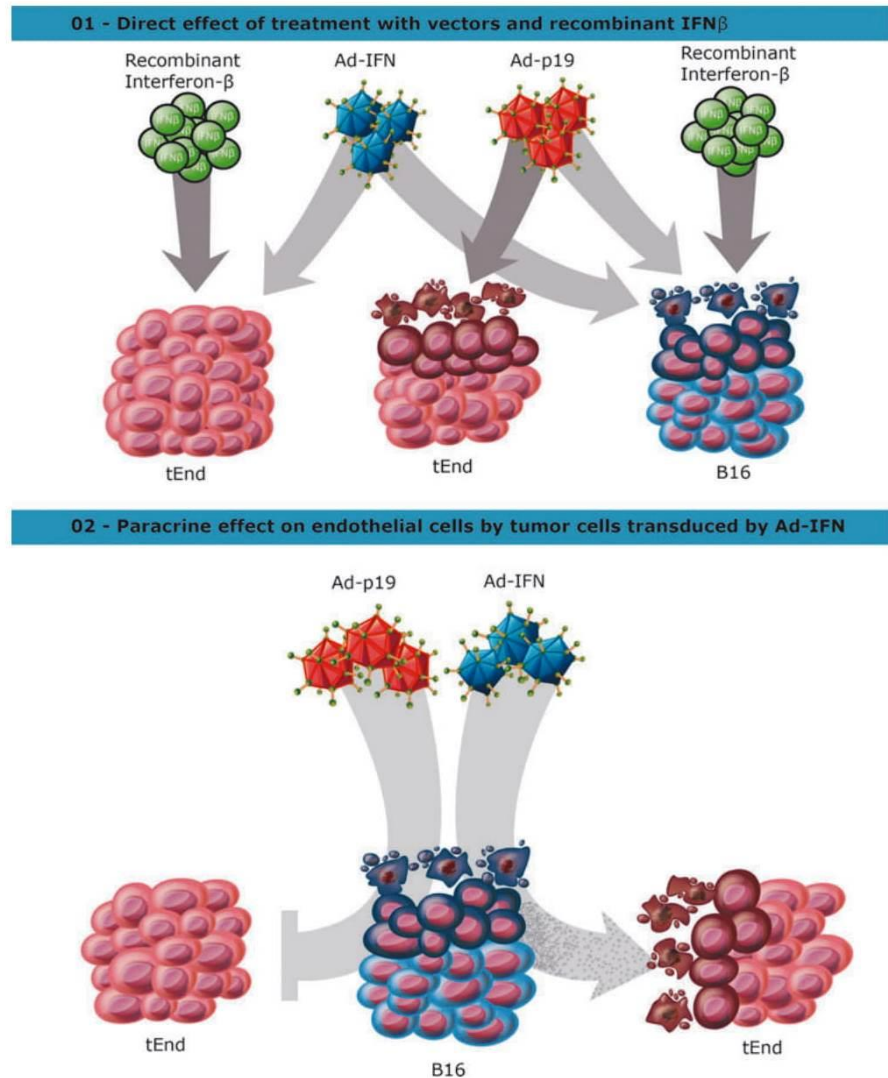

FIG. 6. Schematic representation of cellular response to direct transduction or paracrine exposure to secreted factors. **(01)** Although gene transfer or treatment with rIFNβ induces death in B16 cells, tEnd cells are not affected. **(02)** tEnd cell death is, however, induced by a paracrine effect when exposed to conditioned medium derived from B16 transduced with Ad-IFN or upon cocultivation with B16 transduced with Ad-IFN. Together, these observations suggest that B16 cells transduced with Ad-IFN produce not only IFNbeta, but also additional factors that are necessary for the killing of tEnd cells.

the target cell does not express CAR. Extensive efforts have been made to overcome CAR dependence and to direct adenoviral transduction to specific cell types (Khare and others 2011; Beatty and Curiel 2012; Alonso-Padilla and others 2015; Schmid and others 2018). A well-known approach for altering tropism involves the introduction of the RGD tripeptide in the adenovirus fiber protein, thus alleviating dependence on CAR and directing the virus to interact with cells expressing integrins (Mizuguchi and others 2001).

Since melanomas typically retain wild-type p53, we propose that this may serve as a molecular target of treatment. To this end, we have developed a series of viral vector platforms, including retrovirus, adenovirus, and adeno-associated virus, where transgene expression is under the control of p53 (Strauss and Costanzi-Strauss 2004; Bajgelman and Strauss 2008; Bajgelman and others 2013). Using the AdPG vector (native Ad5 fiber), we showed that the combination of IFNβ+p19Arf is beneficial for killing B16 cells *in vitro* and *in vivo* (Merkel and others 2013) and plays an essential role in NK cell activation and inducing a protective immune response (Medrano and others 2016). We then constructed the AdRGD-PG platform to improve viral tropism and have used these vectors to show the induction of immunogenic cell death in B16 (Hunger and others 2017) and for the stimulation of a protective immune response with critical participation of neutrophils in a mouse model of lung carcinoma (Catani and others 2016). These previous studies highlight the importance of the upregulation of IFNβ+p19Arf combination for the induction of cell death and immune activation.

As presented in this article, we have identified novel aspects of our gene transfer approach. In agreement with our previous work, the vectors were shown to express high levels of the transgene. Although not studied directly, we hypothesize that adenoviral vectors bearing the RGD motif in the adenoviral fiber protein may aid in the transduction of endothelial cells known to present high levels of integrins on the cell membrane (Danhier and others 2012). Certainly, the importance of integrins and the RGD motif for endothelial cell biology (Li and others 2008; Dettin and others 2015) and for the targeting of viral vectors (Mizuguchi and others 2001; Beatty and Curiel 2012; Pesonen and others 2012) has been extensively explored. Although the AdRGD-PG approach was not specifically developed for

targeting tumor vasculature, this may indeed prove to be a benefit when using this vector platform.

Angiogenic characteristics of endothelial cells, such as migration and tube formation, were inhibited upon transduction of the tEnd cells with the Ad-p19. In addition, cell death by a mechanism consistent with apoptosis was also observed in this scenario. Since the expression of critical factors in the p53 pathway was concomitantly increased, we infer that these genes contributed to the cellular response since p21 participates in cell cycle control (el-Deiry and others 1993) and Puma induces apoptosis (Nakano and Vousden 2001; Chipuk and others 2005). Based on these results, we postulate that our gene therapy approach may indeed offer an antiangiogenic benefit when endothelial cells are transduced with a vector encoding p19Arf. Certainly, further exploration is necessary to confirm this point *in vivo*. It has been shown that activation of the p53/Arf pathway can significantly decrease cell motility by repression of phosphoinositide 3-kinase and Rac1 activity and can induce senescence (Guo and others 2003; Debidda and others 2006), additional activities that would favor an approach involving transduction of both the tumor and its microenvironment.

Using coculture or conditioned medium from transduced B16 cells, we observed the induction of tEnd cell death when IFNβ was included in the gene transfer step. Again, our melanoma gene therapy approach may indeed have the desired negative impact on angiogenesis since the secretome generated by the tumor cells treated with IFNβ was lethal for the endothelial cells. Intriguingly, rIFNβ was not sufficient to induce death of tEnd cells, suggesting that additional products secreted by B16 may be necessary for the killing of tEnd cells.

Our study raises several interesting possibilities. First, our melanoma gene therapy approach depends on the cooperation of IFNβ with p19Arf. However, we did not see evidence of cooperation that may yield an antiangiogenic effect, suggesting distinct mechanisms in B16 versus tEnd cells. Even so, the observations made in this study are potentially beneficial for tumor treatment. Second, we saw distinct activities when the endothelial cells were transduced as compared with their exposure to the factors released by the treated tumor cells. This too was not an expected finding, but still points to the antiangiogenic potential of our approach. Finally, we demonstrated that treatment with rIFNβ did not impact tEnd cells, although they were killed when exposed to factors released by transduced B16 cells. This is especially intriguing, suggesting that some factors other than IFNβ may have been responsible for this observation. Recall that transduced tEnd cells produced just as much IFNβ as B16 cells, yet the endothelial cells only responded to the tumor secretome generated after IFNβ gene transfer. Since IFNβ was present in both scenarios, we postulate that the latter involved additional factors, a point that will require extensive investigation in the future.

Based on examples from the literature, we would expect that IFNβ present in conditioned medium should have an impact on the exposed cultured cells. That is to say, we would expect a paracrine or bystander effect mediated by IFNβ. For example, conditioned medium from melanoma cells transfected with a plasmid encoding IFNβ showed a strong paracrine effect, where conditioned medium rich in soluble cytotoxic factors was cytotoxic for nontransfected melanoma cells (Rossi and others 2015). It has been shown that not only are the tumor cells targeted by a paracrine effect mediated by IFNβ, but also other cells in the tumor microenvironment are affected, undergoing apoptosis and reducing angiogenesis due to IFNα/β activity (Benedict and others 2004). *Ex vivo* gene transfer of IFNβ to bladder cancer cells delays growth of the tumor due to the induction of apoptosis of endothelial cells upon implantation *in vivo* (Izawa and others 2002), and IFNβ activates expression of genes related to decline in survival and proliferation (Gomez and Reich 2003). In another study, systemic IFNβ produced by liver-targeted adenoassociated virus (AAV) resulted in maturation of tumor vessels and improved blood flow, yet inhibited tumor growth by impeding further angiogenesis (Dickson and others 2007). In fact, our own work has shown a significant bystander effect where presence of p19Arf sensitized B16 cells to the effects of neighboring B16 cells that had been transduced with the IFNβ vector (Hunger and others 2017). Certainly, further testing, especially *in vivo* studies, will be required to reveal the full impact of our melanoma gene therapy approach on the tumor microenvironment in general and angiogenesis in particular.

With this study we have taken the first steps to show that our gene therapy approach combining upregulation of IFNβ and p19Arf may indeed impact endothelial cells. The use of an adenoviral vector containing the RGD tripeptide in the fiber protein yielded efficient transduction of the tEnd and B16 cell lines, direct transduction with the vector encoding p19Arf or exposure to the tumor secretome produced after transduction with the vector encoding IFNβ both resulted in endothelial cell killing. Although much remains to be elucidated, this study shows that our approach warrants further development and investigation.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1

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Discussão Final

DISCUSSÃO GERAL

As imunoterapias para o câncer estão em alta, essa modalidade tem ganhado a atenção dos pesquisadores e da mídia, que volta e meia divulga alguma nova terapia promissora para o câncer. A linha do tempo do FDA está cheia de novas imunoterapias aprovadas e os trabalhos científicos publicados nessa área só aumentam. O principal objetivo das imunoterapias consiste em superar a supressão imunológica dos tumores e matar as células tumorais por mecanismos imunes, tais como, promover a função das células apresentadoras de antígenos (APCs) e estimular respostas das células T citotóxica e T *helper* (Chen and Mellman 2013).

Nosso grupo propõe a utilização de terapia gênica com vetores adenovirais aperfeiçoados, portadores de IFNβ e ARF, como uma imunoterapia para o câncer. A estratégia consiste em usar o vetor adenoviral sorotipo 5 (Ad5) com design melhorado. Nossos vetores passaram por algumas melhorias como a inserção do tripeptídeo RGD no domínio *Knob* da proteína fibra, com essa modificação o vírus passa a depender da interação com integrinas e não mais das proteínas receptoras de adenovírus para ser internalizado. Essa modificação aumentou a capacidade de transdução do nosso vetor (Hunger et al. 2017a, Mizuguchi et al. 2001). Também foi inserido no material genético do vetor, o promotor forte responsivo a p53 (PGTxβ) utilizado para promover transcrição dos transgenes terapêuticos (Bajgelman and Strauss 2008, Tamura et al. 2016, Strauss, Bajgelman and Costanzi-Strauss 2005). Além das melhorias realizadas na estrutura do no nosso vetor, foram inseridos os transgenes terapêuticos, IFNβ (Ad-mIFN) e Arf (Ad-p19), que conferem ao vetor a característica terapêutica, devido às funções de indução de morte celular e ativação do sistema imune conferido por esses genes (Kamijo et al. 1998, Zhang, Xiong and Yarbrough 1998, Hunger et al. 2017a, Milojkovic et al. 2013).

Os dados publicados pelo grupo com uso da nossa terapia gênica, mostraram efeito anti-tumoral significativo no modelo murino. A combinação da terapia gênica com Ad-mIFN e Ad-p19 induziu morte celular nos modelos tratados, promoveu resposta imune e aumentou da sobrevida dos camundongos com melanoma (Medrano et al. 2017, Catani et al. 2016, Medrano et al. 2016, Hunger et al. 2017a).

Nosso grupo tem ambição de empregar nosso modelo de terapia gênica em humanos. O processo para levar nosso protocolo para humanos é longo, portanto o grupo está evoluindo com investigações básicas que dão a sustentação para alcançar os protocolos com humanos. Os passos básicos atuais representam movimentos necessários para alcançar o objetivo final. Dentro desses passos necessários e alvo desta tese, estão os estudos dos mecanismos de atuação do nosso vetor adenoviral portador do IFNβ no contesto da massa tumoral e do microambiente.

Não menos importante que as células tumorais, as células do microambiente podem ser afetadas pela nossa terapia gênica, afinal, nossos vetores adenovirais podem transduzir uma enorme variedade de células. O microambiente tumoral de tumores sólidos é essencial para a manutenção e sobrevida do tumor. Devido às funções essenciais do microambiente como a produção da matriz extracelular, os vasos sanguíneos para as trocas de gases e nutrientes, entre outras estruturas, o microambiente pode ser visto como um alvo promissor em terapias anti-tumorais (Chen and Mellman 2013, Wang et al. 2017, Carmeliet 2005).

Dedicamos o nosso foco de pesquisa ao tratamento com o vetor Ad-mIFN, devido ao seu potencial anti-tumoral. A via clássica do IFNβ inicia-se quando a proteína se liga à dupla IFNAR1/IFNAR2 de receptores e inicia a ativação da via do IFN (Constantinescu et al. 1994, Müller et al. 1994, Platanias 2005). Para explorarmos os mecanismos autócrino e parácrino já conhecidos da via do IFN, na atuação do nosso modelo de terapia com Ad-mIFN, decidimos utilizar células de melanoma e do microambiente *knockout* para o IFNAR1 (Song et al. 2015).

Nossos dados mostraram que as células *knockout* para o IFNAR1 não respondem ao tratamento com Ad-mIFN e nem ao tratamento com a proteína rIFNβ. Esses resultados elucidaram o mecanismo autócrino do nossa terapia com Ad-mIFN, que necessita da produção e ligação do IFNβ aos receptores cognatos IFNAR1/IFNAR2 para ativar a via do IFNα/β e desencadear efeito de morte celular. Nos ensaios de co-cultura também foi possível observar o efeito parácrino do nosso tratamento, onde as células de melanoma transduzidas pelo Ad-mIFN induzem inibição das células co-cultivadas. A indução do efeito parácrino foi mantida pelas células *knockout* para o IFNAR1, porém estás células não sofreram do efeito parácrino. Esses resultados demonstraram a importância da produção celular do IFNβ e do receptor IFNAR1 nos

mecanismos autócrino e parácrino do no nosso modelo de terapia gênica com Ad-mIFN em células tumorais e do microambiente.

No trabalho publicado com estudo da angiogênese utilizando células endoteliais (tEnd) no tratamento com Ad-mIFN da Cap. 04, foi possível identificar que a nossa linhagem endotelial não responde aos tratamentos com Ad-mIFN e rIFNβ. A eficiência do tratamento anti-tumoral do IFNα/β necessita da integridade da sua via para ter efeito. A resistência ao tratamento com IFNα/β pode ocorrer de duas maneiras, pela modulação negativa dos componentes da via ou por mecanismos contra regulatórios que bloqueariam a via do IFN (Budhwani, Mazzieri and Dolcetti 2018). Dentro dos elementos da via que podem ser inibidos podemos relatar o IFNAR1, que através da ação do VEGF, TNFα, IL-1, IL-6 e falta de oxigenação, podem ser modulados negativamente e causar resistência ao tratamento por IFNα/β através do bloqueio da via do IFN (Wagner et al. 2004). A modulação da sinalização do Jak-STAT também pode causar resistência ao tratamento. O silenciamento epigenético do Jak1, a perda do STAT2 e a ação falha do ISGF3 podem induzir ao bloqueio do sinal do IFN (Dunn et al. 2005, Romero-Weaver et al. 2010). O sucesso do tratamento também depende da presença continua dos IRFs, que na sua ausência subvertem o efeito do tratamento (Bidwell et al. 2012, Zhao et al. 2017). O miRNA conhecido como miR-146a foi encontrado em células de carcinoma hepatocelular que apresentaram resistência ao tratamento com IFNα/β e também a quimioterapia (Tomimaru et al. 2010, Tomokuni et al. 2011, Pogribny et al. 2010).

A linhagem endotelial do trabalho publicado não apresentou resposta aos tratamentos com o Ad-mIFn e rIFNβ, porém ela foi inibida nos ensaios de efeito parácrino na co-cultura com a linhagem B16 transduzida pelo Ad-mIFN e quando recebeu o meio condicionado da linhagem B16 transduzida pelo Ad-mIFN, ou seja, somente o efeito parácrino se mostrou efetivo em inibir a linhagem endotelial. Esses dados sugerem a atuação de outros fatores produzidos pela linhagem B16 transduzida pelo vetor Ad-mIFN que atuaram na inibição da linhagem de células endoteliais utilizada (Vieira et al. 2019). A ativação da via do IFN causada pelo tratamento com IFNα/β pode induzir a produção de uma ampla variedade de fatores biológicos conhecidos por causar efeitos citotóxicos (Stark et al. 1998, Kalie et al. 2008). O ensaio de expressão gênica realizado no Cap. 03 mostrou aumento significativo de TRAIL nas células B16 transduzidas pelo vetor Ad-mIFN Outros trabalhos encontrados na

literatura mostraram que o tratamento com IFNα/β induz a produção de TRAIL em células de carcinoma renal, mieloma múltiplo e linfoma (Clark et al. 2010, Chen et al. 2001, Gong and Almasan 2000). TRAIL é uma citocina produzida por células diversas indutora de apoptose e utilizada como terapia anti-tumoral (von Karstedt et al. 2017, Papageorgiou, Dinney and McConkey 2007). De acordo com os trabalhos da literatura, TRAIL e IFNα/β atual sinergicamente na indução de morte celular (Gong and Almasan 2000, Clark et al. 2010). Frente a essas informações, TRAIL pode ser um candidato em potencial para estar envolvido na indução de morte celular da linhagem endotelial que sofreu efeito parácrino e também estar relacionado à indução de morte celular ocorrida pelo tratamento com Ad-mIFN, porem necessita-se mais investigações para confirmar esse sinergismo.

Conclusão final

O tratamento com Ad-mIFN vai muito além da estimulação do sistema imune e indução de morte imunogênica. Os efeitos autócrinos e parácrino tem o potencial de serem as peças-chave para explicar efeito anti-tumoral observado nos ensaios *in vitro* e *in vivo.* Todos os ensaios publicados pelo grupo mostraram indução de morte das células tratadas diretamente com o Ad-mIFN, e estás células não tiveram contato com o sistema imune. Esses dados demonstram que o efeito de morte não depende do sistema imune, mas pode ser potencializados pela ativação do sistema imune (Cerqueira et al. 2020, Hunger et al. 2017a).

Nos trabalhos desta tese, mostramos que para a concretização do efeito de morte celular pela da terapia gênica com o vetor adenoviral Ad-mIFN, as células transduzidas devem produzir o IFNβ e se ligar a essa proteína para desencadear ativação da via do IFN e posterior morte celular. O tratamento com o Ad-mIFN também atua de forma parácrina, com a inibição das células próximas pela ação principalmente do IFNβ. O IFNAR1 tem papel fundamental no efeito do tratamento, sua ausência bloqueia a célula do efeito direto do tratamento e também da recepção do efeito parácrino. Os efeitos do tratamento com Ad-mIFN diretos ou parácrinos podem ser observados em células tumorais e células do microambiente.

O conjunto de resultados desta tese contribui para o entendimento do mecanismo de ação da terapia gênica com adenovírus portador do IFNβ, em células tumorais e do microambiente. Além disso, este estudo fornece alguns alvos críticos ao tratamento,

como o IFNAR1 que na sua ausência bloqueia o tratamento. A evolução da terapia gênica com IFNβ desenvolvida pelo grupo, agora consta uma nova parcela de conhecimento do mecanismo de ação da nossa terapia, que pode nortear o foco de estudos do grupo para alcançar seus objetivos e o sucesso.

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